

**Staphylococcal Surface Proteins as Vaccine Candidates for the
Control of *Staphylococcal* Mastitis in Dairy Cows**

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Abstract

Bovine mastitis, an inflammation of the mammary gland, is the single most costly disease of dairy cattle. Staphylococci, a major bacteria that cause bovine mastitis, are commonly divided into two major groups: *Staphylococcus aureus* and non-*aureus* staphylococci (NAS), previously known as coagulase-negative *Staphylococcus* species (CNS). Current mastitis control programs are not fully effective against staphylococcal mastitis and antibiotics are not sustainable due to limited success and the emergence of antimicrobial resistant bacteria. Non-antibiotic sustainable control tools, such as effective vaccines, are critically needed. Our lab developed and evaluated *Staphylococcus aureus* surface associated protein (SASP) and *Staphylococcus chromogenes* surface associated protein (SCSP) based experimental vaccines. The objective of this study was to evaluate efficacy of SCSP and SASP vaccines against staphylococcal intramammary infection and mastitis via natural exposure over 300 days of lactation. A total of 45 pregnant Holstein dairy cows were randomly enrolled in the study in three groups of 15 (Group1), 16 (Group 2) and 14 (Group 3) cows. Cows in groups 1 and 2 were vaccinated with 1.2 mg of SASP and SCSP with Emulsigen-D® adjuvant respectively. Cows in group 3 (control) were injected with phosphate buffered saline (pH 7.4) with Emulsigen-D adjuvant. Cows were monitored for increase in antibody titers in serum and milk, milk yield, milk somatic cells count, mastitis, adverse reactions to the vaccinations, and *S. aureus* and non-*aureus* staphylococci count from milk over 300 days of lactation. Our results showed increased milk and serum anti-SASP and -SCSP antibody titers in vaccinated cows compared to controls. The incidence and incidence density of staphylococcal subclinical mastitis were reduced both at the cow (SCSP group) and quarter (SCSP and SASP) levels. Overall, the SCSP vaccine conferred higher protective effects compared to the control and SASP vaccinated groups. Anti-SASP and -SCSP antibody titers began to wane at about 120 DIM indicating the length of immunity is about 4 months. About 3 - 4 doses would be required over 300 days of lactation. In conclusion, with further optimization of vaccination regimen staphylococcal surface associated protein vaccines particularly SCSP is a promising vaccine for the control of staphylococcal mastitis in dairy cows.

Keywords: Bovine mastitis; *Staphylococcus aureus*; non-*aureus* staphylococci, *Staphylococcus chromogenes*; vaccination, immunity, dairy cow; *Staphylococcus aureus* surface associated proteins, *Staphylococcus chromogenes* surface associated proteins;

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Chapter 1: Review of Literature

1. Mastitis

Mastitis, an inflammation of mammary glands, is usually caused by pathogenic microorganisms, such as bacteria, fungi, yeast, and algae. Mastitis is one of the most economically devastating diseases in the dairy industry worldwide. Annual economic losses due to bovine mastitis in the United States alone is estimated to be \$1.7 to 2.0 billion (NMC, 1996) with, on average, clinical mastitis costs being \$444 per case during the first 30 days in milk (Rollin et al., 2015). The cost of mastitis per case was estimated to be \$325.76 and \$426.50 for primiparous and multiparous cows, respectively (Liang et al., 2017). Mastitis incurs severe economic losses to the dairy industry due to loss of milk production, low premium of poor-quality milk, treatment cost, milk disposal during treatment, decreased fertility, culling of non-curable chronically infected cows, and replacement costs (Guimaraes, 2017). In addition to the incremental loss of milk, mastitis affects milk composition. For instance, on average total solids decrease by 1.07% (Janzen, 1970), which include decreased levels of fat and lactose. A study on economic losses due to correlating high SCC from dairy farms in the Midwestern Region of United States showed 5.2 lbs. of milk is lost for every 100,000 cells/mL increase in SCC (Dickerell et al., 2016).

1.1 Bovine Staphylococcal Mastitis

Staphylococci are opportunist and contagious pathogens that colonize the nasal cavity, nasopharynx, skin, and mucus membranes of mammals and birds (Le Loir et al., 2003, Markey, 2013). The most common staphylococci that cause mastitis in dairy cows are *Staphylococcus aureus* and non-*aureus* staphylococci (NAS), previously known as coagulase negative *Staphylococcus* species, such as *S. chromogenes*, *S. epidermidis*, *S. haemolyticus*, *S. simulans*, and *S. xylosus* (Thompson-Crispi et al., 2014, Song et al., 2020, Todar, 2020). *S. aureus* and NAS are in the family of *Staphylococcaceae* and genus *Staphylococcus*. Staphylococci are gram-positive, catalase-positive, coagulase-positive or negative, non-spore forming, oxidase-negative, non-motile, cluster-forming, facultative anaerobes (Takahashi et al., 1999).

S. aureus is one of major contagious mastitis pathogens on U.S. dairy farms (APHIS, 2008). There are several coagulase positive staphylococcus species, but about 95% of coagulase positive *Staphylococci* from bovine mastitis are *S. aureus* (Fox and Hancock, 1989). There are

more than 50 different species of NAS and approximately 15% are associated with bovine mastitis. The most frequently isolated NAS from bovine intramammary infections (IMI) are *S. chromogenes*, *S. epidermidis*, *S. xylosus*, *S. simulans*, and *S. haemolyticus* (Wald et al., 2019). Different strains of *S. aureus* exist (Côté-Gravel and Malouin, 2019), for example, the small colony variant (SCV) internalizes into mammary epithelial cells and avoids clearance by host immune defense and antibiotic treatment (Côté-Gravel and Malouin, 2019). Small colony variants are strains responsible for facilitating recurrent, persistent infections (Côté-Gravel and Malouin, 2019).

Recently, NAS have increasingly been the most prevalent intramammary pathogen isolated from lactating dairy cows (Tenhagen et al., 2006, Thorberg et al., 2009, Condas et al., 2017). The prevalence of NAS in US and European dairy herds ranges from 27 – 55% (Gillespie et al., 2009, De Visscher et al., 2016). The cure rate of staphylococcal mastitis with antibiotic treatment is low and ineffective, but producers continue to use antibiotic treatment for the control of staphylococcal mastitis, leading to increased antibiotic pressure and development of antimicrobial resistant *S. aureus* and NAS (Barkema et al., 2006). Detailed understanding of pathogenesis of bovine staphylococcal mastitis in addition to knowledge of host innate and adaptive immunity required to clear staphylococcal IMI is critically important to develop a sustainable non-antibiotic control tool, such as effective vaccines.

1.2 Staphylococcus aureus Mastitis

S. aureus mainly causes subclinical and chronic mastitis characterized by high somatic cell counts. In general, prevalence of *S. aureus* IMI and occurrence of clinical mastitis significantly decreased in well-managed dairy farms that adopted effective mastitis control measures. However, the prevalence of *S. aureus* IMI may reach 50 – 75% of all herds having at least one *S. aureus* IMI since all farms are not equally adopted effective mastitis control measures (Dufour et al., 2012a). In low-SCC herds prevalence of *S. aureus* IMI in cows ranges from 1 – 10%, but in high-SCC herds prevalence of *S. aureus* IMI may reach up to 50% with quarter infection rates ranging from 10 – 25% (Dufour et al., 2012a, Diseases of the Mammary Gland, 2017). The prevalence of *S. aureus* IMI in heifers at parturition ranges from 5 – 15% (Owens et al., 2001, Diseases of the Mammary Gland, 2017). In herds with an effective mastitis control program, the most common causes of clinical mastitis are environmental mastitis pathogens. However, in some herds with a low rolling SCC, incidence of clinical mastitis is about 190 to 240 cows per 1000 cows per year

with 47% of clinical cases being *S. aureus* (Diseases of the Mammary Gland, 2017). Treatment with an antibiotic is the only available tool for intervention for cows that developed mastitis. However, the cure rate of staphylococcal mastitis with antibiotic treatment is very low. Drugs may not reach bacteria in the purulent inflammatory reaction because of extensive fibrosis and micro abscess formation in the gland. Cows with chronic infection (>3 months) usually do not respond to antibiotic treatment.

Chronic *S. aureus* IMI may form biofilm that serves as a physical barrier against both the host immune defense and antibiotics administered for treatment (Atwood et al., 2016). The antibiotics never reach the bacteria in the middle of the dense biofilm, instead it will only affect those organisms on the outside of the biofilm cluster leaving those deeper in the biofilm unaffected (Atwood et al., 2016). Biofilm formation is one of the most important virulence factors of *S. aureus* that allow bacteria to resist killing by host immune defense and antibiotics (Stewart and Costerton, 2001, Donlan and Costerton, 2002). Slime is an exopolysaccharide layer that protects bacteria from opsonophagocytic killing by host immune defense and antibiotics. Ability to form slime increases adhesion to host cells (Milanov et al., 2010). Mature biofilm can break and re-infect different parts of the host tissue (Melchior et al., 2006b). Biofilm formation can be achieved through different mechanisms including polysaccharide intercellular adhesin (PIA), different staphylococcal surface proteins, teichoic acids, and extracellular DNA (eDNA) (Gotz, 2002, Otto, 2008, Dhanawade et al., 2010). The intercellular adhesion locus encodes different proteins including IcaA, IcaB, IcaC and IcaD that are involved in biofilm formation (Gotz, 2002, Otto, 2008). Evaluation of association of presence of *ica* locus genes, slime production and ability to form biofilm showed that all *S. aureus* isolates tested harbor *icaA* and *icaD* genes (Vasudevan et al., 2003, Szweda et al., 2012) and most of them produce slime (Vasudevan et al., 2003). However, all slime producers cannot form biofilm in vitro (Vasudevan et al., 2003). Biofilm formation increases adhesion ability of *S. aureus* but the *Bap* protein inhibits internalization into host cells (Valle et al., 2012). The presence of *ica* locus genes is associated with biofilm formation ability of *S. aureus*; however, the *ica* gene is not an absolute requirement for biofilm formation since some *ica* negative strains of *S. aureus* can form biofilm through other mechanisms (O’Gara, 2007, Otto, 2013).

S. aureus is not only a mastitis pathogen of dairy cows, but also zoonotic pathogen causing several infections in humans ranging from minor skin infection and food poisoning (Jakobsen et al., 2011, Rola et al., 2016) to life threatening endovascular infections (Pollitt et al., 2018).

S. aureus grows prominently in milk and other dairy products, which contribute to majority of the pathways for human infection (Abril et al., 2020). Human staphylococcal food poisoning is characterized by clinical signs, such as vomiting, diarrhea, nausea, and abdominal cramps beginning 2 - 6 h post consumption of contaminated food (Murray et al., 2005). For children and adults with weakened immune systems, only a few micrograms of toxins are required to cause inflammatory response (Abril et al., 2020). The enterotoxins themselves alter the expression of cytokines within the human as well as the production of those vital immune cells, such as T cells, macrophages, monocytes, and mastocytes (Abril et al., 2020). Reducing Staphylococcal prevalence in dairy products via reduction of infection by utilizing effective control methods, such as a vaccine, overall decreases exposure to the pathogenic bacteria.

S. aureus has the ability to grow and produce staphylococcal enterotoxins (SE) over an extensive range of temperature, pH, salt concentration, and water activity (Adams and Moss, 2008). *S. aureus* can be eliminated by heating the food, but the enterotoxins will remain and can cause staphylococcal food poisoning (Le Loir et al., 2003). Staphylococcal food poisoning is intoxication due to ingestion of improperly prepared or stored food in which staphylococci grew and produced enterotoxins. The severity depends on the amount of food consumed, the quantity of toxin in the consumed food and the general health of the person (Smyth et al., 2004). For children and adults with weakened immune systems, only a few micrograms of toxins are required to cause inflammatory response (Abril et al., 2020). The enterotoxins themselves alter the expression of cytokines within the human as well as the production of those vital immune cells, such as T cells, macrophages, monocytes, and mastocytes (Abril et al., 2020). Staphylococcal food poisoning can be caused by ingestion of preformed enterotoxins ranging from 20 –100 ng (Asao et al., 2003). About 21 staphylococcal enterotoxins (SE) or enterotoxin-like proteins (SEIs) have been identified and designated SEA to SEIV (Thomas et al., 2006, Schlievert and Case, 2007).

The genes encoding enterotoxins are carried by different mobile genetic elements such as prophages, plasmids, pathogenicity islands (SaPIs), enterotoxin gene cluster (egc) and the staphylococcal cassette chromosome (Khan and Novick, 1982, Altboum et al., 1985, Betley and Mekalanos, 1985, Couch et al., 1988, Bayles and Iandolo, 1989, Munson et al., 1998, Zhang et al., 1998, Fitzgerald et al., 2001, Kuroda et al., 2001, Yarwood et al., 2002, Letertre et al., 2003). Enterotoxins are short, water-soluble extracellular proteins that are very stable and are resistant to heat and degrading enzymes (Denny et al., 1966, Genigeorgis, 1989, Le Loir et al., 2003).

Enterotoxins can be eliminated by proteases produced by lactic acid bacteria (LAB), (Hallis et al., 1991, Bore et al., 2007, Chordash and Potter, 2008). The superantigenic enterotoxins induce pyrogenicity, immune suppression, a mitogenic effect on T cells (Le Loir et al., 2003), and toxic shock syndrome (Fraser and Proft, 2008). The *sea* gene is carried by temperate bacteriophages and inserted into the bacterial chromosome as a prophage then behaves like part of the bacterial genome (Betley and Mekalanos, 1985). However, under environmental stress conditions, such as mild food preservation conditions, the prophage can be induced to replicate the phage genome and release new bacteriophages (Wallin-Carlquist et al., 2010). There are six completely sequenced *S. aureus* strains containing different *sea*-carrying prophages including Φ 252B, Φ Mu3, Φ Mu50A, Φ NM3, Φ Sa3ms, and Φ Sa3mw, which frequently carry the genes for enterotoxin A, staphylokinase, and the complement inhibitor (Kuroda et al., 2001, Baba et al., 2002, Holden et al., 2004, Baba et al., 2008, Goerke et al., 2009).

Improperly handled or contaminated foodstuffs or non-pasteurized dairy products are main source of staphylococcal poisoning. Two major factors contributing to the resulting bacterial growth are improper milk storage temperature and inappropriate handling of the foodstuffs (Abril et al., 2020, Khairullah et al., 2020). Higher levels of starch and protein in food, pH, water activity, and warmer temperature increases enterotoxin production (Abril et al., 2020). *S. aureus* optimal growth conditions are not as stringent as other bacteria that commonly cause food poisoning in humans. *S. aureus* can survive in a pH of 4.5 - 7.0, a low water activity of 0.86, and a salt tolerance of up to 20%, that would normally kill bacteria (Valero et al., 2009, Medvedova et al., 2019). Lower pH decreases the ability of *S. aureus* to adhere to solid surfaces, subsequently decreasing the ability to establish and maintain infection (Cunha et al., 2019). *S. aureus* strains, such as methicillin resistant *S. aureus* (MRSA), have the ability to utilize salt and not only survive, but actually grow in unnatural temperatures (Medvedova et al., 2019).

To combat the growth of *S. aureus* in milk and the subsequent enterotoxins production milk cooling systems are used at the producer level to limit growth by continuously cooling and keeping the temperature below optimum level for bacterial growth (Le Loir et al., 2003, Schelin et al., 2011). In the dairy processing industry pasteurization is used to kill bacteria. However, pasteurization temperatures of 92°C, resulted in 45% of samples still containing staphylococcal enterotoxins compared to 72°C which resulted in 87.5% of samples still exhibiting staphylococcal enterotoxins (SE) post pasteurization (Necidova et al., 2016). The prevalence of SE in pasteurized

milk samples is nearly 50%, raising serious concern that consumption of pasteurized milk may still lead to food poisoning (Necidova et al., 2016, Wang et al., 2018).

Different methods are used to detect *S. aureus* in the animal before collecting milk or before enterotoxin can be detected. Conventional methods, such as serological tests, lack sensitivity and specificity and are not able to meet the need of real-time detection (Wu et al., 2016). The most common method to detect SE is Polymerase Chain Reaction (PCR), but PCR is expensive and requires trained professionals (Wu et al., 2016). Immunoassays such as 1) optical, 2) electrochemical, and 3) mass detection techniques can be used (Wu et al., 2016). New technologies, including immunosensors combined with optical, electrical, and mass-based bioassays utilize new recognition agents drastically increasing target molecules such as SE (Wu et al., 2016).

1.3 Mastitis due to Non-*aureus* Staphylococci

Non-*aureus* staphylococci are increasingly reported as cause of mastitis in the dairy farms (Pyörälä and Taponen, 2009, Tomazi et al., 2015). Prevalence of NAS has increased significantly in the past few years on dairy farms, creating larger ubiquitous reservoirs and extensively escalating the risk of transmission from animals to food or humans (Darbaz et al., 2018). The main effect of NAS IMI is increased somatic cells count (Tomazi et al., 2015). Pathogenic NAS predominantly thrives on the skin of the udder or teats and dirty equipment causing infection as an opportunistic pathogen (Bianchi et al., 2019). Although commonly grouped together, NAS have been shown to be drastically different in genetic makeup, pathogenicity, and ability to cause disease in dairy cows (Cameron et al., 2014, Vakkamaki et al., 2017). Some strains of NAS may cause little to no harm, while others cause chronic mastitis and increase SCC. Some of the most common NAS that increasingly cause mastitis include *S. chromogenes*, *S. simulans*, *S. haemolyticus*, *S. xylosus*, and *S. epidermidis* (Vanderhaeghen et al., 2014). The distribution of strains varies; for example, *S. chromogenes* is more prevalent in primiparous cows, whereas *S. epidermidis* is seen more in IMI of multiparous cows (Thorberg et al., 2009). Overall, NAS cause SCC of about 70,000 cells/mL of milk which is far lower than the threshold for major mastitis pathogens which is >200,000 cells/mL milk (Condas et al., 2017).

Many herd-level factors play major roles for occurrence of NAS. Many studies on bulk tank milk samples found that the incidence of NAS in a herd ranged from as low as 43% to as high

as 60 or 90% (De Visscher et al., 2017, Jenkins et al., 2019, Yun et al., 2020). Hygiene is the leading control method for NAS mastitis management (De Visscher et al., 2017). In a herd with a lack of udder health programs, there are many positive cases of subclinical mastitis due to *S. chromogenes*, *S. simulans*, and *S. xylosus* in bulk tank milk samples (De Visscher et al., 2017). For example, herds with free-stall barns tend to have less NAS compared to tie-stall and loose-pack bedding barns (De Visscher et al., 2017). *S. equorum* and *S. cohnii* are two NAS species that may originate from the cow's environment and increase with unsanitary conditions. However, mammary glands pre-colonized by certain strains of NAS may not be infected by major pathogens (De Vliegher et al., 2004, Stevens et al., 2018).

In addition to housing differences, grouping and age differences of animals are a deciding factor for NAS variation and bacterial count (Pyorala and Taponen, 2009). NAS mastitis prevalence has been found to be significantly higher in primiparous cows than in multiparous cows (Tenhagen et al., 2006, Pyorala and Taponen, 2009). A lower prevalence (16%) has been found in prepartum heifers in pasture grazing systems compared to farms with heavy management practices with over 50% NAS prevalence (Pyorala and Taponen, 2009). Multiparous cows were found to be infected with *S. simulans* more frequently and have been known to become infected later in lactation (Taponen et al., 2006). In primiparous cows, the prevalence of *S. chromogenes* is higher compared to other NAS (Rajala-Schultz et al., 2004, Pyorala and Taponen, 2009). Determining a positive infection due to NAS is difficult due to variation among studies and absence of standards for detecting NAS positive case.

1.4 Virulence Factors of *S. aureus* and Non-*aureus* Staphylococci

1.4.1 Universal Staphylococcal Virulence Regulators

In general staphylococci are an opportunistic bacteria that cause diseases that range from common skin infections to the most severe infections such as bloodstream infections, endocarditis, osteomyelitis, and necrotizing fasciitis (Lowy, 1998) in humans. Similarly, in animals staphylococci cause diseases that range from superficial purulent skin infection to severe diseases such as mastitis, necrotizing endometritis, pyometra, exudative epidermitis, cystitis, and otitis (Markey and Markey, 2013). To survive in different environmental niches, *S. aureus* coordinates the expression of different virulence genes (Novick, 2003). Staphylococcal virulence factors

include cell surface components and proteins directly released into the extracellular environment and their function include adherence to host cells, evasion of host defenses, nutrient degradation and acquisition (Jenul and Horswill, 2019). Staphylococcal virulence factors are encoded directly from the chromosome and from mobile elements such as phages, plasmids, and pathogenicity islands (Jenul and Horswill, 2019). The expression of these different virulence factors of *S. aureus* are under the control of some universal virulence regulators (regulons), such as quorum sensing accessory gene regulator (*agr*) (Recsei et al., 1986), the two component regulatory systems (TCS) that comprises 16 different TCS (Somerville and Proctor, 2009, Villanueva et al., 2018), and the staphylococcal accessory regulator A (*SarA*) (Bayer et al., 1996) and its homologues *SarR* (Manna et al., 1998), *SarT*, and *SarU* (Schmidt et al., 2001, Manna and Cheung, 2003) protein family. Some TCS, such as *agrAC* (Recsei et al., 1986), the *S. aureus* exoprotein expression locus (*saeRS*) (Giraud et al., 1994, Liu et al., 2016), the staphylococcal respiratory regulator (*SrrAB*) (under low oxygen condition) (Throup et al., 2001, Yarwood et al., 2001), and the autolysis regulated locus (*ArlRS*) (Fournier and Hooper, 2000, Fournier et al., 2001) are linked to *S. aureus* virulence and regulate many host-impacting secreted proteins. Out of 16 TCS the *WalKR*, is essential for the viability of *Staphylococcus aureus*, playing a central role in controlling cell wall metabolism while the other fifteen have been inactivated in multiple strains (Matsuo et al., 2010, White et al., 2014). Staphylococci, especially *S. aureus*, survives in the host environment by coordinated expression of its cytoplasmic regulators (Somerville and Proctor, 2009) including *SarA* protein family of transcriptional regulators (*SarA*, *Rot*, *MgrA*, etc.), the alternative sigma factors (*SigB* and *SigH*), the quorum sensing *agr* regulon, and TCS.

sarA and *saeRS* work synergistically to diminish protease production and aid in biofilm formation in *S. aureus* (Mrak et al., 2012). *SarA* mutants decreased biofilm formation and susceptibility to antibiotics while increasing the production of alpha toxin, which is a virulence factor of *S. aureus*. The *saeRS* leads to transcription of *fnbA* and other proteins found on the surface of *S. aureus*. The mutation of *saeRS* will reduce the number of surface proteins and biofilm formation (Mrak et al., 2012), increase susceptibility to antibiotics, and increase efficacy of treatment with antibiotics (Rom et al., 2017). The *sarA* mutation accounts for a higher production of extracellular proteases, reducing the ability to bind fibronectin therefore not limiting the accumulation of surface associated proteins.

S. aureus exhibits a flexible and adaptable regulatory circuit to accommodate the

adaptations needed to keep up with the host's immune system (Rom et al., 2017). Changing the ability of *S. aureus* to utilize regulatory loci that limit or enhance the amount of a protein produced can be useful in limiting biofilm formation (Rom et al., 2017). Reducing the ability of *S. aureus* to form biofilm under in vivo condition may limit the number of bacteria in infected animals and improve efficacy of antibiotics. Overall, *S. aureus* and NAS virulence factors can be broadly grouped into two major classes 1) non-secretory or structural components and 2) secretory (secreted) virulence factors

1.4.2 Non-Secretory

Surface proteins of *S. aureus* are adhesins enabling bacteria to colonize host tissues (Pi et al., 2020). Virulence factors associated with the peptidoglycan cell wall express a multitude of functions and participate in staphylococcal pathogenesis. In addition to adhesion, non-secretory proteins assist in the evasion of the immune responses, invasion of host cells and tissues, and formation of physical barriers like biofilms.

Staphylococcal protein A (SpA) - A cell wall protein encoded by the *spa* gene and found in nearly all *S. aureus* strains. It binds Fcγ domains of the IgG antibody and prevent antibody mediated clearance of *S. aureus* from the host (Todar, 2020). The SpA also binds to the Fab domain of IgM, triggering cross-linking between B-cell receptors. This cross-linking between B-cell receptors alters host adaptive immune responses by programming B lymphocytes death (Becker et al., 2014). Thus, antibodies cannot effectively remove *S. aureus* due to this immune evasion effects of protein A (Daum and Spellberg, 2012).

Biofilm-associated protein (Bap) - *S. aureus* strains that cause bovine mastitis may harbor both *ica* and *bap* genes or carry the *ica* gene but be negative for the *bap* gene or negative for both *ica* and *bap* genes (Cucarella et al., 2004). Infection with both *ica* and *bap* positive strains resulted in lower milk somatic cells count than infection with both *ica* and *bap* negative strains. Cucarella et al. (2004) found that *bap*-positive strains of *S. aureus* were more significantly able to colonize and cause IMI and less sensitive to antibiotics when it forms biofilm in vitro which may indicate that Bap enhances biofilm formation and chronic *S. aureus* IMI. A study (Shukla and Rao, 2017) showed that *bap* was more likely (56.6%) to be found in *Staphylococcus* strains isolated from

subclinical mastitis cases compared to clinical cases. The presence of *icaA*, *icaD*, and *bap* genes are associated with biofilm formation; however, most of *S. aureus* isolates without these biofilm-forming genes were able to produce biofilms (Notcovich et al., 2018).

Biofilm formation - Biofilm formation is a virulence mechanism (Melchior et al., 2006a) responsible for mastitis due to *S. aureus* (Hensen et al., 2000b, Szweda et al., 2012). Biofilm formation involves cell attachment and formation of an extracellular matrix that protects bacteria against a hostile environment of the host immune responses and antimicrobial therapy by slowing their growth, reducing metabolism, and reducing penetration of antimicrobial into the biofilm structure (Stewart and Costerton, 2001). The biofilm is made of exopolysaccharide that protect staphylococci from host immune defense and antibiotics (Coelho et al., 2011). Staphylococcal ability to form biofilm enhances their colonization of mammary gland (Coelho et al., 2011) and they protected from killing by host phagocytic cells (Raza et al., 2013). Biofilm attaches to the host tissue surfaces by polysaccharide/ adhesin (PS/A) and polysaccharide intercellular adhesin (Pérez et al., 2020) matrix that prevents bacterial killing by phagocytic cells (Arciola et al., 2015). The biofilm may break and spread infection to different tissues (Shukla and Rao, 2017). Proteases help to detach adherent bacteria and promote internalization or invasion into cells and tissues (Magro et al., 2017). This attachment to host cells and internalization into cells increase creating safe intracellular haven for bacteria to resist effects of intramammary antibiotics and humoral host immune defense. The formation of polysaccharide intercellular adhesin and polysaccharide adhesin is mediated by *ica* operon. The *ica* operon contains different genes including *icaA*, *icaB*, *icaC*, *icaD*, and *icaR* that encode IcaA, IcaB, IcaC, IcaD, and IcaR proteins respectively (Vasileiou et al., 2018, Cantekin et al., 2019, Pérez et al., 2020). Each protein has different role, for example, *icaR* regulates *ica* operon, the mutual expression of *icaA* and *icaD* promote slime production, and *icaC* encodes for proteins that serve as receptors (Bissong and Ateba, 2020). The presence of different genes in this operon also varies by strain. A study found that 24.1% and 21.4% of NAS isolates carry *icaA* and *icaD* genes, respectively (Gajewska and Chajęcka-Wierzchowska, 2020), whereas 100% of *S. aureus* isolates carry *icaD* gene (Gajewska and Chajęcka-Wierzchowska, 2020).

Slime is an additional extracapsular layer of the biofilm, but is not found on all biofilms (Milanov et al., 2010). Slime is essentially a pseudo-capsule produced by bacteria that reduces

effects of antibiotics on bacteria (Cunha et al., 2019). All isolates do not contain genes enabling for the phenotypic expression of a slime layer. The biofilm, slime partnership depends on the bacterial strain. A study found that most *S. aureus* isolates (80%) that produced slime could also form a strong biofilm (Gajewska and Chajęcka-Wierzchowska, 2020). However, for NAS, 87% with and 84.2% without slime production exhibited the ability to produce strong biofilm (Gajewska and Chajęcka-Wierzchowska, 2020). Strong biofilm production is characterized by the ability of staphylococcal isolates to colonize and form large, distinct biofilms completely covering a stainless-steel surface under laboratory conditions (Milanov et al., 2010). When evaluating slime production of 708 staphylococcal strains, 66% of NAS isolates exhibited at least a weak slime production capability (Vasileiou et al., 2018). When breaking down the NAS into species specifics, the *S. epidermidis* strain was a slime producer in 95% of cases compared to the total NAS isolates examined expressing slime production in 65% of cases (Vasileiou et al., 2018). Even within slime-producing species, genes continue to vary in frequency. *S. epidermidis* isolates rely heavily on *icaD* (50%), *eno* (86%), and *clfa* (50%) genes while in *S. simulans*, *icaB* (65%) and *icaC* (50%) genes are the most predominant (Vasileiou et al., 2018).

There are at least six genes found to have at least some link with biofilm formation in NAS (Srednik et al., 2017). These are *icaA* and *icaD*, found in the majority (73.2%) of NAS (Felipe et al., 2017). The *bap* is also important for biofilm formation. However, within the *icaA* and *icaD* positive NAS the majority (81.7%) of them were negative for the *bap* gene (Felipe et al., 2017). In contrast to *S. aureus*, NAS are still able to form biofilm although negative for *icaA* and *icaD* genes, proving that these genes are not always essential for phenotypic mechanisms (Felipe et al., 2017). There are several mechanisms by which staphylococci form biofilm (Pérez et al., 2020). The biofilm components also vary with *Staphylococcus* species and microenvironmental niche (Raza et al., 2013). Further detailed investigations are required to unravel association between biofilm and slime and their role on pathogenicity of staphylococcal mastitis especially under in vivo conditions.

To improve efficacy of antimicrobials on NAS, heavy doses for extended periods of time are required (Ribeiro et al., 2016) to break through layer of biofilm and or slime. The ability to form biofilm and the strength of the defense also varies with species and genes affecting biofilm production. A study tested 90 NAS isolates found that majority (96.7%) of them revealed at least some ability to form biofilm with only a very few (3.3%) unable to produce any biofilm (Srednik

et al., 2017). Similarly, other studies found that 90% of isolates were biofilm positive with at least 11 different species identified in each study (Piessens et al., 2012, Simojoki et al., 2012, Tremblay et al., 2013, Osman et al., 2015). Biofilm producing NAS isolates can further divided into weak (47.8%), moderate (13.3%), and strong (35.6%) producers (Srednik et al., 2017). The ability to form biofilms appears to be highly dependent on species genetic elements with *S. haemolyticus* and *S. deviesei* forming significantly higher amounts of biofilm compared to *S. chromogenes* (Srednik et al., 2017).

Staphylococcal adhesive surface proteins also known as microbial surface components recognizing adhesive matrix molecules (MSCRAMM) enabling the staphylococci to bind to the host tissue allowing adhesion and further biofilm formation (Goetz et al., 2017, Srednik et al., 2017, Vasileiou et al., 2018, Kumar et al., 2020). MSCRAMMs include staphylococcal cell surface proteins, capsular polysaccharides of cell wall, laminin-binding protein (*eno*), elastin binding protein (*ebpS*), and fibronectin-binding protein (*fnbA*) (Gajewska and Chajęcka-Wierzchowska, 2020). A study found that eighty-four (n=84) NAS isolates obtained from milk samples all produce biofilms and contain at least one MSCRAMM gene (Pizauro et al., 2019). Another study on sixty-seven (n=67) NAS isolates found *eno* (32.1%), *fnbA* (12.0%), and *ebpS* (12.0%) genes among isolates (Zuniga et al., 2015, Pizauro et al., 2019). In addition to the *eno* and *fnbA* gene-positive isolates being present at high frequency, *fib* gene-positive isolates accounted for 71.7% of the samples (Zuniga et al., 2015). It can be concluded that *eno*, *fnbA*, and *fib* genes are of utmost significance for biofilm formation and resistance against host immunity.

Fibronectin is an additional glycoprotein located in the extracellular matrix enabling a bridge for *S. aureus* and NAS pathogens leading to the internalization into the mammary epithelial tissue (Dieser et al., 2019). Laminin binding genes work with fibronectin-binding genes to contribute to successful adhesion to host cells and further bacterial adhesion, causing a signal to be generated to begin biofilm production (Nobbs et al., 2009). This glycoprotein was found in 53% of isolates exhibiting strong biofilm production in vitro and a statistically significant effect on biofilm production (Dieser et al., 2019).

Fib has been identified as a virulence gene and can be distinguished between *S. aureus* and NAS by PCR, specifically in *S. epidermidis* (Sunagar et al., 2013). As another glycoprotein, fibrinogen is found in the blood and can be immobilized by *fib* exhibited by various NAS species. Like clumping factors produced by *S. aureus*, *fib* causes coagulation and may lead to production

of additional substances that will interfere with normal host physiology (Felipe et al., 2017). While it has been concluded that the presence of the *fib* gene correlates with biofilm activity, further research still needed to clearly understood the exact role of the protein in relation to the virulence of NAS (Nilsson et al., 1998, Felipe et al., 2017) especially in the pathogenesis of IMI under in vivo conditions.

Monitoring the genotypic and phenotypic requirements of NAS to form biofilm may improve understanding of pathogenesis of IMI due to NAS and allow development of tools to disrupt or reduce preformed biofilm (Felipe et al., 2017). Due to multiple genes acting concurrently to form biofilm, emphasis on an individual genetic trait responsible for biofilm formation would most likely be ineffective for protection against bovine Staphylococcal and NAS mastitis. Blocking vital biofilm virulence genes induction and production of biofilm in a cascade may provide a breakthrough in preventative technology or therapeutic treatment. Eliminating or reducing Staphylococcal and NAS ability to form biofilm or assisting in the removal of the biofilm may be pivotal technology in the prevention and control of bovine Staphylococcal mastitis (Felipe et al., 2017).

Coagulases - Proteins that convert prothrombin in the blood to staphylothrombin which in turn enables the conversion of fibrinogen to fibrin. Fibrin catalyzes blood clots formation, which inhibit bacterial killing by phagocytic cells (McAdow et al., 2012, Crosby et al., 2016)

1.4.3 Secretory

Exotoxins – A group of enzymatic proteins with properties that induce harm to host cells (Dinges et al., 2000). Type I exotoxins function as signaling proteins in the host cell membranes, type II causes disintegration of host cell membranes, and type III enter host cells and change cellular function (Ala'Aldeen and Wooldridge, 2012). Membrane-impairing toxins (pore-forming toxins) cause damage to host cell membranes. The damage in the membrane interferes with selective ion movement across the membrane, resulting in lysis of the cell (Todar, 2020). *S. aureus* exotoxins catalyze covalent changes (BlumJ and Barbieri, 2019). Most *S. aureus* strains from bovine mastitis produce exotoxins, which are enzymes or hemolysins that convert local host tissue into nutrients including hemolysins, nucleases, proteases, lipases, hyaluronidase, and collagenase

(Dinges et al., 2000). Post internalization into the host cells these exotoxins cause damage to cells (Pollard, 2017). The staphylococcal leukocidins (lukS/lukF or γ -hemolysin, lukD/lukE and lukM/lukF–PV(P83) cause lysis of host leukocytes including macrophages, neutrophils, monocytes, and dendritic cells (Thomsen et al., 2014, Magro et al., 2017, Todar, 2020). These leukocidins enable colonization of mammary glands (Pérez et al., 2020). The kinases cause damage to fibrin and clots in the host to prevent infection from being controlled through phosphorylation. Hyaluronidase causes break down of hyaluronic acid of the connective tissue, which helps infection to spread through the connective tissue (Todar, 2020).

Staphylococcal α and β toxins (α and β hemolysins) are membrane-damaging toxins. Hemolysins and phenol soluble modulins cause damage to cell membranes of the immune cells and induce inflammatory processes (Oliveira et al., 2018). Hemolysins and modulins inhibit movement of leukocytes to the site of infection and thereby decreasing inflammatory processes resulting in death and necrosis of tissue at a higher amount. A study found that 69% of hemolytic isolates carry β toxins, which may indicate its role in the virulence and pathogenesis (Coelho et al., 2011). In addition, the α - and β -toxins promote invasion and exacerbate spread of infection (Coelho et al., 2011). The invasion into intracellular area and spread of infection promote chronic recurrent infection (Pérez et al., 2020).

Enterotoxins - Type I exotoxins, which include SEA, SEB, SEC_n, SED, SEE, SEG, SEH, and SEI) (Ortega et al., 2010) that bind to receptors on the host cell surface and induce a cascade of signaling and responses inside the cell. They are superantigens, that bind to the major histocompatibility molecule II outside the antigen binding groove and to T- cell receptors on CD4+ cells (Magro et al., 2017). They then induce potent polyclonal activation of T-cells and massive release of pro-inflammatory cytokines (Magro et al., 2017) that leads to shock and death.

Enterotoxin genes within NAS species are still being studied and there is a lack of detailed knowledge of their enterotoxigenic potential (Podkowik et al., 2013). To date, about 25 staphylococcal enterotoxin (SE) genes have been identified in various NAS and *S. aureus* isolates. A total of 263 NAS isolates causing IMI were found after evaluating 1000 milk samples from 6 different dairy farms (Park et al., 2011). Further species identification using partial 16S rRNA and/or *rpoB* gene sequence analysis (Park et al., 2011) found that of the 25 known SE genes, 18 were identified in IMI causing NAS species including *S. chromogenes* ($n = 190$), *S. xylosus* ($n = 24$), *S.*

haemolyticus ($n = 16$), *S. sciuri* subsp. *carnaticus* ($n = 9$), *S. hyicus* ($n = 8$), *S. simulans* ($n = 7$), *S. caprae* ($n = 3$), *S. epidermidis* ($n = 2$), *S. succinus* ($n = 2$), *S. capitis* ($n = 1$), and *S. hominis* ($n = 1$). Out of the 263 isolates, 82 (31.2%) displayed one or more SE genes with the staphylococcal enterotoxin q like gene (*selq*) gene being the most prevalent (22.4%) and *S. chromogenes* making up the largest percentage of isolates in the bovine IMI samples (45.8%) (Park et al., 2011, Podkowik et al., 2013).

In comparison to *S. aureus* isolates containing SE genes, NAS from bovine IMI appeared to present with unstable genetic elements responsible for harboring SE genes, continually losing stability with proceeding generations (Ubeda et al., 2007, Park et al., 2011). While most foodborne diseases caused by staphylococci were predominantly caused by *S. aureus*, some proportions were caused by NAS that carry the same SE genes. The first documented case of food poisoning caused by staphylococcal enterotoxins was in 1884 in Michigan (USA) by Vaughan and Sternberg due to consumption of contaminated cheese (Hennekinne et al., 2012). It was believed that the outbreak was due to *S. aureus* as the prospect of food poisoning due to NAS in relation to consumption of dairy products is of negligible risk. The low risk is surprising with the extremely high prevalence of NAS on dairy farms and their pathogenic potential due to presence of SE genes. However; the fermentation of foods seems to increase pathogenicity in only a few NAS (particularly in *S. xylosus*, *S. carnosus* and *S. equorum*) presenting increased medical risk for humans (Blaiotta et al., 2004).

It can be concluded that while NAS are most prevalent in human and animal environments, as well as in the food products including dairy, there is a low chance of NAS causing food poisoning. However, due to the high prevalence of NAS, they can be regarded as opportunistic pathogens in both animals and humans especially those with suppressed immune function. To date, no NAS have been isolated as the cause of food poisoning from milk or dairy products or human pathology cases due to dairy product ingestion.

Toxic shock syndrome toxin- is caused by toxic shock syndrome toxin- 1 (TSST-1), which was first identified in *S. aureus* as enterotoxin B. The genes responsible for TSST-1 were first found in NAS isolates from toxic shock syndrome patients (Crass and Bergdoll, 1986). Proinflammatory cytokines such as tumor necrosis factor alpha (TNF- α), interleukin-2 (IL-2), and interferon- γ (IFN- γ) present in high concentrations contribute to the development of the toxic shock syndrome disease characterized by capillary leakage, high fever, and eventual multi-

organ dysfunction. The toxin crosses epithelial cell barriers into the bloodstream and circulated through the body (Kuroishi et al., 2003). The TSST-1 is rare and in a recent study only one strain of *S. haemolyticus* was positive (Martins et al., 2017).

The roles of different secretory and non-secretory virulence factors of staphylococci in the pathogenesis of Staphylococcal mastitis in dairy cows under in vivo condition still requires detailed investigation since most of Staphylococcal isolates from cases of bovine mastitis are known to test positive for these virulence factors. However, their exact roles or contribution to mastitis are not well defined.

2. Mammary Gland Defenses

2.1 Natural defense

Physical barriers - The teat canal is closed by the smooth muscle sphincter, also called Rosette of Frustenburg (Paulrud, 2005, Ezzat Alnakip et al., 2014), and keratin plug which is a waxy substance produced by stratified squamous epithelium of the teat duct that traps invading bacteria and limits their entry into intramammary area (Paulrud, 2005). Keratin contains bacteriostatic esterified and non-esterified fatty acids, including lauric, palmitoleic, myristic, and linoleic acids (Zigo et al., 2021), and fibrous structural proteins (scleroproteins) produced by stratified epithelial cells lining the teat canal (Bragulla and Homberger, 2009, Smolenski et al., 2015, Wang et al., 2016). Fibrous proteins bind electrostatically to microorganisms and cause changes in the cell wall that make them susceptible to osmotic pressure leading to death (Paulrud, 2005). Fibrous proteins also have an inhibitory effect against *Staphylococcus aureus* and *Streptococcus agalactiae* (Hibbitt and Benians, 1971), similar to proteins isolated from bovine neutrophils (Gennaro et al., 1983). The lipid content and composition of teat duct keratin vary throughout the milking process (Bitman et al., 1991) between lactating and dry cows (Bitman et al., 1988). Oxytocin, the hormone for milk letdown, is produced by the hypothalamus and transferred to the posterior pituitary gland, where it is secreted into blood circulation upon stimulation by pre-milking teat dipping in antiseptic solution and wiping for milking or by calf suckling. The oxytocin induces contraction of myoepithelial cells that line milk-producing epithelial cells forcing milk flow through the lactiferous duct, gland cistern, teat cistern, and finally through the teat canal and teat opening, breaking the keratin plug. During milking, keratin is

flushed out with distention of the teat canal (Rainard and Riollot, 2006). Damage of keratin by improper intramammary infusion (Nickerson, 1987) or faulty machine milking (Capuco et al., 1994) increases susceptibility to bacterial invasion and colonization (Bramley and Dodd, 1984). The teat canal stays open post milking for approximately 2 h, making it susceptible to the entry of pathogenic bacteria from outside into the intramammary area (Capuco et al., 1992, Sharma, 2010, Trevisi and Minuti, 2018).

Despite these defenses, *S. aureus* can enter intramammary areas and cause infection during dry periods as well as during lactation as confirmed by a controlled experimental studies (Merrill et al., 2019, Kerro Dego et al., 2020), with continued survival of *S. aureus* deposited a few mm inside the teat canal for several days (Newbould and Neave, 1965, Craven and Williams, 1985, Jones, 2019). The microorganisms at the teat end can also be propelled into the teat cistern during mechanical milking, which is considered the major mechanism for the spread of contagious mastitis pathogens (Griffin et al., 1983).

2.2 Immunity

Infection of mammary glands by pathogenic microorganisms, such as bacteria induces immune responses (Van Saun, 2016, Ruegg, 2021). There are two types of immunity, innate and adaptive (Thompson-Crispi and Mallard, 2012). Both are critically important for immune-based mammary glands' protection against invading pathogenic microorganisms.

2.2.1 Innate immunity

Once the physical barriers have been crossed, the innate immune response, or second line of defense, will be induced. The teat canal tissue has been reported to express toll-like receptors (TLRs) and produce cytokines and antimicrobial peptides (Rinaldi et al., 2010, Whelehan et al., 2011). The most common innate immune responses are 1) cellular innate immunity (Leukocytes: neutrophils, macrophages, lymphocytes, mammary epithelial cells) and 2) humoral innate immunity (Lactoferrin, transferrin, Lysozyme, Lactoperoxidase and myeloperoxidase, complement systems, cytokines, chemokines, host defense peptides) (Rich et al., 2012, Alnakip et al., 2014).

I. Cellular innate immunity

Neutrophils- The most abundant leukocytes that increases in numbers by up to 80% when an infection is present. Neutrophils move into the site of inflammation following proinflammatory signaling molecules (chemoattractants), such as C5a, C3a, and IL-8, from the site of infection as part of the inflammatory response (Nussenblatt, 2010, Margraf et al., 2022). Staphylococcal lipoteichoic acid (LTA) can trigger the production of chemoattractants which attract phagocytic cells (PMNs) and monocytes to the site of infection through inflammatory responses (Fournier and Philpott, 2005). The neutrophils are produced in the bone marrow and move to the site of infection following chemical gradient. At a high concentration of chemoattractants, neutrophils slow down their mobility through blood by binding to their ligand on endothelial surfaces and exit to the site of infection by squeezing themselves (diapedesis) through areas between endothelial cells (Nussenblatt, 2010).

Lymphocytes are also produced in the bone marrow and migrate to thymus and became mature naïve T cells and enter the blood circulation and exit from blood into regional lymph nodes at high endothelial venules. The naïve T cells will interact with antigen presented by antigen presenting cells in the medulla region of lymph node. After binding to the antigen, T cells become activated and produce cytokines that induce B-cells to produce antibodies to promote phagocytosis. If staphylococci are not controlled by phagocytic cells (neutrophils or macrophages) and survive in the intracellular area in the phagocytic cells, the natural killer cells (NK) kill phagocytic cells and release intracellular bacteria for another chance of killing by phagocytic cells (Bröker et al., 2016). Some *S. aureus* strains can survive killings by neutrophils (Murphy et al., 2019). If the innate immune system failed to control *S. aureus* the adaptive immune response with the help of antibodies specifically produced for infecting *S. aureus* will clear the infection by opsonophagocytic killing of phagocytic cells. Murphy et al. (2019) evaluated the amount of IL-6 and IL-8 and found that IL-8 is the most important chemokine for neutrophils based quick response (Murphy et al., 2019). The *S. aureus* strain CC97 induced significantly higher IL-6 production compared to other strains (e.g., strain CC151) (Murphy et al., 2019). A quick and effective neutrophil response is required to control staphylococcal IMI from progressing into mastitis.

II. Humoral innate immunity

Lactoferrin- Also seen in the adaptive immune response, lactoferrin exhibits another feature in fighting against infectious agents. As an innate immune mechanism, lactoferrin deprives the inflamed area off the iron, causing oxidative stress, inhibiting bacterial growth and helping host cells survive (Actor et al., 2009).

The complement system- a proteolytic cascade consisting of 30 proteins in plasma and on cell surfaces that produce proinflammatory mediators, opsonins, and membrane attack complexes (Dunkelberger and Song, 2010). The complement system has three pathways for activation and removal of the pathogen, 1) the classical, 2) lectin, and 3) alternative pathways (Tizard, 2018). The C3a and C5a are anaphylatoxins responsible for releasing histamine, vasodilation, inflammation, and eliminating or removing pathogens (Tizard, 2018). The fragments then activate those phagocytes for immediate elimination of the targeted pathogen. The membrane attack complex of the complement system destroys the invading bacteria's cell membrane. The membrane attack complex of the proteins breaks holes, or pores, into the lining causing irreparable damage (Janeway Jr et al., 2001).

Antimicrobial peptides (AMPs) - Small peptides between 10 and 60 amino acids that widely exist in nature in animals (mammals, amphibians, insect, aquatic), plants and microorganisms that have a wide range of antimicrobial effects against bacteria, fungi, parasites and viruses (Malkoski et al., 2001, Schitteck et al., 2001, Brogden et al., 2003, Ganz, 2003, Brogden, 2005, Lai et al., 2007, Huan et al., 2020). Almost all AMPs are cationic, but some anionic AMPs also exist (Smolenski et al., 2015, Huan et al., 2020).

AMPs exist in several tissues including polymorphonuclear leukocytes, macrophages, and mucosal epithelial cells. Major AMPs known to exist in cattle include defensins, cathelicidins, and anionic peptides (Brogden et al., 1997). There are several cationic AMPs and a few groups of anionic AMPs in domestic animals (Brogden et al., 2003). Other mammalian AMPs include histatins (Tsai and Bobek, 1998) and dermcidin (Schitteck et al., 2001). AMPs have different mechanisms of killing microbes. For example, defensins induce ion channel formation (Kagan et

al., 1990), anionic peptides cause flocculation of intracellular contents (Brogden et al., 1996), and bactericins affect transport and energy metabolism (Skerlavaj et al., 1990).

β -defensins are AMPs largely produced by polymorphonuclear cells (Schroder, 1999, Zasloff, 2002, Goldammer et al., 2004). The stimulation of mammary epithelial cells with lipopolysaccharide (LPS) and lipoteichoic acid (LTA) triggers the production of β -defensins (Strandberg et al., 2005). In humans, β -defensins form the main class of AMPs that exist in epithelial cells (Ganz, 2003). There are three types of β -defensins in human skin which include hBD-1, hBD-2, and hBD-3 (Fulton et al., 1997, Harder et al., 1997, Harder et al., 2001). Interleukin-1 triggers the production of β -defensin-2 during inflammatory process of skin (Liu et al., 2003). The hBD-1 is produced constitutively (Zhao et al., 1996) and has been shown to increase in production during the inflammatory process (Hiratsuka, 2003). The hBD-3 is shown to increase during inflammatory processes of skin and epithelia (Harder et al., 2001, Wehkamp et al., 2003). The microbial products are believed to induce activation of lymphocytes and monocytes, which induce epidermal production of all three defensins (Sørensen et al., 2005). The hBD-3 induction is through transactivation of epidermal growth factor (Sørensen et al., 2005).

Type 3 Immunity- There are innate and adaptive type 3 immune systems that have recently been shown to function at epithelial and mucosal surfaces (Eberl et al., 2015). Lymphoid cells (ILCs) that do not express a TCR and innate antigen-specific CD4+(T17) and CD8+T (Tc17) or $\gamma\delta$ T cells have been identified (Rainard et al., 2020). CD8 and CD4 lymphocytes with memory phenotypes were found in the milk of infected, healthy mammary glands (Taylor et al., 1997, Riollot et al., 2000). The CD4 T cells producing IL-17A or expressing ROR γ t have been found in mouse mammary glands, but ROR γ t+CD8+T cells have failed to be reported (Porcherie et al., 2016, Betts et al., 2018). Antigen presentation by APCs is required for these lymphocytes with MHC class I or class II molecules. Within the innate immune system, the T17 cells and $\gamma\delta$ T cells express receptors, such as toll-like receptors TLR1, TLR2, TLR3, TLR4 and dectin 1, which allow them to respond to mammary associated molecular patterns (MAMPs) (Bonneville et al., 2010, Baldwin and Telfer, 2015). They can also secrete IL-22 and IL17A without the activation of the TCR in the presence of IL-23 and IL-1 β . In the bovine species, WC1+ $\gamma\delta$ T cells, CD4+(T17), and CD8+T cells have been shown to produce IL-17A (Peckham et al., 2015, Steinbach et al., 2016, Wattedgera et al., 2017, Elnaggar et al., 2018). At periphery tissues and within organs, most

bovine $\gamma\delta$ T cells express the WC1-phenotype and function differently from the WC1+phenotype (Baldwin and Telfer, 2015). Cells, such as $\gamma\delta$ T, are recruited in milk during infection with selective recruitment of subsets being indicated by a few studies (Soltys and Quinn, 1999, Riollot et al., 2001). Other type 3 immune cells belong to the innate arm of immunity and do not possess TCR, including the ILCs that come in three types, ILC1, ILC2, and ILC3 as the most recently described (Eberl et al., 2015). The ILCs are the innate versions of the T1, T2, and T17 adaptive T cells. ILC3 cells reside in the mucosal epithelia and parenchymal tissues where they function as regulation of inflammation, resistance to pathogens infection and tissue homeostasis (Klose and Artis, 2016). Bovine ILCs have not been described yet because they are difficult to isolate from their niche environment. Human ILCs do, or cannot, react like mice ILCs therefore it is unknown if they can respond to MAMPs. ILC3 produce effector cytokines after responding to IL-23 and IL-1 α or IL-1 β (IL17A, IL-17F, IL-22) (Klose and Artis, 2016).

2.2.2 Adaptive immunity

The second branch of immunity is adaptive or acquired immunity. The adaptive (acquired) immune system comprises 1) humoral (antibody-mediated) and 2) cellular (cell-mediated) immunity. The innate immune response create the basis for the induction of adaptive immune response through phagocytosis, processing, and presenting of antigens from infecting staphylococci to the immune system (Wilson et al., 2018). Due to this process of antigen processing and presentation by antigen presenting cells to the immune system the adaptive immunity takes 5 - 7 days to respond to infecting staphylococci. The adaptive immune response requires antigen processing and presentation by antigen presenting cells such as macrophages, B-lymphocytes, and dendritic cells that transport antigens to their cell surface while attached to the major histocompatibility molecule II (MHC-II). The naïve T cells that are circulating in the blood exit from blood to regional lymph nodes at high endothelial venules, bind to foreign antigens attached to MHC-II by its T cell receptor (TCR), and become activated T helper cells of Th1, Th2, Th17, Tfh, Treg, or Cytotoxic T cells. The helper T cells activate B-cells to produce antibodies or activate other T-cells to become cytotoxic effector cells depending on type and location of antigen in the body (Shishido et al., 2012).

Once the staphylococcal bacterium enters the body, it replicates and starts releasing antigens, such as the staphylococcal superantigen analogous to SSL5 and SSL10. First, the

neutrophils, specifically, are recruited by the innate immune system (Thomer et al., 2016). Although these staphylococcal superantigens can work as a protective measure for the *S. aureus* bacteria, they also will serve as a source of alarm for B and T cells. To protect the body from future infections by the same pathogen, the adaptive immune system generates a percentage of its helper T-cells to serve as memory T cells (Krzywińska et al., 2012). For intracellular pathogen the helper T cells activate CD8⁺ T cells to become cytotoxic T cells that kills infected cells. For extracellular pathogens the T helper cells activate B-cells to produce pathogen specific antibodies that bind to the pathogen and subject it to removal by the opsonophagocytic mechanism (Alberts et al., 2002) or block bacterial binding to host tissue surface receptors (Thomer et al., 2016).

The adaptive immune system mainly produces effectors (antibodies or activated cytotoxic T cells) that remove pathogens and memory cells, which will retain the information about a specific pathogen. If new antigen is injected into a cow, for example, the antigen will be taken up by APCs and broken into small peptides and loaded onto MHC-II molecules then transported to the cell surface where they are displayed to naïve T cells in the regional lymph nodes. The naïve T- cells exit from the thymus into blood circulation to the lymph node at high endothelial venules. In the lymph node naïve T- cells binds to peptides presented by MHC-II molecules on APC through its T-cell receptor (TCR). This binding induces primary stimulation. Subsequently, the CD28 molecule on the surface of T- cells binds to CD80 or CD86 on the surface of APC which induces co-stimulation. The primary stimulation is through TCR binding to peptide-MHC-II molecule followed by a co-stimulatory signal from CD28 on the surface of T-cells binding to a CD80 or CD86 molecule on the surface of APC activated T-cells to become activated and differentiated into active helper cells. The activated cells then produce IL-2 that induce proliferation of antigen specific repertoire of T-cells. The activated T-helper cells on the Th2 pathway induce activation of B-cells to become plasma cells that produce antibodies specific to the antigen injected into the cow. If the injected antigen is from a mastitis causing pathogenic bacteria, the induced immune response will attack future exposure to the same pathogen, thus indicating the importance of vaccines in controlling infectious pathogens (Thompson-Crispi and Mallard, 2012).

3. Pathogenesis of Staphylococcal IMI

Almost all mastitis causing pathogens get into intramammary area through teat opening, except *Mycoplasma bovis* mastitis which can spread to mammary gland systemically from the

primary site of infection. The mammary pathogenic bacteria are prevented from entering udder by physical barriers, such as the teat sphincter muscle and keratin plug. The antibacterial fatty acids in the keratin such as myristic, lauric, palmitoleic, and linoleic acids also serve as natural defense against mammary pathogenic bacteria. Fibrous proteins that make up the keratin also specifically bind to the cell wall of bacteria and subject them to damage. Damage to keratin by incorrect intramammary infusion or by faulty milking machines increases the potential for teat canal colonization by bacteria (Dinges et al., 2000, Coelho et al., 2011, BlumJ and Barbieri, 2019). *S. aureus* enters the mammary gland either through progressive colonization from teat opening area or during fluctuation of milking machine vacuum and then attaches and internalizes or invades gland tissues to resist washing out of the gland by milk during milking (Côté-Gravel and Malouin, 2019). Staphylococci have several virulence factors, such as adhesive cell surface proteins, the ability to form biofilm or small colony variants (SCV) or L-forms, and several other virulence factors that help the bacteria to evade host immunological defense and treatment with antibiotics (Côté-Gravel and Malouin, 2019). *Staphylococcus aureus* binds to α -5B1 integrin on the host through fibronectin-binding proteins (FnBPs) (Josse et al., 2017). The FnBP production is vital for adhesion, but its production may vary by Staphylococcal strain. (Josse et al., 2017). This initial binding leads to actin polymerization, cytoskeleton formation, and entry of bacterium into the host cell (Josse et al., 2017). The NAS can also utilize its cell surface adhesive proteins (e.g., laminin-binding proteins) to attach to host cells (Gomes et al., 2016).

Attachment of the *S. aureus* and NAS to host cells leads to the secretion of different virulence factors (Côté-Gravel and Malouin, 2019), which triggers an inflammatory response that induces production of proinflammatory chemokines and cytokines, inducing production and chemotaxis of phagocytic cells to the site of infection which clear bacteria by phagocytic killing. However, Staphylococci have several virulence factors and mechanisms that help them to evade host immune defenses. The extracellular fibrinogen-binding proteins (Efb) can mask the surface of staphylococci to avoid the binding of antibodies, and the leukotoxin subunit (LukM) can eliminates the leukocytes by interacting with its target receptor on the surface of the neutrophils (Boerhout et al., 2015). Staphylococci can form a biofilm that prevents killing by phagocytic cells (Otto, 2012). Infection during early lactation may result in the peracute form of mastitis with gangrene formation in the udder. Infection during the mid and late stages of lactation or dry period usually result in chronic or acute form of disease. Pathogenesis of acute and chronic *S. aureus*

mastitis is the same except the level of involvement of mammary tissue, but each start with acute forms characterized by the multiplication of *S. aureus* in the duct. Acute mastitis is characterized by severe inflammatory processes and blockage of ducts and regions of the gland, whereas chronic mastitis is characterized by less severe inflammation and a mild inflammatory reaction in the epithelial lining of the duct. Chronic *S. aureus* mastitis is characterized by cyclical shedding of bacteria and paralleling cyclical rise and fall in the number of polymorphonuclear cells in the milk and their capacity to phagocytose bacteria.

4. Clinical Manifestation of Staphylococcal Mastitis

Mastitis causes physical, chemical, and microbial changes in the milk due to pathological alterations in the mammary gland tissue (Hussein et al., 2018). Staphylococcal IMI follows the same pattern of infection, and the consequential inflammatory signs are displayed locally in milk and udder tissue or systemically in the infected animal (Markey, 2013). The majority of *S. aureus* IMI are subclinical (Diseases of the Mammary Gland, 2017). Acute and peracute *S. aureus* mastitis are characterized by sudden swelling of the gland with fever, purulent inflammation with thick milk clots, and pus formation. Peracute *S. aureus* mastitis usually occurs suddenly in the first few days after calving potentially developing into gangrene in the gland and on the teat and is highly fatal. Infected animals develop systemic clinical signs, such as increased body temperature, pulse and respiratory rates, anorexia, toxemia, muscle weakness, and ruminal stasis. Chronic *S. aureus* mastitis characterized by high SCC, gradual inflammatory process, necrosis, fibrous tissue growth, atrophy of the udder, decrease in milk production, occasional clots in milk, and milk wateriness. Clinical staphylococcal mastitis manifests as redness, heat, pain, swelling, and altered or reduced milk production of the mammary glands (Merrill et al., 2019).

There are multitudes of host, pathogen, and environmental/managerial risk factors for mastitis. The host factors include parity, days in milk, local defense mechanisms, colonization with minor pathogens, presence of other diseases, heredity, and immune system. The pathogen risk factors include virulence factors (Anderson et al., 2006, Melchior et al., 2006b, Oliveira et al., 2006, Oliveira et al., 2007, Atalla et al., 2008, Fournier et al., 2008, Graber et al., 2009, Capurro et al., 2010, Oliveira et al., 2011, Piccinini et al., 2012, Lundberg et al., 2014, Snel et al., 2014), genotype of the strain (Anderson et al., 2006, Graber et al., 2009, Capurro et al., 2010), number, frequency of exposure, adhesion and invasion ability, and antimicrobial resistance. The

environmental and management factors include post-milking teat dipping, clean and dry bedding, thorough disinfection of teat orifice before administration of intramammary infusion, milking clinical cases last, proper maintenance of milking machines, avoiding udder trauma, and climate. Parity is a large determining factor, especially with a cow on her third lactation or greater, significantly increasing the prospect of developing clinical mastitis (Elghafghuf et al., 2014). The increase in the number of lactations runs parallel with the increased probability of exposure to mastitis causative agents and, separately, the exacerbation of previous cases (Elghafghuf et al., 2014). Cows have a significantly higher likelihood of developing clinical mastitis within the first 30 days in milk (DIM), with >50% of mastitis infection cases occurring during this period as opposed to the remaining days of lactation (Green et al., 2002). However, 80% of the clinical mastitis cases occurring > 30 DIM were due to new infections instead of reintroducing previous cases (Green et al., 2002). Bacterial species have a large influence on many facets of clinical mastitis, most importantly, infection duration, severity, treatment outcomes, and milk yield. Over 50% of recurring cases of clinical mastitis are caused by the same pathogen responsible for previous cases in the same animal (Bradley and Green, 2001).

Local clinical mastitis may become systemic acute or peracute mastitis characterized by increased vital signs (temperature, pulse, and respiratory rates), anorexia, and fluid loss (dehydration) (Martins et al., 2019). Subclinical mastitis does not manifest abnormal changes in the milk and/or udder but decreases milk quality and quantity. The incidence of subclinical mastitis is 15–40 times higher than clinical mastitis (Ashraf and Imran, 2018). *S. aureus* mastitis is usually subclinical and chronic with limited cure rates with antibiotics treatment. The recommended management is to cull the infected cows before the infection spreads through the whole herd (Rocha et al., 2019).

5. Diagnosis of Staphylococcal Mastitis

Clinical signs- Clinical mastitis can be detected based on the clinical symptoms expressed in either the cow's milk or udder (Kandeel et al., 2019). Clinical mastitis causes inflammation and subsequent damage to the blood-milk barrier in the mammary epithelial lining and broken tight junctions resulting in the leakage of blood, cells, and other extracellular fluid and components (Kandeel et al., 2019). The increased recruitment of white blood cells to the infected gland to fight

off infection and dead bacteria, cells, and tissue during the fight form purulent inflammatory fluid or pus are often seen when stripping out the foremilk prior to milking. The swelling is due to influx of fluid and white blood cells, redness/hyperemia is due to increased flow of blood to the gland, increased heat is due to an increased flow of blood to the infected area, pain is due to pressure caused by swelling on local nerve fibers, and decreased or loss of milk production is due to death of milk-producing cells which constitute cardinal visible clinical signs of mastitis (Kandeel et al., 2019).

Bacteriological culture- The best method for diagnosis of *S. aureus* IMI, but because of cyclical shedding of bacteria more than one sampling may be required to increase the sensitivity of culture result. The sensitivity of composite milk culture can be as low as 53%, but individual quarter milk cultures have higher sensitivity (Mahmmod et al., 2013). The sensitivity of bacteriological culture is affected by type, volume, and time between repeated samplings. Quarter milk sampling at 1 and 3- or 4-days interval with 0.1 mL volume culturing separately are expected to have sensitivities of 90% to 95% and 94% to 99%, respectively. Daily quarter milk sampling and culturing separately provides a sensitivity of 97% and a specificity of 97% to 100%. Different On-Farm Culture Systems are available for dairy farmers. The Minnesota Easy Culture System (Laboratory for Udder Health, University of Minnesota, College of Veterinary Medicine, Veterinary Diagnostic Laboratory, Saint Paul, MN), is one of the On-Farm Culture Systems developed to eliminate the need to transport samples from a farm to a laboratory, which might be located far away from the farm. The On-Farm Culture System provides positive culture results in a short time, which makes this system a quick, simple, and inexpensive method to diagnose the bacterial cause of clinical mastitis to help guide clinical mastitis treatment decisions. The Minnesota Easy Culture System uses the Tri-plate system consisting of three types of media. These include MacConkey media (selects for Gram-negative organisms, mainly coliforms: *E. coli*, *Klebsiella* spp. and *Enterobacter* spp., but inhibit Gram-positive bacteria), Factor media (selects for Gram-positive bacteria such as *Staphylococcus*, *Streptococcus*, *Bacillus* and *Corynebacterium*, but inhibit Gram-negative bacteria), and Modified TKT media (selects for *Streptococcus*-like bacteria, such as *Enterococcus* spp. and *Aerococcus* spp.) (Royster et al., 2014).

In samples from clinical mastitis cases, *S. aureus* appears in high concentrations (10^4 – 10^5 CFU /mL), however, only one colony is required for a positive result due to its classification as a

major pathogen (Juronen et al., 2018). However, *S. aureus* and non-aureus staphylococci can be isolated from udder quarters of dairy cows without increase in somatic cells count (Petersson-Wolfe et al., 2010, Rall et al., 2014). NAS colony forming unit (CFU/mL) counts from subclinical and clinical mastitis cases ranged from 10^3 – 10^4 and 10^5 – 10^6 , respectively (Wald et al., 2019). Overall, a sample containing at least 10 CFU/mL of milk with SCC > 100,000 cells/mL is considered subclinical mastitis. Culturing of composite milk instead of individual quarter milk increases the number of false-negative results but culturing 0.05 mL volume than that recommended by NMC increase sensitivity. Freezing of milk samples either has no effect on *S. aureus* count or increase count by approximately 200% due to release of intracellular bacteria when cells lysed. Positive coagulase and catalase test are used to differentiate *Staphylococci* from other gram-positive cocci, especially Streptococci. *S. aureus* causes an outer zone of incomplete hemolysis on blood agar with an inner zone of complete hemolysis resulting in double hemolysis (Graber et al., 2013), which is positive diagnosis for *S. aureus*. If no double hemolysis on blood agar occurs, a tube coagulase test must be conducted with *S. aureus* being positive with 100% specificity. Differentiation of coagulase positive *Staphylococcus* species is best done by inoculation of colony from blood agar with complete hemolysis after 24 h culturing into *S. aureus* CHROMagar plates which gives mauve to rose colonies (Graber et al., 2013) or by MALDI-TOF.

MALDI-TOF MS- is a good quick diagnostic method that can differentiate *S. aureus* from the other 7 coagulase positive *Staphylococcus* spp. (Rodrigues et al., 2017). Few tests offer high precision when identifying specific mastitis causing pathogens making the Matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS) a highly evolved, upcoming technology in the agriculture industry. The MALDI-TOF MS assay promotes the bio-typing of pathogens isolated from samples, producing swift, accurate microorganism identification, and has begun replacing techniques that currently dominate the industry. In a study testing 152 strains of staphylococcal species, 99.3% were correctly identified, leading to a highly accurate tool for identifying staphylococcal strains (Dubois et al., 2010). An additional study found that the MALDI-TOF MS attained 100% specificity and sensitivity when characterizing staphylococcal species isolates, both coagulase-positive and negative strains (da Motta et al., 2014). While not as accurate as elongation factor (*tuf*) gene sequencing (Li et al., 2012), the newer assay is the preferred detection method due to the lower cost in comparison without sacrificing the

high accuracy (Dubois et al., 2010). The fault with MALDI-TOF MS is the lack of non-clinical isolates in the comprehensive database for comparison in cases such as NAS mastitis (Rodrigues et al., 2017). With further research and usage of this test, the catalog of organisms will naturally expand, allowing for a constantly increasing opportunity to identify microbial ecologies from isolated mastitis samples. Other methods such as the Pathoproof mastitis PCR assay is also good method with a sensitivity of 91% and a specificity of 99% on cow level composite milk samples (Mahmmod et al., 2013). Numerous immunological tests have been developed, but still because of specificity, sensitivity, and cost there is no preferred method for diagnosis (Fabres-Klein et al., 2014).

Somatic cell count (SCC)- The SCC threshold of $\geq 200,000$ cells/mL of milk in either a quarter or composite milk samples indicates an inflammatory reaction (Middleton et al., 2014, Busanello et al., 2017). The International Dairy Federation deems a $SCC > 200,000$ cells/mL as a case of subclinical mastitis aside from any determination of presence of microorganisms. In addition to monitoring SCC at bulk tank milk level to achieve premium prices, individual cow SCCs are used to identify and subsequently treat or segregate specific animals to continue to ensure high milk quality and low transmission of pathogens during milking. Current technology for indirect detection of mastitis cases based on SCC increase in milk includes the Sodium Lauryl Sulphate Test (SLST), California Mastitis Test (CMT), White Side Test (WST), electric conductivity (EC), pH Multistix strips, detection of enzymes, Tanuchek kits, DeLaval Cell Counter (DCC), flow cytometry, and Surf Field Mastitis Test (SFMT).

The SLST and CMT are extremely similar and use the same reagent (detergent): 3% sodium lauryl sulfate, which disrupts the cell membrane of somatic cells and releases the DNA then forming a gelatinous product (Sharma et al., 2010, Chen and Liou, 2018). The test results are scored on scale of 0 - 5, with a score of 0 or trace being considered negative for subclinical mastitis and score of 1, 2 or 3 is considered positive for subclinical mastitis (Mishra et al., 2018). The CMT results of 1, 2 or 3 to diagnose *S. aureus* IMI provide a range of sensitivities from 0.47 to 0.96 and specificities of 0.41 to 0.80 (Mahmmod et al., 2013). While both assays are accessible in the parlor, the subjective element of assigning an appropriate score proves to be insufficient and inaccurate to standardize for different farmers. Similarly, the WST requires a small volume of milk to be mixed with a 4% Sodium Hydroxide (NaOH) reagent that damages white blood cells

releasing DNA that form a viscous gel (Hussain et al., 2017). Heat can affect results by damaging the physiology of the leukocytes, resulting in the lack of normal interaction with the NaOH. Due to negative effects of heat, tests on post pasteurization samples would not be accurate (Dunn et al., 1943). The WST is another test applicable in the field on dairy farms. However, a similar scoring system to that of CMT and SLST is used on a scale of no reaction to strong positive reaction based on coagulation, making the accuracy also subjective (Islam et al., 2020). Another very similar human biased test, the Surf Field Mastitis Test, is another assay that utilizes a detergent to detect enhanced levels of leukocytes, however the substrate used is much more economically achievable and maintains similar sensitivity as WST and CMT (Muhammad et al., 2010).

The Tanuchek SCC Kit developed by the Translational platform for veterinary biologicals (TRPVB), for example, is a diagnostic test with the ability to detect high numbers of SCC via color-changing technology with darker blue hues indicating more severe subclinical mastitis (Revathi et al., 2020). The rapid, portable DeLaval cell counter is an expensive technology that computes SCC with high accuracy, but due to the high cost of the machine and cartridges required for each sample, there are few facilities in which it is economically feasible for on-farm use (Kandeel et al., 2017). Increase of acute phase proteins, such as haptoglobin (Hg) and mammary associated serum amyloid A (MSAA), in milk and serum of cows with *S. aureus* mastitis can also be used for diagnosis of mastitis (Eckersall et al., 2006)

Flow cytometry is an approach for identifying populations of cells within a sample by determining chemical and physical markers. The levels of milk leukocytes are increased in cases of subclinical mastitis and employing flow cytometry will allow for the classification of subpopulations (Souza et al., 2020). High levels of T lymphocytes (CD4+, CD8+) and polymorphonuclear leukocytes (PMN) are present in quarters experiencing subclinical IMI, in contrast to a higher level of monocytes and macrophages in healthy noninflamed quarters (Souza et al., 2020). Specifically, numbers of CD4+ T lymphocytes appear to be inflated in nonspecific subclinical cases of mastitis indicating the importance of this cell type in healthy mammary tissue (Souza et al., 2020). Staphylococcal infected quarters express an elevated number of CD4+ T cells and CD8+ T lymphocytes, possibly allowing for future development of assays quickly and accurately identifying specific cell types in samples collected and processed in the field (Souza et al., 2020).

6. Prevention and Control of Staphylococcal Mastitis

6.1 Management

A 5-point mastitis control measure was initially developed by the National Institute for Research into Dairying in England (Neave et al., 1969) and later adopted by NMC as a 5-point mastitis management program known as the “5-Point Plan” for mitigating contagious mastitis (Hillerton et al., 1995, Blowey, 2010, Ruegg, 2017). The “5-Point Plan” includes 1) effective post-milking teat dipping, 2) use of antibiotic dry cow therapy in every quarter at the end of each lactation, 3) appropriate treatment of clinical cases, 4) culling of chronically infected cows, and 5) maintenance of milk equipment to ensure stable teat end vacuum (Ruegg, 2017). After implementing this plan as a basis for management in the dairy industry, infection rates have decreased by up to 50%, allowing cow to cow transfer to also decrease gradually (Ruegg, 2017). The NMC improved the 5-point plan to 10-point plan which includes 1) Establishment of goals for udder health, 2) maintenance of a clean, dry, comfortable environment, 3) proper milking procedures, 4) proper maintenance and use of milking equipment, 5) good record keeping, 6) appropriate management of clinical mastitis during lactation 7) effective dry cow management, 8) maintenance of biosecurity for contagious pathogens and marketing of chronically infected cows, 9) regular monitoring of udder health status and 10) periodic review of the mastitis control program (Middleton et al., 2014). Despite the adoption of these control measures mastitis caused by *S. aureus* continues to be the most common disease of dairy cattle throughout the world. For the prevention and control of *S. aureus* mastitis, nearly the same techniques are used in the parlor and for herd management. Failure to control *S. aureus* is mainly due to failure to implement the 10-point plan (Barkema et al., 1998).

The cure rate of staphylococcal mastitis with antibiotics is low and ineffective and the current effort must focus on preventing transmission from infected to non-infected cows. *S. aureus* is highly contagious so hygiene in the milking parlor, early identification, culling, and segregation are of paramount importance. Current *S. aureus* control program includes ensuring optimal teat condition, hygienic washing and drying of udders before milking, wearing disposable gloves during milking, regular milking-machine maintenance, teat dipping after milking, dry cow therapy on all cows, culling cows with chronic mastitis, milking infected cows last. Teat dipping in antiseptic solution before and after milking reduce new IMI by 50% to 65% than control cows

without dipping teats and the antiseptic carrier glycerin is helpful for teat-orifice condition (Azizoglu et al., 2013).

Parlor and milker hygiene are also vital to prevent or at least mitigate NAS mastitis. The *eno* and *bap* positive NAS have been found in liners and milkers' hands (Otto et al., 2013), as well as in the mouths and nostrils of calves and other areas of the farm (Otto et al., 2013). The presence of the *bap* virulence gene is extremely important in the environments where cross-contamination can occur between different animal groups due to poor hygiene in milking practices (Otto et al., 2013, Felipe et al., 2017). Some studies from poor hygienic milking systems indicated that the number of NAS were higher on milkers' nasal swabs (11.8%) than in milk (9.1%) which suggested milk contamination during milking process (Gizaw et al., 2020). The authors also reported NAS from udders, buckets, the milk tank, and milkers' nasal swab, and hands indicating ubiquitous reservoirs on dairy farms (Gizaw et al., 2020). The overall prevalence of bovine mastitis causing NAS is approximately between 11.3% and 78% (Rajala-Schultz et al., 2004, Piepers et al., 2007, Gillespie et al., 2009, Pyorala and Taponen, 2009, Schukken et al., 2009, Supre et al., 2011).

A large component of hygiene on dairy farms is the type of bedding used and its impact on milk quality and overall health of the animals. Bedding types are associated with cleanliness of specific body parts of cows and hygiene is based on the management practices of the farm. A study found that out of different bedding types tested sand was the driest with lower bacterial counts (Robles et al., 2020). There was correlation between wider stalls and deeper bedding management systems with significantly lower bulk milk bacterial counts and lower mean SCC (Robles et al., 2020).

6.2 Use of Antibiotics

6.2.1 Therapeutic

The prudent antimicrobial use in the dairy farms is strongly recommended to slow down the exponential increase of antimicrobial resistance. Antimicrobial treatment for mastitis can be administered either systemically or locally through intramammary infusion (IMM). Cure rates for *S. aureus* mastitis vary considerably with treatment regimens and cow- and pathogen-factors (Sol et al., 1994, 1997, Wilson et al., 1999, Sol et al., 2000, Deluyker et al., 2005) with cure rates for

subclinical *S. aureus* mastitis range from 4 to 92% (Schällibaum et al., 1981, Ziv and Storper, 1985, Owens et al., 1988, Owens et al., 1997). Treatment regimens, and cow- and pathogen-related factors have a strong impact on the probability of cure (Sol et al., 1994, 1997, Wilson et al., 1999, Sol et al., 2000, Deluyker et al., 2005), but are often not included in the consideration to treat a cow with *S. aureus* mastitis. Additionally, estimates for cure rates may differ because of differences in study design, such as definition of cases and cure and length of follow up (Barkema et al., 2006). Variations in cure rate can be due to various reasons including treatment regimen, parity, SCC, bacterial species, bacterial load, and other environmental and pathogenic factors (Barkema et al., 2006). Management based mastitis control measures have been developed and implemented with mild success on reducing contagious mastitis pathogens, such as *S. aureus* and *Streptococcus agalactiae* mastitis (Bekuma and Galmessa, 2018, Bhakat et al., 2020, Rowe et al., 2021), but limited success is due to the application disparities across mastitis management systems (Stevens et al., 2019).

Bacteriologic cure rates with antibiotic treatment rarely exceed 50% and *S. aureus* IMI usually persist throughout the lactations potentially due to limited antibiotic access to the site of infection, survival of *S. aureus* in phagocytic cells (Yancey et al., 1991, Mullarky et al., 2001), *S. aureus* L-forms (Owens and Nickerson, 1989, Brouillette et al., 2004), small colony variants (Atalla et al., 2008), β -lactamase production, formation of micro-abscess and fibrosis (Ziv and Storper, 1985, Sordillo et al., 1989, Erskine et al., 2003), and invasion into mammary epithelial cells (Kerro Dego et al., 2002). The β -lactamase confers resistance to β -lactam antimicrobial agents, such as penicillin G, penethamate hydroiodide, ampicillin, and amoxicillin (Sakwinska et al., 2011). When using culturing methods, pathogen isolation, identification, and susceptibility testing is not available. The knowledge of local epidemiological factors is needed to determine treatment (Roberson, 2003). Selection of antibiotics for treatment based on *in vitro* sensitivity testing may not be effective *in vivo*, but for cases of less than 2 weeks' time with *in vitro* sensitivity test may be used as predictor of a cure, but not for chronic cases (Owens et al., 1997). The importance of antibiotic sensitivity testing for the treatment of clinical mastitis is debatable (Constable and Morin, 2003), but most agree that it is better to do sensitivity testing (Ziv and Storper, 1985, Taponen et al., 2003, Pyörälä, 2005, Barkema et al., 2006) rather than treating without testing, especially for subclinical mastitis.

Cloxacillin and nafcillin are effective against gram-positive bacteria, but less effective against non-lactamase staphylococci. Clavulanic acid with amoxicillin or cloxacillin with ampicillin overcomes β -lactamase resistance and this made its use as a popular intramammary formulation. First- and third generation cephalosporins and erythromycin are effective against β -lactamase-producing staphylococci. One study found a cephalosporin (1st generation cephalosporin) dry cow product administered to heifers with *S. aureus* infections cleared infection in their first lactation (Owens and Ray, 1996, Owens et al., 2001). Since *S. aureus* usually produce β -lactamase, cloxacillin or ampicillin are considered the preferred initial treatment for *S. aureus* mastitis.

Antibiotic therapy for *S. aureus* subclinical mastitis during the lactating period is not economically feasible because of low cure rate, milk withdrawal during treatment, and lack of increased milk production after treatment (Barkema et al., 2006). Dry cow therapy is more effective with a 40 – 70% successful clearance rate (Shephard et al., 2004). The cure rate of lactating cow with antibiotic treatment depends on duration of infection, number of quarters infected, type of quarter (hind or front) infected, strain of *S. aureus* (β -lactamase producer), the immune status of the cow, antibiotic administered, and duration of treatment with better cure rate with longer treatment (Owens et al., 1988). Current recommendations are to combine intramammary and parenteral antibiotic treatment or use extended intramammary treatments alone for 4 to 8 days. Penicillin G is the antibiotic of choice for penicillin sensitive *S. aureus* strains. The intramammary use of pirlimycin is effective when administered for 8 days (Barlow et al., 2013). Extended treatment with pirlimycin decreased transmission of *S. aureus* IMI and strain-specific clinical mastitis within the herd (Barlow et al., 2013).

A study on subclinical *S. aureus* mastitis treatment with commercial intramammary infusion showed no significant difference between treated and untreated controls with the bacteriologic cure rates of erythromycin (65%), penicillin (65%), cloxacillin (47%), amoxicillin (43%), and cephalosporin (43%). Another study on clinical *S. aureus* mastitis treatment with extended cefquinome intramammary infusion showed improved clinical cure from 60% to 84%, but did not alter bacteriologic cure (Swinkels et al., 2013). The treatment of subclinical *S. aureus* IMI with simultaneous IMM of amoxicillin and intramuscular administration of procaine penicillin G achieved a cure rate of 50%. The final choice of the antibiotic for treatment should be based on a culture and susceptibility test. The susceptibility test is required to determine whether *S. aureus* strain is a β -lactamase producer.

6.2.2 Prophylactic

For well managed ideal dairy farms, cows are in lactation for about 300 days and then dried off for about 60 days before they calve again, then the lactation cycle continues again. Dairy cows are susceptible to IMI most frequently during early non-lactating (dry period) and transition (three weeks before and three weeks after calving) periods (Drackley, 1999, Pinedo et al., 2012, Esposito et al., 2014). The IMI is high during the early dry period because of the absence of hygienic milking practices known to reduce bacterial colonization and IMI. The high incidence of mastitis during periparturient period is associated with parturition inducing immunosuppressive hormones (e.g., cortisol), negative energy balance during early lactation to meet the high energy demand for increased milk production, and parturition related physical stress (Esposito et al., 2014).

Naturally IMI during the dry period is expected to be low due to involution and closure of teat opening after drying off by the formation of a keratin plug in the teat canal. However, teat canal closure after drying off vary from animal to animal (Williamson et al., 1995). Some bacteria may enter the intramammary area crossing the keratin plug or enter when keratin plug is broken by intramammary infusion. Dry cow therapy has been the major management tool for the prevention of IMI, as well as for the treatment of subclinical mastitis established during the previous lactation (Williamson et al., 1995, USDA APHIS, 2008a). The intramammary infusion (IMM) of antimicrobials at drying off is of growing concern because this practice exposes healthy animals to antimicrobials allowing for antimicrobial selection pressure on commensal bacteria and other opportunistic bacteria to develop antimicrobial resistance (Abdi et al., 2018).

S. aureus is one of the pathogens with a known ability to develop antimicrobial resistance, and established *S. aureus* infections are persistent and difficult to clear. Once *S. aureus* infection is established, it is persistent and difficult to clear, due to its ability to form biofilm that is protected against the immune system and poorly accessible by therapeutics (Wellnitz and Bruckmaier, 2012). The emergence of aggressive, antibiotic-resistant *S. aureus* strains (such as methicillin-resistant *S. aureus* – MRSA) has become a significant problem, as these strains have spread beyond their typical hospital milieu to the environment and are commonly isolated from bovine sources (Holmes and Zadoks, 2011, Fitzgerald, 2012b, a). This evolution is linked to the transfer of mobile genetic elements within the microbial population (Brody et al., 2008, Otto, 2010). The failure to control these infections leads to the presence of reservoirs in dairy herds, which ultimately leads

to the spread of the infection and the culling of chronically infected cows (Barkema et al., 2006, McDougall et al., 2009).

During the periparturient period dairy cows experience various metabolic, immunological, and physiological changes increasing the risk of periparturient diseases development (Redfern et al., 2021). Blanket dry cow therapy (BDCT) is the IMM of long-acting antibiotic at drying off for all quarters of lactating cows on the farm at drying off. Blanket dry cow therapy is the most common form of usage in over 90% of dairy farms in the United States (Bonsaglia et al., 2017). According to United States Department of Agriculture survey result, 85% of conventional dairy farms use BDCT (FDA, 2015), which estimated to be accounted for one-third of the total antibiotics used on conventional dairy farms in the United States (Armstrong, 2020). While use of dry cow therapy is beneficial for dairy farmers to clear existing IMI, cure subclinical mastitis, and prevent new IMI during dry period, there is growing concern on increased usage of antibiotics on all cows for enhancing emergence and spread of antimicrobial resistant bacteria (Abdi et al., 2018). Antibiotics usage for treatment of mastitis account for 85.4% of antibiotics usage on U.S. dairy farms (USDA, 2016). However, with growing concern of the use of antibiotics in food animals, current strategies including blanket antibiotic dry cow therapy in the dairy industry are being heavily reviewed and has motivated research into alternative practices (Ekakoro et al., 2018). Finding alternatives for preventive antibiotics use at dry-off is key in controlling mastitis and easing concerns of antibiotics resistance.

Alternatives to antibiotics, such as internal teat sealants (ITS) are shown to reduce IMI during the dry period (McParland et al., 2019) and reduce new IMI after calving when used with or without antibiotics (Rabiee and Lean, 2013). Selective dry cow therapy (SDCT) is selectively treating only the quarters with an infection at drying-off. Despite reduction in antibiotic usage, SDCT was only applied on 10% of U.S. dairy operations (USDA, 2016). The concern is due to increased risk of IMI that could influence herd health and profitability (Berry and Hillerton, 2002, Scherpenzeel et al., 2016) SDCT need to be evaluated extensively before fully implementing throughout dairy operation. Despite reduction of antibiotic use, the risk of missing IMI exists when compared to blanket dry cows therapy (BDCT) where all cows in the farm are infused with long acting antibiotic at drying off (Browning et al., 1994, Cameron et al., 2014, Rowe et al., 2020). Another alternative is supplementing nutritional additives to the cows' diet to boost the immune system. Well-known dietary ingredients in the dairy industry, Vitamin E and selenium

(Se), when fed daily promotes immune competency and reduce duration of clinical mastitis (Weiss et al., 1997, Spears and Weiss, 2008).

The goal of dry cow therapy (DCT) is to eliminate the existing IMI and for prevention of new IMI during the dry period. Additional benefits of successful DCT includes milk not being discarded and high bacteriologic cure rates. The probability of cure of an infected quarter decreases when SCC increases ($>250,000$ cells/mL) (Swinkels et al., 2013), cows age, another quarter of the cow has an IMI, a hindquarter is infected, and a high prevalence is present of *S. aureus* IMI before drying off. Antibiotics are not only used to treat animals but also in the form of feed additives for growth promotion, which continue to be unregulated in developing countries without stringent restrictions (Manyi-Loh et al., 2018). In the United States, the adoption of the Veterinary Feed Directive has restricted use of antimicrobials in agricultural water and feed for therapeutic uses under the approval of a licensed veterinarian and antibiotics for therapeutic use only prescribed by licensed veterinarian and not allowed to be sold as over the counter since January 2017 (Ekakoro et al., 2019). Wastewater, for example, is a reservoir of antimicrobial resistance genes and able to undergo one of the pathways in which genes are acquired across microorganisms (Li and Webster, 2018). The use of antibiotics in the agriculture field contributes to residues as seen in pollutants, finding their way into reservoirs such as soil and wastewater (Fouz et al., 2020).

6.2.3 Antimicrobial resistance

Specific antibiotics usage data from producers are not available from dairy farms in the United States. Thus, it is not possible to know all the doses of each antibiotic given to dairy cattle, the length of the treatment, and the diseases for which antibiotics were prescribed. However, the United States Food and Drug Administration (FDA) report showed more than 16,155 kg of medically important antimicrobials intended for intramammary therapy were sold in 2019 (FDA, 2018). The previous review on the impact of antibiotic use in adult dairy cows indicated that common antibiotic use in dairy farms did not lead to the widespread occurrence of resistance among mastitis pathogens (Oliver et al., 2011). However, some studies showed that use of antibiotics for mastitis treatment is associated with antimicrobial resistance development and changes in the diversity of mastitis pathogens (Myllys et al., 1994, Lago et al., 2011). Another study (Pol and Ruegg, 2007) reported a positive relationship between use of antibiotics, such as pirlimycin, ampicillin, erythromycin, and tetracycline and increased resistance among gram-

positive mastitis pathogens. Similarly a study (Tikofsky et al., 2003) reported a significantly higher proportion of resistant mastitis pathogens from conventional dairy farms that use antibiotics (ampicillin, erythromycin, penicillin, and tetracycline) than organic dairy farms which do not use antibiotics, indicating antibiotics usage increases development of antimicrobial resistance. A study (Abdi et al., 2018) on *S. aureus* isolates from cases of mastitis in East Tennessee, reported that about 34.3% were resistant to at least 1 of the 10 tested antimicrobials. The authors also reported a trend of rising antimicrobial resistance in *S. aureus* for some antimicrobials (e.g., tetracycline).

There are different mechanisms by which bacteria may develop resistance to antibiotics including spontaneous mutations, which is acquiring DNA coding for antibiotic resistance gene. The most common deterrent of antimicrobials for bacteria is the outer membrane (Li and Webster, 2018). Antibiotic resistance genes encode products that are helpful in increasing resistance in this membrane (Zhang and Feng, 2016). Presence of antimicrobial resistance genes is predictor for antibiotic resistance among alternate pathogens (Zhang and Feng, 2016). Metabolic processes involving enzymes and transferases establish inherent resistance once the intrinsic genes are activated and are subsequently expressed at heightened levels (Zhang and Feng, 2016). The four pathways antibiotic resistance can be gained include transformation, transduction, conjugation, and mutation (Li and Webster, 2018).

Penicillin resistance is the most well-known antibiotic resistance of *S. aureus*. The prevalence of penicillin resistant *S. aureus* appears to be decreased in the United States in recent years (1994 to 2001) (Erskine et al., 2002, Makovec and Ruegg, 2003), but resistance levels differ considerably between countries (Costa et al., 2000, De Oliveira et al., 2000) and within a country. Prevalence of penicillin-resistant *S. aureus* from bovine mastitis in the United States range from just over 30% (Makovec and Ruegg, 2003) to more than 70% (De Oliveira et al., 2000, Abdi et al., 2021). Resistance of *S. aureus* to macrolide antibiotics ranged from 14 to 17% based on phenotypic testing (Erskine et al., 2004).

NAS are the most prevalent pathogen found in cows with subclinical mastitis (Persson et al., 2011). Both *S. aureus* and NAS are highly resistant to β -lactam antibiotics, such as penicillin G and ampicillin (Botrel et al., 2010, Persson et al., 2011). Methicillin resistant *S. aureus* (MRSA) is not the only staphylococcus species known to be resistant to methicillin, but cases of methicillin resistant NAS (MRNAS) being found to be of the same prevalence with MRSA. Evaluation of 121 NAS isolates from bovine mastitis found that methicillin resistance was frequently observed

among *S. epidermidis* and *S. haemolyticus* isolates (Fessler et al., 2010). Multiple isolates of NAS have a mechanism for methicillin resistance due to presence of specific genes, *mecA* or *mecC*, located on the staphylococcal cassette chromosome (SCC*mec*) (Srednik et al., 2017). One main gene, *mecA* and its variant *mecC*, are responsible for methicillin resistance. However, use of the *mecA* solely as a resistance marker provided false positives due to the ancestor of *mecA* naturally occurring in NAS (Couto et al., 1996). Aside from methicillin, cephalosporins (cefotaxime, ceftriaxone, and ceftazidime) are the most marketed class of antibiotics utilized on dairies in the form of Spectramast® Lactating Cow and Dry Cow treatment (Zoetis, Kalamazoo, MI). A study found that of the NAS isolates from bovine mastitis at least 33% (cefoperazone) and up to 97% (chloramphenicol) were resistant to 11 groups of antibiotics tested (Gupta et al., 2019).

6.3 Genetic Selection

Genetic selection is still a progressive field that has been undergoing improvement with room for advancement as technology and knowledge becomes readily available. Collecting and documenting health data from farms and testing genomics of the herd will allow for predictions of beneficial traits for health and wellness as a selection tool. Having a large database of genetic selections will allow for the herd health to improve over long term, even in the area of mastitis prevention (Vukasinovic et al., 2017).

Genomic selection was performed based on the assumption that specific alleles can be inherited leading to phenotypes that can be quantified; however, the substantial genotyping costs makes for an inadequate procedure for continuation on genotyping progeny (Miles and Huson, 2021). To date, over 2,000 Cattle Quantitative Trait Locus (QTL) have been associated with phenotypic mastitis and documented in a database (Gutierrez-Gil et al., 2018). Unfortunately, QTLs are polygenic and complex, implicating intertwined associated pathways with innate immunity mechanisms responsible for varying responses via cytokines and inflammatory mediators due to pathogen-dependent variation in responses. In 2017, Zoetis integrated their Wellness Trait index for the first time including mastitis resistance in their genomic prediction toolbox (Zoetis, 2017). Genomic reliabilities for compound traits, like mastitis, have increased substantially from 35.3% to 75% due to improved selection strategies (VanRaden et al., 2018). Future research will be needed to enhance knowledge of cellular networks to understand the complex genetics of mastitis and identify potential markers crucial in resistance selection for

progeny. Moreover, the interconnected relation between specific pathogen and innate immune response needs to further be evaluated to find blanket genetic markers that will allow for nonspecific mastitis pathogen protection through phenotypic resistance of mastitis through genomic selection.

6.4 Vaccines

Different vaccines based on cellular or soluble antigens with and without adjuvants were tested in dairy cows, but protection against IMI and clinical disease is limited in the field trials (Pereira et al., 2011). Vaccines evaluated for the control of *S. aureus* mastitis in dairy cows include autogenous bacterins, recombinant protein, DNA-recombinant protein, or contain one or more *S. aureus* strains (Pereira et al., 2011). Vaccination with simultaneous antibiotic administration during lactation (Sears and McCarthy, 2003, Luby and Middleton, 2005) showed some results but no significant effects. A combined administration of 3 doses of a polyvalent *S. aureus* bacterin over 21 days with IMM of pirlimycin in all 4 quarters once daily for 5 days (16–20 d) eliminated 40% of *S. aureus* IMI compared to 9% in untreated controls (Smith et al., 2006). Vaccination with autogenous toxoid-bacterin of lactating cows with *S. aureus* mastitis resulted in a significant decrease of SCC and significantly higher (27%) cure of quarters in the vaccinated group compared to the controls (5%) (Hwang et al., 2000). Response to vaccination may be strain-specific, which could result in farm-specific or regional differences in vaccine efficacy (Guidry et al., 1998).

There are two commercially available bacterin vaccines marketed for the control of *S. aureus* mastitis in dairy cows. These are 1) a commercial bacterin vaccine composed of different strains used in the United States and 2) a polyvalent commercial bacterin vaccine used in Europe and some other countries. The commercial bacterin vaccine used in the US contains three inactivated serovars of *S. aureus* in the form of a bacterin marketed to reduce severity of chronic infections caused by *S. aureus*. The most recent study evaluating the efficacy of the commercial bacterin staphylococcal vaccine utilized a herd consisting of multiparous and primiparous animals all assessed on the lactating cow model and found no significant difference between vaccinated and control groups (Middleton et al., 2006). Another study that evaluated immune response in cows vaccinated with the commercial bacterin staphylococcal vaccine concluded that no significant differences were determined in anti-*S. aureus* antibody levels between vaccinated and unvaccinated cows (Luby et al., 2007). Similarly vaccination with the commercial bacterin

staphylococcal vaccine coupled with simultaneous antibiotic treatment did not reduce SCC in the vaccinated animals (Luby and Middleton, 2005). In conclusion, the commercial bacterin staphylococcal vaccine did not decrease incidence of IMI or clinical mastitis due to *S. aureus* nor provide sufficient protective immunity through antibody production indicating the lack of efficacy of the commercial bacterin staphylococcal vaccine.

The commercial polyvalent bacterin used in Europe and some other countries contains inactivated *E. coli* J5 strain and an inactivated high biofilm forming *S. aureus* strain with label claims of reducing subclinical cases of mastitis due to *S. aureus*, NAS, *E. coli*, and other coliforms. The main mechanisms of reduction include affecting biofilm formation of *S. aureus* and limited numbers of NAS while inducing antibody production against coliform pathogens, including *E. coli*. Many studies have evaluated the efficacy of the polyvalent bacterin staphylococcal vaccine on both lactating cows and heifers with contradictory results. Some authors concluded that vaccination with the polyvalent bacterin staphylococcal vaccine reduced incidence, severity of clinical disease, and duration of mastitis (Schukken et al., 2014, Bradley et al., 2015, Piepers et al., 2017). Contrarily, on observed efficacy, others concluded that vaccination with the polyvalent bacterin staphylococcal vaccine did not induce significant reduction in staphylococcal IMI between vaccinated and unvaccinated groups (Prenafeta et al., 2010, Landin et al., 2015, Freick et al., 2016, Tashakkori et al., 2020). There was an overwhelming consensus, or most authors concluded that the polyvalent bacterin staphylococcal vaccine did not prevent new *S. aureus* mastitis (Schukken et al., 2014, Bradley et al., 2015). There was no significant difference in SCC between animals vaccinated with the polyvalent bacterin staphylococcal vaccine and controls (Freick et al., 2016).

An unregulated noncommercial staphylococcal vaccine contains inactivated *Staph. aureus*, *Strep. agalactiae*, *Strep. uberis*, *Strep. dysgalactiae*, *E. coli* J5, *Trueperella pyogenes*, and *Strep. pyogenes*. Cows vaccinated with the noncommercial staphylococcal vaccine showed no significant difference in rate of infection nor severity of infection compared to control cows (Tashakkori et al., 2017). Contrarily, another study showed protective effect of the noncommercial staphylococcal vaccine with significant reduction in SCC, staphylococcal bacteria counts, prevention of clinical mastitis, and overall increase in quality of milk produced in the vaccinated group (Kajaysri et al., 2014). These two studies had drastically different sample sizes which may be responsible for inconsistent results (Kajaysri et al., 2014, Tashakkori et al., 2017).

An experimental vaccine currently being studied exploits herd specific strains of *S. aureus* isolated from clinical cases in the herd, inactivated then suspended in adjuvant to create an autologous vaccine. Frick et al. (2016) examined the protective effects, milk yield, rate of severe clinical infection, SCC, and staphylococcal bacterial count (Freick et al., 2016) showing no significant difference of incidence of clinical mastitis nor milk yield due to vaccination with this experimental vaccine (Freick et al., 2016). However, the quarter level SCC and *S. aureus* prevalence were significantly lower in vaccinated animals compared to controls (Freick et al., 2016).

In the United States, based on published vaccine efficacy studies, currently available vaccines cannot be recommended as part of the routine measures for controlling *S. aureus* mastitis. Challenges with developing an effective vaccine include the intracellular location of *S. aureus*, ability to form a biofilm, strain variation with each strain having its own genetic makeup and virulence factors (Lin et al., 2009) responsible for different clinical signs (Daum and Spellberg, 2012), and insufficient vaccine-induced opsonizing antibodies in milk to facilitate phagocytosis and clearance of *S. aureus* from infected mammary gland tissue (Middleton et al., 2006, Middleton et al., 2009). Proper vaccine-adjuvant formulation and optimization of a vaccination regimen is also critical factor to induce an optimal immune response required to formulate an effective vaccine (Burakova et al., 2018).

Development of an effective *S. aureus* vaccine remains one of the most important issues confronting the control of infectious diseases of dairy cows. The goal of developing an effective staphylococcal vaccine is not to prevent IMI, but to increase the bacteriological cure rate, prevent clinical disease, and production losses. Our lab developed two novel vaccines against staphylococcal mastitis and currently evaluating their efficacies via controlled experimental vaccination and challenge studies during the dry period (Merrill et al., 2019), early lactation, and over 300 days of lactation. The experimental vaccine containing *S. chromogenes* surface associated proteins (SCSP) conferred promising efficacy against clinical and subclinical *S. aureus* mastitis during the dry period (Merrill et al., 2019) and early lactation (Kerro Dego, unpublished 2019). Further optimization of a vaccination regimen and evaluation of efficacy over a longer period is required to improve observed efficacy.

Chapter 2: Evaluation of Efficacy of Novel Staphylococcal Surface Protein Vaccines Against Staphylococcal Mastitis in Dairy Cows

Abstract

Mastitis is an inflammation of the mammary gland, usually caused by bacterial infection. Annual economic losses due to mastitis are estimated to be \$2 billion in the United States alone. *Staphylococcus aureus* is one of the major bacteria that cause mastitis in dairy cows. Non-aureus staphylococci (NAS) are the most frequent bacteria isolated from intramammary infection. *S. chromogenes* is one of the NAS increasingly reported as a cause of mastitis. Current mastitis control programs are not fully effective against staphylococcal mastitis and treatment with antibiotics are not sustainable because of limited success and the emergence of antimicrobial-resistant bacteria. There is no effective vaccine against *S. aureus* mastitis in dairy cows that prevents clinical disease and production losses. Non-antibiotic sustainable control tools, such as effective vaccines are critically needed. Our lab developed *Staphylococcus aureus* surface associated proteins (SASP) and *Staphylococcus chromogenes* surface associated proteins (SCSP) vaccines. Evaluation of efficacies of the SASP and SCSP vaccines through controlled experimental vaccination and challenge studies showed that the SASP and SCSP vaccines conferred partial protective effects. We hypothesized that vaccination with SCSP or SASP would reduce the incidence of staphylococcal mastitis throughout the lactation period. The objective of this study was to evaluate the efficacy of SCSP and SASP vaccines against staphylococcal mastitis under natural exposure over 300 days of lactation. A total of 45 pregnant Holstein dairy cows were randomly enrolled in this study in three groups of 15 (Group 1), 16 (Group 2), and 14 (Group 3) cows. Cows in groups 1 and 2 were vaccinated with 1.2 mg of SASP and SCSP with Emulsigen-D adjuvant, respectively. Cows in group 3 (control) were injected with phosphate buffered saline (pH 7.4) with Emulsigen-D adjuvant. Vaccine injection was given subcutaneously at 60, 40 and 20 days before expected calving. Booster vaccinations were given at 120 and 240 days in milk (DIM). All cows were monitored for any adverse reactions to the vaccines for three consecutive days and at 7- and 14-days post each vaccination. Cows were monitored for increase in serum and milk anti-SASP and -SCSP antibody titers, milk yield, milk somatic cell counts, mastitis incidence, *S. aureus* and non-aureus staphylococci count in milk over 300 days of lactation. Our results showed increased milk and serum anti-SASP and -SCSP antibody titers in vaccinated cows

compared to control cows. Post-calving health monitoring over 300 days of lactation showed that the incidence of staphylococcal subclinical mastitis was significantly reduced both at the cow (SCSP group) and quarter (SCSP and SASP) levels in vaccinated cows compared to unvaccinated control cows. The SCSP group conferred a significant reduction of the incidence of staphylococcal mastitis over 300 days of lactation. Anti-SASP and -SCSP antibody titers began to wane at about 120 DIM indicating the duration of immunity is about 4 months. In conclusion, the SASP and SCSP vaccines conferred partial protective effects. The optimization of vaccination regimen (dose, adjuvant, route and time of vaccination) may further improve the efficacy of these vaccines for the control of staphylococcal mastitis in dairy cows.

Keywords: *Staphylococcus aureus* surface associated proteins, *Staphylococcus chromogenes* surface associated proteins, *Staphylococcus aureus*; *Staphylococcus chromogenes*; non-aureus staphylococci; vaccine; antibody; dairy cow; natural exposure; vaccination; immunity; bovine staphylococcal mastitis;

1. Introduction

Mastitis, an inflammation of mammary glands, is usually caused by pathogenic microorganisms, such as bacteria, fungi, yeast, and algae. *Staphylococcus aureus* is typically found in the infected mammary gland and is one of the most prevalent major contagious mastitis pathogen on U.S. dairy farms (APHIS, 2008). There are several coagulase positive *Staphylococcus* species, but approximately 95% of coagulase positive staphylococci from cases of bovine mastitis are due to *S. aureus* (Fox and Hancock, 1989). Many different strains of *S. aureus* exist (Aarestrup et al., 1995, Zadoks et al., 2002, Smith et al., 2005), but one strain predominates in a herd due to its contagious nature and inability to coexist with other strains (Zadoks et al., 2000). There is strain variation in ability to spread in herd (Smith et al., 1998), ability to increase SCC and cause clinical mastitis or persistent IMI (Zadoks et al., 2000, Haveri et al., 2005), milk production losses (Middleton and Fox, 2002), resistance with *in vivo* killing by neutrophils (Mullarky et al., 2001), biofilm formation (Fox et al., 2005), formation of small colony variants (Atalla et al., 2008), and invasion of mammary epithelial cells (Hensen et al., 2000a). *S. aureus* mainly causes subclinical and chronic mastitis characterized by high somatic cell counts (Barkema et al., 2006). Annual economic loss due to mastitis is estimated to be \$2 billion in the United States alone (NMC, 1996).

Non-aureus staphylococci (NAS), previously known as coagulase negative staphylococcus species (CNS), are a group of more than 50 species comprised of several increasingly isolated bacteria from milk samples of dairy cows and associated mainly with subclinical mastitis and mild clinical mastitis (Gillespie et al., 2009, Kalińska et al., 2017, Krishnamoorthy et al., 2017, Sender et al., 2017, Srednik et al., 2017, Schabauer et al., 2018, Jenkins et al., 2019, Kim et al., 2019, De Buck et al., 2021). Recently, mastitis caused by NAS has increasingly become more problematic in dairy herds (Taponen et al., 2007, Taponen et al., 2008, Gillespie et al., 2009, Pyörälä and Taponen, 2009, Taponen et al., 2017).

In U.S. dairy herds, NAS are the most prevalent bacterial isolates from milk with some increasingly cause subclinical mastitis and occasionally cause clinical mastitis (Gillespie et al., 2009, Jenkins et al., 2019). *Staphylococcus chromogenes* is one NAS increasingly reported as a causative agent of mastitis in dairy cows (Gillespie et al., 2009, Pyorala and Taponen, 2009, Taponen and Pyorala, 2009, Piessens et al., 2011, Dufour et al., 2012b, Fry et al., 2014, Taponen et al., 2017). *S. chromogenes* exists in the environment as well as in the infected mammary gland (Bradley, 2002, Piessens et al., 2011) with a higher rate of isolation from mammary secretions than

from the environment (Gillespie et al., 2009, Piessens et al., 2011). *S. chromogenes* is consistently isolated from the cow's udder and teat skin (Taponen et al., 2008) and causes long-lasting, persistent subclinical infections (Taponen and Pyorala, 2009). In a study on conventional and organic Canadian dairy farms, NAS were found in 20% of the clinical samples (Levison et al., 2016). *S. chromogenes*, along with other NAS, have been shown to cause subclinical infections in dairy cows that reduce the prevalence of contagious mastitis pathogens in their herds (Bradley, 2002). *S. chromogenes* also inhibit *in vitro* growth of major mastitis pathogens, such as *S. aureus*, *S. uberis*, and *S. dysgalactiae* via the production of bacteriocin (De Vliegher et al., 2004, Pyörälä and Taponen, 2009).

Current staphylococcal mastitis control programs are not fully effective with antibiotics not being sustainable due to limited success (Barkema et al., 2006, McDougall et al., 2009) and the emergence of antimicrobial resistant bacteria (Holmes and Zadoks, 2011, Fitzgerald, 2012a, 2012b). No effective vaccine exists against staphylococcal mastitis in dairy cows that prevents clinical disease and production losses. However, two commercially available vaccines are marketed for *S. aureus* mastitis control in dairy cows: a staphylococcal bacterin vaccine available in the United States and a polyvalent bacterin vaccine with a coliform prevention element available in Europe and some other countries.

The staphylococcal bacterin vaccine contains three inactivated serovars of *S. aureus* in the form of a bacterin and is marketed to reduce severity of chronic infections caused by *S. aureus*. A recent study evaluating the efficacy of the staphylococcal bacterin vaccine utilized a herd consisting of multiparous and primiparous animals all assessed on the lactating cow model found no significant difference between vaccinated and control animals (Middleton et al., 2006). Another study that evaluated immune response in cows vaccinated with the staphylococcal bacterin vaccine concluded no significant differences in anti-*S. aureus* antibody levels between vaccinated and unvaccinated cows (Luby et al., 2007). Similarly, vaccination with the staphylococcal bacterin vaccine coupled with simultaneous antibiotic treatment did not reduce SCC in the vaccinated animals (Luby and Middleton, 2005). Overall, the staphylococcal bacterin vaccine did not decrease incidence of IMI due to *S. aureus* nor provide sufficient protective immunity through antibody production indicating the lack of efficacy of the staphylococcal bacterin vaccine.

The alternate bacterin vaccine is a multivalent vaccine containing an inactivated *E. coli* J5

strain and an inactivated high biofilm forming *S. aureus* strain SP 140 labelled with claims of reducing subclinical cases of mastitis due to *S. aureus*, NAS, *E. coli*, and other coliforms. The main mechanisms of reduction include affecting biofilm formation of *S. aureus* and limited numbers of NAS while inducing antibody production against coliform pathogens, including *E. coli*. Many studies have evaluated the efficacy of the polyvalent bacterin vaccine on both lactating cows and heifers producing contradictory results. Some authors concluded that vaccination with the polyvalent bacterin vaccine reduced incidence, severity of clinical disease, and duration of mastitis (Schukken et al., 2014, Bradley et al., 2015, Piepers et al., 2017). On the contrary to observed efficacy others concluded that vaccination with the polyvalent bacterin vaccine did not induce a significant reduction in staphylococcal IMI between vaccinated and unvaccinated groups (Prenafeta et al., 2010, Landin et al., 2015, Freick et al., 2016, Tashakkori et al., 2020). A large portion of the literature concluded that the polyvalent bacterin vaccine did not prevent new *S. aureus* mastitis (Schukken et al., 2014, Bradley et al., 2015). Additionally, a significantly lower SCC was observed in the vaccinated group compared to the unvaccinated control group (Freick et al., 2016).

Several staphylococcal vaccine efficacy trials showed that vaccination with bacterin vaccines induced increased antibody titers in the serum and milk that are associated with partial protection (Rivas et al., 2002, Leitner et al., 2003, Shkreta et al., 2004, Middleton et al., 2006, Smith et al., 2006, Chang et al., 2008, Schukken et al., 2014, Bradley et al., 2015, Piepers et al., 2017) or no protection at all (Middleton et al., 2009, Landin et al., 2015, Freick et al., 2016). Different vaccines based on cellular or soluble antigens with and without adjuvants were tested in dairy cows, but protection against IMI and clinical disease is limited in the field trials (Pereira et al., 2011). Vaccines evaluated for the control of *S. aureus* mastitis in dairy cows include autogenous bacterins, recombinant protein, DNA-recombinant protein, or contain one or more *S. aureus* strains (Pereira et al., 2011). Vaccination with simultaneous antibiotic administration during lactation (Sears and McCarthy, 2003, Luby and Middleton, 2005) showed some differences, but no significant effects. A combined administration of 3 doses of a polyvalent *S. aureus* bacterin over 21 days with IMM of pirlimycin in all 4 quarters once daily for 5 days (16–20 d) eliminated 40% of *S. aureus* IMI compared to 9% in untreated controls (Smith et al., 2006). Vaccination with autogenous toxoid-bacterin of lactating cows with *S. aureus* mastitis resulted in a significant decrease of SCC and significantly higher (27%) cure of quarters in the vaccinated group compared

to the controls (5%) (Hwang et al., 2000). Response to vaccination may be strain-specific, which could result in farm-specific or regional differences in vaccine efficacy (Guidry et al., 1998).

In the United States, based on published vaccine efficacy studies, currently available vaccines cannot be recommended as part of the routine measures for controlling *S. aureus* mastitis. Several factors affect successful development of effective vaccines against *S. aureus* mastitis including several host-pathogen associated factors that enable bacterium to evade host immunological defense and effect of antimicrobials administered as treatment. Pathogen associated factors include adhesion and invasion of *S. aureus* into mammary epithelial cells (Hensen et al., 2000a) or survival of *S. aureus* in intracellular location in phagocytic cells include (Yancey et al., 1991, Mullarky et al., 2001) the ability to form a biofilm (Fox et al., 2005, Melchior et al., 2006a, Wellnitz and Bruckmaier, 2012), the ability to form L-forms (Owens and Nickerson, 1989, Brouillette et al., 2004) or small colony variant (Atalla et al., 2008), and strain variation with associated variation of virulence factors (Aarestrup et al., 1995, Peterson, 1996, Zadoks et al., 2000, Zadoks et al., 2002, Smith et al., 2005, Lin et al., 2009, Bien et al., 2011, Daum and Spellberg, 2012, Lindsay, 2014, Putz et al., 2020, Bohl et al., 2021). Host associated factors include insufficient vaccine-induced opsonizing antibody in milk (Middleton et al., 2006, Middleton et al., 2009), dilution of intramammary immune effectors by large volume of milk (Guidry et al., 1994, Yancey, 1999) that is usually removed at least twice daily, milk being a rich source of fat molecules frequently engulfed by phagocytic cells (decreasing bactericidal effects), (Russell et al., 1977), mammary glands of dairy cows not being part of the mucosal immune system, and the intramammary immune response depending on systemic immune responses in the body to cross from the general systemic circulation into the mammary glands.

Moreover, proper vaccine-adjuvant formulation and optimization of vaccination regimen is also critical factor to induce optimal immune response required for an effective vaccine (Burakova et al., 2018). Development of an effective *S. aureus* vaccine remains one of the most important critical issues confronting the control of infectious diseases of dairy cows. The goals of developing effective staphylococcal vaccines are not to prevent IMI but to increase the cure rate, prevent clinical disease, and reduce production losses. Our lab developed two novel vaccines against staphylococcal mastitis and are currently evaluating efficacies via controlled experimental vaccination and challenge studies during the dry period (Merrill et al., 2019), early lactation, and over 300 days of lactation. The experimental vaccine containing *S. chromogenes* surface

associated proteins (SCSP) conferred partial cross protective effects against *S. aureus* mastitis during the dry period (Merrill et al., 2019) and early lactation (non-published data). Further optimization of a vaccination regimen and subsequent evaluation of efficacy over a longer period is required to improve observed efficacy.

2. Materials and Methods

2.1 Vaccines preparation

A total of two vaccines were prepared for the current study. The *Staphylococcus aureus* surface proteins (SASP) and *Staphylococcus chromogenes* surface proteins (SCSP) extraction and vaccine preparation were made as described in previous studies (Abdi et al., 2019, Merrill et al., 2019). The SASP vaccine strain was one of the dominant isolates of *S. aureus* from cases of bovine mastitis across east Tennessee dairy farms collected 2005 - 2012 (Abdi et al., 2019, Vaughn et al., 2020). We used the *S. chromogenes* isolate that prevented mammary gland colonization by our challenge strain of *S. aureus* under *in vivo* conditions during an experimental challenge infection model development (Kerro Dego et al., 2020). A total of 1.2 mg of SASP or SCSP in 1.5 mL of PBS (pH 7.4)] was mixed with 1.5 mL of Emulsigen-D® (Phibro Animal Health Corporation, Teaneck, NJ), making a total volume of 3 mL. The control vaccine was 1.5 mL of PBS (pH 7.4) mixed with 1.5 mL of Emulsigen-D® resulting in a total volume of 3 mL.

Briefly, each strain was streaked on Tryptic Soy Agar with 5% sheep blood plates (Becton, Dickinson and Company, Franklin Lakes, NJ) and incubated overnight (16 - 18h) at 37°C, 5% CO₂: 95% air balanced. After overnight growth, three colonies of each species were suspended in 450 mL of Tryptic soy broth (TSB) and grown to mid-log phase in 37 °C, incubator with shaking at 125 rpm. Each culture was centrifuged for 10 minutes at 500 Xg at 4°C then the pellet was resuspended in 30 mL of 1% cholic acid (w/v) (Sigma-Aldrich, St. Luis, MO) and incubated at room temperature (25°C) for 2 h with shaking at 125 rpm. Bacterial suspension was centrifuged at 10,000Xg for 30 min at 4°C and the supernatant containing the proteins was buffer exchanged to PBS using Cetriprep Ultracel- 10K YM concentrators (10 kDa cutoff) (EMD Millipore Corporation, Billerica, MA). The proteins were filter sterilized and concentration was measured using a nanodrop spectrophotometer (ThermoFisher Scientific, Waltham, MA).

2.2 Study animals and vaccination schedule

Utilizing EpiTools Epidemiological Calculator (Ausvet©, Sargent, ESG, 2018), sample size was calculated at a 95% confidence level at desired power of 80%. Prior to enrollment in the study, cows had to be healthy and free of mastitis due to major pathogens, such as *S. aureus*, streptococci (*S. uberis*, *S. agalactiae*, *S. dysgalactiae*), and coliform bacteria (*E. coli* and *Klebsiella* spp.). Individual quarter milk somatic cell count (SCC) of less than 200,000 cells/mL of milk was required. Additionally, all four quarters had to be free of clinical and subclinical mastitis caused by NAS, but in the presence of NAS, individual quarter somatic cell count had to be less than 80,000 cells/mL of milk. Cows were screened weekly starting at four weeks prior to drying off at 28, 21, 14, and 7 days before drying off. A total of 45 dairy cows in their 1st - 6th lactation from the East Tennessee AgResearch and Education Center Little River Dairy herd were randomly enrolled in three groups of 15 (Group 1), 16 (Group 2) and 14 (Group 3) cows. Cows in groups 1 (SASP) and 2 (SCSP) were vaccinated with 1.2 mg of SASP and SCSP vaccines respectively with Emulsigen-D® adjuvant at 60, 40, and 20 days before expected calving date (C -60, C -40 and C -20). The booster vaccinations were given at 120 and 240 days in milk (DIM). Cows in the group 3 (Control) were injected with PBS (pH 7.4) mixed with Emulsigen-D® at similar time points. All cows were vaccinated subcutaneously (SC) in the neck area.

For Groups 1, 2, and 3, a series of three subcutaneous vaccinations were given on alternate sides of the neck roughly halfway between the top of the shoulder and the base of the ear. Injections were given utilizing sterile disposable needles and syringes. The first vaccination was given on the upper left side of the neck, the second on the right side, and the third was on the lower left side of the neck. The fourth and fifth booster vaccinations were given on lower right and upper left side of the neck. Vaccine groups were masked to all personnel administering the vaccinations and conducting future assays. Treatments were revealed after the completion of data collection and sample analyses. (Table 1).

All experimental cows were housed at the East Tennessee AgResearch and Education Center- Little River Animal and Environmental Unit (ETREC-LRAEU) dairy herd facility at Walland, TN, under the same herd care for the duration of the study. Cows were housed in freestalls containing loose-pack sand bedding with management adding new bedding weekly. Experimental cows were kept under similar management with other cows in the farm and usually fed a mixed total ration and other supplements required for dairy cows following the normal

Table 1. Immunization protocol

Group	# Cows	Vaccine/adjuvant	Vaccine dose/ total volume	Frequency
1	15	SASP/Em-D	1.2 mg/3 mL	60, 40, 20 days before expected calving with 2 more booster doses at 120 and 240 DIM
2	16	SCSP/Em-D	1.2 mg/3 mL	
3	14	None-Em-D	3 mL	

standard operating protocol of the farm. This study was approved by the University of Tennessee, Institutional Animal Care and Use Committee (IACUC) (Registration Number: 2655-1118).

2.2.1 Blood samples

Blood samples were collected prior to vaccination at 60, 40 and 20 days before expected calving, at calving (C), and monthly from 30 – 300 DIM. About 20 mL of blood was collected into 10 mL plain uncoated tubes with clot activator (ThermoFisher Scientific) from the jugular vein at each time points. Samples were kept on ice and transported to the lab and serum was prepared within 1 - 2 h of blood collection. For serum preparation, tubes were centrifuged at 2,500 RPM at 4°C for 30 min to separate serum from blood components. The top serum layer was collected aseptically into sterile 96 well serum blocks and stored at -80°C until ELISA assay conducted.

2.2.2 Milk samples

During screening of cows prior to enrollment in the study, individual quarter milk samples (3 mL) for bacteriological culture were aseptically collected into sterile 15 mL conical Falcon tubes weekly at 28, 21, 14 and 7 days before drying off (D-28, D-21, D-14 and D-7). At similar time points individual quarter milk samples (50 mL) for somatic cell counts were collected into 50 mL vials with preservative pills containing potassium dichromate (Capitol Vial™, Auburn, AL).

For cows enrolled in the study, individual quarter milk samples for bacteriological analysis (3 mL) and for somatic cell counts (50 mL) were collected at dry off (60 days before expected calving [C -60]), at calving (C), 7 days in milk (7 DIM), and monthly from 30 – 300 DIM. Composite milk samples (50 mL, collected from all quarters at equal proportion) for measuring antibody titers were collected into sterile 50 mL Falcon tubes immediately before first vaccine injection at 60 days before expected calving (C -60), calving (C), 7 DIM, and monthly from 30 – 300 DIM. Individual quarter milk samples for bacteriological analysis were collected aseptically with gloved hands after pre-dipping of teats into antiseptic solution (OPI blue dodecyl benzene sulfonic acid solution, DeLaval, Inc., Kansas, MO) and thorough cleaning with an individual towel and wiping of teat opening with 70% ethanol (disposable alcohol impregnated swabs, Covidien Webcol™, Salt Lake City, Utah). Milk samples were not collected during dry period at 40 and 20 days before expected calving to avoid the break of natural teat canal closure by keratin plug and entry of pathogenic bacteria. Milk samples collected for bacteriological culture were placed on ice and transported to the lab and cultured immediately or stored at -20°C until processed. Milk

samples for antibody titers evaluation were processed into skim milk and stored at -80°C until measuring antibody titers by enzyme-linked immunosorbent assay (ELISA).

To detect clinical mastitis during study period, abnormal inflammatory changes in the udder tissue and milk were examined and recorded as described previously (Merrill et al., 2019). Briefly, abnormal inflammatory changes in the udder tissue were determined by clinical evaluation of udder for swelling, pain, redness (hyperemic), increased heat during milking, and reduction or loss in milk yield. The abnormal inflammatory changes in the mammary gland tissue were scored on scale of 0 (normal) to 3 (severe abnormal inflammatory changes) (Merrill et al., 2019). Similarly, abnormal inflammatory changes in milk such as clots, change in color (bloody milk, watery milk, etc.) and change in consistency (viscous milk or purulent inflammation with pus like discharge) were examined and recorded on scale of 0 to 3 as 0 (normal), 1 (few flakes), 2 (large clots/ slugs), and 3 (bloody, watery, stringy) (Merrill et al., 2019). Clinical mastitis was defined as receiving a score of 2 in both milk and udder tissue categories or receiving a score of 3 in either the milk or udder tissue categories for three consecutive days. Both mammary gland and milk examination and scoring were performed by research team member who is also stationed at ETREC- LRAEU farm.

I. Somatic cell counts

The SCC was determined at the Dairy Herd Improvement Association (DHIA) Laboratory (Knoxville, TN, USA) using the Soma Count 300 (Bentley Instruments Inc., Chaska, MN, USA)

II. Milk bacteriological culture and isolation and identification of bacteria

Bacteriological analysis was performed following National Mastitis Council (NMC) guidelines (Oliver et al., 2004) with slight modification. Briefly, 100 µL of milk sample was spread on individual blood agar plate and incubated at 37°C, 5% CO₂: 95% air balanced for 24 to 48 h. At 24 and 48 h plates were examined for growth of colonies and characteristics of each colony such as size, morphology, hemolysis, and pigment production. One colony of pure culture was Gram-stained and differentiated into Gram-positive or Gram-negative bacteria with identification of bacterial morphology as cocci or rods. Gram positive cocci were further tested by catalase to differentiate staphylococci from streptococci. Catalase positive cocci are believed to be

staphylococci and were further tested by the tube coagulase using rabbit plasma to differentiate the coagulase positive *Staphylococcus* from coagulase negative staphylococcus species.

Gram positive rods with spore formation were identified as presumptive *Bacillus* spp. non-spore forming, catalase positive bacteria were identified as perspective *Corynebacterium* spp. (48 h growth). The oxidase test was used to differentiate Gram negative rods into the *Enterobacteriaceae* or Gram-negative non-*Enterobacteriaceae* organisms.

I. Skim milk preparation

Skim milk was prepared from composite milk by centrifuging milk at 10,000 RPM at 4°C for 25 min. The solid sediments and the fat from top layer were discarded. Skim milk was stored at -80°C until ELISA was conducted.

2.2.3 Vaccines safety monitoring

Cows were monitored throughout the study for adverse reactions to vaccines through rectal temperature. Similarly, vaccine injection site reaction was measured in volume by taking three-dimensional measurements, height (depth), width (dorsal/ventral), and length (cranial/caudal) in millimeters (mm). The standard ellipsoid volume ($V = \pi * L * W * H / 6$) was calculated in cubic centimeter (Powers et al., 2007). Measurements were taken immediately prior to vaccination, daily for three consecutive days after each vaccine injection, days 7 and 14 post each vaccine injection, and monthly between vaccinations. Cows were monitored daily by the research team member, who is also ETREC-LRAEU staff, for any clinical signs of mastitis and other diseases and associated abnormal behavioral manifestations including lethargy, loss of appetite, decreased or loss of milk production, or any other complications.

2.2.4 Milk Production

Milk yield was recorded automatically by the BouMatic (BouMatic LLC, Madison, WI). and SmartDairy parlor system (Valley Agricultural Software, Tulare, CA) with the PCDart Interface (Dairy Records Management Systems, Raleigh, NC). The weight (kg) of milk yield per day over three days before and after the actual sample collection date, including the yield of actual sample collection date (total 7 days) were obtained from the recorded data and used to evaluate

effect of vaccination on milk yield over the study period. Milk yield was recorded on -60, C0, 7, 30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 DIM.

2.2.5 Enzyme linked Immunosorbent Assay

Milk and serum anti-SASP and -SCSP IgG, IgG1, IgG2, and IgA antibody titers were determined using indirect enzyme-linked immunosorbent assay (ELISA), as previously described (Merrill et al., 2019). Briefly, 96 well polystyrene Immulon® 2 HB plates (Thermo Fisher Scientific) were coated with 1µg/mL of SASP or SCSP in a sodium bicarbonate (NaHCO₃) coating buffer (200 µL/ well) and incubated overnight (16 - 18 h) at 4°C. The coating buffer was removed, and plates were washed 5X using an automated 405 touch screen (TS) microplate washer (Biotek Instruments Inc., Winooski, VT) with PBS-T (phosphate-buffered saline containing 0.05% Tween 20 (v/v) (Thermo Fisher Scientific). The plates were blocked with PBS-T containing 1% gelatin (w/v) (PBS-TG) (Thermo Fisher Scientific) for 2 h. The plates were washed again 5X with PBS-T (Thermo Fisher Scientific). Serum and skim milk samples were serially diluted four folds and incubated at room temperature (25°C) for 1 h. Post incubation, plates were washed again 5X with PBS-T. A 1:10,000 dilution of horseradish peroxidase-conjugated polyclonal sheep anti-bovine IgG or IgA (heavy + light chain) or monoclonal sheep anti-bovine IgG1 or IgG2 antibody (Bethyl Laboratories, Inc. Montgomery, TX) in PBS-TG were added (100 µL/ well) and incubated at room temperature (25°C) for 1 h. Post incubation, plates were washed 5X with PBS-T and ABTS™ horseradish peroxidase substrate (SeraCare Life Sciences, Milford, MA) was added (100 µL/well); plates were incubated at room temperature for 20 minutes. Absorbance of each plate was read at a wavelength of 405 nm (A405) using a Synergy H1 Microplate Reader (Biotek Instrument Inc.). Data were exported to Excel (Microsoft Corporation, Redmond, WA) and the blank row's average + 2 standard deviations (avg +2stdev) was used to calculate the cutoff point for titer determination. Both serum and milk titers were calculated by the intersection of least-square regression of A405 versus the logarithm of the dilution.

2.3 Statistical Analysis

Outcomes measured at a cow level (e.g., total milk yield, rectal temperature, size of vaccination site reaction and cow level mastitis incidence and incidence density) were modelled as two-level data structure with repeated measurements nested within a cow. Outcomes measured

either at the individual quarter (SCC, quarter level mastitis incidence and incidence density) or had replicate measurements (antibody titers) were modeled as a 3-level data structure to account for the repeated measurements within each quarter and clustering of observations within a cow. Quantitative data with two or three levels were analyzed with mixed effects linear regression model. Mean rectal temperature, size of site reaction following vaccine injection, and amount of milk yield between the vaccinated and control cows were compared with mixed effects linear regression model. Treatment group, sampling day, and treatment by day interaction were included as fixed effects while cow ID and quarter were included as a random effect to account for the repeated measurements within a cow. Somatic cell count was $\log_{10}$ transformed and analyzed by including quarter nested within animal as a random effect into the model.

Prior to analysis of variance (ANOVA), the univariate procedure was utilized to assess continuous outcomes for ANOVA assumptions. Somatic cell counts (SCC) were natural log transformed to meet assumption of normality of residuals and equal variance. To test if vaccination (SASP, SCSP, Control), day of sampling (C -60, C -40, C -20) before expected calving, calving (C) and 7 days in milk (DIM) and monthly from 30 - 300 DIM, or the interaction affected milk production or SCC, a mixed model ANOVA and mean separation were used (SAS 9.4).

The overall subclinical mastitis (SCM) status of cows and quarters were modelled as a binary outcome, using logistic regression. Odd ratio [OR]: with its 95% confidence interval [CI] was used to assess the measure of association between vaccination and SCM incidence during the entire study period. Relative risks comparing vaccinated groups to the unvaccinated group was determined by fitting generalized linear model with binomial distribution and log link. Vaccine efficacy (VE) was calculated based on relative risk in each treatment group vs. control using the formula: $VE = 1 - RR_{\text{vac vs control}}$ (Orenstein et al., 1985). To assess if treatment groups differed in development of subclinical or clinical mastitis, initial numbers and proportions of illness were assessed within the frequency procedure. Incidence of CM and SCM, and subsequent relative risks among treatment groups were determined in the Glimmix procedure using log linear models with binomial distribution and log link. Incidence density was determined as incidence/ time at risk. The Glimmix procedure of SAS was used to assess if incidence density differed between vaccine groups using Poisson distribution and log link.

Mean NAS count was analyzed by multilevel mixed effects negative binomial regression by including cow and quarter as random effects. The marginal output was obtained as $\log_{10}$ to

obtain the mean NAS counts from vaccinated and unvaccinated control cows over the course of the study.

Serum and milk antibody titers were $\log_{10}$ transformed and analyzed with three-level hierarchical data structure by including triplicate measurements per cow as additional random variable. To assess the significance of treatment (vaccination group), sampling day, and their interaction term, we used STATA's '*testparm*' post estimation of their effects. For all statistical analyses, the level of significance was set at $\alpha = 0.05$. Data were analyzed in STATA 16.1 (STATA Corporation, College Station, TX) or SAS 9.4 (SAS Institute Inc., Cary, NC).

3. Results

3.1 Overall animal health and retention in the study over 300 days of lactation

Out of the sixteen cows enrolled in the SCSP group, two cows were removed due to a left displacement of the abomasum (LDA), and another two cows were removed due to hip dislocation. The remaining twelve cows completed the study. Out of the fifteen cows originally randomized into the SASP group, a total of three cows were removed from the study due to hip injury ($n=1$), an enlarged cecum and loss of rumination ($n=1$), and late-stage abortion due to high environmental temperatures ($n=1$). Out of the fourteen cows in the control group, one cow was removed due to hip injury and another cow was removed due to milk fever. Overall, twelve cows completed the study in each vaccine group (Table 2). The median parity number did not significantly differ by treatment group ($P = 0.5755$).

3.2 Vaccine Safety

Mean rectal temperature did not significantly vary by vaccination ($P = 0.0727$); however, day had a significant ($P < 0.0001$) effect. Mean rectal temperature did not significantly ($P = 0.667$) vary by group between first three consecutive days (acute phase) and 7 and 14 days (delayed phase) after vaccine injection. Both time periods ($P < 0.0001$) and their interactions with vaccination ($P = 0.0331$) were significantly associated with the mean reaction size at the sites of injections, with no main effect of vaccination ($P = 0.5750$). Both mean rectal temperature and sizes of the injection site reactions temporarily increased immediately (i.e., during the acute phase) following

Table 2. Description of study cows for the evaluation of efficacy of SASP and SCSP vaccines against staphylococcal mastitis. SASP= Staphylococcus aureus surface proteins; SCSP= Staphylococcus chromogenes surface proteins

Parameters	Group		
Cows	1 (SASP)	2 (SCSP)	3 (Control)
No. randomized	15	16	14
Parity, median (range)	3 (1-6)	3 (1-6)	2 (1-6)
No. removed	3	4	2
No. completed study	12	12	12

the primary (1st – 3rd) and booster (4th and 5th) vaccinations, after which it declined during the delayed phase (Figure 1).

3.3 Serum and milk anti-SASP and -SCSP antibody titers

3.3.1 Effects of vaccinations on serum anti-SASP antibody titers

Vaccination of dairy cows with SASP or SCSP had a significant vaccination effect on serum anti-SASP IgA ($P= 0.0012$), IgG1 ($P= 0.0465$), and IgG2 ($P< 0.0001$) titers. SASP vaccinated cows had significantly higher serum anti-SASP IgG titers at C0 ($P= 0.037$), 150 DIM ($P< 0.0001$), 270 DIM ($P= 0.004$); -IgG1 titers at 150 DIM ($P= 0.012$); -IgG2 titers at -20C ($P= 0.028$), C0 ($P= 0.005$) and 150 DIM ($P= 0.002$) compared to the control cows. SASP vaccinated cows had significantly higher cross-reacting serum anti-SCSP IgG titers at 270 DIM ($P= 0.011$) compared to the control cows. The SCSP vaccinated cows also had significantly higher cross-reacting serum anti-SASP IgG titers at 150 DIM ($P= 0.031$) (Figure 2A – D).

3.3.2 Effects of vaccinations on serum anti-SCSP antibody titers

The SCSP vaccinated cows had significantly higher serum anti-SCSP IgG titers at 30 DIM ($P= 0.032$), 60 DIM ($P= 0.026$); IgG2 titers at -40C ($P= 0.013$), -20C ($P= 0.007$) compared to the control cows. The SCSP vaccinated cows had significantly higher cross-reacting serum anti-SASP IgG titers at C0 ($P<0.0001$) and 30 DIM ($P= 0.029$) compared to the control cows (Figure 3A - D). Vaccination of dairy cows with SASP or SCSP had no significant vaccination effect on serum anti-SCSP IgA titers ($P= 0.8593$).

3.3.3 Effects of vaccinations on milk anti-SASP antibody titers

SASP vaccinated cows had significantly higher milk anti-SASP IgG titers at C0 ($P= 0.012$), 150 DIM ($P= 0.037$); IgG2 titers at 150 DIM ($P= 0.002$), 270 DIM ($P= 0.002$) titers compared to control cows. SASP vaccinated cows had significantly higher cross-reacting milk anti-SCSP IgG titers at C0 ($P< 0.0001$), 150 DIM ($P< 0.0001$); IgG1 titers at 150 DIM ($P= 0.018$) compared to control cows (Figure 4A – D).

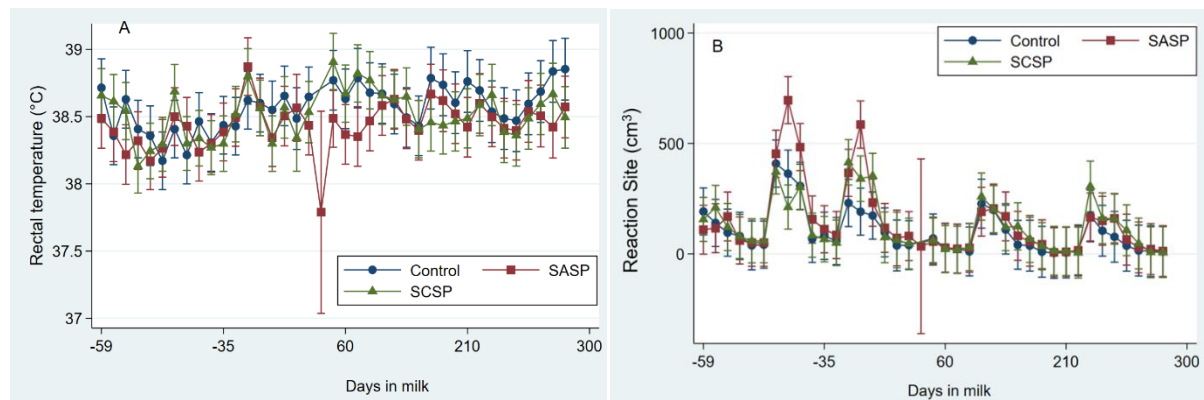


Figure 1. Effects of vaccinations on rectal temperature (A) and injection site reactions (B) over 300 days of lactation. SASP: *Staphylococcus aureus* surface associated proteins; SCSP: *S. chromogenes* surface associated proteins; -59: 59 days before expected calving date; -35: 35 days before expected calving date

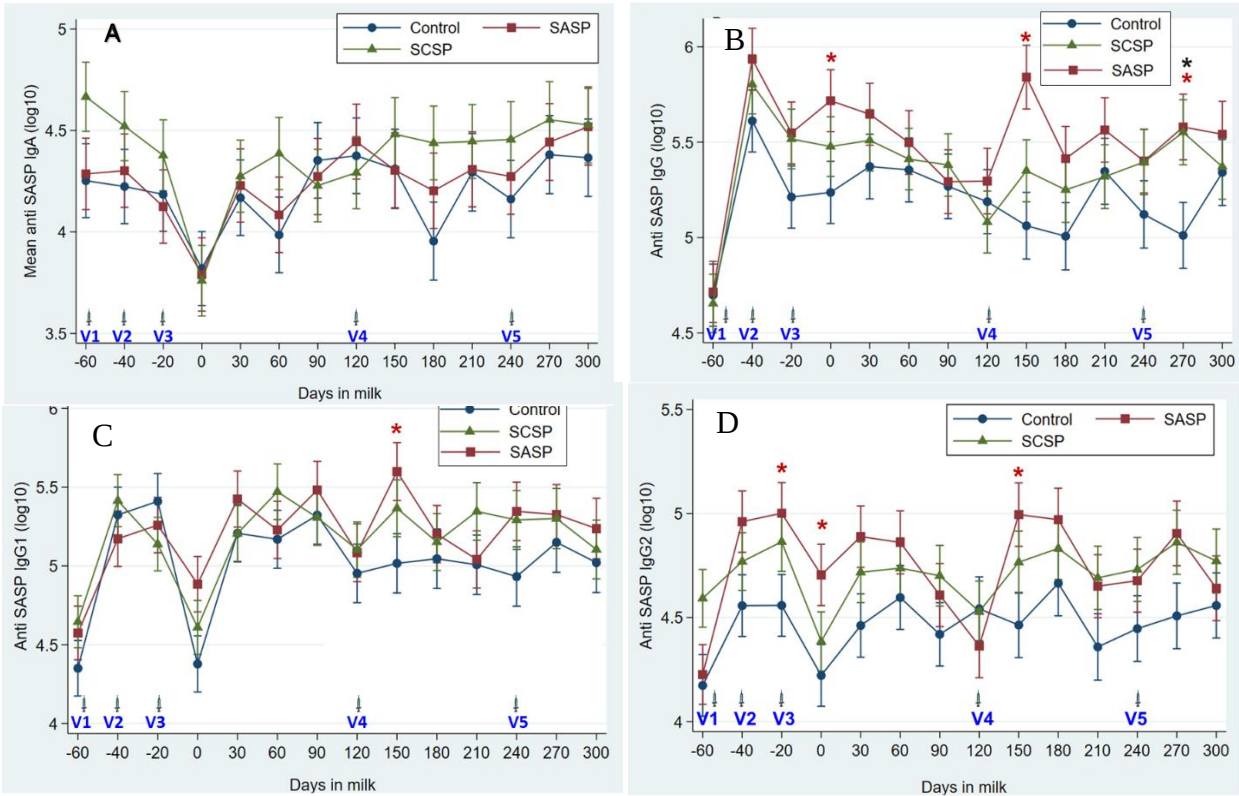


Figure 2: Mean serum anti-SASP antibody titers over 300 days of lactation. A) IgA; B) IgG; C) IgG1; D) IgG2; SASP: *Staphylococcus aureus* surface associated proteins; SCSP: *S. chromogenes* surface associated proteins; -60: 60 days before expected calving date; -40: 40 days before expected calving; -20: 20 days before expected calving; 0: calving day, V1 – V3: 1st to 3rd vaccinations, V4 and V5: 4th and 5th booster vaccinations.

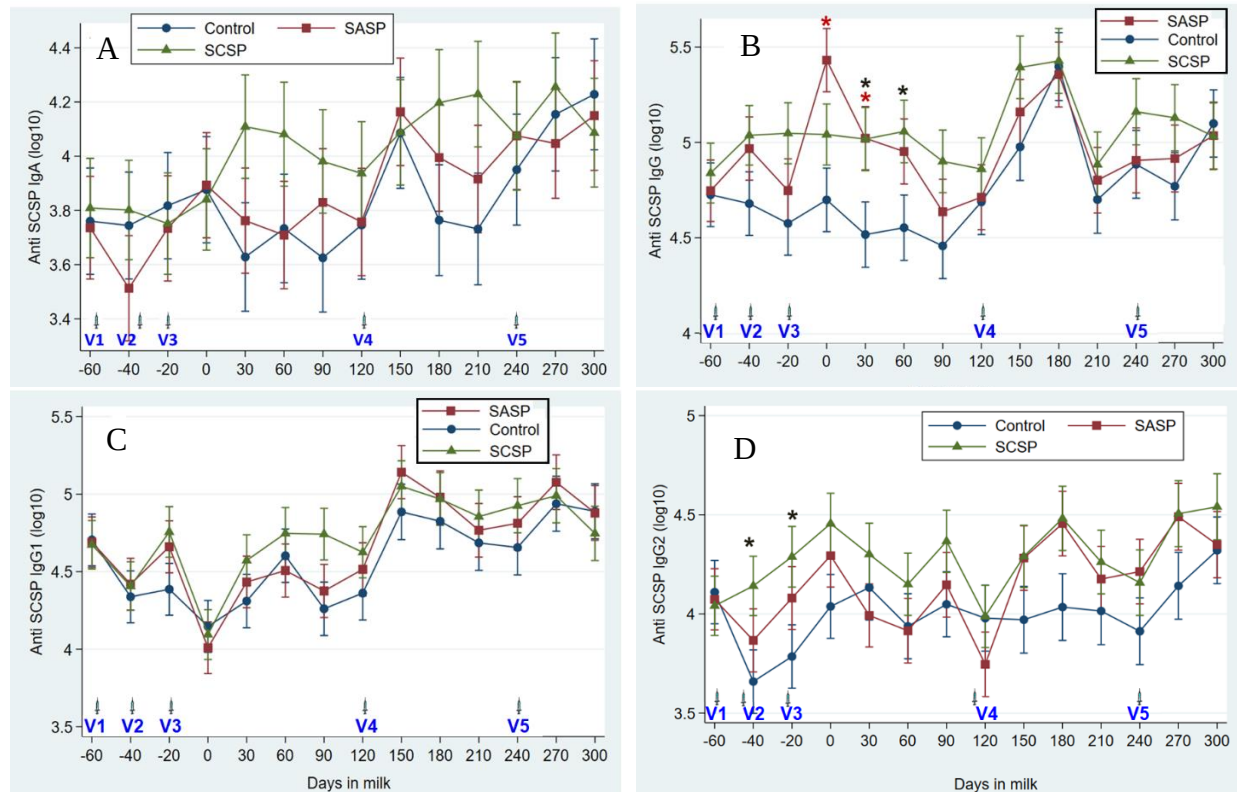


Figure 3: Mean serum anti-SCSP antibody titers over 300 days of lactation. A) IgA; B) IgG; C) IgG1; D) IgG2; SASP: *Staphylococcus aureus* surface associated proteins; SCSP: *S. chromogenes* surface associated proteins; -60: 60 days before expected calving date; -40: 40 days before expected calving; -20: 20 days before expected calving; 0: calving day; V1 – V3: 1st to 3rd vaccinations, V4 and V5: 4th and 5th booster vaccinations.

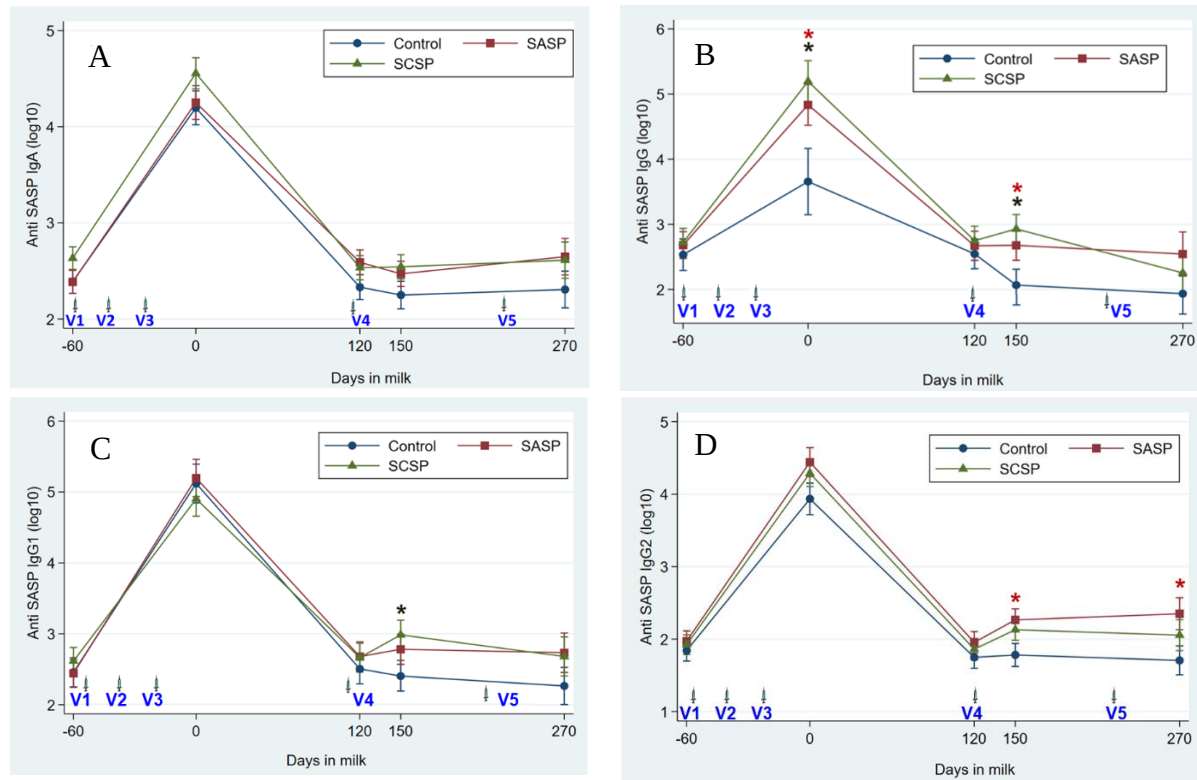


Figure 4: Mean milk anti-SASP antibody titers over 300 days of lactation. A) IgA; B) IgG; C) IgG1; D) IgG2; SASP: *Staphylococcus aureus* surface associated proteins; SCSP: *S. chromogenes* surface associated proteins; -60: 60 days before expected calving date; 0: calving day, V1 – V3: 1st to 3rd vaccinations, V4 and V5: 4th and 5th booster vaccinations.

3.3.4 Effects of vaccinations on milk anti-SCSP antibody titers

SCSP vaccinated cows had significantly higher cross-reacting milk anti-SASP IgG titers at C0 ($P=0.005$) compared to the control cows. SASP vaccinated cows had no significantly higher milk anti-SCSP titers compared to control cows (Figure 5A – D).

3.4 Detection of *Staphylococcus aureus* and non-aureus staphylococci

Non-aureus staphylococci (NAS) were identified from 18.6% ($n=2,025$) of quarter milk samples tested during the study time of 300 days of lactation across the vaccine groups. A total of 377 NAS isolates were identified from individual quarter samples (Control: $n=133$; SASP: $n=129$; SCSP: $n=115$). While vaccination ($P=0.5825$) and day ($P=0.4800$) did not have any significant effects, vaccination by day interaction had a significant ($P=0.0305$) effect on the mean NAS count (Figure 6). *Staphylococcus aureus* was counted only from five quarters of four cows (two each from control and SASP groups). One of the control cows had 20 CFU/mL in the two front quarters on 300 DIM; the other cow had 80 CFU/mL in the left front quarter on 180 DIM. Two cows in the SASP group had 50 and 150 CFU/mL, respectively, in their right front quarters at calving.

3.5 Detection of other bacteria from milk samples

Other bacteria were isolated and identified from milk samples (Table 3). Overall, there was an even spread of the identified species across groups. Unidentified *Streptococcus* spp. were isolated from individual quarter samples.

3.6 Effect of vaccinations on milk production and Somatic Cell Count

Vaccination ($P=0.9375$) had no significant effect on milk production (Figure 7A). The mean $\log_{10}$ SCC did not significantly differ among vaccine groups ($P=0.4393$) (Figure 7B).

3.7 Staphylococcal Mastitis Status

3.7.1 Cow level clinical and subclinical Staphylococcal mastitis

At cow level, a total of 3 cows developed clinical mastitis (CM) (6.6%, $n=45$), of which 14.2% ($n=14$) and 6.6% ($n=15$) were in control and SASP vaccinated cows, respectively with no significant (Fisher's exact test $P=0.1934$) vaccination effect. None of the SCSP vaccinated cows developed clinical mastitis. Fifteen cows (33.3%; $n=45$) developed subclinical mastitis (SCM), of

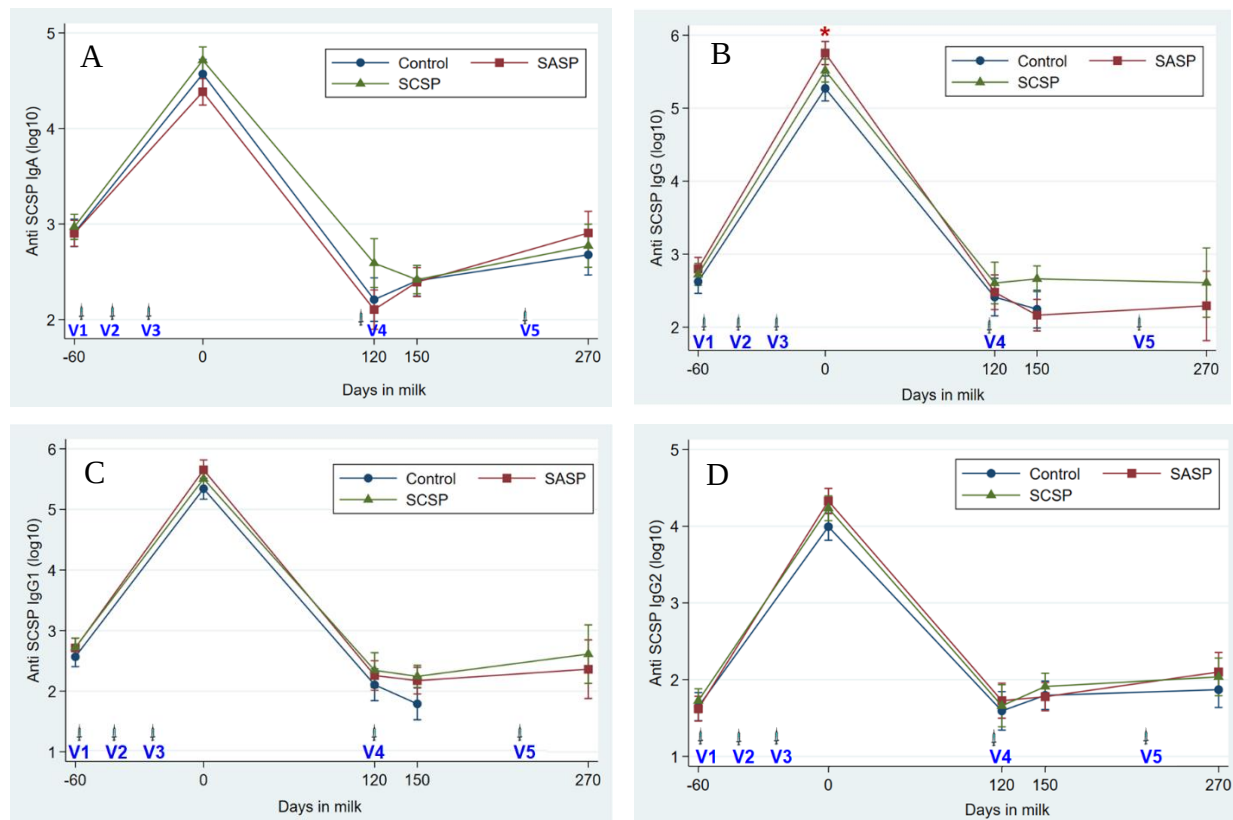


Figure 5: Mean milk anti-SCSP antibody titers over 300 days of lactation. A) IgA; B) IgG; C) IgG1; D) IgG2; SASP: *Staphylococcus aureus* surface associated proteins; SCSP: *S. chromogenes* surface associated proteins; -60: 60 days before expected calving date; 0: calving day; V1 – V3: 1st to 3rd vaccinations, V4 and V5: 4th and 5th booster vaccinations.

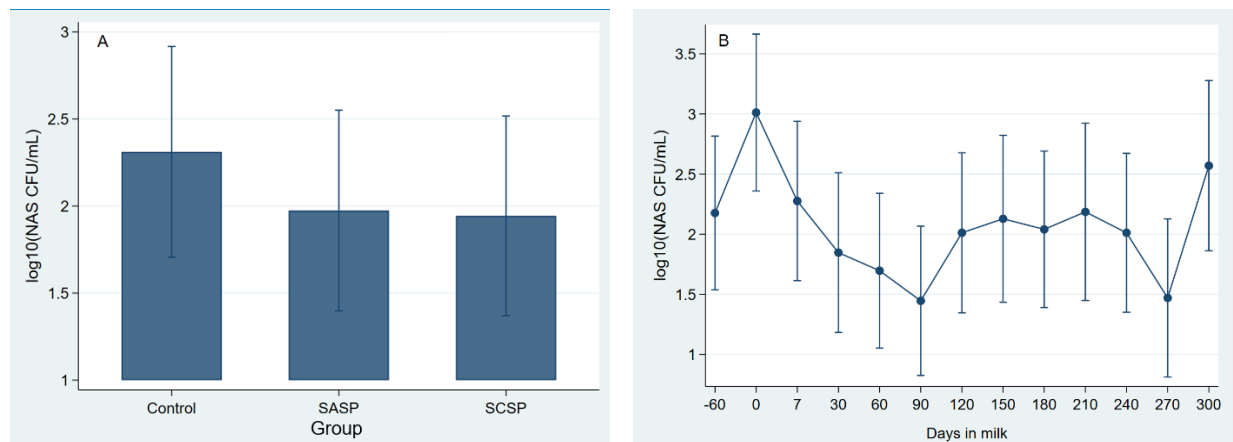


Figure 6. Effect of vaccination on mean counts of non-aureus staphylococci by vaccine group (A) and day (B) over 300 days of lactation (SCSP only). SASP: *Staphylococcus aureus* surface associated proteins; SCSP: *S. chromogenes* surface associated proteins; -60: 60 days before expected calving date; 0: calving day.

Table 3. Isolation and identification of other bacteria from milk samples.

Group	<i>Bacillus</i> spp.	Gram Negative Rod	Gram Positive Rod	Strep. spp.	<i>Coryne- bacterium</i> spp.	Mold	Yeast
SASP	78	12	1	32	5	3	5
SCSP	74	12	1	27	10	3	16
Control	64	13	1	21	6	1	5

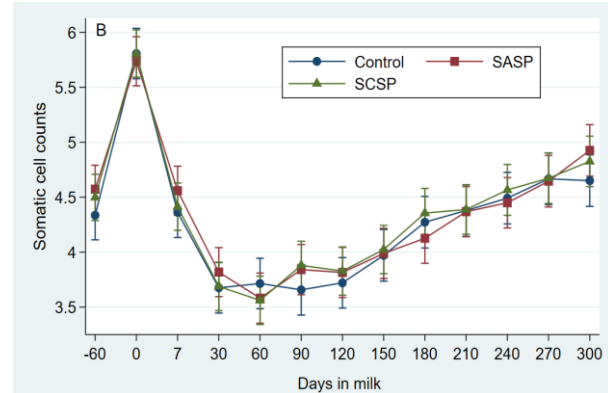
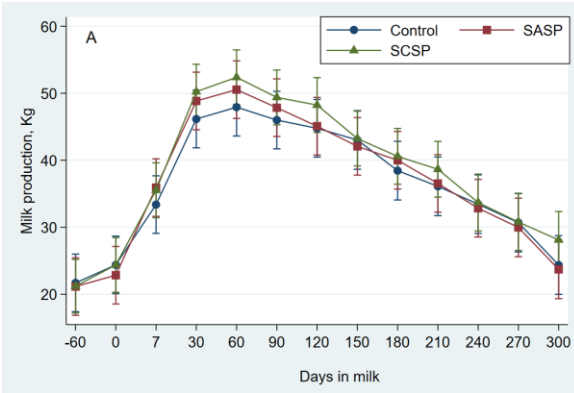


Figure 7. Effects of vaccinations on milk yield (A) and somatic cell counts (B) over 300 days of lactation. SASP: *Staphylococcus aureus* surface associated proteins; SCSP: *S. chromogenes* surface associated proteins; -60: 60 days before expected calving date, 0: calving date.

which 64.3% (n=14) in the control group, 33.3% (n=15) in the SASP group, and 6.3% (n=16) in the SCSP group with a significant (Fisher's exact test $P=0.0031$) vaccination effect. Binary logistic regression analysis showed that vaccination with SCSP significantly ($P=0.005$; OR = 0.04; 95% CI = 0.004-0.4) reduced cow-level incidence of SCM compared to the control group. However, vaccination with SASP did not have a significant ($P=0.101$) effect on the incidence of SCM compared to the control group (Figure 8A). Risk ratio analysis showed that SCSP vaccine significantly ($P=0.018$) reduced the risk of staphylococcal SCM by 10-fold whereas that of SASP was not significant ($P=0.114$). The SCSP showed 90% (95% CI = 30 – 99%) vaccine efficacy with significant protective effect; SASP vaccine had a vaccine efficacy of 48% (95% CI = -17 – 77%), although this was not statistically significant (Table 4). Incidence density was calculated using cow days at risk, beginning at the time of enrollment until either first incidence of staphylococcal SCM, or end of the study, whichever occurs first. The SAS GLIMMIX Procedure was utilized to determine if the incidence densities of SCM differed by vaccine group. Only the SCSP vaccine group had a significant ($P=0.0096$) effect on the incidence density; SASP ($P=0.057$) was not significant. In the control group, we observed 3.8 new cases of SCM per 1000 cow days; in the SASP group, we observed 1.3 new cases of SCM per 1000 cow days; and in the SCSP group, we observed 0.2 new cases of SCM per 1000 cow days (Table 5).

3.7.2 Quarter-level clinical and subclinical staphylococcal mastitis

Throughout the study time of 300 days in lactation, clinical mastitis caused by *Staphylococcus* species was observed in four quarters in the control (n=2) and the SASP (n=2) groups. The two quarter cases from the same cow in the SASP group were observed on milk collection days, while the other two cases in the control group were observed during the intervals between subsequent monthly milk samplings. Overall, 85 cumulative quarter-level staphylococcal subclinical mastitis incidences were observed during the study. Mixed effects logistic regression model showed that the main effect of vaccination was significantly ($P=0.0005$) associated with quarter-level SCM. Both SASP ($P=0.006$) and SCSP ($P<0.001$) vaccines significantly reduced the incidence of SCM compared to the control group. The cumulative incidence of SCM was 11.6% (control), 2.4% (SASP) and 0.5% (SCSP) by group (Figure 8B). Both the SCSP ($P=0.0007$) and SASP ($P=0.001$) vaccines had a significant effect on the incidence density. In the control group, we observed 22 new cases of SCM per 10,000 quarter days; in the SASP group, we observed 4.8

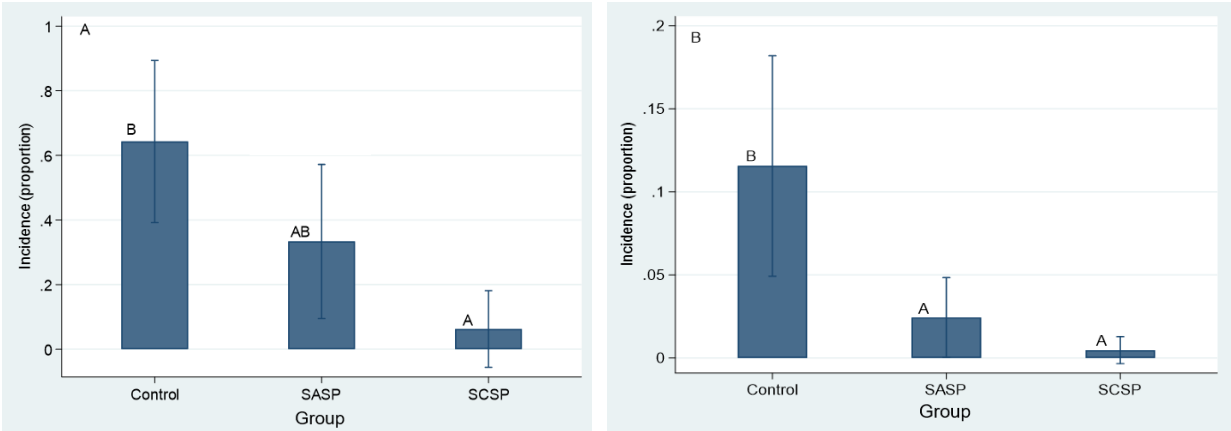


Figure 8. Effect of vaccination on the cumulative incidence of cow-level (A) and quarter-level (B) subclinical mastitis over 300 days of lactation. SASP: *Staphylococcus aureus* surface associated proteins; SCSP: *S. chromogenes* surface associated proteins.

Table 4. Vaccine efficacy

Group	Efficacy	Significance
SASP	48% (-17%, 77%)	P= 0.114
SCSP	90% (30%, 99%)	P= 0.018

Table 5. Incidence density of subclinical mastitis at cow level

Group	New Cases	Significance Compared to Control
Control	3.8 per 1000 cow days	-
SASP	1.3 per 1000 cow days	P = 0.057
SCSP	0.2 per 1000 cow days	P = 0.0096

new cases of SCM per 10,000 quarter days; and in the SCSP group, we observed 0.7 new cases of SCM per 10,000 quarter days (Table 6).

4. Discussion

Vaccine safety was evaluated for both rectal temperature and injection site reaction volume to ensure the reactions were all within normal limits. While there were no differences between vaccinated and control groups in either the rectal temperature or the site reaction volume, there were slight increases in both categories after each vaccination. The increased rectal temperatures and site reaction volumes were expected and followed published literature based on control challenge study utilizing the same vaccines and control formulation (Merrill et al., 2019).

These results are similar to our previous findings where vaccination of dairy cows with SASP and SCSP vaccines induced significantly increased SASP- specific serum IgG2 and SCSP-specific serum IgG1 titers at post-vaccination days during the dry period (Merrill et al., 2019) and during early lactation (Kerro Dego's Lab 2018, non-published data). In line with our results of previous studies, the results of our current study showed that SCSP vaccine conferred better protection compared to SASP vaccine and control group. The incidence rate of staphylococcal mastitis was significantly reduced by SCSP and SASP vaccines at quarter level and only by SCSP vaccine at cow level compared to the control cows. However, detailed close evaluation results showed that in the current study SASP- and SCSP-specific IgG, IgG1, IgG2 and IgA titers in the serum and milk were higher than that of control at almost all time points throughout the 300 days of lactation but significantly higher only at few time points. The overall higher titers indicate a trend of higher titers in the SCSP vaccinated group compared to the control group. These differences could be due to several reasons, which include in the previous efficacy studies, vaccinations were followed by experimental challenges and evaluation of protective effects of vaccine-induced immunity was conducted for a shorter period. In the current study, cows were not challenged but monitored for naturally occurring IMI infections post-vaccination over 300 days of lactation. One of the two previous studies was conducted during the dry period, whereas the other previous study was conducted during early lactation (Merrill et al., 2019).

Both the SASP and SCSP vaccines were previously evaluated in an early dry period challenge study which found both produced immunogenic effects with the SCSP vaccinated group eliciting higher protective effects against the control compared to the SASP group against the

Table 6. Incidence density of subclinical mastitis at quarter level

Group	New Cases	Significance Compared to Control
Control	22 per 10,000 quarter days	-
SASP	5 per 10,000 quarter days	P= 0.001
SCSP	0.7 per 10,000 quarter days	P= 0.0007

control (Merrill et al., 2019). While there is an observed protective effect amongst the vaccinated groups compared to the control group, the type of immune response is unknown. Potentially with cytokine analysis, a more definitive determination of cytokine expression and subsequent protective effects may be determined along with type of immune response, cell mediated (Th1), antibody mediated (Th2), or a combination of both immune responses. Similarly, the commercialized polyvalent bacterin staphylococcal showed some protective effects during a controlled challenge study; however, the route of protection is also unknown (Piepers et al., 2017). While the commercialized polyvalent bacterin staphylococcal vaccine was a challenge study compared to the current natural exposure study, both studies found a decreased inflammatory response in vaccinated cows in comparison to the control cows (Piepers et al., 2017). In contrast to the Peipers et al. study, the current study expressed no difference in the serum anti-SCSP and serum anti-SASP IgG1 titers (Th2 immune response) in the vaccinated group compared to the control group. The serum anti-SCSP and serum anti-SASP IgG2 titer levels (Th1 immune response) were significantly increased compared to the control cows, similar to the previous SCSP and SASP challenge study and the commercialized polyvalent bacterin staphylococcal vaccine (Piepers et al., 2017, Merrill et al., 2019). The significant serum anti-SCSP and serum anti-SASP IgG2 titers suggests the Th1 immune response may be the cause of the reduction of subclinical staphylococcal mastitis among the SCSP group at a cow level and the subclinical staphylococcal mastitis among the SCSP and the SASP groups at quarter level compared to the control group.

In this study, initial prevalence of staphylococcal bacteria was relatively low for *S. aureus* at a cow level and majority of the cows displayed a high prevalence of NAS in at least one quarter. The total colony counts of NAS across treatment groups were high in both vaccinated groups as well as the control group, coinciding with findings from studies performed on the distribution of NAS on United States and Canadian dairy farms (Jenkins et al., 2019, Wuytack et al., 2020). The number of *S. aureus* naturally causing infection in the herd was low and ranged below 10%. Number of *S. aureus* isolates were too low to run statistical analysis on them. Still, there is no significant difference in the count of non-*aureus* staphylococci among SCSP, SASP, and control groups. Contrary to our observation, Schukken et al. (Schukken et al., 2014) vaccinated dairy cows with the commercialized polyvalent bacterin staphylococcal c and post-vaccination monitoring showed that the number of *S. aureus* and NAS count in milk decreased significantly leading to a reduced severity of staphylococcal IMI as well as diminished spread in the herd and

subsequently lower incidence of new cases. Even though our vaccines are different, the number of animals in the study was larger in the Schukken et al. (2014) study compared to ours, which were less than 20 cows per group. The difference in number of animals per group are most likely responsible for the observed differences in addition to the vaccine being a bacterin in the aforementioned study compared to our surface associated protein vaccines.

There was no difference in milk production among groups in the current study which did not align with Merrill et al. (2019) observation utilizing the same SCSP and SASP vaccines for milk production analysis. Due to the nature of a natural exposure study, the prevalence of staphylococcal bacteria and number of cows in the study may have influenced the significant association of SCC and milk production among groups. Similar to the lack of identification of clinical mastitis cases, future studies may employ more animals in a dairy farm environment with a higher prevalence of staphylococcal bacteria in order to clarify findings.

Throughout the study, the SCC was not significantly different between any groups, although the SCC followed an expected trend along a lactation curve (Madouasse et al., 2012). Milk production did not differ significantly among groups, but similarly to SCC milk production followed the expected lactation curve for dairy cows (Lee et al., 2020). The high level of SCC at calving, regardless of the group should be noted that SCC was high due to the natural physiology of the dairy cow and the colostrum sample used for SCC naturally has very high somatic cell count (Pandey et al., 2021). Neither of the vaccines were definitively linked to decreased SCC nor decreased milk production losses with increased antibody titers from any commercially available staphylococcal vaccine and subsequent significant protection against staphylococcal pathogens (Middleton et al., 2006, Schukken et al., 2014). Similar to some of these observations, our current study showed no significant difference in somatic cell counts, and milk yield between SCSP and SASP vaccinated cows and unvaccinated control cows. Looking at staphylococcal clinical mastitis cases developed throughout the study, too few incidences occurred in total to compare to any other published studies. However, subclinical staphylococcal mastitis incidence was able to be overall identified between groups. The SCSP vaccinated group had significantly lower incidence of subclinical Staphylococcal mastitis at cow level compared to the control groups, similar to a previous studies in a controlled experimental challenge (Merrill et al., 2019). The incidence was further divided into quarter level subclinical mastitis resulting in both the SCSP and SASP vaccinated groups having lower incidence of staphylococcal subclinical mastitis similar to the

challenge study using the same vaccines in which the SCSP vaccinated group reduced incidence of subclinical and clinical cases of staphylococcal mastitis (Merrill et al., 2019). Overall, increasing the number of animals in the study may be beneficial in determining a difference in clinical mastitis cases among groups. Additionally, utilizing a farm with a higher prevalence of staphylococcal mastitis cases may help to access the efficacy of SCSP and SASP vaccines.

Aside from the commercially available polyvalent bacterin staphylococcal vaccine, the other vaccine commercially available is a bacterin staphylococcal marketed for the reduction of staphylococcal mastitis in dairy cattle. The bacterin staphylococcal vaccine is akin to the polyvalent bacterin staphylococcal vaccine in the formulation uses inactivated strain whole cell technology. Multiple studies published contradictory findings with a study by Nickerson et al. showing that boosters of the bacterin staphylococcal vaccine every six months beginning at six months of age until calving reduced the incidence of non-*aureus* Staphylococcus species (NAS) (Nickerson et al., 1999). Similarly, the bacterin staphylococcal vaccine was found to decrease incidence of *S. aureus* intramammary infection at calving compared to control cows (Nickerson et al., 2008). Another study performed by Middleton et al. found, during a controlled challenged study, the group of heifers vaccinated the bacterin staphylococcal vaccine expressed no protection against *S. aureus* compared to the control group (Middleton et al., 2006).

Like incidence evaluation during the study, incidence density was calculated for the rate at which each of the vaccinated groups developed staphylococcal subclinical mastitis. At cow level, the SCSP vaccinated group developed subclinical mastitis significantly slowly than the control. The rate at which staphylococcal subclinical mastitis develops at a quarter level was significantly slower in the SCSP and SASP vaccinated group compared to the control, which is a unique finding from the controlled challenge study that did not take place over the same amount of time. The current study evaluated development of staphylococcal mastitis over 300 days of lactation providing results for the staphylococcal surface protein vaccines that have yet to be found. Overall, the efficacy of the SCSP vaccine was found to have a significant efficacy coinciding with the controlled challenge study utilizing the same vaccine (Merrill et al., 2019).

Results of several efficacy studies on commercial staphylococcal mastitis vaccines showed that vaccination of dairy cows with these commercial vaccines increased serum and milk antibody titers and subsequent challenge infection with *S. aureus* reduced inflammatory response, decreased milk production losses, especially in primiparous cows compared to multiparous cows, but neither

vaccine significantly exhibited dependable protection against staphylococcal IMI (Middleton et al., 2006, Nickerson et al., 2008, Schukken et al., 2014). However, unlike most of these previous efficacy studies, our results showed that the SCSP vaccine significantly reduced the incidence and incidence rate of subclinical staphylococcal mastitis at cow and quarter levels.

Due to the commercially available vaccines utilizing Bacterin technology, the whole cell of a *S. aureus* strain is used when developing the vaccine. While there was shown to be an immune response in some studies for both commercially available bacterin staphylococcal vaccine, the reduction in staphylococcal mastitis incidence was not achieved (Middleton et al., 2006, Middleton et al., 2009, Schukken et al., 2014). In order to optimize an effective vaccine, a smaller scope needs to be utilized while still maintaining a broad enough spectrum to elicit immune responses and subsequent protective effects in dairy cows. The SCSP and SASP vaccines may have the ability to provide protective effects with further identification and evaluation of specific surface associated proteins. Additionally, potential optimization of the adjuvant by choosing a different dosage or even another adjuvant may prove to be beneficially in eliciting a stronger immune response. Due to complete lack of independent trials for the SCSP or SASP vaccines, treatment groups may behave similarly in titer levels, SCC, milk yield, and other areas able to be evaluated. Overall, future studies need to be completed using a larger cohort of cows in a more heavily *S. aureus* prevalent farm vaccinated with more closely identified proteins from the SCSP and SASP groups to clarify further the potential protective effects expressed within this study.

Conclusions

Vaccination of dairy cows with *Staphylococcus aureus* surface associated proteins (SASP) and *Staphylococcus chromogenes* surface associated proteins (SCSP) vaccines during dry period induced significantly increased SASP- and SCSP-specific antibody titers in serum and milk of vaccinated cows compared to control cows during lactation. Subsequent monitoring of vaccinated and control cows for staphylococcal mastitis due to natural exposure to the staphylococci over 300 days of lactation showed that the incidence of subclinical staphylococcal mastitis was significantly reduced at quarter level by both vaccines and cow level by SCSP vaccine. The duration of immunity was about four months. The SCSP vaccine conferred more protection than SASP vaccine. The optimization of vaccination regimen (dose, adjuvant, route, and

time of vaccination) may further improve these vaccines efficacy for the control of staphylococcal mastitis in dairy cows.

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Appendices

Appendix 1

Table 7. Summary comparing vaccination effects on cow level subclinical mastitis

Measure	Group		
	1 (SASP)	2 (SCSP)	3 (Control)
Absolute risk, %	33.3 (9.5, 57.2)	6.3 (0,18.1)	64.3 (43.5, 95.0)
Odds ratio, compared to control	0.28 (0.06,1.3)	0.04 (0.004, 0.37)	1
Risk difference, control-vaccine group	-0.31 (-0.66, -0.04)	-0.58 (-0.86, -0.30)	0
Risk ratio, relative to control	0.52 (0.23, 1.17)	0.1 (0.01, 0.7)	1
Vaccine efficacy, relative to control	0.48 (-0.17, 0.77)	0.9 (0.3, 0.99)	0
Incidence density ratio, new cases/1000 cows	1.3 (0.52, 3.2)	0.2 (0.03, 1.6)	3.8 (1.9, 7.5)

Appendix 2

Table 8. Table for pairwise comparison of treatments by day

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SASP	IgA	(SASP#C0) vs (SASP#D0)	-0.4951686	0
Blood	SASP	IgA	(SASP#C0) vs (SASP#D21)	-0.5111023	0
Blood	SASP	IgA	(SASP#C120) vs (SASP#C0)	0.654187	0
Blood	SASP	IgA	(SASP#C150) vs (SASP#C0)	0.5131909	0
Blood	SASP	IgA	(SASP#C210) vs (SASP#C0)	0.517548	0
Blood	SASP	IgA	(SASP#C240) vs (SASP#C0)	0.482456	0
Blood	SASP	IgA	(SASP#C270) vs (SASP#C0)	0.6521881	0
Blood	SASP	IgA	(SASP#C300) vs (SASP#C0)	0.7275492	0
Blood	SASP	IgA	(SCSP#C0) vs (SCSP#D0)	-0.9060377	0
Blood	SASP	IgA	(SCSP#C0) vs (SCSP#D21)	-0.7614697	0
Blood	SASP	IgA	(SCSP#C0) vs (SCSP#D42)	-0.6175292	0
Blood	SASP	IgA	(SCSP#C30) vs (SCSP#C0)	0.5149814	0
Blood	SASP	IgA	(SCSP#C60) vs (SCSP#C0)	0.6266104	0
Blood	SASP	IgA	(SCSP#C90) vs (SCSP#C0)	0.4685705	0
Blood	SASP	IgA	(SCSP#C120) vs (SCSP#C0)	0.532314	0
Blood	SASP	IgA	(SCSP#C150) vs (SCSP#C0)	0.7220597	0
Blood	SASP	IgA	(SCSP#C180) vs (SCSP#C0)	0.6785045	0
Blood	SASP	IgA	(SCSP#C210) vs (SCSP#C0)	0.685975	0
Blood	SASP	IgA	(SCSP#C240) vs (SCSP#C0)	0.6960354	0
Blood	SASP	IgA	(SCSP#C270) vs (SCSP#C0)	0.7931784	0

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SASP	IgA	(SCSP#C300) vs (SCSP#C0)	0.7673278	0
Blood	SASP	IgA	(SASP#C30) vs (SASP#C0)	0.4379224	0.002
Blood	SASP	IgA	(SCSP#C90) vs (SCSP#D0)	-0.4374672	0.002
Blood	SASP	IgA	(SASP#C300) vs (SASP#C60)	0.4340544	0.011
Blood	SASP	IgA	(SASP#C180) vs (SASP#C0)	0.4121583	0.015
Blood	SASP	IgA	(SCSP#C30) vs (SCSP#D0)	-0.3910563	0.015
Blood	SASP	IgA	(SCSP#C120) vs (SCSP#D0)	-0.3737237	0.034
Blood	SASP	IgA	(SASP#C300) vs (SASP#D42)	0.4019917	0.046
Blood	SASP	IgG	(SASP#C150) vs (Control#C150)	0.7793667	0
Blood	SASP	IgG	(SASP#D21) vs (SASP#D0)	1.219789	0
Blood	SASP	IgG	(SASP#D42) vs (SASP#D0)	0.833516	0
Blood	SASP	IgG	(SASP#C0) vs (SASP#D0)	1.002466	0
Blood	SASP	IgG	(SASP#C30) vs (SASP#D0)	0.9315824	0
Blood	SASP	IgG	(SASP#C60) vs (SASP#D0)	0.783191	0
Blood	SASP	IgG	(SASP#C90) vs (SASP#D0)	0.5778918	0

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SASP	IgG	(SASP#C120) vs (SASP#D0)	0.5809762	0
Blood	SASP	IgG	(SASP#C150) vs (SASP#D0)	1.126387	0
Blood	SASP	IgG	(SASP#C180) vs (SASP#D0)	0.6990528	0
Blood	SASP	IgG	(SASP#C210) vs (SASP#D0)	0.8491623	0
Blood	SASP	IgG	(SASP#C240) vs (SASP#D0)	0.6859772	0
Blood	SASP	IgG	(SASP#C270) vs (SASP#D0)	0.8646967	0
Blood	SASP	IgG	(SASP#C300) vs (SASP#D0)	0.826953	0
Blood	SASP	IgG	(SASP#C90) vs (SASP#D21)	-0.6418972	0
Blood	SASP	IgG	(SASP#C120) vs (SASP#D21)	-0.6388127	0
Blood	SASP	IgG	(SASP#C180) vs (SASP#D21)	-0.5207361	0
Blood	SASP	IgG	(SASP#C240) vs (SASP#D21)	-0.5338117	0
Blood	SASP	IgG	(SASP#C150) vs (SASP#C90)	0.5484951	0
Blood	SASP	IgG	(SASP#C150) vs (SASP#C120)	0.5454107	0
Blood	SASP	IgG	(SCSP#D21) vs (SCSP#D0)	1.148917	0

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SASP	IgG	(SCSP#D42) vs (SCSP#D0)	0.8625515	0
Blood	SASP	IgG	(SCSP#C0) vs (SCSP#D0)	0.82247	0
Blood	SASP	IgG	(SCSP#C30) vs (SCSP#D0)	0.8553872	0
Blood	SASP	IgG	(SCSP#C60) vs (SCSP#D0)	0.7566354	0
Blood	SASP	IgG	(SCSP#C90) vs (SCSP#D0)	0.7260402	0
Blood	SASP	IgG	(SCSP#C150) vs (SCSP#D0)	0.6955676	0
Blood	SASP	IgG	(SCSP#C180) vs (SCSP#D0)	0.5954989	0
Blood	SASP	IgG	(SCSP#C210) vs (SCSP#D0)	0.6650189	0
Blood	SASP	IgG	(SCSP#C240) vs (SCSP#D0)	0.7413755	0
Blood	SASP	IgG	(SCSP#C270) vs (SCSP#D0)	0.8976834	0
Blood	SASP	IgG	(SCSP#C300) vs (SCSP#D0)	0.7167742	0
Blood	SASP	IgG	(SCSP#C120) vs (SCSP#D21)	-0.7219959	0
Blood	SASP	IgG	(SCSP#C180) vs (SCSP#D21)	-0.5534176	0
Blood	SASP	IgG	(SCSP#C210) vs (SCSP#D21)	-0.4838976	0.001
Blood	SASP	IgG	(SCSP#C150) vs (SCSP#D21)	-0.4533489	0.002
Blood	SASP	IgG	(SCSP#C270) vs (SCSP#C120)	0.4707628	0.003

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SASP	IgG	(SASP#C270) vs (Control#C270)	0.5681343	0.004
Blood	SASP	IgG	(SCSP#C120) vs (SCSP#D42)	-0.4356309	0.004
Blood	SASP	IgG	(SCSP#C120) vs (SCSP#D0)	0.4269206	0.005
Blood	SASP	IgG	(SASP#C60) vs (SASP#D21)	-0.4365979	0.007
Blood	SASP	IgG	(SASP#C240) vs (SASP#C150)	-0.4404097	0.008
Blood	SASP	IgG	(SCSP#C120) vs (SCSP#C30)	-0.4284666	0.008
Blood	SASP	IgG	(SCSP#C90) vs (SCSP#D21)	-0.4228763	0.009
Blood	SASP	IgG	(SCSP#C270) vs (Control#C270)	0.5406755	0.011
Blood	SASP	IgG	(SCSP#C300) vs (SCSP#D21)	-0.4321423	0.011
Blood	SASP	IgG	(SASP#C90) vs (SASP#C0)	-0.4245744	0.013
Blood	SASP	IgG	(SASP#C180) vs (SASP#C150)	-0.4273341	0.017
Blood	SASP	IgG	(SASP#C120) vs (SASP#C0)	-0.4214899	0.022
Blood	SASP	IgG	(SCSP#C60) vs (SCSP#D21)	-0.3922811	0.028
Blood	SASP	IgG	(SCSP#C120) vs (SCSP#C0)	-0.3955494	0.028

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SASP	IgG	(SCSP#C150) vs (SASP#C150)	-0.4912648	0.031
Blood	SASP	IgG	(SCSP#C240) vs (SCSP#D21)	-0.407541	0.032
Blood	SASP	IgG	(SASP#C0) vs (Control#C0)	0.4807476	0.037
Blood	SASP	IgG	(SASP#D42) vs (SASP#D21)	-0.3862729	0.048
Blood	SASP	IgG1	(SASP#D21) vs (SASP#D0)	0.5981916	0
Blood	SASP	IgG1	(SASP#D42) vs (SASP#D0)	0.6846472	0
Blood	SASP	IgG1	(SASP#C30) vs (SASP#D0)	0.8500502	0
Blood	SASP	IgG1	(SASP#C60) vs (SASP#D0)	0.653973	0
Blood	SASP	IgG1	(SASP#C90) vs (SASP#D0)	0.9072621	0
Blood	SASP	IgG1	(SASP#C150) vs (SASP#D0)	1.024601	0
Blood	SASP	IgG1	(SASP#C180) vs (SASP#D0)	0.6273525	0
Blood	SASP	IgG1	(SASP#C240) vs (SASP#D0)	0.7716195	0
Blood	SASP	IgG1	(SASP#C270) vs (SASP#D0)	0.751867	0
Blood	SASP	IgG1	(SASP#C300) vs (SASP#D0)	0.6625156	0

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SASP	IgG1	(SASP#C90) vs (SASP#C0)	0.5977425	0
Blood	SASP	IgG1	(SASP#C150) vs (SASP#C0)	0.7150814	0
Blood	SASP	IgG1	(SCSP#D21) vs (SCSP#D0)	0.768624	0
Blood	SASP	IgG1	(SCSP#C30) vs (SCSP#D0)	0.5604627	0
Blood	SASP	IgG1	(SCSP#C60) vs (SCSP#D0)	0.8248157	0
Blood	SASP	IgG1	(SCSP#C90) vs (SCSP#D0)	0.6612447	0
Blood	SASP	IgG1	(SCSP#C150) vs (SCSP#D0)	0.7202161	0
Blood	SASP	IgG1	(SCSP#C210) vs (SCSP#D0)	0.7001483	0
Blood	SASP	IgG1	(SCSP#C240) vs (SCSP#D0)	0.6459432	0
Blood	SASP	IgG1	(SCSP#C270) vs (SCSP#D0)	0.6548344	0
Blood	SASP	IgG1	(SCSP#C0) vs (SCSP#D21)	-0.2757261	0
Blood	SASP	IgG1	(SCSP#C30) vs (SCSP#C0)	0.5962426	0
Blood	SASP	IgG1	(SCSP#C60) vs (SCSP#C0)	0.8605957	0
Blood	SASP	IgG1	(SCSP#C90) vs (SCSP#C0)	0.6970247	0
Blood	SASP	IgG1	(SCSP#C150) vs (SCSP#C0)	0.7559961	0
Blood	SASP	IgG1	(SCSP#C210) vs (SCSP#C0)	0.7359283	0
Blood	SASP	IgG1	(SCSP#C240) vs (SCSP#C0)	0.6817232	0

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SASP	IgG1	(SCSP#C270) vs (SCSP#C0)	0.6906144	0
Blood	SASP	IgG1	(SASP#C30) vs (SASP#C0)	0.5405306	0.001
Blood	SASP	IgG1	(SASP#C210) vs (SASP#C150)	-0.2532087	0.001
Blood	SASP	IgG1	(SCSP#C0) vs (SCSP#D42)	-0.5286779	0.001
Blood	SASP	IgG1	(SCSP#C180) vs (SCSP#C0)	0.541684	0.001
Blood	SASP	IgG1	(SCSP#D42) vs (SCSP#D0)	0.4928979	0.003
Blood	SASP	IgG1	(SCSP#C180) vs (SCSP#D0)	0.505904	0.004
Blood	SASP	IgG1	(SASP#C120) vs (SASP#D0)	0.5293671	0.006
Blood	SASP	IgG1	(SCSP#C120) vs (SCSP#C0)	0.4939031	0.006
Blood	SASP	IgG1	(SASP#C150) vs (SASP#C120)	0.5151843	0.009
Blood	SASP	IgG1	(SASP#C150) vs (Control#C150)	0.5825436	0.012
Blood	SASP	IgG1	(SCSP#C300) vs (SCSP#C0)	0.4941995	0.015
Blood	SASP	IgG1	(SCSP#C120) vs (SCSP#D0)	0.4581231	0.02
Blood	SASP	IgG1	(SASP#C210) vs (SASP#D0)	0.7713923	0.027

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SASP	IgG1	(SCSP#C300) vs (SCSP#D0)	0.4584196	0.044
Blood	SASP	IgG2	(SASP#D21) vs (SASP#D0)	0.7344909	0
Blood	SASP	IgG2	(SASP#D42) vs (SASP#D0)	0.7749559	0
Blood	SASP	IgG2	(SASP#C0) vs (SASP#D0)	0.4786161	0
Blood	SASP	IgG2	(SASP#C30) vs (SASP#D0)	0.662559	0
Blood	SASP	IgG2	(SASP#C60) vs (SASP#D0)	0.6347215	0
Blood	SASP	IgG2	(SASP#C150) vs (SASP#D0)	0.7684562	0
Blood	SASP	IgG2	(SASP#C180) vs (SASP#D0)	0.7439601	0
Blood	SASP	IgG2	(SASP#C210) vs (SASP#D0)	0.4243587	0
Blood	SASP	IgG2	(SASP#C240) vs (SASP#D0)	0.4509234	0
Blood	SASP	IgG2	(SASP#C270) vs (SASP#D0)	0.677948	0
Blood	SASP	IgG2	(SASP#C120) vs (SASP#D21)	-0.5973691	0
Blood	SASP	IgG2	(SASP#C120) vs (SASP#D42)	-0.6378341	0
Blood	SASP	IgG2	(SASP#C120) vs (SASP#C30)	-0.5254373	0

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SASP	IgG2	(SASP#C120) vs (SASP#C60)	-0.4975997	0
Blood	SASP	IgG2	(SASP#C150) vs (SASP#C120)	0.6313344	0
Blood	SASP	IgG2	(SASP#C180) vs (SASP#C120)	0.6068384	0
Blood	SASP	IgG2	(SASP#C270) vs (SASP#C120)	0.5408262	0
Blood	SASP	IgG2	(SCSP#C0) vs (SCSP#D42)	-0.4790419	0
Blood	SASP	IgG2	(SCSP#C180) vs (SCSP#C0)	0.4471607	0
Blood	SASP	IgG2	(SCSP#C270) vs (SCSP#C0)	0.4762879	0
Blood	SASP	IgG2	(SASP#C300) vs (SASP#D0)	0.4145276	0.001
Blood	SASP	IgG2	(SCSP#C0) vs (SCSP#D21)	-0.3838901	0.001
Blood	SASP	IgG2	(SASP#C150) vs (Control#C150)	0.5306218	0.002
Blood	SASP	IgG2	(SASP#C90) vs (SASP#D42)	-0.3933612	0.003
Blood	SASP	IgG2	(SASP#C90) vs (SASP#D0)	0.3815947	0.004
Blood	SASP	IgG2	(SCSP#C150) vs (SCSP#C0)	0.3808358	0.004
Blood	SASP	IgG2	(SASP#C0) vs (Control#C0)	0.4829335	0.005
Blood	SASP	IgG2	(SCSP#C300) vs (SCSP#C0)	0.053839	0.005

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SASP	IgG2	(SASP#C150) vs (SASP#C90)	0.158049	0.007
Blood	SASP	IgG2	(SCSP#C60) vs (SCSP#C0)	0.3521384	0.014
Blood	SASP	IgG2	(SASP#C180) vs (SASP#C90)	0.2243739	0.021
Blood	SASP	IgG2	(SASP#C90) vs (SASP#D21)	-0.3528962	0.026
Blood	SASP	IgG2	(SASP#C300) vs (SASP#D42)	-0.3604282	0.026
Blood	SASP	IgG2	(SASP#D42) vs (Control#D42)	0.4428154	0.028
Blood	SASP	IgG2	(SASP#C210) vs (SASP#D42)	-0.3505972	0.029
Blood	SASP	IgG2	(SCSP#C120) vs (SCSP#D42)	-0.3358169	0.037
Blood	SASP	IgG2	(SCSP#C30) vs (SCSP#C0)	0.3325967	0.039
Blood	SASP	IgG2	(SCSP#C240) vs (SCSP#C0)	0.013247	0.043
Blood	SCSP	IgA	(SASP#C150) vs (SASP#D21)	0.6507739	0
Blood	SCSP	IgA	(SASP#C240) vs (SASP#D21)	0.5624333	0
Blood	SCSP	IgA	(SASP#C270) vs (SASP#D21)	0.5340656	0
Blood	SCSP	IgA	(SASP#C300) vs (SASP#D21)	0.6368808	0
Blood	SCSP	IgA	(SCSP#C270) vs (SCSP#D42)	0.5039327	0

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SCSP	IgA	(SASP#C180) vs (SASP#D21)	0.4820686	0.001
Blood	SCSP	IgA	(SCSP#C210) vs (SCSP#D42)	0.4775859	0.001
Blood	SCSP	IgA	(SCSP#C270) vs (SCSP#D21)	0.4539866	0.003
Blood	SCSP	IgA	(SASP#C150) vs (SASP#C60)	0.4547746	0.004
Blood	SCSP	IgA	(SCSP#C270) vs (SCSP#D0)	0.4465953	0.004
Blood	SCSP	IgA	(SCSP#C180) vs (SCSP#D42)	0.4458603	0.004
Blood	SCSP	IgA	(SCSP#C210) vs (SCSP#D21)	0.4276398	0.006
Blood	SCSP	IgA	(SASP#C150) vs (SASP#D0)	0.4274791	0.009
Blood	SCSP	IgA	(SASP#C150) vs (SASP#D42)	0.4299339	0.009
Blood	SCSP	IgA	(SCSP#C210) vs (SCSP#D0)	0.4202485	0.009
Blood	SCSP	IgA	(SASP#C300) vs (SASP#C60)	0.4408815	0.012
Blood	SCSP	IgA	(SCSP#C270) vs (SCSP#C0)	0.4148882	0.022
Blood	SCSP	IgA	(SASP#C300) vs (SASP#D0)	0.4135859	0.026
Blood	SCSP	IgA	(SASP#C300) vs (SASP#D42)	0.4160408	0.027

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SCSP	IgA	(SASP#C210) vs (SASP#D21)	0.4028373	0.032
Blood	SCSP	IgA	(SCSP#C180) vs (SCSP#D21)	0.3959143	0.033
Blood	SCSP	IgA	(SASP#C150) vs (SASP#C30)	0.4014992	0.034
Blood	SCSP	IgA	(SCSP#C180) vs (SCSP#D0)	0.388523	0.045
Blood	SCSP	IgA	(SCSP#C210) vs (SCSP#C0)	0.3885414	0.047
Blood	SCSP	IgG	(SASP#C0) vs (Control#C0)	0.7331524	0
Blood	SCSP	IgG	(SASP#C0) vs (SASP#D0)	0.6852841	0
Blood	SCSP	IgG	(SASP#C180) vs (SASP#D0)	0.6102889	0
Blood	SCSP	IgG	(SASP#C0) vs (SASP#D42)	0.6842114	0
Blood	SCSP	IgG	(SASP#C180) vs (SASP#D42)	0.6092162	0
Blood	SCSP	IgG	(SASP#C90) vs (SASP#C0)	-0.7955473	0
Blood	SCSP	IgG	(SASP#C120) vs (SASP#C0)	-0.7191563	0
Blood	SCSP	IgG	(SASP#C210) vs (SASP#C0)	-0.6298346	0
Blood	SCSP	IgG	(SASP#C240) vs (SASP#C0)	-0.52614	0
Blood	SCSP	IgG	(SASP#C270) vs (SASP#C0)	-0.5161251	0

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SCSP	IgG	(SASP#C150) vs (SASP#C90)	0.5238033	0
Blood	SCSP	IgG	(SASP#C180) vs (SASP#C90)	0.720552	0
Blood	SCSP	IgG	(SASP#C180) vs (SASP#C120)	0.6441611	0
Blood	SCSP	IgG	(SASP#C210) vs (SASP#C180)	-0.5548394	0
Blood	SCSP	IgG	(SCSP#C150) vs (SCSP#D0)	0.5546377	0
Blood	SCSP	IgG	(SCSP#C180) vs (SCSP#D0)	0.5884074	0
Blood	SCSP	IgG	(SCSP#C150) vs (SCSP#C90)	0.4931967	0
Blood	SCSP	IgG	(SCSP#C180) vs (SCSP#C90)	0.5269663	0
Blood	SCSP	IgG	(SCSP#C150) vs (SCSP#C120)	0.5336167	0
Blood	SCSP	IgG	(SCSP#C180) vs (SCSP#C120)	0.5673864	0
Blood	SCSP	IgG	(SCSP#C210) vs (SCSP#C150)	-0.5093368	0
Blood	SCSP	IgG	(SCSP#C210) vs (SCSP#C180)	-0.5431065	0
Blood	SCSP	IgG	(SASP#C0) vs (SASP#D21)	0.4638605	0.001
Blood	SCSP	IgG	(SASP#C60) vs (SASP#C0)	-0.4792699	0.001

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SCSP	IgG	(SASP#C240) vs (SASP#C180)	-0.4511447	0.004
Blood	SCSP	IgG	(SASP#C150) vs (SASP#C120)	0.4474123	0.005
Blood	SCSP	IgG	(SASP#C270) vs (SASP#C180)	-0.4411299	0.01
Blood	SCSP	IgG	(SASP#C150) vs (SASP#D0)	0.4135402	0.014
Blood	SCSP	IgG	(SASP#C30) vs (SASP#C0)	-0.4098111	0.014
Blood	SCSP	IgG	(SASP#C150) vs (SASP#D42)	0.4124674	0.018
Blood	SCSP	IgG	(SCSP#C180) vs (SCSP#C30)	0.4098695	0.025
Blood	SCSP	IgG	(SCSP#C60) vs (Control#C60)	0.5049463	0.026
Blood	SCSP	IgG	(SASP#C30) vs (Control#C30)	0.5053767	0.029
Blood	SCSP	IgG	(SCSP#C30) vs (Control#C30)	0.5012193	0.032
Blood	SCSP	IgG	(SASP#C180) vs (SASP#C60)	0.4042747	0.035
Blood	SCSP	IgG	(SCSP#C180) vs (SCSP#D21)	0.3903893	0.036
Blood	SCSP	IgG1	(SASP#C0) vs (SASP#D0)	-0.6806992	0
Blood	SCSP	IgG1	(SASP#C150) vs (SASP#D21)	0.7221752	0

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SCSP	IgG1	(SASP#C180) vs (SASP#D21)	0.5588329	0
Blood	SCSP	IgG1	(SASP#C270) vs (SASP#D21)	0.6573764	0
Blood	SCSP	IgG1	(SASP#C0) vs (SASP#D42)	-0.6510747	0
Blood	SCSP	IgG1	(SASP#C120) vs (SASP#C0)	0.5057687	0
Blood	SCSP	IgG1	(SASP#C150) vs (SASP#C0)	1.132236	0
Blood	SCSP	IgG1	(SASP#C180) vs (SASP#C0)	0.9688937	0
Blood	SCSP	IgG1	(SASP#C210) vs (SASP#C0)	0.7575945	0
Blood	SCSP	IgG1	(SASP#C240) vs (SASP#C0)	0.8027315	0
Blood	SCSP	IgG1	(SASP#C270) vs (SASP#C0)	1.067437	0
Blood	SCSP	IgG1	(SASP#C300) vs (SASP#C0)	0.8691931	0
Blood	SCSP	IgG1	(SASP#C150) vs (SASP#C30)	0.6166809	0
Blood	SCSP	IgG1	(SASP#C180) vs (SASP#C30)	0.5347564	0
Blood	SCSP	IgG1	(SASP#C270) vs (SASP#C30)	0.5561134	0
Blood	SCSP	IgG1	(SASP#C150) vs (SASP#C60)	0.6343294	0

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SCSP	IgG1	(SASP#C270) vs (SASP#C60)	0.5695307	0
Blood	SCSP	IgG1	(SASP#C150) vs (SASP#C90)	0.765971	0
Blood	SCSP	IgG1	(SASP#C180) vs (SASP#C90)	0.6026287	0
Blood	SCSP	IgG1	(SASP#C270) vs (SASP#C90)	0.7011722	0
Blood	SCSP	IgG1	(SASP#C150) vs (SASP#C120)	0.6264672	0
Blood	SCSP	IgG1	(SASP#C270) vs (SASP#C120)	0.5616684	0
Blood	SCSP	IgG1	(SCSP#C0) vs (SCSP#D0)	-0.5789502	0
Blood	SCSP	IgG1	(SCSP#C150) vs (SCSP#D21)	0.6433286	0
Blood	SCSP	IgG1	(SCSP#C180) vs (SCSP#D21)	0.561404	0
Blood	SCSP	IgG1	(SCSP#C240) vs (SCSP#D21)	0.5183661	0
Blood	SCSP	IgG1	(SCSP#C270) vs (SCSP#D21)	0.582761	0
Blood	SCSP	IgG1	(SCSP#C0) vs (SCSP#D42)	-0.6614972	0
Blood	SCSP	IgG1	(SCSP#C60) vs (SCSP#C0)	0.6526004	0
Blood	SCSP	IgG1	(SCSP#C90) vs (SCSP#C0)	0.6474172	0
Blood	SCSP	IgG1	(SCSP#C120) vs (SCSP#C0)	0.5303601	0
Blood	SCSP	IgG1	(SCSP#C150) vs (SCSP#C0)	0.9556062	0

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SCSP	IgG1	(SCSP#C180) vs (SCSP#C0)	0.8736816	0
Blood	SCSP	IgG1	(SCSP#C210) vs (SCSP#C0)	0.7603485	0
Blood	SCSP	IgG1	(SCSP#C240) vs (SCSP#C0)	0.8306437	0
Blood	SCSP	IgG1	(SCSP#C270) vs (SCSP#C0)	0.8950386	0
Blood	SCSP	IgG1	(SCSP#C300) vs (SCSP#C0)	0.6517283	0
Blood	SCSP	IgG1	(SASP#C150) vs (SASP#D42)	0.4811613	0.001
Blood	SCSP	IgG1	(SASP#C60) vs (SASP#C0)	0.4979065	0.001
Blood	SCSP	IgG1	(SASP#C300) vs (SASP#C90)	0.5029282	0.001
Blood	SCSP	IgG1	(SCSP#C30) vs (SCSP#C0)	0.4778702	0.001
Blood	SCSP	IgG1	(SCSP#C150) vs (SCSP#C30)	0.477736	0.001
Blood	SCSP	IgG1	(SASP#C180) vs (SASP#C60)	0.4709871	0.003
Blood	SCSP	IgG1	(SASP#C180) vs (SASP#C120)	0.4631249	0.004
Blood	SCSP	IgG1	(SCSP#C210) vs (SCSP#D21)	0.4480709	0.004
Blood	SCSP	IgG1	(SASP#C150) vs (SASP#D0)	0.4515367	0.005

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SCSP	IgG1	(SASP#C300) vs (SASP#D21)	0.4591324	0.006
Blood	SCSP	IgG1	(SASP#C300) vs (SASP#C30)	0.3128031	0.011
Blood	SCSP	IgG1	(SCSP#C150) vs (SCSP#C120)	0.425246	0.011
Blood	SCSP	IgG1	(SASP#C30) vs (SASP#C0)	0.4242625	0.012
Blood	SCSP	IgG1	(SASP#C240) vs (SASP#C90)	0.4364666	0.014
Blood	SCSP	IgG1	(SASP#C0) vs (SASP#D21)	-0.4100608	0.023
Blood	SCSP	IgG1	(SCSP#C270) vs (SCSP#C30)	0.4171684	0.036
Blood	SCSP	IgG1	(SASP#C270) vs (SASP#D42)	0.4163625	0.042
Blood	SCSP	IgG2	(SASP#C180) vs (SASP#D21)	0.5887628	0
Blood	SCSP	IgG2	(SASP#C270) vs (SASP#D21)	0.623249	0
Blood	SCSP	IgG2	(SASP#C300) vs (SASP#D21)	0.4825676	0
Blood	SCSP	IgG2	(SASP#C120) vs (SASP#C0)	-0.5480358	0
Blood	SCSP	IgG2	(SASP#C180) vs (SASP#C30)	0.463102	0
Blood	SCSP	IgG2	(SASP#C270) vs (SASP#C30)	0.4975882	0

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SCSP	IgG2	(SASP#C180) vs (SASP#C60)	0.5402234	0
Blood	SCSP	IgG2	(SASP#C270) vs (SASP#C60)	0.5747096	0
Blood	SCSP	IgG2	(SASP#C150) vs (SASP#C120)	0.5358036	0
Blood	SCSP	IgG2	(SASP#C180) vs (SASP#C120)	0.7095195	0
Blood	SCSP	IgG2	(SASP#C240) vs (SASP#C120)	0.4676186	0
Blood	SCSP	IgG2	(SASP#C270) vs (SASP#C120)	0.7440057	0
Blood	SCSP	IgG2	(SASP#C300) vs (SASP#C120)	0.6033243	0
Blood	SCSP	IgG2	(SCSP#C180) vs (SCSP#D0)	0.4405642	0
Blood	SCSP	IgG2	(SCSP#C270) vs (SCSP#D0)	0.4643826	0
Blood	SCSP	IgG2	(SCSP#C300) vs (SCSP#D0)	0.4993367	0
Blood	SCSP	IgG2	(SCSP#C120) vs (SCSP#C0)	-0.4676143	0
Blood	SCSP	IgG2	(SCSP#C180) vs (SCSP#C120)	0.4938517	0
Blood	SCSP	IgG2	(SCSP#C270) vs (SCSP#C120)	0.5176701	0
Blood	SCSP	IgG2	(SCSP#C300) vs (SCSP#C120)	0.5526242	0

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SCSP	IgG2	(SASP#C0) vs (SASP#D21)	0.4272792	0.001
Blood	SCSP	IgG2	(SCSP#C0) vs (SCSP#D0)	0.4143268	0.001
Blood	SCSP	IgG2	(SASP#C300) vs (SASP#C60)	0.4340282	0.002
Blood	SCSP	IgG2	(SASP#C210) vs (SASP#C120)	0.4299509	0.002
Blood	SCSP	IgG2	(SASP#C150) vs (SASP#D21)	0.4150469	0.003
Blood	SCSP	IgG2	(SASP#C270) vs (SASP#D0)	0.4162946	0.004
Blood	SCSP	IgG2	(SCSP#D42) vs (Control#D42)	0.5028677	0.007
Blood	SCSP	IgG2	(SASP#C270) vs (SASP#D42)	0.4100813	0.007
Blood	SCSP	IgG2	(SASP#C120) vs (SASP#C90)	-0.1591654	0.008
Blood	SCSP	IgG2	(SCSP#C300) vs (SCSP#D21)	0.3989572	0.008
Blood	SCSP	IgG2	(SCSP#C120) vs (SCSP#C90)	-0.3785167	0.012
Blood	SCSP	IgG2	(SCSP#D21) vs (Control#D21)	0.4821183	0.013
Blood	SCSP	IgG2	(SASP#C180) vs (SASP#D0)	0.3818084	0.013
Blood	SCSP	IgG2	(SASP#C60) vs (SASP#C0)	-0.3787397	0.018

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Blood	SCSP	IgG2	(SCSP#C300) vs (SCSP#C60)	0.390745	0.018
Blood	SCSP	IgG2	(SASP#C180) vs (SASP#D42)	0.3755951	0.021
Blood	SCSP	IgG2	(SCSP#C270) vs (SCSP#D21)	0.3640031	0.043
Blood	SCSP	IgG2	(SASP#C150) vs (SASP#C60)	0.3665075	0.044
Blood	SCSP	IgG2	(SCSP#C300) vs (SCSP#C240)	0.3826417	0.047
Milk	SASP	IgA	(SASP#C0) vs (SASP#D0)	1.865457	0
Milk	SASP	IgA	(SASP#C120) vs (SASP#C0)	-1.661602	0
Milk	SASP	IgA	(SASP#C150) vs (SASP#C0)	-1.781974	0
Milk	SASP	IgA	(SASP#C270) vs (SASP#C0)	-1.603269	0
Milk	SASP	IgA	(SCSP#C0) vs (SCSP#D0)	1.921965	0
Milk	SASP	IgA	(SCSP#C120) vs (SCSP#C0)	-2.021612	0
Milk	SASP	IgA	(SCSP#C150) vs (SCSP#C0)	-2.012275	0
Milk	SASP	IgA	(SCSP#C270) vs (SCSP#C0)	-1.943273	0
Milk	SASP	IgG	(SCSP#C0) vs (Control#C0)	1.539002	0

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Milk	SASP	IgG	(SCSP#C150) vs (Control#C150)	0.8618925	0
Milk	SASP	IgG	(SASP#C0) vs (SASP#D0)	2.153508	0
Milk	SASP	IgG	(SCSP#C0) vs (SCSP#D0)	2.473021	0
Milk	SASP	IgG	(SCSP#C120) vs (SCSP#C0)	-2.445676	0
Milk	SASP	IgG	(SCSP#C150) vs (SCSP#C0)	-2.266236	0
Milk	SASP	IgG	(SCSP#C270) vs (SCSP#C0)	-2.9419	0
Milk	SASP	IgG	(SCSP#C270) vs (SCSP#C150)	-0.6756638	0.011
Milk	SASP	IgG	(SASP#C0) vs (Control#C0)	1.178243	0.012
Milk	SASP	IgG	(SASP#C150) vs (Control#C150)	0.6106588	0.037
Milk	SASP	IgG1	(SASP#C0) vs (SASP#D0)	2.754581	0
Milk	SASP	IgG1	(SASP#C120) vs (SASP#C0)	2.517252	0
Milk	SASP	IgG1	(SASP#C150) vs (SASP#C0)	-2.414087	0
Milk	SASP	IgG1	(SASP#C270) vs (SASP#C0)	-2.462696	0
Milk	SASP	IgG1	(SCSP#C120) vs (SCSP#C0)	-2.237553	0

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Milk	SASP	IgG1	(SCSP#C150) vs (SCSP#C0)	-1.917324	0
Milk	SASP	IgG1	(SCSP#C270) vs (SCSP#C0)	-2.221555	0
Milk	SASP	IgG1	(SCSP#C150) vs (SCSP#D0)	0.3692824	0.001
Milk	SASP	IgG1	(SASP#C150) vs (SASP#D0)	0.340494	0.005
Milk	SASP	IgG1	(SCSP#C150) vs (Control#C150)	0.5805373	0.018
Milk	SASP	IgG1	(SCSP#C150) vs (SCSP#C120)	0.3202287	0.021
Milk	SASP	IgG2	(SASP#C0) vs (SASP#D0)	2.468843	0
Milk	SASP	IgG2	(SASP#C120) vs (SASP#C0)	-2.487616	0
Milk	SASP	IgG2	(SASP#C150) vs (SASP#C0)	-2.177032	0
Milk	SASP	IgG2	(SASP#C270) vs (SASP#C0)	-2.092478	0
Milk	SASP	IgG2	(SCSP#C0) vs (SCSP#D0)	2.365145	0
Milk	SASP	IgG2	(SCSP#C120) vs (SCSP#C0)	-2.428401	0
Milk	SASP	IgG2	(SCSP#C150) vs (SCSP#C0)	-2.159645	0
Milk	SASP	IgG2	(SCSP#C270) vs (SCSP#C0)	-2.234838	0

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Milk	SASP	IgG2	(SASP#C150) vs (Control#C150)	0.4832907	0.002
Milk	SASP	IgG2	(SASP#C270) vs (Control#C270)	0.6435876	0.002
Milk	SASP	IgG2	(SASP#C150) vs (SASP#C120)	0.3105839	0.002
Milk	SASP	IgG2	(SASP#C150) vs (SASP#D0)	0.2918109	0.003
Milk	SASP	IgG2	(SCSP#C150) vs (SCSP#C120)	0.2687563	0.013
Milk	SASP	IgG2	(SASP#C270) vs (SASP#C120)	0.3951375	0.027
Milk	SASP	IgG2	(SASP#C270) vs (SASP#D0)	0.3763644	0.046
Milk	SCSP	IgA	(SASP#C0) vs (SASP#D0)	1.480599	0
Milk	SCSP	IgA	(SASP#C120) vs (SASP#D0)	-0.7979745	0
Milk	SCSP	IgA	(SASP#C150) vs (SASP#D0)	-0.5104552	0
Milk	SCSP	IgA	(SASP#C270) vs (SASP#C120)	0.8019032	0
Milk	SCSP	IgA	(SCSP#C0) vs (SCSP#D0)	1.74406	0
Milk	SCSP	IgA	(SCSP#C150) vs (SCSP#D0)	-0.5520508	0
Milk	SCSP	IgA	(SCSP#C120) vs (SCSP#C0)	-2.123073	0

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Milk	SCSP	IgA	(SCSP#C150) vs (SCSP#C0)	-2.29611	0
Milk	SCSP	IgA	(SCSP#C270) vs (SCSP#C0)	-1.941745	0
Milk	SCSP	IgA	(SASP#C270) vs (SASP#C150)	0.3802782	0.001
Milk	SCSP	IgG	(SASP#C0) vs (SASP#D0)	2.95544	0
Milk	SCSP	IgG	(SASP#C150) vs (SASP#D0)	-0.6354182	0
Milk	SCSP	IgG	(SASP#C120) vs (SASP#C0)	-3.277169	0
Milk	SCSP	IgG	(SASP#C150) vs (SASP#C0)	-3.590858	0
Milk	SCSP	IgG	(SASP#C270) vs (SASP#C0)	-3.46373	0
Milk	SCSP	IgG	(SCSP#C0) vs (SCSP#D0)	2.792886	0
Milk	SCSP	IgG	(SCSP#C120) vs (SCSP#C0)	-2.911301	0
Milk	SCSP	IgG	(SCSP#C150) vs (SCSP#C0)	-2.85355	0
Milk	SCSP	IgG	(SCSP#C270) vs (SCSP#C0)	-2.905753	0
Milk	SCSP	IgG	(SASP#C0) vs (Control#C0)	0.4843195	0.005
Milk	SCSP	IgG1	(SASP#C0) vs (SASP#D0)	2.93924	0

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Milk	SCSP	IgG1	(SASP#C150) vs (SASP#D0)	-0.5395205	0
Milk	SCSP	IgG1	(SASP#C120) vs (SASP#C0)	-3.393752	0
Milk	SCSP	IgG1	(SASP#C150) vs (SASP#C0)	-3.478761	0
Milk	SCSP	IgG1	(SASP#C270) vs (SASP#C0)	-3.290004	0
Milk	SCSP	IgG1	(SCSP#C0) vs (SCSP#D0)	2.781669	0
Milk	SCSP	IgG1	(SCSP#C150) vs (SCSP#D0)	-0.4782434	0
Milk	SCSP	IgG1	(SCSP#C120) vs (SCSP#C0)	-3.160337	0
Milk	SCSP	IgG1	(SCSP#C150) vs (SCSP#C0)	-3.259912	0
Milk	SCSP	IgG1	(SCSP#C270) vs (SCSP#C0)	-2.892343	0
Milk	SCSP	IgG1	(SASP#C120) vs (SASP#D0)	-0.4545118	0.023
Milk	SCSP	IgG2	(SASP#C0) vs (SASP#D0)	2.706901	0
Milk	SCSP	IgG2	(SASP#C120) vs (SASP#C0)	-2.602896	0
Milk	SCSP	IgG2	(SASP#C150) vs (SASP#C0)	-2.550782	0
Milk	SCSP	IgG2	(SASP#C270) vs (SASP#C0)	-2.228803	0

Table 8 (Continued)

Sample Type	Protein	Antibody	Treat/ Day Comparison	Contrast	P-Value
Milk	SCSP	IgG2	(SCSP#C0) vs (SCSP#D0)	2.508358	0
Milk	SCSP	IgG2	(SCSP#C120) vs (SCSP#C0)	-2.574408	0
Milk	SCSP	IgG2	(SCSP#C150) vs (SCSP#C0)	-2.323595	0
Milk	SCSP	IgG2	(SCSP#C270) vs (SCSP#C0)	-2.198186	0

Vita

Jessica Jean Vidlund was born in Oakland, MI on February 9th, 1992. Jessica grew up in Newport, MI where she graduated from Airport High School located in Carleton, MI in 2010. Jessica received her Bachelors of Science in Animal Science from Michigan State University in 2016. During her undergraduate career, Jessica worked at the Michigan State Dairy Cattle Teaching and Research Center. After graduation, Jessica pursued a full-time job at the University of Tennessee East Tennessee Research and Education Center, Little River Animal and Environmental Unit where she became Head Milker. Jessica then began her Masters program in the Animal Science Department at the University of Tennessee Institute of Agriculture in August 2018. Upon completion of her Masters of Science in Animal Health and Welfare, Jessica would like to pursue career opportunities in the areas of dairy health and preventative management of pathogens.