Immunological Responses and Protection in Dairy Cows Vaccinated with *Staphylococcus aureus* Surface Proteins (SASP) and *Staphylococcus chromogenes* Surface Proteins (SCSP)

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Caitlin Elizabeth Merrill
August 2018

ACKNOWLEDGEMENTS

I would first like to thank the University of Tennessee Department of Animal Science for allowing me to complete my Master's degree program. I wish to express my deepest gratitude to my major professor, Dr. Oudessa Kerro Dego, for giving me the opportunity to further my graduate education at The University of Tennessee Knoxville. I would like to thank him for his unwavering support and guidance throughout the completion of my degree. Most importantly, I would like to thank him for always encouraging me to chase my educational dreams. In addition to Dr. Kerro Dego, I would like to thank the other members of my committee, Drs. Jun Lin and Gina Pighetti, for their overall guidance, support, constructive comments and corrections.

None of this would have been possible without the assistance of Barbara Gillespie and Dr. Reta Abdi. Their invaluable help and advice was essential to my graduate program and I am extremely grateful for them. I am also thankful for the time and assistance of the University of Tennessee Institute of Agriculture, East Tennessee Research and Education Center -Little River Animal and Environmental Unit (UTIA-ETREC-LRAEU) personnel.

Above all, I wish to thank my parents, Dean and Kari Merrill. Without their love and support, none of this would have been possible. I am truly blessed to have parents that are consistently encouraging me to pursue my dreams and helping me persevere through any obstacle that comes my way. Additionally, I would like to thank my siblings, David, Dean, Amy, and Laura, for not only being an incredible support system but also being such amazing role models. As well as my in-laws Katy, Devan, Dan, and Don. Though they will never be able to read this, thank you to my dogs, Belle and Briar Rose, for the companionship during late night studying and writing sessions.

Last but not certainly not least, I would like to thank all of my friends and fellow graduate students, especially Hannah Bailey, for their love, laughs, and

unfailing support. I am incredibly grateful for the friendships and memories I have made over the past two years.

ABSTRACT

Bovine mastitis is the major cause of economic losses in dairy production worldwide. *Staphylococcus aureus* is a major causative agent that possesses multiple virulence factors responsible for successful colonization of mammary glands. Despite the adoption of current mastitis control measures, *S. aureus* continues to be one of the most prevalent mastitis pathogens throughout the world. Lysigin® (Boehringer Ingelheim Vetmedica, Inc, St. Joseph, MO) is a commercial *S. aureus* vaccine currently available in the US and Startvac® (Hipra, Girona, Spain) is a commercial *S. aureus* vaccine in Europe. Although some studies evaluated efficacy of these vaccines reported reduction in the duration and intensity of clinical signs, none of them prevent *S. aureus* intramammary infection (IMI) in either field trials or under controlled experimental studies. Because of the tendency of this organism to cause chronic IMI, treatment with antibiotics is of limited success. Therefore, there has been an increasing demand for alternative control measures, such as a vaccine to effectively prevent *S. aureus* IMI. The objectives of this study were to evaluate the immune responses and protection against *S. aureus* IMI in dairy cows vaccinated with *Staphylococcus aureus* surface proteins (SASP) and *Staphylococcus chromogenes* surface proteins (SCSP). A total of 18 pregnant Holstein dairy cows ranging from heifers to 3rd lactation cows were divided into three groups of 6 animals each. Animals in Groups 1 and 2 were vaccinated with 1.2 mg/dose of SASP and SCSP proteins with Emulsigen-D adjuvant, respectively. Animals in Group 3 were injected with PBS mixed with Emulsigen-D at equal proportion (1. 5 ml each) and used as control. Animals were vaccinated subcutaneously in the neck area during late lactation at 28 (D-28) and 14 (D-14) days before drying off, and at drying off (D0). Subsequently, each animal was challenged with *S. aureus* strain 60 by teat dipping in bacterial suspension at 10^7 CFU/ml culture medium. Results showed that vaccinated cows had increased milk and serum antibody titers and reduced bacterial shedding through milk. Interestingly, SCSP vaccine

cross-protected against *S. aureus* clinical mastitis thus suggesting its potential as immunogenic antigens to control bovine *S. aureus* mastitis.

TABLE OF CONTENTS

CHAPTER ONE LITERATURE REVIEW....1
INTRODUCTION....2
MASTITIS....4
Etiology of mastitis....6
Responses of dairy cows to bacterial intramammary infections....10
Control and prevention of mastitis....12
Vaccines....14
CHAPTER TWO IMMUNOLOGICAL RESPONSE OF DAIRY COWS VACCINATED WITH *STAPHYLOCOCCUS AUREUS* SURFACE PROTEINS (SASP) AND *STAPHYLOCOCCUS CHROMOGENES* SURFACE PROTEINS (SCSP) AND PROTECTION UPON SUBSEQUENT EXPERIMENTAL INTRAMAMMARY CHALLENGE (INFECTION)....17
ABSTRACT....18
INTRODUCTION....19
MATERIALS AND METHODS....22
Animals....22
Vaccine strain selection, antigen identification and preparation, and vaccine formulation....22
Vaccination schedule....23
Experimental challenge (infection)....23
Clinical examination of challenged cows....25
Mammary gland secretion and blood collection....27
Milk sample evaluation....29
Vaccine safety....29
Evaluation of vaccine induced immune response by ELISA....30
STATISTICAL ANALYSES....31
RESULTS....31
Vaccine safety....31
Immune responses to vaccines....32
Protective effects of SASP and SCSP vaccines....34
The relationship among serum or milk anti-SCSP and –SASP antibody titers and number of S. aureus shedding through milk during challenge period....39
The relationship among anti-SCSP and –SASP antibody titers and somatic cell count (SCC) during challenge period....39
The relationship among serum and milk antibody titers and milk production status....40
DISCUSSION....40
CONCLUSION....44
REFERENCES....45
APPENDICES....55
VITA....65

LIST OF TABLES

Table 1. Vaccination protocol 24
Table 2. Scoring scheme for abnormal changes in milk or dry secretion 26
Table 3. Scoring scheme for abnormal changes in the mammary gland tissue 26
Table 4. Sample collection and data recording schedule 28

LIST OF FIGURES

Figure 1. Serum anti-SASP and -SCSP antibody titers. SASP = Staphylococcus aureus surface proteins, SCSP = Staphylococcus chromogenes surface proteins, D-28 = 28 days before drying off, D-14 = 14 days before drying off, D0 = at drying off, Ch = Challenge, Ch0 = right before challenge, Ch+7 = on day 7 of challenge, Ch+14 = on day 14 of challenge, Ctrl = Control. The different letters represent statistically different titers ($P < 0.05$). 33
Figure 2. Response of cows to *S. aureus* challenge over the period of 14 days. *Staphylococcus aureus* surface proteins (SASP) vaccinated group, *Staphylococcus chromogenes* surface proteins (SCSP) vaccinated group. 35
Figure 3. Number of *S. aureus* shedding through milk over 14 days of challenge period. Ch = Challenge (infection), SASP = *Staphylococcus aureus* surface proteins vaccinated group, SCSP = *Staphylococcus chromogenes* surface proteins vaccinated group, Ch+1 – Ch+7 = Day 1 to Day 7 of challenge and at days 10 and 14 (Ch+10 & Ch+14) of challenge, each symbol on the curve showed mean of *S. aureus* counts in CFU/ml of milk per group per day and $P > 0.05$. 36
Figure 4. Number of *S. aureus* shedding through milk/mammary secretion and somatic cell counts (SCC) for SASP vaccinated and subclinically infected cows or for SCSP vaccinated cows. *Staphylococcus aureus* counts in mammary secretion from SASP vaccinated and subclinically infected cows (A), somatic cell counts (SCC) from SASP vaccinated and subclinically infected cows (B), number of *S. aureus* counts from SCSP vaccinated cows (C) and somatic cell counts (SCC) from SCSP vaccinated cows (D). SASP = *Staphylococcus aureus* surface proteins, SCSP = *Staphylococcus chromogenes* surface proteins, Ch0 = day of challenge, Ch+ 1 – Ch+7, Ch+10 and Ch+14 = Days 1 – 7 of challenge and days 10 and 14 of challenge. 37
Figure 5. Number of *S. aureus* shedding through mammary secretion and somatic cell count (SCC) for control cows at certain time points during challenge. (A) Number of *Staphylococcus aureus* counts on days 1 – 7, 10 and 14 of challenge, (B) somatic cell counts (SCC) immediately before challenge (Ch0) and on days 6 (Ch+6) and 14 (Ch+14) of challenge. Ctr = Control group. 38

CHAPTER ONE
LITERATURE REVIEW

INTRODUCTION

Bovine mastitis is one of the most costly diseases of the dairy industry worldwide. Mastitis is usually caused by bacterial pathogens. Economic losses due to bovine mastitis is estimated to be $2 billion in the United States (NMC, 2005), $400 million in Canada (Canadian Bovine Mastitis and Milk Quality Research Network-CBMQRN) and $130 million in Australia (Ismail, 2017) per year. These losses are due to decreased milk production, decreased milk quality, and the treatment cost of infected animals (Petrovski et al., 2006). Mastitis is also a serious public health concern because these pathogens or products of these pathogens, have the potential to enter the food supply and cause foodborne diseases, especially through the consumption of raw milk (Oliver et al., 2005). Guimarães et al. (2017) evaluated the economic impacts of mastitis at the herd level from February 2011 to January 2012 on a Holstein dairy herd in tropical conditions and found that the cost of mastitis was $61,623.13, with the most prevalent components of these losses were due to reduced milk production and milk disposal. The economic impact of mastitis on this herd from February 2012 to January 2013 was estimated to be $91,552.69 (Guimarães et al., 2017). Overall, at a herd level, the component that had the biggest effect on cost of mastitis was the reduction in milk production (Guimarães et al., 2017).

Bovine mastitis can be classified into clinical and subclinical intramammary infection (IMI). Clinical mastitis is characterized by visible signs of inflammation including udder swelling, redness, heat, pain and change in the consistency (presence of clots or flakes) and color of milk. Subclinical mastitis does not show obvious signs of inflammation but is manifested by high somatic cell count (SCC) and shedding of bacteria through milk. The subclinical mastitis cause greater economic losses because the infected cows are not as readily detected and treated or culled (Halasa et al., 2007).

Bacteria that cause mastitis are usually categorized into environmental or contagious mastitis pathogens. This classification depends upon their distribution in their natural habitat and mode of transmission from their natural habitat to the mammary glands of dairy cows (Calvinho and Oliver, 1998). Environmental bacteria exist in the

cow's environment and can cause infection at any time. The most common mastitis causing environmental bacteria include coliform bacteria (*Escherichia coli, Klebsiella spp*., *Enterobacter spp*. *and Citrobacter spp*.), environmental *Streptococci (Streptococcus uberis and Streptococcus dysgalactiae), Trueperella pyogenes,* which was previously called *Arcanobacterium pyogenes* or *Corynebacterium pyogenes* and environmental coagulase negative *Staphylococcus spp*. (CNS) (*Staphylococcus chromogenes, Staphylococcus simulans, Staphylococcus epidermidis, etc.*) (De Vliegher et al., 2012). Contagious bacterial pathogens primarily exist on the cow's teat skin and/or infected mammary glands and most commonly spread from infected to non-infected mammary glands during non-hygienic milking practices. The most frequent mastitis causing contagious bacterial pathogens are *Staphylococcus aureus, Streptococcus agalactiae, Mycoplasma bovis* and *Corynebacterium bovis* (De Vliegher et al., 2012). The prevalence of these different bacterial mastitis pathogens varies depending on herd management practices, geographical location, and other environmental conditions (Oliver and Mitchell, 1984). These different bacterial causative agents of mastitis have a multitude of virulence factors that make treatment and prevention of mastitis difficult.

The National Mastitis Council developed a 5-point mastitis control program in 1969 to control the incidence rate of mastitis. This 5-point mastitis control program includes dipping teats in an antiseptic solution before and after milking, proper cleaning and maintenance of milking equipment, early detection and treatment of infected animals, dry cow therapy with long acting antibiotics to reduce duration of existing infection and to prevent new intramammary infection, and finally culling chronically infected animals (Blowey and Edmondson, 2010; Neave et al., 1969). Later, it was updated to a 10-point plan, which includes more steps such as establishing udder health goals, maintain clean, dry and comfortable environment, proper milking procedures, proper maintenance and use of milking equipment, good record keeping, management of clinical mastitis during lactation, effective dry cow management including blanket dry cow therapy, maintenance of good biosecurity for contagious pathogens and marketing chronically infected cows, regular monitoring of udder health

status and periodic review of mastitis control program (Middleton et al., 2014). Though these hygienic milking practices and control measures decrease bacterial spreading, transmission, and subsequent infection, it does not fully prevent infections from establishing. Dairy farmers utilize antimicrobials as a prophylactic treatment for the prevention of mastitis or as therapeutics to treat cases of mastitis (USDA APHIS, 2008a).

There has been a growing concern with the extensive use of antimicrobials in production animals, especially non-therapeutic usage such as dry cow therapy in the case of dairy production, because of potential emergence and spread of antimicrobial resistant bacteria. There has been an increased incidence of antimicrobial resistant bacteria both in human and animal medical services. Therefore, alternative and sustainable control measures such as effective vaccines are required to prevent mastitis in dairy cows. Currently, there are two commercial bacterin vaccines, Lysigin® (Boehringer Ingelheim Vetmedica, Inc, St. Joseph, MO) in US and Startvac® (Hipra, Girona, Spain) in Europe that are claimed to have some effects against *S. aureus* mastitis in dairy cows. These vaccines are bacterin based made up of a suspension of killed bacteria (Leitner et al., 2011). In some trials, vaccination with these vaccines were reported to decrease production losses, clinical severity, and *S. aureus* shedding (Freick et al., 2016) through milk. Other studies reported that these vaccines had no protective effects both under controlled experimental and field studies and also did not prevent new IMI (Bradley et al., 2015; Middleton et al., 2009; Middleton et al., 2006; Piepers et al., 2017; Schukken et al., 2014).

MASTITIS

Mastitis is increasingly becoming a public health concern due to the ability of the causative bacterial pathogens and/or their products, such as enterotoxins, to enter the food supply and cause foodborne diseases (Hennekinne et al., 2012; Oliver et al., 2005). The Center for Disease Control (CDC) estimates that roughly 48 million people in the United States a year become sick from foodborne diseases (CDC, 2016). Foodborne pathogens have been detected in bulk tank milk in multiple studies (Jayarao

and Henning, 2001; Oliver et al., 2005; STEELE et al., 1997; Van Kessel et al., 2004). These authors found that the number of foodborne pathogens detected in bulk tank milk vary with location, management practices, hygiene, and number of animals on the farm (Oliver et al., 2005). Similarly, a study on bulk tank milk from east Tennessee and southwest Virginia by Rohrbach et al. (1992) showed that 32.5% of the samples analyzed contained one or more foodborne pathogens. Even dairy producers who used proper hygienic milking practices, pre- and post-milking teat disinfectant and antibiotic dry cow therapy, had foodborne pathogens in their bulk tank milk (Jayarao and Henning, 2001). The isolation of these foodborne pathogens from bulk tank milk samples across the United States demonstrate the threat that mastitis pathogens and zoonotic mastitis causing pathogens create on public health if raw milk is consumed or if these pathogens make it through processing.

There are host, pathogen, and environmental risk factors that predispose dairy cows to mastitis. The host risk factors include age and parity, stage of lactation, somatic cell counts, breed, anatomy of the mammary glands/morphology of udder and teat (diameter of teat canal & conformation of udder) and immune-competence (immunity) (Sordillo and Streicher, 2002). The environmental risk factors include status of milking machine function, udder trauma, sanitation, climate, nutrition, management, season and housing condition (Hogan and Smith, 1987). The pathogen risk factors include type, number, virulence, frequency of exposure, ability to resist flushing out of the glands by milk (adhesion and invasion), zoonotic potential and resistance to antimicrobials (Bradley, 2002). The warm, humid, and moist climate favors the growth of bacteria and increases the chances of IMI and disease development (Hogan and Smith, 1987). The incidence of mastitis varies from farm to farm due to the combined effects of these different factors that increase the risk of disease development. Dairy cows are most susceptible to IMI during the early dry period and the periparturient period because of the absence of hygienic milking procedures during early dry period (Oliver and Mitchell, 1983) and parturition related immunosuppression and negative energy balance during the periparturient period (Drackley, 1999; Esposito et al., 2014).

Mastitis can be divided into clinical and subclinical infections. Clinical infections are characterized by visible abnormalities in the mammary gland tissue such as redness, swelling, pain, increased heat and abnormal changes in milk color and consistency (clots or flakes) (Blowey and Edmondson, 2010). Subclinical infections are those with nonvisible abnormalities such as a high somatic cell count and shedding of causative bacteria (Blowey and Edmondson, 2010). The increase in somatic cell count during subclinical infections leads to a decrease in useful components in the milk such as lactose and casein (Malek dos Reis et al., 2013). Lactose is the sugar found in milk and casein is one of the major proteins in milk and decreases in these two components affect quality and quantity of milk yield (Blowey and Edmondson, 2010). During mastitis there is an increase in lipase and plasmin, which have a detrimental effect on quantity and quality of milk due to the breakdown of milk fat and casein (Blowey and Edmondson, 2010). Subclinical infections can reduce milk production by 10 – 12% when just one quarter is infected (Akers and Nickerson, 2011). These subclinical infections cause some of the greatest unseen economic (Almeida et al., 2015b) losses because of their detrimental impact on production and milk quality without showing visible signs of infection (Akers and Nickerson, 2011).

Etiology of mastitis

The bacterial pathogens that cause mastitis can be classified into environmental and contagious pathogens. The environmental bacterial pathogens are organisms that can be found anywhere in the cow's environment and can infect mammary glands at any time. Some of the most common environmental mastitis pathogens are coliform (bacteria that can utilize lactose) bacteria (*E. coli*, *Klebsiella spp*., etc.), environmental *Streptococci* (*S. uberis* and *S. dysgalactiae*), *Trueperella pyogenes* and coagulase negative *Staphylococcus* (CNS) *spp*. (*S. chromogenes*, *S. haemolyticus*, *S. epidermidis*, *S. simulans,* etc*.)* (Blowey and Edmondson, 2010; Bradley, 2002; Piessens et al., 2011). In general, it is believed that mastitis pathogens gain entrance to the teat canal during reverse flow of milk due to vacuum pressure fluctuation (Blowey and Edmondson, 2010). However, the mechanism of mastitis pathogens colonization in the

mammary gland may vary among species of bacteria and the virulence factors associated with strains in each species. An example of this is in some cases, it has been shown that *E. coli* has the ability to penetrate the teat canal without the reverse flow of milk (Blowey and Edmondson, 2010). Some of the major mastitis pathogens such as *E. coli* (Dogan et al., 2006)*, Staphylococcus aureus* and *Streptococcus uberis* (Almeida et al., 2015a; Almeida et al., 1999; Patel et al., 2009) have ability to adhere to and subsequently invade into the mammary epithelial cells. This adherence and subsequent invasion ability allows them to persist in the intracellular area and escape attack from the host immune defenses and antibiotics (Almeida et al., 2011; Almeida et al., 1996; Bayles et al., 1998; Craven and Anderson, 1984; Dogan et al., 2006; Zhao et al., 2017). Dogan et al., (2006) compared *E. coli* strains known to cause chronic infections with strains known to cause acute infections and found that chronic strains were more invasive to the epithelial cells, leading to the difficulty in clearance and persistent infection compared to acute strains.

Streptococcus uberis is one of the environmental mastitis pathogens that accounts for a significant proportion of subclinical and clinical mastitis in lactating and non-lactating cows and heifers (Smith et al., 1985). This organism is commonly found in the bedding material, which facilitate infection of mammary glands at any time (Bramley, 1982). *S. uberis* has various mechanisms of virulence that increases the chances of this organism establishing infection. These include a capsule, which avoids phagocytosis, adherence to and invasion into mammary epithelial cells (Almeida and Oliver, 1993; Oliver et al., 1998). *S. uberis* adheres to epithelial cells using different mechanisms including the formation of pedestals (Matthews et al., 1994) and bridge formation through *Streptococcus uberis* adhesion molecule (SUAM) and lactoferrin (Almeida et al., 2015a; Almeida et al., 1999; Patel et al., 2009). This attachment is specific and mediated through a bridge formation between *Streptococcus uberis* adhesion molecule (SUAM) (Almeida et al., 2006; Fang and Oliver, 1999) on *S. uberis* surface and lactoferrin which is in the mammary secretion and has a receptor on the mammary epithelial surface (Almeida et al., 2015a; Patel et al., 2009). These factors increase the pathogenicity of *S. uberis* to cause mastitis.

Staphylococcus chromogenes is another common environmental pathogen that is classified as a coagulase-negative *Staphylococcus* species (CNS) (Bradley, 2002; Piessens et al., 2011). This pathogen is most commonly isolated from mammary secretions rather than from the environment itself (Gillespie et al., 2009; Piessens et al., 2011). *S. chromogenes* consistently isolated from the cow's udder and teat skin (Taponen et al., 2008) and some studies showed that it causes long-lasting, persistent subclinical infections (Taponen and Pyörälä, 2009). *S. chromogenes*, along with other CNS species, have been shown to cause subclinical infections in dairy farms that reduce the prevalence of contagious mastitis pathogens in their herds (Bradley, 2002). The CNS species caused high somatic cell counts in milk on some dairy farms (Fry et al., 2014; Gillespie et al., 2009). Woodward et al. (1987) evaluated the normal teat skin flora, and found that 25% of the isolates exhibited the ability to prevent growth of some mastitis pathogens. An in vitro study conducted on *S. chromogenes* showed that this organism had the ability to inhibit the growth of major mastitis-causing pathogens such as *S. aureus, S. dysgalactiae,* and *S. uberis* (De Vliegher et al., 2004). In a study conducted on conventional and organic Canadian dairy farms, CNS species were found in 20% of the clinical samples (Levison et al., 2016). Recently, mastitis caused by CNS species increasingly became more problematic in dairy herds (Pyörälä and Taponen, 2009; Taponen et al., 2008; Taponen et al., 2007; Taponen et al., 2017). However, mastitis caused by CNS species is less severe compared to mastitis caused by *Staphylococcus aureus* (Taponen and Pyörälä, 2009).

Contagious mastitis pathogens, such as *Staphylococcus aureus, Streptococcus agalactiae*, *Corynebacterium bovis* and *Mycoplasma spp*., especially *M. bovis,* are organisms that colonize mammary glands of cows or the skin of teat and/or udder. These organisms can be transmitted from infected glands or skin to non-infected mammary glands of dairy cows (Blowey and Edmondson, 2010) during milking process. Both *Staphylococcus aureus* and *Streptococcus agalactiae* have strong adhesive properties, which allow them to attach to the skin tissue at teat opening and progressively grow up through the teat canal (Akers and Nickerson, 2011).

Staphylococcus aureus is one of the most common contagious mastitis pathogens, with an estimated incidence rate of 43 -74% (Riekerink et al., 2010; USDA APHIS, 2008a, 2008b, 2009a, 2014). *Staphylococcus aureus* is primarily found on the skin of the udder of the cow or in the infected mammary glands of a cow. The mode of transmission from infected mammary glands and/or colonized udder skin to healthy mammary glands is through contact during milking procedures with milker's hand, towel, milking machine (Zadoks et al., 2002). *S. aureus* usually causes subclinical or chronic infections and is difficult to clear with antibiotic treatment (Carter and Kerr, 2003). Virulence factors of *S. aureus* that contribute to its pathogenicity include: protein A, exopolysaccharides (capsule, slime, and biofilms) (Donlan and Costerton, 2002; Gotz, 2002; Otto, 2008; Stewart and Costerton, 2001), adhesion to and invasion into epithelial cells (Josse et al., 2017), intracellular survival in macrophages (Fowler et al., 2000) and epithelial cells, and production of enterotoxins (Aydin et al., 2011), hemolysins, enzymes and super antigens (Kerro-Dego et al., 2012; Kerro and Nederbragt, 2002). The ability of this organism to survive within epithelial cells and macrophages allow them to avoid detection by the host immune system and resist treatment with antibiotics (Almeida et al., 1996). Due to its poor response to treatments, *S. aureus* infections often become chronic with a low cure rate (Abdi et al., 2018). Treatment of *Staphylococcus aureus* mastitis with cloxacillin cured only a 25% of clinical cases and 40% of subclinical cases (Tyler and Baggot, 1992). *Staphylococcus aureus* also has a known ability to form biofilms (Donlan and Costerton, 2002; Melchior et al., 2006; Stewart and Costerton, 2001) and acquire antimicrobial resistant genes via horizontal resistance gene transfer which enables this bacterium to develop antimicrobial resistance (Brüssow et al., 2004; Owens et al., 1997).

The most important virulence factor of *S. agalactiae* is the capsular polysaccharide (Emaneini et al., 2016), which protect this bacterium from being engulfed by macrophages and subsequently phagocytosed (Emaneini et al., 2016). Another virulence factor of *S. agalactiae* is the Rib protein, which confers resistance to proteases. Emaneini et al. (2016) found that the Rib encoding gene (*rib)* was detected in 89% of the isolates from bovine origin. *Streptococcus agalactiae* causes persistent

infections that usually difficulty to clear without antibiotic treatment (Farnsworth, 1987). Though *Streptococcus agalactiae* is highly contagious, it has good response to treatment with antibiotics, which makes it possible to eliminate from herds with current mastitis control measures (Tyler and Baggot, 1992). Since the adoption of hygienic milking practices, the incidence of mastitis caused by *S. agalactiae* has dramatically decreased and is now rarely observed in dairy herds (Hillerton and Berry, 2003).

Mastitis caused by *Mycoplasma* spp. is a growing concern in the United States. It is believed that this organism has been underreported due to the difficulty of isolation by culture method (Nicholas et al., 2007). The incidence of *Mycoplasma* mastitis varies across the globe, with a 3.2% prevalence rate in the United States that may increase to 14.4% in a larger herd size of greater than 500 cows (USDA APHIS, 2008a, 2008b, 2009a, 2014). A risk factor for *Mycoplasma* mastitis increase with herd size and most of the *Mycoplasma* mastitis cases are subclinical infections with outbreaks linked to asymptomatic carriers (Fox, 2012). Pathogenesis of most *mycoplasma* spp. infection is characterized by adherence to and internalization into host cells resulting in colonization of the host with immune modulation without causing severe disease (Fox, 2012). *Mycoplasma* species lack a cell wall, thus not sensitive to beta-lactam antibiotics but showed sensitivity to non-beta-lactam antibiotics (Jasper, 1981).

Responses of dairy cows to bacterial intramammary infections

When the teat canal defenses fail to prevent entry of bacteria, there are several udder defenses in place that are characterized as intrinsic defense mechanisms and inducible systems (Korhonen et al., 2000). Intrinsic defense mechanisms consist of lactoferrin, complement, immunoglobulins, and the cellular immune response. Lactoferrin is an intrinsic defense mechanism because its function is to remove iron from the milk, which deprives bacteria from receiving this nutrient which is essential for bacterial growth (Smith and Oliver, 1981). The complement system is activated in order to clear bacteria. This system consists of a series of proteins, which act together, in a cascade to kill bacteria by triggering phagocytosis, inflammation, or rupturing the cell wall of the bacteria by membrane attack complex (Korhonen et al., 2000).

Immunoglobulins mark bacteria by opsonization which is the process by which an antibody attaches to the bacteria through its variable chain (fragment), and the other constant chain (fragment) portion of the antibody has receptor for phagocytic cells (FCγR) that upon binding activates opsonophagocytic killing of a bacterium. These phagocytic white blood cells (neutrophils and macrophages), when activated, promote phagocytosis of the bacterium. In the process of phagocytosis, the bacteria is engulfed and destroyed (Paape et al., 2003).

One of the functions of immunoglobulins is to work with the complement fraction C3b in order to induce phagocytosis of bacteria (Howard et al., 1980; Korhonen et al., 2000; Targowski, 1983). Various types of cells such as macrophages, polymorphonuclear neutrophils (PMNs), and lymphocytes are present in the milk and contribute to the intrinsic cellular response. The number of these cells present in the milk makes up the somatic cell count (SCC). A high SCC (≥ 200,000 cells/ml) in the composite milk of an individual cow serves as an indicator for a possible infection. Somatic cells include polymorphonuclear leukocytes (neutrophils, eosinophils, mast cells, and basophils), monocytes, macrophages, lymphocytes and some mammary epithelial cells (Harmon, 1994). One of the functions of these cells is to recognize bacteria and then activate the inducible systems, which will cause a larger and stronger host immune response. The inducible systems in the udder consist of the release of chemotaxins to attract phagocytic cells and induce an inflammatory response (Rainard and Riollet, 2006). When polymorphonuclear neutrophils (PMNs) and macrophages phagocytose bacteria, pieces of the destroyed bacteria and then those pieces are released. The fragments of bacteria cause the release of chemical mediators, or chemotaxins. The release of chemotaxins causes a major influx of PMNs, which is the beginning of the inflammatory response (Rainard and Riollet, 2006).
Immunoglobulins are produced by plasma cells and involved in the clearance of pathogens by recognizing and binding specific antigens. The immunoglobulins identified in bovine species are IgA, IgM, IgG (subclasses IgG1, IgG2 and IgG3), and a protein that has characteristics similar to that of IgE (Butler, 1983; Korhonen et al., 2000). Massive selective transport of IgG1 in the mammary gland is one of the unique features

of the bovine immune system (Butler, 1983). Both IgG1 and IgG2 are able to fix complement, meaning they can activate the complement system, in bovine serum (Korhonen et al., 2000; McGuire et al., 1979). Early studies on the role of IgG1 and IgG2 antibodies in ruminants showed that polymorphonuclear leukocytes were able to phagocytose when these antibodies (McGuire et al., 1979) opsonize the antigen. These antibodies play an important role in detecting the presence of microbes and marking them for destruction. Though these natural defenses assist in preventing bacteria from gaining entry and establishing infection, they are not always fully effective.

Control and prevention of mastitis

In the 1960s, the National Institute for Research in Dairying (NIRD) developed a five-point plan for mastitis control. These recommendations include treating and recording all clinical cases, dipping teats in disinfectants after milking, utilizing dry cow therapy at the end of lactation, culling chronic mastitis cases, and maintaining milking machine equipment regularly (Middleton et al., 2014). The five-point control program later extended to the 10-point control program (Middleton et al., 2014). The additions to the five-point plan included establishment of udder health goals, maintaining a clean environment, proper milking procedures, good record-keeping, monitoring udder health, and periodical review of the mastitis control program (Middleton et al., 2014). The presence of contagious or environmental bacterial pathogens of mastitis, is directly correlated to the incidence of mastitis. Hygienic practices are important in reducing the number of pathogens that cows are exposed to during milking practices. In the parlor, hygienic milking practices consist of three different steps: pre-milking cleansing, sanitizing milking equipment between uses, and then applying antiseptic solution to the teats post-milking (Bushnell, 1984). The pre-milking cleansing step is important to prevent environmental bacterial pathogens from gaining entry into intramammary area. The other two steps, disinfecting milking equipment between uses and the post-milking teat dipping in antiseptic solution, are important to prevent entry of contagious bacterial pathogens into intramammary area that can spread from infected to healthy mammary glands during milking (Bushnell, 1984). Sometimes even the best control measures do

not prevent establishment of infection, therefore farmers rely on antimicrobials for prevention as dry cow therapy or as therapeutics to treat cases of mastitis (Saini et al., 2012a; Saini et al., 2012b).

As a routine prophylactic mastitis control measure, dairy farmers utilize a blanket dry cow antimicrobial therapy in an attempt to prevent bacterial colonization of mammary glands during dry period. In the U.S, more than 90% of dairy farms use antimicrobials at drying off (USDA APHIS, 2008a). The intramammary infusion of long acting antimicrobials at drying off is important to prevent establishment of new intramammary infections during dry period and to cure existing chronic and/or subclinical infections.

The most common antibiotics used to treat mastitis include cephalosporin (53.2%), followed by lincosamide (19.4%) and non-cephalosporin β-lactam antibiotics (19.1%) (USDA APHIS, 2008a). The problem with the use of non-selective blanket antimicrobials administration to dairy cows as a prophylactic control of mastitis is that they put selective pressure on both mastitis-causing bacteria as well as commensal bacteria in the animals' body (Barber et al., 2003; Barbosa and Levy, 2000). The ultimate result may not be different but the exposure level to antibiotics and its biotransformed products are different for the bacteria in the gut and in the mammary glands during treatment of mastitis. Antibiotics infused into the mammary glands excreted through milk and/or enter the serum biotransformed (pharmacokinetics) in the liver and/or kidney and excreted from the body through urine or feces into the environments. Therefore, both parenteral and intramammary administration of antibiotics have significant impact on other commensals or opportunistic bacteria in the gastrointestinal tract of dairy cows, some of which are major human pathogens. However, the level of exposure to antibiotics seems high for bacteria in the mammary glands than bacteria in other parts of the animal body during intramammary infusion of antibiotics. This selective pressure can result in antimicrobial resistant bacteria that become difficult to clear and persistent on farms and spread among animals (Normanno et al., 2007). The antimicrobial resistant bacteria or their genes may spread from these sources to human or animals or to other bacteria. McAllister et al., (2001) found that

CNS spp. could potentially transfer penicillin, cephalosporin, and fluoroquinolones resistant genes to *S. aureus*. The transfer of these antibiotic resistance genes could lead to the development of antimicrobial resistant bacteria including methicillin-resistant *S. aureus* (MRSA) (McAllister et al., 2001). Until recently, MRSA was a common antimicrobial resistant strain mainly found in human hospitals; however, recent findings indicated that it has also been increasingly isolated from cattle herds (Haran et al., 2012). The major problem with MRSA is that it is mostly resistant to multiple commonly used antimicrobials (multidrug resistant) and difficult to control and eliminate (Holmes and Zadoks, 2011). Waller et al. (2011), evaluated the antimicrobial susceptibility of CNS species, and found a difference across the species on β-lactamase production. Similarly, Sawant et al., (2009) found that 18% and 46% of the *S. chromogenes* and *S. epidermidis* isolates produce β-lactamase respectively. Sampimon et al. (2009) also found a 70% resistance to penicillin in *S. epidermidis*, but more importantly found that 30% of the CNS spp. were resistant to more than one antimicrobial. Therefore, in light of the foregone it is critically important to develop non-antibiotic feasible and sustainable control measures such as vaccines to control staphylococcal infections in animals and human.

Vaccines

The physiological nature of mammary glands where induced systemic immune responses need to cross from the body into the mammary glands, the dilution of effector immune responses by large volume of milk coupled with the ability of mastitis causing bacteria to develop resistance to antimicrobials makes control of mastitis very difficult. Therefore, the development of an alternative preventive tool such as vaccine, which can overcome these limitations, has been a crucial focus of current research to decrease not only the incidence of mastitis but also the usage of antimicrobials in dairy cattle production. Several vaccine studies were conducted over the years as controlled experimental and field trials. Some of the most common mastitis pathogens that have been targeted for vaccine development are *S. aureus*, *S. agalactiae, S. uberis* and *E. coli* (Ismail, 2017). Most of these experimental and some commercial vaccines are

bacterins which are inactivated whole organism, and some vaccines contained subunits of the organism such as surface proteins, toxins, or polysaccharides. The *E. coli* vaccine studies predominantly used the mutant core antigen J5 for vaccines (Ismail, 2017). Several studies have been conducted analyzing the efficacy of the commercial and experimental vaccines on udder health and protection from mastitis.

The Startvac® (Hipra, Girona, Spain) is the commercially available vaccine in Europe and is a polyvalent vaccine that contains *E. coli* J5 and *S. aureus* strain SP140 (Ismail, 2017). In a field trial, Freick et al. (2016) compared the efficacy of Startvac® with Bestvac® (IDT, Dessau-Rosslau, Germany) another herd-specific autologous commercial vaccine in a dairy herd with high prevalence of *S. aureus* and found that the herd prevalence of *S. aureus* mastitis was lower in the Startvac® and Bestvac® vaccinated cows compared to the control cows. However, there were no other differences in terms of improvement of udder health. These authors (Freick et al., 2016) concluded that vaccination with Startvac® and Bestvac®, did not improve udder health. In another field efficacy study on Startvac® in the UK, Bradley et al. (2015) found that Startvac® vaccinated cows had clinical mastitis with reduced severity and higher milk production compared to non-vaccinated control cows (Bradley et al., 2015). Similarly, Schukken et al. (2014), evaluated effect of Startvac® on the development of new IMI and the duration of infections caused by *S. aureus* and CNS. These authors (Schukken et al., 2014) found that vaccinated cows had decreased incidence rate and a shorter duration of *S. aureus* and CNS mastitis. Piepers et al. (2017), also tested the efficacy of Startvac® through vaccination and subsequent challenge with a heterologous killed *S. aureus* strain and found that the inflammatory response in the vaccinated cows was less severe compared to the control cows. These authors (Piepers et al., 2017) suggested that Startvac® elicited a strong Th2 immune response against *S. aureus* in vaccinated cows and was more effective at clearing bacteria compared to the control cows. Contrary to these observations, Landin et al. (2015), evaluated the effects of Startvac® on milk production, udder health, and survival on two Swedish dairy herds with *S. aureus* mastitis problems and found no significant differences between the Startvac® vaccinated and non-vaccinated control cows on the health parameters they evaluated.

An experimental *S. aureus* vaccine made up of a combination of plasmids encoding fibronectin-binding motifs of fibronectin binding protein (FnBP) and clumping factor A (ClfA), and plasmid encoding bovine granulocyte-macrophage-colony stimulatory factor, was used as vaccine with subsequent challenge with bacteria to test its protective effects (Shkreta et al., 2004). These authors (Shkreta et al. 2004), found that their experimental vaccine induced immune responses in the heifers that was partially protective upon experimental challenge (Shkreta et al., 2004). Another controlled experimental vaccine efficacy study was conducted on the slime associated antigenic complex (SAAC) which is an extracellular component of *Staphylococcus aureus,* as vaccine antigen in which one group of cows were vaccinated with a vaccine containing low amount of SAAC and another group with a high amount of SAAC and unvaccinated group served as a control (Prenafeta et al., 2010). Upon intramammary infusion (challenge) with *S. aureus*, no difference in the occurrence of mastitis among all three groups despite the fact that the vaccine with high SAAC content induced higher production of antibodies compared to the vaccine with low amount of SAAC (Prenafeta et al., 2010). Similarly, Pellegrino et al. (2008), vaccinated dairy cows with an avirulent mutant strain of *S. aureus* and subsequently challenged with *S. aureus* 20 days after the second vaccination which resulted in no significant differences in number of somatic cell count (SCC) or number of bacteria shedding through milk despite increased IgG antibody titer in the vaccinated cows compared to the control cows.

CHAPTER TWO IMMUNOLOGICAL RESPONSE OF DAIRY COWS VACCINATED WITH *STAPHYLOCOCCUS AUREUS* SURFACE PROTEINS (SASP) AND *STAPHYLOCOCCUS CHROMOGENES* SURFACE PROTEINS (SCSP) AND PROTECTION UPON SUBSEQUENT EXPERIMENTAL INTRAMAMMARY CHALLENGE (INFECTION)

ABSTRACT

Bovine mastitis is a major cause of economic losses in the dairy industry. One of the most prevalent pathogens, *Staphylococcus aureus,* possesses various virulence factors that contribute to its success. Current mastitis control measures have decreased incidence of *S. aureus* mastitis, however, it remains a problem in dairy herds. There are two *S. aureus* vaccines on the market, Lysigin® and Startvac®. These vaccines claim to control *S. aureus* mastitis, however they did not prevent new *S. aureus* IMI in field trials or controlled experimental studies. The objectives of this study were to evaluate the immune responses elicited in dairy cows vaccinated with *Staphylococcus aureus* surface proteins (SASP) and *Staphylococcus chromogenes* surface proteins (SCSP) and protection upon subsequent challenge with *S. aureus* suspension. A total of 18 Holstein dairy cows were divided into three groups. Group 1 & 2 were vaccinated with SASP and SCSP with Emulsigen-D adjuvant respectively. Group 3 was injected with PBS mixed with Emulsigen-D and served as a control. Cows were vaccinated at 28 (D-28) and 14 (D-14) days before drying off, and at dry off (D0). Two weeks after last vaccination, each animal was challenged by teat dipping in *S. aureus* strain 60 culture for 14 consecutive days. Milk and serum antibody titers were evaluated during vaccination and challenge period. Milk was also evaluated for bacterial shedding and somatic cell counts. Out of 5 cows vaccinated with SASP, 2 were infected clinically, 2 were infected subclinically, and the remaining cow was not infected but shedding low number of *S. aureus*. Out of 6 control cows, 2 were infected clinically and 4 cows were infected subclinically. Out of 6 SCSP vaccinated cows there were none infected but cows were shedding relatively low number of *S. aureus* through milk. Statistical analysis showed no significant difference ($P > 0.05$) in clinical frequency among the SCSP, SASP, and control groups. The SCSP vaccine cross-protected against *S. aureus* by inducing increased immune response, reducing number of *S. aureus* shedding in milk and decreasing somatic cell counts (SCC), suggesting its potential as an effective immunogenic antigens to control bovine *S. aureus* mastitis.

INTRODUCTION

One of the most costly diseases affecting the dairy industry worldwide is bovine mastitis. Bovine mastitis is a disease that is primarily caused by a bacterial infection in the mammary gland. Economic losses due to mastitis is roughly $2 billion annually in the United States (NMC, 2005). The National Mastitis Council has recommended a 10-point control program to reduce the rate of mastitis (Middleton et al., 2014). The implementation of this program has reduced the incidence rates of mastitis, but it has not been successful in preventing new infections from establishing. Dairy farmers in the United States and many other parts of the globe rely on the prophylactic intramammary infusion of long acting antimicrobials at drying off to prevent new infections during dry period or to treat existing infections (USDA APHIS, 2008a). The intramammary administration of antimicrobials at drying off is a growing issue since this practice exposes large number of healthy animals to antimicrobials. This exposure puts pressure on commensal bacteria and other opportunistic bacteria to develop antimicrobial resistance.

Dairy cows are more susceptible to intramammary infection during early dry and transition (periparturient) periods. The incidence of IMI is high during early dry period because of absence of hygienic milking practices (teat washing and drying, as well as pre- and post-milking teat dipping in antiseptic solutions) that are known to reduce teat end colonization and infection. The high incidence of mastitis during transition (periparturient) period is due to combined effects of parturition hormones causing immunosuppression, negative energy balance at early lactation and parturition related physical stress (Esposito et al., 2014). In general, immunoglobulins play an important role in the host immune system for the clearance of pathogens and fighting off infections. In the adaptive immune system, each specific immunoglobulin binds to specific epitope on the bacteria that induced its production. This binding triggers opsonization, which is an antibody attaches to the antigen (bacteria) to mark it for destruction by phagocytic cells (Paape et al., 2003). Immunoglobulin binds to epitopes on an antigen through its variable chain and the constant chain end of the

immunoglobulin binds to phagocytic cells through specific receptor (FCγR) resulting in opsonophagocytic killing of bacteria by phagocytic cells. The most prevalent antibodies in bovine serum are IgG (subclasses IgG1 and IgG2), IgA, and IgM (Butler, 1983). The immunoglobulin G2 (IgG2) plays an important role in the bovine immune system by marking microbes (opsonizing) for destruction and removal by polymorphonuclear neutrophils (PMNs) (McGuire et al., 1979). *Staphylococcus aureus* is one of the most frequent pathogens implicated in mastitis (Bradley, 2002). There are two commercial vaccines for *Staphylococcus aureus* mastitis on the market, Lysigin® gin in the United States and Startvac® in Europe (Freick et al., 2016). Several field trials and controlled experimental studies have been conducted testing the efficacy of Lysigin® and Startvac® and results from those studies have shown some results including reduced incidence, severity and duration of mastitis in vaccinated cows compared to non-vaccinated control cows (Bradley et al., 2015; Piepers et al., 2017; Schukken et al., 2014). Contrary to these observations in other studies, these vaccines had no effect on improving udder health or showed no difference between vaccinated and non-vaccinated control cows (Freick et al., 2016; Landin et al., 2015). None of these bacterin-based vaccines prevents new *S. aureus* IMI (Bradley et al., 2015; Middleton et al., 2009; Middleton et al., 2006; Schukken et al., 2014). Therefore, developing an effective vaccine to control *S. aureus* IMI is a sustainable alternative approach for controlling mastitis rather than prophylactic use of antibiotics. Differences found in these studies are mainly due to methodological differences (vaccination schedule, route of vaccination, challenge model, herd size, time of lactation, etc.) in testing the efficacy of these vaccines. It is critically important to have a good infection model that mimics natural infection and a model that has 100% efficacy in causing infection. Without a good challenge model, the results from vaccine efficacy will be inaccurate.

There are different ways to test the efficacy of experimental vaccines through either natural exposure based field studies or controlled experimental studies. Experimental intramammary infection of *S. aureus* can be induced by either intramammary infusion of bacteria or teat dipping in bacterial suspension. The intramammary infusion of *S. aureus* is a reliable method in terms of causing infection

but it is an unrealistic method in terms of mimicking naturally occurring intramammary infections because: 1) large number of bacteria are directly delivered into the intramammary area bypassing non-specific natural defenses, as well as inducible innate and acquired immune responses, 2) since the host natural and innate defense systems were bypassed the host's acquired immune responses did not get enough time to process antigen and deliver protective effector molecules and cells to kill the invading pathogen, 3) because of the above mentioned reasons intramammary infusion challenge method overwhelms the host innate and acquired immune defenses and is not good model for testing vaccine efficacy. Therefore, a challenge model that is closely resembles natural infection is necessary to evaluate efficacy of an experimental vaccine against *S. aureus* mastitis. Prior to this vaccine efficacy study, our lab developed experimental *S. aureus* mastitis infection model by teat dipping in *S. aureus* bacterial suspension. This teat dip infection model was utilized in this particular vaccine efficacy study.

Interestingly, our group observed that prior colonization of the mammary gland by one strain of *S. chromogenes* prevented growth and colonization by *S. aureus* strain 60 (SAUT2) under *in vivo* conditions. It is unknown whether this prevention of colonization is related to bactericidal/bacteriostatic product from this strain or related to inducible innate or acquired immune responses. However, other studies showed that bactericidal/static proteins induced strong protective immune response characterized by increased production of interferon gamma and IgA in mice (Wang et al., 2017). While we were still evaluating bactericidal/static effect of *S. chromogenes* surface proteins (SCSP) and secretory proteins, we thought that these proteins may have similar effect seen during mice study elsewhere (Wang et al., 2017). Thus, we decided to further evaluate this interesting observation by vaccinating dairy cows with SCSP and evaluate protection through experimental challenge.

MATERIALS AND METHODS

Animals

18 pregnant Holstein dairy cows including 8 heifers of about 23 months (almost 2 years of age), 6 cows of 1st lactation (about 3 years of age), 3 cows of 2nd lactation (about 4 years of age) and 1 cow of 3rd lactation (about 5 years of age) were divided into three groups of 6 cows each. Cows in Group 1 (3 cows of 1st lactation, 1 cow of 2nd, 1 cow of 3rd and 1 heifer) and cows in Group 2 (2 cows of 2nd lactation, 3 cows of 1st lactation and 1 heifer) were vaccinated with *Staphylococcus aureus* surface proteins (SASP) and *Staphylococcus chromogenes* surface proteins (SCSP) with Emulsigen-D adjuvant, respectively. Cows in Group 3 (all heifers) were injected with PBS mixed with Emulsigen-D (Phibro Animal Health Corporation, Omaha, NE) adjuvant at equal proportion (1.5 ml each), and used as control. Prior to enrolment in the study two cows (4431 and 4449) had more than 250,000 SCC per mL of milk in all quarters and 4 cows had more than 250,000 SCC (4488-LR, 4438-RR, 4420-RR and 4358-LF) per mL of milk in one of their four quarters with no bacterial growth from milk. The remaining quarters of all cows had less than 250,000 SCC per ml of milk prior to enrolment in the study. On average, all animals had a background serum and milk LS mean log titers of about 3 to 4. Experimental and control cows were under the same herd management throughout the study and housed at the East Tennessee Research and Education Center-Little River Animal and Environmental Unit (ETREC-LAEU, Walland, TN).

Vaccine strain selection, antigen identification and preparation, and vaccine formulation

Staphylococcus aureus surface proteins (SASP) and *Staphylococcus chromogenes* surface proteins (SCSP) were extracted using 1% cholic acid (Sigma Aldrich, St. Luis, MO). *Staphylococcus aureus* strain selection was based on analysis of pulsed field gel electrophoresis (PFGE) results of *S. aureus* isolates from cases of bovine mastitis across Tennessee. The criteria for selection was dominance among strains or the most frequent isolates among identified PFGE types. One of the dominant

strains (*S. aureus* strain 38 or SAUT1) was selected for SASP vaccine preparation. The *S. chromogenes* strain was selected for SCSP vaccine preparation because of its ability to prevent the mammary gland colonization by *S. aureus* through in vivo inducible effects.

For antigen preparation, selected strains were streaked on blood agar plates and incubated at 37ºC overnight. After incubation, 3 isolated colonies were suspended in 450 ml of tryptic soy broth (TSB) and grown to mid-log phase with shaking at 125 rpm at 37ºC in 5% CO_2: 95% air balanced incubator, until absorbance of 0.5 nm at OD_{600} was achieved (~ 3 – 4 h of incubation). Then we centrifuged the culture at 500xg for 10 min at 4ºC and the pellet was resuspended in 1% cholic acid (Sigma-Aldrich Co) and incubated at room temperature for 2 h with shaking (125 rpm). After incubation, bacterial suspension was centrifuged at 1000 xg for 30 min at 4ºC and proteins in the supernatant were concentrated using Centriprep Ultracel-10K YM concentrators with 10 KDa cut off (EMD Millipore Corporation, Billerica, MA). Protein concentration was measured using a BCA protein assay kit (Pierce-Thermofisher Scientific, Waltham, MA). Vaccine was prepared, using either SASP or SCSP (1200 µg in 1.5 ml of PBS [pH 7.2]) mixed with 1.5 ml of Emulsigen-D, resulting in a total final volume of 3 ml.

Vaccination schedule

All cows were given three series of vaccinations at about 14 days interval subcutaneously (SQ) on alternate sides of the neck area, approximately midway between the base of the ear and the point of the shoulder at 28 days before drying off (D-28), 14 days before drying off (D-14) and at drying off (D0) (Table 1).

Experimental challenge (infection)

S. aureus strain 60 (SAUT2), which is different from our vaccine strain (*S. aureus* strain 38 or SAUT1), was used as our heterologous challenge strain. This strain was originally isolated from a dairy cow with mastitis was another dominant strain frequently isolated from different farms(Abdi et al., 2018). An aliquot from a frozen vial of *S. aureus* strain UT60 (SAUT2) stored at -80^{0}C in 50% tryptic soy broth / glycerol was inoculated

Table 1. Vaccination protocol

Group	# of cows	Vaccine	Adjuvant	Route	Dose	Total volume (ml)	Frequency
1	6	SASP	Em-D	SQ	1.2mg	3	3X at 14 d interval at D-28, D-14 & D0
2	6	SCSP	Em-D	SQ	1.2 mg	3	“
3	6	PBS	Em-D	SQ	1.5 ml PBS + 1.5 ml Em-D	3	“

Legend: SASP = *Staphylococcus aureus* surface proteins, SCSP = *Staphylococcus chromogenes* surface proteins, EM-D = Emulsigen D, SQ = subcutaneous, D-28 = 28 days before drying off, D-14 = 14 days before drying off, D0 = at drying off.

to a blood agar plate and grown overnight (23 to 24 h) at 37^0C. After incubation, five colonies were inoculated into 2 liters of tryptic soy broth (TSB) and grown for 3.5 h at 37^0C to create our challenge culture. Prior to challenge, teats were cleaned thoroughly with a mild non-bactericidal dish detergent and dried with an individual paper towel. Each teat was challenged two weeks after dry off by teat dipping in a *S. aureus* culture suspension using a single cup per cow, containing approximately 100 ml of *S. aureus* suspension at 1×10^7 CFU/ml of culture media for approximately 15 sec. The number of *S. aureus* in the challenge suspension was determined using a viable plate count before and after the challenge. Each cow was challenged once a day for 14 consecutive days, or until removal due clinical mastitis for 3 consecutive days. Challenged teats were allowed to air dry for about 10 min prior to releasing cows from the parlor and the floor of the parlor was disinfected by bleach at 1:10 dilution.

Clinical examination of challenged cows

During the challenge period, rectal temperature, clinical assessment of inflammatory changes in the mammary glands tissue, and milk/mammary secretion were clinically examined and the findings were recorded by qualified laboratory personnel. The following milk scoring system was used to determine severity of changes: 0 = Normal, 1 = Flakes, 2 = Clots, 3 = Stringy/watery/bloody (Table 2). Inflammatory changes in the mammary glands were scored as follows: 0 = Normal; the udder is pliable, no detection of heat, pain, redness, and/or swelling, 1 = Slight swelling; the udder is less pliable, some firmness detected, heat, pain, redness, and/or swelling not necessarily detected, 2 = Moderate swelling; the udder is firm, redness and heat detected, discomfort detected, 3 = Severe swelling; the udder is very hard, red and hot, noticeable difference compared to other quarters and the cow exhibits signs of irritation (Table 3).

Rectal temperature of each cow was taken daily during challenge period to monitor for potential systemic reactions to challenge with *S. aureus.* A cow was declared to have clinical mastitis when a score of 2 was achieved for both abnormal

Table 2. Scoring scheme for abnormal changes in milk or dry secretion

Score	**Milk or dry secretion appearance**
0	Normal
1	Flakes
2	Slugs / clots
3	Stringy / watery / bloody

Table 3. Scoring scheme for abnormal changes in the mammary gland tissue

Score	**Mammary Gland Appearance**
0	Normal; the udder is pliable. Heat, pain, redness, and/or swelling are not detectable. Cow exhibits no signs of discomfort.
1	Slight swelling; the udder is less pliable with some firmness or heavier in weight. Redness, heat and pain are generally not detectable.
2	Moderate swelling; the udder is definitely firm, heavy, reddened and warm to the touch. The cow generally exhibits signs of discomfort (irritable, performs a stepping motion with feet and/or kicks) during evaluation.
3	Severe swelling; the udder is very hard, heavy, red and hot, and noticeably larger than other quarters. The cow is extremely uncomfortable, very irritable and manifests pain by kicking and stepping.

inflammatory changes in milk and udder tissue or a score of 3 was achieved either for abnormal inflammatory changes in milk or in udder tissue for three consecutive days. For a cow in lactation with somatic cell count of ≥200,000 SCC/mL of composite milk from quarters or >100,000 SCC/mL of milk from individual quarter and positive isolation of the challenge strain from milk during 3 consecutive days, with no clinical signs, we declared subclinically infected. For dry cows, these scores are higher because of increase in SCC due to decrease in secretion volume. So in this study, SCC of ≥250,000 cells/ml of milk, with positive isolation of the challenge strain from milk for 3 consecutive days and no clinical signs, was declared subclinically infected. Clinically infected cows were removed from challenge and treated with antibiotics (Ceftiofur hydrochloride/Spectramast DC) (Zoetis Inc., Kalamazoo, MI) following manufacturer instructions for dry cow treatment. The antibiotic sensitivity patterns of the challenge strain 60 (SAUT2) was conducted prior to the beginning of the challenge study during challenge model development and the selected challenge strain 60 (SAUT2) was sensitive to ceftiofur hydrochloride. The clearance of infection was checked by culturing milk samples collected on day of calving (C) and three days after calving (C+3).

Mammary gland secretion and blood collection

Mammary gland secretion samples were collected 1 week before the beginning of the study (D-35), at 28 and 14 days before drying off (D-28 and D-14) and at drying off (D0), and daily during challenge period at days 0 – 7 (Ch0 – Ch+7) and at days 10 and 14. Individual quarter milk samples collected on day of calving (C) and 3 days after calving (C+3) to evaluate presence of *S. aureus* challenge strain (Table 4). Samples were collected aseptically in sterile 15 ml tubes, placed on ice and transported to the laboratory. At the lab, mammary gland secretions were placed at – 20^{o}C until processed.

Blood samples were collected 1 week before the beginning of the study (D-35) and immediately prior to each vaccination at D-28, D-14, and D0, and then during challenge period of days 0 – 7 (Ch0 – Ch+ 7) and on days 10 and 14 (Table 4).

Table 4. Sample collection and data recording schedule

Sample type	**Group**	**Sample collection time in days**
Blood and mammary secretion for antibody titers	All animals	D-35, D-28, D-14, D0, Ch0 - Ch+7, Ch+10, Ch+14
Mammary secretion for bacteriological culture	All animals	D-28, D-14, D0, Ch0 – Ch+7, Ch+10, Ch+14, C0, C+3 and about 2 milk samples following antibiotic treatment for animals removed due to mastitis
Mammary secretion for somatic cells count (SCC)	All animals	D-28, D-14, D0, Ch0 - Ch+7, Ch+10, Ch+14
Vaccination	All animals	D-28, D-14, D0
Rectal temperature	All animals	D-29, D-28, D-27, D-26, D-25, D-22, D-14, D-13, D-12, D-11, D-7, D0, D+1, D+2, D+3, Ch0, Ch+1, Ch+2, Ch+3, Ch+4, Ch+5, Ch+6, Ch+7, Ch+8, Ch+9, Ch+10, Ch+11, Ch+12, Ch+13, Ch+14
Injection site reaction	All animals	D-29, D-28, D-27, D-26, D-25 D-22, D-14, D-13, D-12, D-11, D-7, D0, D+1, D+2 D+3, D+7, D+14
Challenge (teats dipping)	All animals	Ch0 – Ch+13
Milk score	All animals	Ch0 – Ch+14
Mammary gland score	All animals	Ch0 – Ch+14

Immediately after collection, samples were centrifuged at 2,500 rpm for 20 min at 4ºC and serum was separated out and stored at -20ºC until evaluated by ELISA assay. Skim milk was prepared from milk or mammary secretion samples by centrifugation at 20, 000 x g for 30 min to remove fat and cellular debris. Skim samples were stored at -20ºC until antibody titers were analyzed by ELISA.

Milk sample evaluation

Somatic cell count of milk samples was determined by the Dairy Herd Improvement Association Laboratory (Knoxville, TN). Bacteriological analysis were conducted following the National Mastitis Council guidelines as described by Oliver et al. (Oliver et al., 2004). Briefly, 100 µl of milk was streaked on tryptic soy agar with 5% sheep blood (blood agar plates) (Becton, Dickinson and Company, Franklin Lakes, NJ) and incubated at 37ºC for 24 h to 48 h until colony growth was observed. Colony characteristics such as morphology, color, and hemolysis on blood agar were recorded. Those suspected to be *Staphylococcus* spp. were further tested by gram staining and catalase test to differentiate them from *Streptococcus* spp. The coagulase tube test using rabbit plasma was used to differentiate *S. aureus* from coagulase negative staphylococcus (CNS) species. Those which were catalase positive and coagulase positive were identified as *Staphylococcus aureus*.

Vaccine safety

In order to monitor for adverse reactions, rectal temperature and injection site reaction were measured and recorded. Rectal temperatures were taken 24 h prior to vaccination, immediately before vaccination, and for 3 consecutive days following vaccination and at days 7 and 14 (Table 4). Injection site reactions were monitored at the same time points by measuring the length (cranial/caudal), width (dorsal/ventral), and height (thickness) in millimeters (mm). All vaccinated animals were closely monitored for loss of appetite or any other complications at 24 h, daily for 1 – 3 days and at days 7 and 14 after each vaccination.

Evaluation of vaccine induced immune response by ELISA

Serum- and milk-anti-SASP and -SCSP IgG, IgG1, IgG2, and IgA antibody titers were determined using indirect enzyme-linked immunosorbent assay (ELISA) as described elsewhere (Kerro Dego et al., 2006). Briefly, 96 well polystyrene plates (Immulon® 2 HB) (ThermoScientific, Rochester, NY) were coated with 1 µg/ml of SASP or SCSP in a sodium bicarbonate (NaHCO3) coating buffer [1.6 grams sodium carbonate (Na_2CO3), 2.9 grams sodium bicarbonate (NaHCO3) in a total volume of 1L with laboratory grade water and pH adjust to 9.6] and incubated overnight (16 h) at 4ºC. The coating buffer was removed and plates were washed 5X using an automated 405 touch screen (TS) microplate washer (Biotek instrument Inc, Winooski, VT) with PBS containing 0.05% tween 20 (v/v) (PBS-T) and blocked with PBS-T containing 1% gelatin (W/V) (PBS-TG) for 2 h. The Plates were washed 5X with PBS-T and serum was serially diluted four-fold with PBS-TG starting from 1:100 dilution.

Milk or skim samples were serially four-fold diluted with PBS-TG starting from 1:10 dilution and incubated for 1h at room temperature. Then plates were washed 5X and 100 µl of 1:10,000 diluted (in PBS-TG) horseradish peroxidase-conjugated polyclonal sheep anti-bovine IgG or IgA (heavy + Light Chain) or monoclonal sheep anti-bovine IgG1 or IgG2 (Bethyl Laboratories, Inc. Montgomery, TX) were added and incubated for 1h at room temperature. After incubation, plates were washed 5X with PBS-T, and 100 µl of ABTS@ horseradish peroxidase substrate (1 Component, KPL-SeraCare Life Sciences, Milford, MA) were added and incubated for 20 min at room temperature.

The absorbance was read at wavelength of 405 nm using a Synergy H1 Microplate reader (Biotek instrument Inc, Winooski, VT). Data was exported to Excel (Microsoft Corporation) and the average + 2 standard deviation (avg+2 stdev) of the blank row, which received everything except our primary antibody (serum or skim milk), were used to determine cutoff point for titer calculation. Serum or skim titers were calculated by the intersection of least-square regression of A_{405} versus logarithm of dilution.

STATISTICAL ANALYSES

To assess the effect of SASP and SCSP vaccines on antibody responses, antibody impacts on experimental intramammary infection and measures of infection post-challenge, a mixed model ANOVA was used (SAS 9.4). Continuous measures were assessed using a mixed model ANOVA evaluating the fixed effects of SASP and SCSP vaccines (treatments) at certain time points post vaccination (e.g. D-28, D-14, D0, Ch0, Ch+1 - Ch+3, Ch+7, & Ch+14), and the interaction of the treatment and day. A significant effect was declared when $P \leq 0.05$. Multiple comparisons among treatment means were evaluated with Fisher's least significant difference (LSD). To accomplish this, linear regression models were conducted within treatment groups to evaluate combinations of serum and milk anti-SCSP and -SASP IgG, IgG1, IgG2, and IgA antibody titers, somatic cell count (SCC), and *S. aureus* shedding through milk. Regression models were conducted within treatment groups to evaluate the relationship among milk and serum antibody titers and 2017 calendar year milk production patterns (7 – 47 days in milk (DIM), 47 – 87 DIM, 87 – 127 DIM, milk per day, and peak milk) with 2016 milk production added as a covariate.

To assess the effect of SASP and SCSP vaccines on somatic cell count, *S. aureus* bacterial shedding through milk and infection status during the challenge period, a mixed model ANOVA was used (SAS 9.4). Continuous measures were assessed using a mixed model ANOVA, evaluating the fixed effects of SASP and SCSP vaccines (treatments) at different time points (e.g. D-28, D-14, D0, Ch0, Ch+1 - Ch+3, Ch+7, & Ch+14). A significant effect was declared when $P \leq 0.05$. Fisher's exact test was used to evaluate the clinical frequency of post challenge infection status.

RESULTS

Vaccine safety

Out of 6 SASP vaccinated cows, one cow was removed from the study and euthanized due to physical injury that resulted in permanent lameness. Only five cows completed the study in SASP vaccinated group. There were no signs of systemic

reactions to the vaccine throughout the vaccination period. All vaccinated cows developed local reactions at injection sites characterized by slight to moderate swelling. There was no significant difference ($P > 0.05$) in regards to size (mm) of injection site reaction among the SASP (39.12 ± 2.20), SCSP (42.46 ± 2.10), and the control (40.24 ± 2.10) groups. All vaccinated cows had normal rectal body temperature at 24 h prior to vaccination, immediately before vaccination and for 3 consecutive days (1 – 3 days) after each vaccination and at 7 and 14 days after each vaccination (data not shown). Rectal body temperatures were also monitored each day throughout the 14 days of the challenging period. There was no significant difference ($P > 0.05$) in regards to the mean rectal temperatures (°F) among the SASP (100.96 ± 0.12), SCSP (100.97 ± 0.12), and the control (101.2 ± 0.12) groups.

Immune responses to vaccines

Serum anti-SASP IgG2 titers were significantly higher ($P < 0.05$) in SASP vaccinated group compared to control group at D-14, Ch0, and Ch+7 (Fig. 1, panel A). There was no significant difference ($P > 0.05$) in serum anti-SASP IgG2 titers at D-28, D0, or Ch+14. Serum anti-SCSP IgG1 titers were significantly higher ($P < 0.05$) in vaccinated group compared to control group at D-14, Ch0, Ch+7 and Ch+14 (Fig. 1, panel B). There was no significant difference ($P > 0.05$) in serum anti-SCSP IgG1 titers at D-28 or D0. There was an overall significant difference ($P < 0.05$) in serum anti-SASP IgG1 titers in the vaccinated cows compared to the control cows. There was no significant differences ($P > 0.05$) in serum anti-SASP IgG and IgA. There was no significant differences ($P > 0.05$) in serum anti-SCSP IgG, IgG2, and IgA. There was no significant difference in milk anti-SASP antibody titers IgG, IgG1, IgG2, or IgA in the vaccinated cows compared to the control cows ($P > 0.05$). As well as no significant difference in milk anti-SCSP antibody titers IgG, IgG1, IgG2, and IgA in the vaccinated cows compared to the control cows ($P > 0.05$).

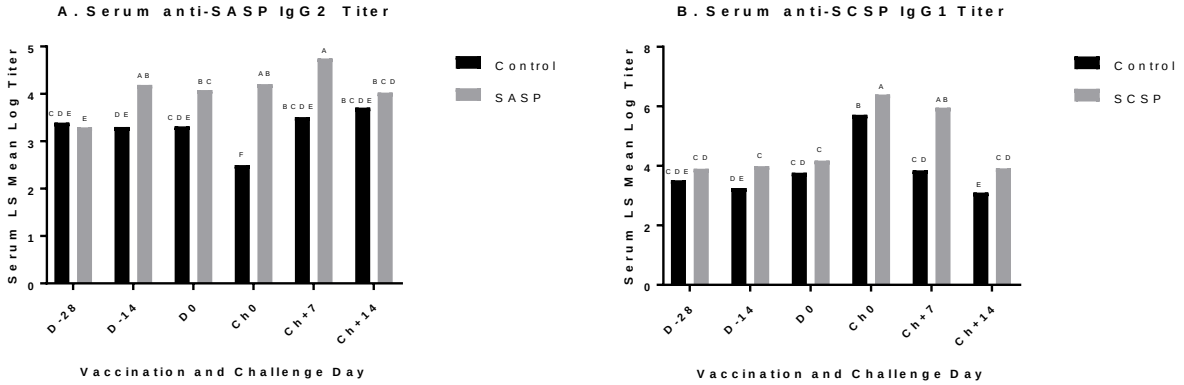


Figure 1. Serum anti-SASP and -SCSP antibody titers. SASP = Staphylococcus aureus surface proteins, SCSP = Staphylococcus chromogenes surface proteins, D-28 = 28 days before drying off, D-14 = 14 days before drying off, D0 = at drying off, Ch = Challenge, Ch0 = right before challenge, Ch+7 = on day 7 of challenge, Ch+14 = on day 14 of challenge, Ctrl = Control. The different letters represent statistically different titers ($P < 0.05$).

Protective effects of SASP and SCSP vaccines

Of the 5 cows in SASP vaccine group, 2 were infected clinically and another 2 were infected subclinically and the remaining one cow was neither clinically or subclinically infected but shed low number of *S. aureus* through milk on Ch+1 (Figs. 2 and 3, Table 8). Of 6 control cows, 2 were infected clinically and the 4 cows were infected subclinically (Figs. 2 and 3, Table 8). Out of 6 SCSP vaccinated cows all cows were neither clinically nor subclinically infected but they were shedding relatively low number of *S. aureus* through milk during challenge time of 14 days (Figs. 2 and 3, Table 8). Further evaluation on number of *S. aureus* shedding through milk and somatic cell count (SCC) showed that SASP vaccinated and subclinically infected cows were shedding relatively lower number of *S. aureus* (Fig. 4A) through milk and had lower number of somatic cell counts (SCC) (Fig. 4B) compared to control cows (Fig. 5 A&B). The SCSP vaccinated cows shed lowest number of *S. aureus* through milk (Fig. 4C) and had lowest number of somatic cell count (SCC) (Fig. 4D) compared to SASP vaccinate cows (Fig. 4A&B) and control cows (Fig. 5A&B). However, statistical analysis results showed that there were no significant difference ($P > 0.05$) in the clinical frequency among the SCSP (0%), SASP (33.33%), and control (33.33%) groups.

Overall, there was no significant difference in bacterial shedding in the vaccinated cows compared to the control cows over the 14 days of challenge period ($P > 0.05$). However, vaccination of dairy cows at early dry period with SASP or SCSP proteins resulted in a relatively lowest *S. aureus* counts in CFU/ml of milk in SCSP vaccinated group followed by lower *S. aureus* counts in SASP vaccinated group compared to the control group that had highest counts during experimental challenge period of 14 days (Fig. 3).

The somatic cell counts from the dry cow secretion is higher than SCC from milk of a cow in lactation because of the decrease in volume of fluid in the mammary gland during dry period. Therefore, SCC is not good criteria for evaluation of intramammary infection in dry cows but infected cows still have relatively higher count compared to non-infected cows. There was a significant difference in the somatic cell count (SCC) of

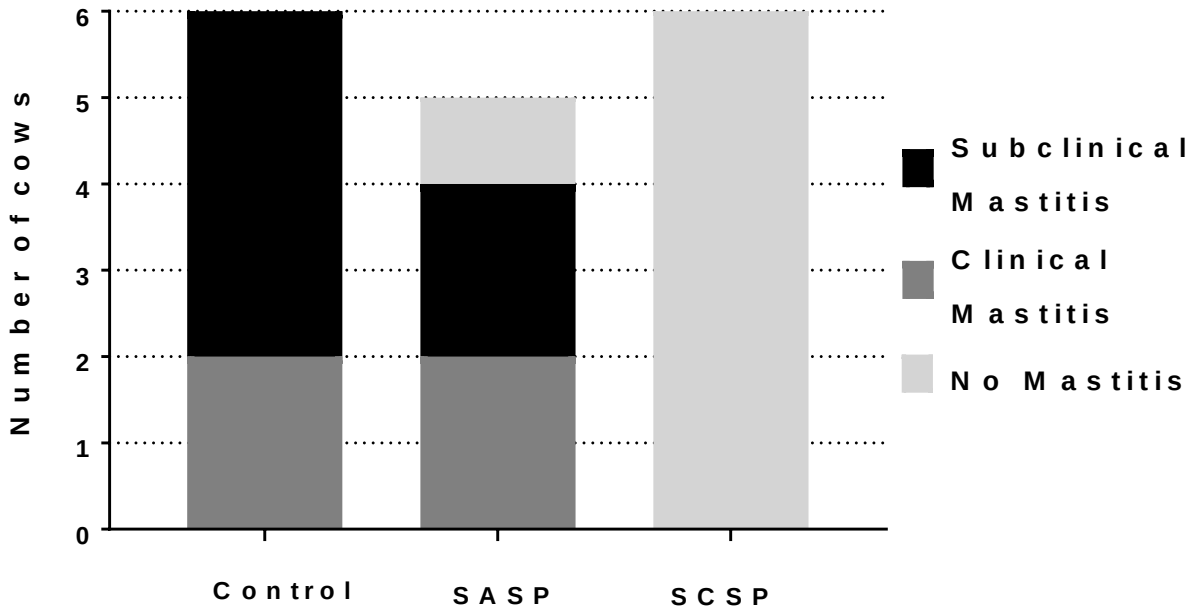


Figure 2. Response of cows to *S. aureus* challenge over the period of 14 days. *Staphylococcus aureus* surface proteins (SASP) vaccinated group, *Staphylococcus chromogenes* surface proteins (SCSP) vaccinated group.

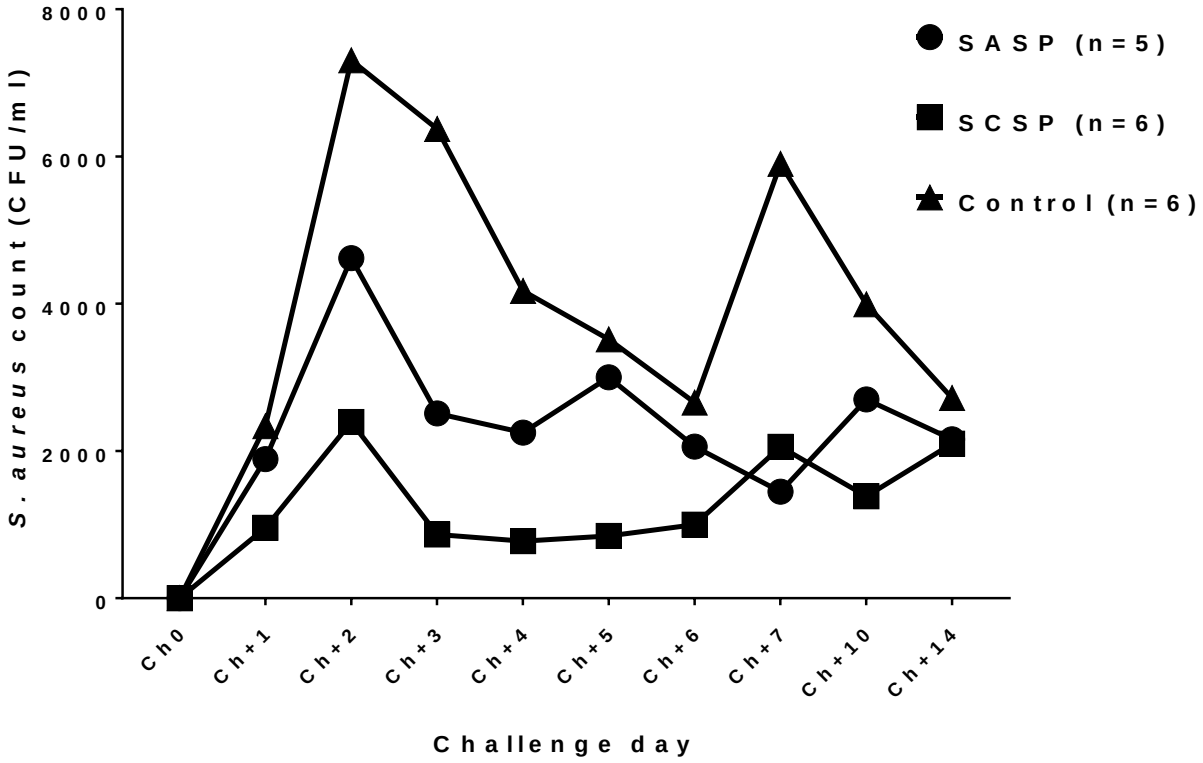


Figure 3. Number of *S. aureus* shedding through milk over 14 days of challenge period. Ch = Challenge (infection), SASP = *Staphylococcus aureus* surface proteins vaccinated group, SCSP = *Staphylococcus chromogenes* surface proteins vaccinated group, Ch+1 – Ch+7 = Day 1 to Day 7 of challenge and at days 10 and 14 (Ch+10 & Ch+14) of challenge, each symbol on the curve showed mean of *S. aureus* counts in CFU/ml of milk per group per day and $P > 0.05$.

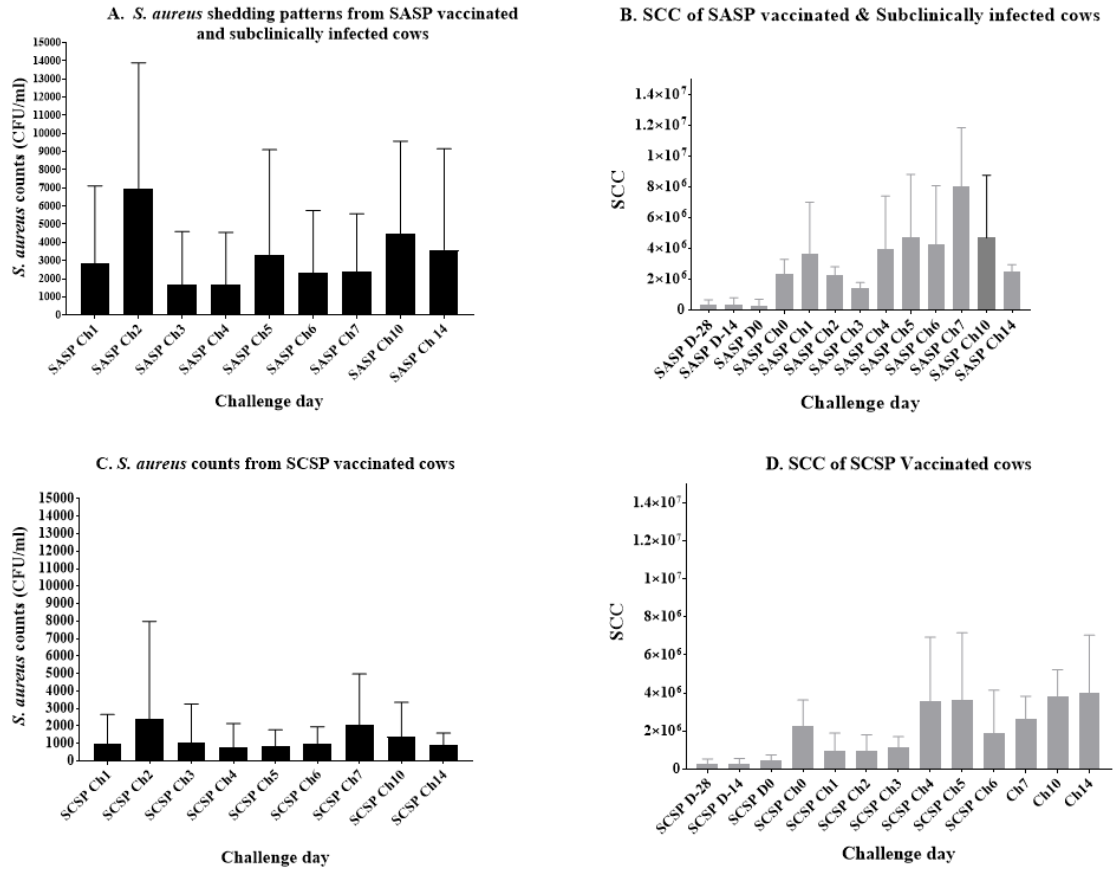


Figure 4. Number of *S. aureus* shedding through milk/mammary secretion and somatic cell counts (SCC) for SASP vaccinated and subclinically infected cows or for SCSP vaccinated cows. *Staphylococcus aureus* counts in mammary secretion from SASP vaccinated and subclinically infected cows (A), somatic cell counts (SCC) from SASP vaccinated and subclinically infected cows (B), number of *S. aureus* counts from SCSP vaccinated cows (C) and somatic cell counts (SCC) from SCSP vaccinated cows (D). SASP = *Staphylococcus aureus* surface proteins, SCSP = *Staphylococcus chromogenes* surface proteins, Ch0 = day of challenge, Ch+ 1 – Ch+7, Ch+10 and Ch+14 = Days 1 – 7 of challenge and days 10 and 14 of challenge.

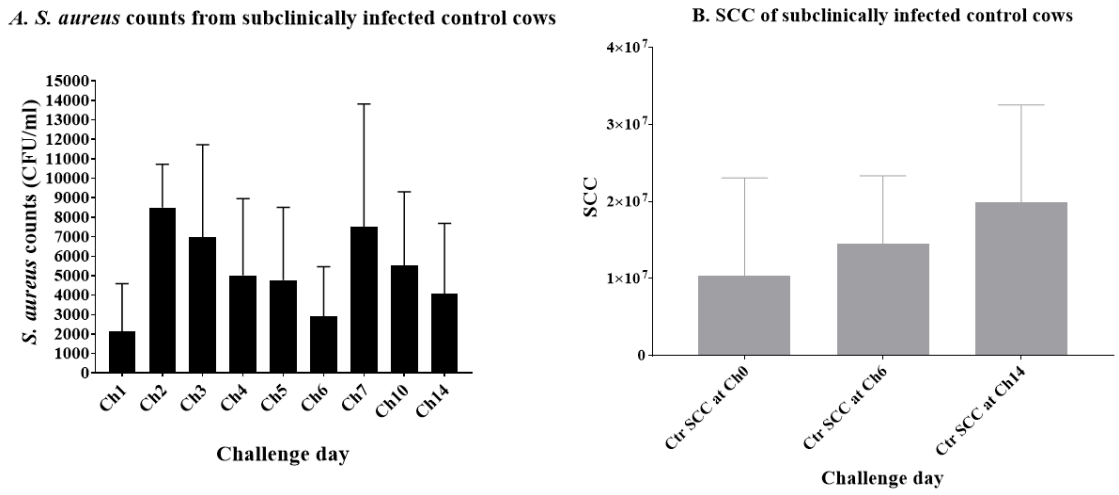


Figure 5. Number of *S. aureus* shedding through mammary secretion and somatic cell count (SCC) for control cows at certain time points during challenge. (A) Number of *Staphylococcus aureus* counts on days 1 – 7, 10 and 14 of challenge, (B) somatic cell counts (SCC) immediately before challenge (Ch0) and on days 6 (Ch+6) and 14 (Ch+14) of challenge. Ctr = Control group.

the SASP vaccinated cows and SCSP vaccinated cows compared to the control cows ($P < 0.05$). Somatic cell count (SCC) were relatively higher in subclinically infected control cows compared to SASP vaccinated and subclinically infected cows and SCSP vaccinated and non-clinically or –subclinically infected cows that were shedding *S. aureus*. Most heifers and few quarters of some cows don't have enough secretion for SCC during the beginning of the study than during the challenge period. Some days during challenge, not enough mammary secretion was obtained from some heifers in the control group. Because of that, only Ch0, Ch+6 and Ch+14 samples were analyzed for SCC.

The relationship among serum or milk anti-SCSP and –SASP antibody titers and number of S. aureus shedding through milk during challenge period

Evaluation of relationship among number of *S. aureus* shedding through milk and titers showed a significant ($P < 0.05$) relationship between serum anti-SCSP IgG antibody titer and average number of *S. aureus* shedding in SCSP vaccinated cows compared to the control cows. This significance means that without treatment, the bacterial shedding would increase, thus the serum anti-SCSP IgG titer kept bacterial shedding at a constant level. There were no significant ($P > 0.05$, data not shown) relationship among milk anti-SCSP IgG or serum or milk anti-SCSP IgG1, IgG2 and IgA antibody titers of SCSP vaccinated cows and *S. aureus* shedding through their milk during challenge period. Similarly, there were no significant ($P > 0.05$, data not shown) relationship among serum anti-SASP IgA or serum or milk anti-SASP IgG, IgG1, IgG2, or IgA antibody titers of SASP vaccinated cows and *S. aureus* shedding through their milk during challenge period (Appendix 1).

The relationship among anti-SCSP and –SASP antibody titers and somatic cell count (SCC) during challenge period

Significant relationships ($P < 0.05$) existed among serum anti-SASP IgG, IgG1 and milk anti-SASP IgG1 titers and somatic cell counts (SCC) of SASP vaccinated cows during challenge period. Which means when these serum anti-SASP IgG and IgG1

titers and milk anti-SASP IgG1 titers increased, the SCC of those SASP vaccinated cows decreased. There were no significant ($P > 0.05$, data not shown) relationship among serum and mammary secretion of anti-SCSP IgG, IgG1, IgG2 and IgA antibody titers and somatic cell count (SCC) during challenge period. Similarly, there were no significant ($P > 0.05$) relationship among serum anti-SASP IgG2, IgA and milk anti-SASP IgG, IgG2 and IgA antibody titers and somatic cell count (SCC) during challenge period (Appendix 2).

The relationship among serum and milk antibody titers and milk production status

For the purpose of this study, milk production was evaluated during 7 – 47 days in milk, 47 – 87 days in milk, and 87 – 127 days in milk, milk per day, and peak milk. A significant ($P < 0.05$, Appendix 3) relationship was found between serum anti-SASP IgG1 titer at Ch0 and milk production during 7 – 47 DIM in the SASP vaccinated group compared to the control group. There were no significant ($P > 0.05$, data not shown) relationship between the milk or serum anti-SASP and anti-SCSP antibody titers at D-28 and milk production status.

DISCUSSION

The goal of this study was to test the protective effects of two experimental *S. aureus* mastitis vaccines through an infection challenge study during early dry period and transition (periparturient) period. Proper implementation of current control measures have reduced the incidence of mastitis infections but do not prevent new infections from establishing. A major problem with the currently available *S. aureus* commercial vaccines (Lysigin® and Startvac®) is their limited to no protective effects in field trials and controlled experimental studies (Bradley et al., 2015; Middleton et al., 2009; Middleton et al., 2006; Schukken et al., 2014). It was also shown that the vaccination with Startvac® (Hippra, Spain) did not have any beneficial effects on udder health, milk production, or rate of culling in dairy herds with *S. aureus* mastitis (Landin et al., 2015).

The SASP and SCSP vaccines both induced an increased immune response in the vaccinated cows compared to the control cows. There was a significant increase in the serum anti-SCSP and anti-SASP IgG1 titers in the vaccinated cows compared to the control cows. As well as a significant increase in the serum anti-SASP IgG2 titers in vaccinated cows compared to control cows. These findings correspond with findings in other studies where there was an overall increase in the serum total antibody titers in vaccinated groups compared to non-vaccinated control groups (Gurjar et al., 2013; Wilson et al., 2007). Early studies of the role of IgG1 and IgG2 in ruminants show that when these antibodies are present, polymorphonuclear leukocytes were able to phagocytose antigens effectively (McGuire et al., 1979). With increases in the IgG2 antibody, there is an increased detection of the presence of microbes to mark them for destruction by phagocytic cells. This was indicated with results found in a study conducted by Wilson et al. (2007), where there was no clear switch toward IgG1 or IgG2 dominated responses. However, there was a significant increase in both IgG1 and IgG2 titers of vaccinated cows over the dry period compared to non-vaccinated control cows (Wilson et al., 2007). In the SCSP vaccine group, there were no clinical or subclinical infections. With the significant increase in IgG1, the protection from clinical infection may be achieved by IgG1 and/or IgG2 mediated opsonophagocytic removal of *S. aureus* from the mammary gland. High background titers were recorded at the starting baseline of the study in all cows. This could be due to prior exposure, since the cows in this study were not free from exposure to *S. aureus* or other similar bacterial pathogens before this study. However, there were increases in titers from baseline titers after vaccination in all vaccinated cows compared to control cows. These results indicate that our vaccines did induce an increased immune response in the vaccinated cows.

The protection efficiency of our two experimental vaccines (SASP and SCSP) was tested through teat dip challenge method with *S. aureus* culture. The teat dip challenge model was used to mimic natural infection. Results of this challenge study showed that SASP vaccine did not induce protection, since 2 of the 5 cows were clinically infected and 2 were subclinically infected. However, the remaining one cow was shedding low number of *S. aureus* in the milk but was not infected clinically or subclinically. Further

study with increased number of animals is required to determine the protective effect of SASP vaccine. The SCSP vaccinated group was protected from both clinical and subclinical intramammary infections. This observation is very interesting and requires further evaluation with increased number of animals to confirm these findings. In the control group, 2 of the 6 cows were clinically infected and the remaining 4 were subclinically infected. These results suggest that the SCSP vaccine is effective at controlling *S. aureus* intramammary infection.

Bacterial shedding in the SASP and SCSP vaccination groups and control groups were not significantly different. However, there was a relatively lower number of *S. aureus* shedding through milk of the vaccinated cows compared to the control cows. Even more so, between the two vaccination groups, the SCSP vaccinated cows had lower bacterial shedding throughout the challenge period compared to the SASP vaccinated cows. These results showed that the SCSP vaccine was better at reducing *S. aureus* shedding than the SASP vaccine as well as control group. Low number of *S. aureus* shedding throughout the challenging period, without increase in somatic cell count may be due to daily challenge with *S. aureus*. This bacteria could have been unable to colonize glands and removed with milk rather than establishing infection, in which *S. aureus* is growing and multiplying inside the mammary gland. Monitoring cows over longer period of time after challenge period before treating them with antibiotics would clarify this findings.

This study was conducted during the dry period, which resulted in a relatively increased SCC due to decreased volume of secretion samples. Some of the cows enrolled in this study were heifers, which made it even more difficult to collect enough sample for SCC. Due to this, enough sample for SCC in the control group was only collected on Ch0, Ch+6, and Ch+14. There was lower SCC in the SASP and SCSP vaccinated group compared to control group. The SCC of the cows that were categorized as subclinically infected in the SASP vaccination group was compared to the SCC of all the cows in SCSP vaccination, since none were clinically or subclinically infected, and there was a relatively lower SCC in the SCSP vaccination group. A meta-analysis conducted by Djabri et al. (2002), found that SCC for *S. aureus* infections were

roughly 357,000 cells/ml and for CNS infections were roughly 138,000 cells/ml. The results from Djabri et al. (2002) showed that overall, *S. aureus* infection caused a greater increase in SCC compared to CNS infections. In contradiction, Sharma et al. (2011), found that both *S. aureus* and *S. chromogenes* had relatively similar increase in SCC compared to other species. The high SCC during the dry period, the differences in parity (Sharma et al., 2011) of the cows enrolled in this study, and/or the infection status all contributed to increased SCC and differences of SCC in the treatment groups. So use of cows in the same parity would aide in avoiding this possibility.

Further analysis on the relationship between the serum and milk antibody titers with bacterial shedding showed that increases in serum anti-SCSP IgG antibody titer kept the *S. aureus* shedding at a constant level and that without treatment, the bacterial shedding numbers would increase. This could indicate that IgG play a role in reducing *S. aureus* bacterial shedding. Overall, there were no significant differences among treatment groups for either IgG or IgA, and there was no significant difference in *S. aureus* shedding. However, this relationship showed that IgG antibody plays a role in lowering the bacterial shedding in the mammary gland.

Furthermore, the relationship between antibody titers and SCC was analyzed. Significant relationships were found in serum anti-SASP IgG and IgG1, titers. This relationship represents that when these antibody titers increased, the SCC decreased. Interestingly, there was no relationship found with anti-SCSP antibody titers and SCC. However, this vaccine group had no clinical or subclinical infections. However, as indicated previously, the use of SCC as a measurement for IMI for dry cows is problematic since SCC already high due to reduction in secretion volume. Though there were increased immune responses in both vaccination groups, there was little correlation of the increased immune responses with decreasing SCC.

The relationship between antibody titers and milk production was analyzed to see whether our vaccines had any effect on milk production following this study. For this analysis we used milk produced at 7 - 47 days in milk, 47 - 87 days in milk, and 87-127 days in milk, milk per day, and peak milk. With an increase in serum anti-SASP IgG1 antibody titer, there was a decrease in milk production at 7- 47 days in milk. In regards

to the SCSP vaccine, there was neither an increase nor decrease in milk production during those time points. This means the immune responses induced by this vaccine did not have a negative impact on the milk production. Further detail study over longer period of time is required to determine conclusive findings on this observation.

CONCLUSION

In conclusion we observed that three consecutive vaccinations of dairy cows at 28 and 14 days before drying off, and at drying of with SASP and SCSP induced a significant increase in immune responses in vaccinated cows compared to control cows. The subsequent experimental challenge of vaccinated cows with the heterologous strain of *S. aureus* resulted in reduced number of bacterial shedding in milk in vaccinated cows compared to control cows. More interestingly, SCSP vaccine cross-protected vaccinated cows from *S. aureus* mastitis indicating that SCSP seems to perform better than SASP as a vaccine to control *Staphylococcus aureus* mastitis in dairy cows. Further detailed studies using different antigen doses (antigen dose titration) with different adjuvants, routes of vaccination coupled with monitoring of duration of immunity while evaluating efficacy by natural exposure may optimize the efficacy of SCSP vaccine to control *S. aureus* mastitis.

REFERENCES

Abdi, R.D., Gillespie, B.E., Vaughn, J., Merrill, C., Headrick, S.I., Ensermu, D.B., D'Souza, D.H., Agga, G.E., Almeida, R.A., Oliver, S.P., 2018. Antimicrobial resistance of Staphylococcus aureus isolates from dairy cows and genetic diversity of resistant isolates. Foodborne pathogens and disease.

Akers, R.M., Nickerson, S.C., 2011. Mastitis and its impact on structure and function in the ruminant mammary gland. Journal of mammary gland biology and neoplasia 16, 275-289.

Almeida, R., Oliver, S., 1993. Antiphagocytic effect of the capsule of Streptococcus uberis. Zoonoses and Public Health 40, 707-714.

Almeida, R.A., Dego, O.K., Headrick, S.I., Lewis, M.J., Oliver, S.P., 2015a. Role of Streptococcus uberis adhesion molecule in the pathogenesis of Streptococcus uberis mastitis. Veterinary microbiology 179, 332-335.

Almeida, R.A., Dogan, B., Klaessing, S., Schukken, Y.H., Oliver, S.P., 2011. Intracellular fate of strains of Escherichia coli isolated from dairy cows with acute or chronic mastitis. Veterinary research communications 35, 89-101.

Almeida, R.A., Fang, W., Oliver, S.P., 1999. Adherence and internalization of Streptococcus uberis to bovine mammary epithelial cells are mediated by host cell proteoglycans. FEMS microbiology letters 177, 313-317.

Almeida, R.A., Kerro-Dego, O., Prado, M.E., Headrick, S.I., Lewis, M.J., Siebert, L.J., Pighetti, G.M., Oliver, S.P., 2015b. Protective effect of anti-SUAM antibodies on Streptococcus uberis mastitis. Veterinary research 46, 133.

Almeida, R.A., Luther, D.A., Park, H.-M., Oliver, S.P., 2006. Identification, isolation, and partial characterization of a novel Streptococcus uberis adhesion molecule (SUAM). Veterinary microbiology 115, 183-191.

Almeida, R.A., Matthews, K.R., Cifrian, E., Guidry, A.J., Oliver, S.P., 1996. Staphylococcus aureus invasion of bovine mammary epithelial cells. Journal of Dairy Science 79, 1021-1026.

Aydin, A., Sudagidan, M., Muratoglu, K., 2011. Prevalence of staphylococcal enterotoxins, toxin genes and genetic-relatedness of foodborne Staphylococcus aureus strains isolated in the Marmara Region of Turkey. Int J Food Microbiol 148, 99-106.

Barber, D.A., Miller, G.Y., McNAMARA, P.E., 2003. Models of antimicrobial resistance and foodborne illness: examining assumptions and practical applications. Journal of food protection 66, 700-709.

Barbosa, T.M., Levy, S.B., 2000. The impact of antibiotic use on resistance development and persistence. Drug resistance updates 3, 303-311.

Bayles, K.W., Wesson, C.A., Liou, L.E., Fox, L.K., Bohach, G.A., Trumble, W., 1998. Intracellular staphylococcus aureusescapes the endosome and induces apoptosis in epithelial cells. Infection and immunity 66, 336-342.

Blowey, R.W., Edmondson, P., 2010. Mastitis control in dairy herds. Cabi.

Bradley, A.J., 2002. Bovine mastitis: an evolving disease. The veterinary journal 164, 116-128.

Bradley, A.J., Breen, J., Payne, B., White, V., Green, M.J., 2015. An investigation of the efficacy of a polyvalent mastitis vaccine using different vaccination regimens under field conditions in the United Kingdom. Journal of dairy science 98, 1706-1720.
Bramley, A.J., 1982. Sources of Streptococcus uberis in the dairy herd: I. Isolation from bovine faces and from straw bedding of cattle. Journal of Dairy Research 49, 369-373.
Brüssow, H., Canchaya, C., Hardt, W.-D., 2004. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. Microbiology and molecular biology reviews 68, 560-602.
Bushnell, R.B., 1984. The importance of hygienic procedures in controlling mastitis. The Veterinary clinics of North America. Large animal practice 6, 361-370.
Butler, J.E., 1983. Bovine immunoglobulins: An augmented review. Veterinary Immunology and Immunopathology 4, 43-152.
Calvinho, L.F., Oliver, S.P., 1998. Characterization of mechanisms involved in uptake of Streptococcus dysgalactiae by bovine mammary epithelial cells. Veterinary Microbiology 63, 261-274.
Carter, E., Kerr, D., 2003. Optimization of DNA-based vaccination in cows using green fluorescent protein and protein A as a prelude to immunization against staphylococcal mastitis. Journal of dairy science 86, 1177-1186.
Craven, N., Anderson, J.C., 1984. Phagocytosis of Staphylococcus aureus by bovine mammary gland macrophages and intracellular protection from antibiotic action in vitro and in vivo. Journal of dairy research 51, 513-523.
De Vliegher, S., Fox, L.K., Piepers, S., McDougall, S., Barkema, H.W., 2012. Invited review: Mastitis in dairy heifers: Nature of the disease, potential impact, prevention, and control. Journal of Dairy Science 95, 1025-1040.
De Vliegher, S., Opsomer, G., Vanrolleghem, A., Devriese, L.A., Sampimon, O.C., Sol, J., Barkema, H.W., Haesebrouck, F., de Kruif, A., 2004. In vitro growth inhibition of major mastitis pathogens by Staphylococcus chromogenes originating from teat apices of dairy heifers. Veterinary Microbiology 101, 215-221.
Djabri, B., Bareille, N., Beaudeau, F., Seegers, H., 2002. Quarter milk somatic cell count in infected dairy cows: a meta-analysis. Veterinary research 33, 335-357.
Dogan, B., Klaessig, S., Rishniw, M., Almeida, R., Oliver, S., Simpson, K., Schukken, Y., 2006. Adherent and invasive Escherichia coli are associated with persistent bovine mastitis. Veterinary microbiology 116, 270-282.
Donlan, R.M., Costerton, J.W., 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 15, 167-193.
Drackley, J.K., 1999. Biology of dairy cows during the transition period: The final frontier? Journal of dairy science 82, 2259-2273.
Emaneini, M., Jabalameli, F., Abani, S., Dabiri, H., Beigverdi, R., 2016. Comparison of virulence factors and capsular types of Streptococcus agalactiae isolated from human and bovine infections. Microbial pathogenesis 91, 1-4.

Esposito, G., Irons, P.C., Webb, E.C., Chapwanya, A., 2014. Interactions between negative energy balance, metabolic diseases, uterine health and immune response in transition dairy cows. Animal Reproduction Science 144, 60-71.
Fang, W., Oliver, S.P., 1999. Identification of lactoferrin-binding proteins in bovine mastitis-causing Streptococcus uberis. FEMS microbiology letters 176, 91-96.
Farnsworth, R. 1987. Indications of contagious and environmental mastitis pathogens in a dairy herd. In: Annual meeting-National Mastitis Council, Inc (USA).
Fowler, T., Wann, E.R., Joh, D., Johansson, S., Foster, T.J., Hook, M., 2000. Cellular invasion by Staphylococcus aureus involves a fibronectin bridge between the bacterial fibronectin-binding MSCRAMMs and host cell beta1 integrins. Eur J Cell Biol 79, 672-679.
Fox, L.K., 2012. Mycoplasma mastitis: causes, transmission, and control. Veterinary Clinics: Food Animal Practice 28, 225-237.
Freick, M., Frank, Y., Steinert, K., Hamedy, A., Passarge, O., Sobiraj, A., 2016. Mastitis vaccination using a commercial polyvalent vaccine or a herd-specific Staphylococcus aureus vaccine. Tierärztliche Praxis G: Großtiere/Nutztiere 44, 219-229.
Fry, P.R., Middleton, J.R., Dufour, S., Perry, J., Scholl, D., Dohoo, I., 2014. Association of coagulase-negative staphylococcal species, mammary quarter milk somatic cell count, and persistence of intramammary infection in dairy cattle. Journal of Dairy Science 97, 4876-4885.
Gillespie, B., Headrick, S., Boonyayatra, S., Oliver, S., 2009. Prevalence and persistence of coagulase-negative Staphylococcus species in three dairy research herds. Veterinary microbiology 134, 65-72.
Gotz, F., 2002. Staphylococcus and biofilms. Mol Microbiol 43, 1367-1378.
Guimarães, J.L.B., Brito, M.A.V.P., Lange, C.C., Silva, M.R., Ribeiro, J.B., Mendonça, L.C., Mendonça, J.F.M., Souza, G.N., 2017. Estimate of the economic impact of mastitis: A case study in a Holstein dairy herd under tropical conditions. Preventive Veterinary Medicine 142, 46-50.
Gurjar, A.A., Klaessig, S., Salmon, S.A., Yancey, R.J., Schukken, Y.H., 2013. Evaluation of an alternative dosing regimen of a J-5 mastitis vaccine against intramammary Escherichia coli challenge in nonlactating late-gestation dairy cows. Journal of dairy science 96, 5053-5063.
Halasa, T., Huijps, K., Østerås, O., Hogeveen, H., 2007. Economic effects of bovine mastitis and mastitis management: A review. Veterinary Quarterly 29, 18-31.
Haran, K., Godden, S., Boxrud, D., Jawahir, S., Bender, J., Sreevatsan, S., 2012. Prevalence and characterization of Staphylococcus aureus, including methicillin-resistant Staphylococcus aureus, isolated from bulk tank milk from Minnesota dairy farms. Journal of Clinical Microbiology 50, 688-695.
Harmon, R., 1994. Physiology of mastitis and factors affecting somatic cell counts1. Journal of dairy science 77, 2103-2112.
Hennekinne, J.-A., De Buyser, M.-L., Dragacci, S., 2012. Staphylococcus aureus and its food poisoning toxins: characterization and outbreak investigation. FEMS microbiology reviews 36, 815-836.

Hillerton, J.E., Berry, E.A., 2003. The management and treatment of environmental streptococcal mastitis. Veterinary Clinics of North America: Food Animal Practice 19, 157-169.
Hogan, J., Smith, K., 1987. A practical look at environmental mastitis. The Compendium on continuing education for the practicing veterinarian (USA).
Holmes, M.A., Zadoks, R.N., 2011. Methicillin resistant S. aureus in human and bovine mastitis. Journal of mammary gland biology and neoplasia 16, 373-382.
Howard, C., Taylor, G., Brownlie, J., 1980. Surface receptors for immunoglobulin on bovine polymorphonuclear neutrophils and macrophages. Research in veterinary science 29, 128-130.
Ismail, Z.B., 2017. Mastitis vaccines in dairy cows: Recent developments and recommendations of application. Veterinary world 10, 1057.
Jasper, D.E., 1981. Bovine mycoplasmal mastitis. Advances in veterinary science and comparative medicine 25, 121-157.
Jayarao, B.M., Henning, D.R., 2001. Prevalence of Foodborne Pathogens in Bulk Tank Milk1. Journal of Dairy Science 84, 2157-2162.
Josse, J., Laurent, F., Diot, A., 2017. Staphylococcal Adhesion and Host Cell Invasion: Fibronectin-Binding and Other Mechanisms. Front Microbiol 8, 2433.
Kerro-Dego, O., Prysliak, T., Perez-Casal, J., Potter, A.A., 2012. Role of GapC in the pathogenesis of Staphylococcus aureus. Veterinary microbiology 156, 443-447.
Kerro, O.D., Nederbragt, H., 2002. Factors involved in the early pathogenesis of bovine Staphylococcus aureus mastitis with emphasis on bacterial adhesion and invasion. A review. The veterinary quarterly 24, 181-198.
Korhonen, H., Marnila, P., Gill, H.S., 2000. Milk immunoglobulins and complement factors. Br J Nutr 84, 75-80.
Landin, H., Mörk, M.J., Larsson, M., Waller, K.P., 2015. Vaccination against Staphylococcus aureus mastitis in two Swedish dairy herds. Acta Veterinaria Scandinavica 57, 81.
Leitner, G., Krifucks, O., Kiran, M.D., Balaban, N., 2011. Vaccine development for the prevention of staphylococcal mastitis in dairy cows. Veterinary Immunology and Immunopathology 142, 25-35.
Levison, L.J., Miller-Cushon, E.K., Tucker, A.L., Bergeron, R., Leslie, K.E., Barkema, H.W., DeVries, T.J., 2016. Incidence rate of pathogen-specific clinical mastitis on conventional and organic Canadian dairy farms. Journal of Dairy Science 99, 1341-1350.
Malek dos Reis, C.B., Barreiro, J.R., Mestieri, L., Porcionato, M.A.d.F., dos Santos, M.V., 2013. Effect of somatic cell count and mastitis pathogens on milk composition in Gyr cows. BMC Veterinary Research 9, 67.
Matthews, K., Almeida, R., Oliver, S., 1994. Bovine mammary epithelial cell invasion by Streptococcus uberis. Infection and immunity 62, 5641-5646.
McAllister, T., Yanke, L., Inglis, G., Olson, M., 2001. Is antibiotic use in dairy cattle causing antibiotic resistance. Adv Dairy Technol 13, 229-247.

McGuire, T., Musoke, A., Kurtti, T., 1979. Functional properties of bovine IgG1 and IgG2: interaction with complement, macrophages, neutrophils and skin. Immunology 38, 249-256.
Melchior, M., Vaarkamp, H., Fink-Gremmels, J., 2006. Biofilms: a role in recurrent mastitis infections? The Veterinary Journal 171, 398-407.
Middleton, J.R., Luby, C.D., Adams, D.S., 2009. Efficacy of vaccination against staphylococcal mastitis: a review and new data. Veterinary microbiology 134, 192-198.
Middleton, J.R., Ma, J., Rinehart, C.L., Taylor, V.N., Luby, C.D., Steevens, B.J., 2006. Efficacy of different Lysigin™ formulations in the prevention of Staphylococcus aureus intramammary infection in dairy heifers. Journal of dairy research 73, 10-19.
Middleton, J.R., Saeman, A., Fox, L.K., Lombard, J., Hogan, J.S., Smith, K.L., 2014. The National Mastitis Council: a global organization for mastitis control and milk quality, 50 years and beyond. Journal of mammary gland biology and neoplasia 19, 241-251.
Neave, F., Dodd, F., Kingwill, R., Westgarth, D., 1969. Control of mastitis in the dairy herd by hygiene and management. Journal of dairy science 52, 696-707.
Nicholas, R., Ayling, R., McAuliffe, L., 2007. Mycoplasma mastitis. Veterinary record 160, 382-382.
NMC 2005. The cost of mastitis: Dairy insight research 2005/2006: Final report. In National Mastitis Advisory Committee.
Normanno, G., La Salandra, G., Dambrosio, A., Quaglia, N., Corrente, M., Parisi, A., Santagada, G., Firinu, A., Crisetti, E., Celano, G., 2007. Occurrence, characterization and antimicrobial resistance of enterotoxigenic Staphylococcus aureus isolated from meat and dairy products. International journal of food microbiology 115, 290-296.
Oliver, S., Almeida, R., Calvinho, L., 1998. Virulence factors of Streptococcus uberis isolated from cows with mastitis. Zoonoses and Public Health 45, 461-471.
Oliver, S., Gonzalez, R., Hogan, J., Jayarao, B., Owens, W., 2004. Microbiological procedures for the diagnosis of bovine udder infection and determination of milk quality. Verona WI, USA: National Mastitis Council.
Oliver, S., Jayarao, B., Almeida, R. 2005. Foodborne pathogens, mastitis, milk quality, and dairy food safety. In: NMC Annual Meeting Proceedings, 3-27.
Oliver, S., Mitchell, B., 1983. Susceptibility of Bovine Mammary Gland to Infections During the Dry Period1. Journal of Dairy Science 66, 1162-1166.
Oliver, S., Mitchell, B., 1984. Prevalence of Mastitis Pathogens in Herds Participating in a Mastitis Control Program1. Journal of dairy science 67, 2436-2440.
Otto, M., 2008. Staphylococcal biofilms. Curr Top Microbiol Immunol 322, 207-228.
Owens, W., Ray, C., Watts, J., Yancey, R., 1997. Comparison of Success of Antibiotic Therapy During Lactation and Results of Antimicrobial Susceptibility Tests for Bovine Mastitis1. Journal of dairy science 80, 313-317.
Paape, M.J., Bannerman, D.D., Zhao, X., Lee, J.-W., 2003. The bovine neutrophil: Structure and function in blood and milk. Vet. Res. 34, 597-627.

Patel, D., Almeida, R.A., Dunlap, J.R., Oliver, S.P., 2009. Bovine lactoferrin serves as a molecular bridge for internalization of Streptococcus uberis into bovine mammary epithelial cells. Veterinary microbiology 137, 297-301.
Pellegrino, M., Giraudo, J., Raspanti, C., Nagel, R., Odierno, L., Primo, V., Bogni, C., 2008. Experimental trial in heifers vaccinated with Staphylococcus aureus avirulent mutant against bovine mastitis. Veterinary Microbiology 127, 186-190.
Petrovski, K., Trajcev, M., Buneski, G., 2006. A review of the factors affecting the costs of bovine mastitis. Journal of the South African Veterinary Association 77, 52-60.
Piepers, S., Prenafeta, A., Verbeke, J., De Visscher, A., March, R., De Vliegher, S., 2017. Immune response after an experimental intramammary challenge with killed Staphylococcus aureus in cows and heifers vaccinated and not vaccinated with Startvac, a polyvalent mastitis vaccine. Journal of dairy science 100, 769-782.
Piessens, V., Van Coillie, E., Verbist, B., Supré, K., Braem, G., Van Nuffel, A., De Vuyst, L., Heyndrickx, M., De Vliegher, S., 2011. Distribution of coagulase-negative Staphylococcus species from milk and environment of dairy cows differs between herds. Journal of dairy science 94, 2933-2944.
Prenafeta, A., March, R., Foix, A., Casals, I., Costa, L., 2010. Study of the humoral immunological response after vaccination with a Staphylococcus aureus biofilm-embedded bacterin in dairy cows: Possible role of the exopolysaccharide specific antibody production in the protection from Staphylococcus aureus induced mastitis. Veterinary Immunology and Immunopathology 134, 208-217.
Pyörälä, S., Taponen, S., 2009. Coagulase-negative staphylococci—Emerging mastitis pathogens. Veterinary microbiology 134, 3-8.
Rainard, P., Riollet, C., 2006. Innate immunity of the bovine mammary gland. Vet. Res. 37, 369-400.
Riekerink, R.G.O., Barkema, H.W., Scholl, D.T., Poole, D.E., Kelton, D.F., 2010. Management practices associated with the bulk-milk prevalence of Staphylococcus aureus in Canadian dairy farms. Preventive veterinary medicine 97, 20-28.
ROHRBACH, B.W., DRAUGHON, F.A., DAVIDSON, P.M., OLIVER, S.P., 1992. Prevalence of Listeria monocytogenes, Campylobacter jejuni, Yersinia enterocolitica, and Salmonella in bulk tank milk: risk factors and risk of human exposure. Journal of Food Protection 55, 93-97.
Saini, V., McClure, J.T., Léger, D., Dufour, S., Sheldon, A., Scholl, D., Barkema, H., 2012a. Antimicrobial use on Canadian dairy farms. Journal of dairy science 95, 1209-1221.
Saini, V., McClure, J.T., Scholl, D., DeVries, T., Barkema, H., 2012b. Herd-level association between antimicrobial use and antimicrobial resistance in bovine mastitis Staphylococcus aureus isolates on Canadian dairy farms. Journal of dairy science 95, 1921-1929.
Sampimon, O.C., 2009. Coagulase-negative staphylococci mastitis in Dutch dairy herds. Utrecht University.

Sawant, A., Gillespie, B., Oliver, S., 2009. Antimicrobial susceptibility of coagulase-negative Staphylococcus species isolated from bovine milk. Veterinary microbiology 134, 73-81.
Schukken, Y., Bronzo, V., Locatelli, C., Pollera, C., Rota, N., Casula, A., Testa, F., Scaccabarozzi, L., March, R., Zalduendo, D., 2014. Efficacy of vaccination on Staphylococcus aureus and coagulase-negative staphylococci intramammary infection dynamics in 2 dairy herds. Journal of dairy science 97, 5250-5264.
Sharma, N., Singh, N., Bhadwal, M., 2011. Relationship of somatic cell count and mastitis: An overview. Asian-Australasian Journal of Animal Sciences 24, 429-438.
Shkreta, L., Talbot, B.G., Diarra, M.S., Lacasse, P., 2004. Immune responses to a DNA/protein vaccination strategy against Staphylococcus aureus induced mastitis in dairy cows. Vaccine 23, 114-126.
Smith, K., Oliver, S., 1981. Lactoferrin: a component of nonspecific defense of the involuting bovine mammary gland. Advances in experimental medicine and biology 137, 535-554.
Smith, K.L., Todhunter, D., Schoenberger, P., 1985. Environmental mastitis: Cause, prevalence, prevention1, 2. Journal of Dairy Science 68, 1531-1553.
Sordillo, L.M., Streicher, K.L., 2002. Mammary gland immunity and mastitis susceptibility. Journal of mammary gland biology and neoplasia 7, 135-146.
STEELE, M.L., McNAB, W.B., POPPE, C., GRIFFITHS, M.W., CHEN, S., DEGRANDIS, S.A., FRUHNER, L.C., LARKIN, C.A., LYNCH, J.A., ODUMERU, J.A., 1997. Survey of Ontario Bulk Tank Raw Milk for Food-Borne Pathogens. Journal of Food Protection 60, 1341-1346.
Stewart, P.S., Costerton, J.W., 2001. Antibiotic resistance of bacteria in biofilms. Lancet 358, 135-138.
Taponen, S., Björkroth, J., Pyörälä, S., 2008. Coagulase-negative staphylococci isolated from bovine extramammary sites and intramammary infections in a single dairy herd. Journal of dairy research 75, 422-429.
Taponen, S., Koort, J., Björkroth, J., Saloniemi, H., Pyörälä, S., 2007. Bovine intramammary infections caused by coagulase-negative staphylococci may persist throughout lactation according to amplified fragment length polymorphism-based analysis. Journal of Dairy Science 90, 3301-3307.
Taponen, S., Liski, E., Heikkilä, A.-M., Pyörälä, S., 2017. Factors associated with intramammary infection in dairy cows caused by coagulase-negative staphylococci, Staphylococcus aureus, Streptococcus uberis, Streptococcus dysgalactiae, Corynebacterium bovis, or Escherichia coli. Journal of dairy science 100, 493-503.
Taponen, S., Pyörälä, S., 2009. Coagulase-negative staphylococci as cause of bovine mastitis—Not so different from Staphylococcus aureus? Veterinary Microbiology 134, 29-36.
Targowski, S., 1983. Role of immune factors in protection of mammary gland. Journal of dairy science 66, 1781-1789.
Tyler, J., Baggot, J., 1992. Antimicrobial therapy of mastitis.

USDA APHIS, U., 2008a. United States Department of Agriculture, Animal Plant Health Inspection Service National Animal Health Monitoring System. Antibiotic use on U.S. dairy operations, 2002 and 2007 (Info Sheet, 5p, October, 2008). 2008a. Available at http://nahms.aphis.usda.gov/dairy/index.htm#dairy2007.

USDA APHIS, U., 2008b. United States Department of Agriculture, Animal Plant Health Inspection Service National Animal Health Monitoring System. Highlights of Dairy 2007 Part III: reference of dairy cattle health and management practices in the United States, 2007 (Info Sheet 4p, October, 2008). 2008b. Available at http://nahms.aphis.usda.gov/dairy/index.htm#dairy2007, Accessed April, 23, 2010. (Online.)

USDA APHIS, U., 2009a. United States Department of Agriculture, Animal Plant Health Inspection Service National Animal Health Monitoring System. Injection practices on U.S. dairy opera tions, 2007 (Veterinary Services Info Sheet 4 p, February 2009). 2009a. Available at http://nahms.aphis.usda.gov/dairy/index.htm#dairy2007, accessed April 23, 2010. (Online.)

USDA APHIS, U. 2014. Part III: Health Management and Bio¬security in US Feedlots, 1999. US Department of Agriculture; 2000. Available from: http://www.aphis.usda.gov/animal_health/nahms/feedlot/downloads/feedlot99/Feedlot99_dr_PartIII.pdf. In.

Van Kessel, J., Karns, J., Gorski, L., McCluskey, B., Perdue, M., 2004. Prevalence of Salmonellae, Listeria monocytogenes, and fecal coliforms in bulk tank milk on US dairies. Journal of Dairy Science 87, 2822-2830.

Waller, K.P., Aspán, A., Nyman, A., Persson, Y., Andersson, U.G., 2011. CNS species and antimicrobial resistance in clinical and subclinical bovine mastitis. Veterinary microbiology 152, 112-116.

Wang, M., Gong, S., Du, S., Zhu, Y., Rong, F., Pan, R., Di, Y., Li, C., Ren, D., Jin, N., 2017. The effect of immunoregulation of Streptococcus lactis L16 strain upon Staphylococcus aureus infection. BMC microbiology 17, 130.

Wilson, D.J., Mallard, B.A., Burton, J.L., Schukken, Y.H., Gröhn, Y.T., 2007. Milk and serum J5-specific antibody responses, milk production change, and clinical effects following intramammary Escherichia coli challenge for J5 vaccinate and control cows. Clinical and Vaccine Immunology 14, 693-699.

Woodward, W., Besser, T., Ward, A., Corbeil, L., 1987. In vitro growth inhibition of mastitis pathogens by bovine teat skin normal flora. Canadian Journal of Veterinary Research 51, 27.

Zadoks, R., Van Leeuwen, W., Kreft, D., Fox, L., Barkema, H., Schukken, Y., Van Belkum, A., 2002. Comparison of Staphylococcus aureus isolates from bovine and human skin, milking equipment, and bovine milk by phage typing, pulsed-field gel electrophoresis, and binary typing. Journal of clinical microbiology 40, 3894-3902.

Zhao, S., Gao, Y., Xia, X., Che, Y., Wang, Y., Liu, H., Sun, Y., Ren, W., Han, W., Yang, J., 2017. TGF-β1 promotes Staphylococcus aureus adhesion to and invasion into

bovine mammary fibroblasts via the ERK pathway. Microbial pathogenesis 106, 25-29.

APPENDICES

Appendix 1. Mammary secretion and mammary gland tissue physical clinical examination results during challenge

VG	CID	Q	Milk/mammary secretion score							Mammary gland score							Infection status
			Ch 0	Ch 1	Ch 2	Ch3	Ch 4	Ch 5	Ch 6	Ch 0	Ch 1	Ch 2	Ch 3	Ch 4	Ch 5	Ch 6	
SASP	4358	RF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	SCM & shedding large number of S. aureus from ch1 – ch6
		RR	0	0	0	0	0	0	1	0	0	0	0	1	0	0	
		LR	0	0	0	0	0	0	1	0	0	0	0	0	0	0	
		LF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	4431	RF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	SCM & shedding *S. aureus* on ch2 & Ch6
		RR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
		LR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
		LF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	4493	RF	0	0	0	0	0	0	0	0	0	1	1	0	0	0	Not infected but shedding low No. of *S. aureus on ch1*
		RR	0	0	0	0	0	0	0	0	0	1	1	0	0	2	
		LR	0	0	0	0	0	0	0	0	0	1	1	0	0	3	
		LF	0	0	0	0	0	0	0	0	0	1	1	0	0	3	
	4488	RF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Developed CM and shedding large No. of S. aureus and treated
		RR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
		LR	0	**2**	**3**	**3**	**3**	Rx		0	**2**	**3**	**3**	**3**	Rx		
		LF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	4592	RF	0	**2**	0	**1**	**1**	**1**		0	0	0	0	0	0	0	Developed CM & shedding large No. of *S. aureus* on Ch5 & Ch6 and treated
		RR	0	0	0	0	0	0		0	0	0	0	**1**	**2**	**3** RX	
		LR	0	0	0	0	0	0		0	0	0	0	**3**	**3**	**3** Rx	
		LF	0	**2**	0	**1**	0	**1**		0	0	**1**	0	0	0	0	
SCSP	4420	RF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No mastitis but shedding large No. of *S. aureus* on Ch2 and then very low S. aureus on ch4, 5 & 6
		RR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
		LR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
		LF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	4438	RF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No mastitis but shedding low No. of *S. aureus* on Ch5 & Ch6
		RR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
		LR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
		LF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	4449	RF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No mastitis but shedding very low No. of *S. aureus* on Ch3, 4 & 6.
		RR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
		LR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
		LF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	4519	RF	0	0	0	0	0	0	0	0	0	0	0	**1**	0	**1**	No mastitis but shedding large No. of S. aureus on Ch1 and then very low number on ch3, 4, 5 & 6.
		RR	0	0	0	0	0	0	0	0	0	0	0	**1**	0	0	
		LR	0	0	0	0	0	0	0	0	0	0	0	**1**	0	0	
		LF	0	0	0	0	0	0	0	0	0	0	0	**1**	0	0	
	4521	RF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No mastitis but shedding Low No. of *S. aureus on ch2, 4, 5 & 6.*
		RR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
		LR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
		LF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	4610	RF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	No Mastitis but shedding large No. of *S. aureus* on Ch3 and then very low S. aureus on Ch4 & 6
		RR	0	0	0	0	0	0	0	0	0	0	0	0	0	**2**	
		LR	0	0	0	0	0	0	0	0	0	0	0	0	0	**2**	
		LF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Control	4579	RF	0	**3**	**3**	**3**	**3**R x			0	**3**	**3**	**3**	**3**R x			Developed CM and shedding large No. of S. aureus from ch1 - Ch4 and treated
		RR	0	0	0	0	0			0	0	0	0	0			
		LR	0	0	0	0	0			0	0	0	0	0			
		LF	0	0	0	0	0			0	0	0	0	0			
	4604	RF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Developed CM and shedding S. aureus on Ch1 -3, 5 and 6. large No. of S. aureus on Ch2, 5 & 6 and treated
		RR	0	0	0	0	0	**2**	0	0	0	0	0	0	**2**	**3** Rx	
		LR	0	0	0	0	0	**2**	0	0	0	0	0	0	**2**	**3** Rx	
		LF	0	0	0	0	0	0	0	0	0	0	0	0	0	**3** Rx	
	4608	RF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	SCM and shedding S. aureus from Ch2 – Ch6
		RR	0	0	0	0	0	0	0	0	0	0	0	**1**	**1**	**1**	
		LR	0	0	0	0	0	0	0	0	0	0	0	**1**	**1**	**1**	
		LF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	4612	RF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	SCM and shedding few S. aureus on ch1 and large no. S. aureus on Ch2 & Ch6
		RR	0	0	0	0	0	0	0	0	0	0	0	0	**1**	0	
		LR	0	0	0	0	0	0	0	0	0	0	0	0	**1**	0	
		LF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	4619	RF	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	SCM and shedding large No. of S. aureus from Ch1 – Ch6.
		RR	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	
		LR	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	
		LF	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	

	4620	RF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	SCM and shedding large No. of S. aureus from Ch1 – Ch6
		RR	0	0	0	0	0	0	**1**	0	0	0	0	0	**1**	**2**	
		LR	0	0	0	**1**	**1**	0	0	0	0	0	0	0	**1**	**1**	
		LF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

Legends: V = vaccine, CID = Cow ID, QR = Quarter, SASP = *Staphylococcus aureus* surface proteins, SCSP = *Staphylococcus chromogenes* surface proteins, Ch = Challenge, Ch0 = day of challenge, Ch1 – Ch6 = Day 1 – Day 6 of challenge, SCM = Subclinical mastitis, CM = Clinical mastitis, Ctrl = Control, RF = Right front, RR = Right rear, LR = Left rear, LF = Left front, 0 = normal, 1 = mild changes, 2 = moderate change, 3 = Severe changes.

Appendix 2. Rectal body temperature during challenge

V	CID	Rectal body temperature															
		P-Ch	Ch0	Ch1	Ch2	Ch3	Ch4	Ch5	Ch6	Ch7	Ch8	Ch9	Ch10	Ch11	Ch12	Ch13	Ch14
SASP	4358	101.3	100.5	101.4	100.7	100.4	99.1	100.1	100.2	100.7	100.4	100.6	99.1	100.2	100.4	99.7	98.1
	4431	100.1	100.2	100.4	101.6	100.7	100.7	100.9	100.4	100.4	100.8	100.7	100.2	99.9	100.5	99.6	99.7
	4493	101.9	100.7	101.0	101.8	101.1	101.3	100.7	101.1	101.3	101.4	101.4	100.5	100.5	101.1	100.2	100.9
	4488	100.8	101.5	101.6	102.1	101.0	101.2	100.9	100.9	101.0	101.5	100.8	100.7	101.5	101.6	101.1	99.8
	4592	102.5	100.6	101.4	101.3	100.5	100.7	100.9	100.8	100.8	100.9	100.8	99.6	100.9	100.9	100.8	100.3
SCSP	4420	100.7	100.5	101.2	101.1	99.6	100.2	100.5	100.2	100.5	100.3	100.9	100.4	99.7	99.0	99.0	100.2
	4438	100.6	100.2	101.1	101.8	100.4	100.4	100.3	101.0	101.0	101.2	100.5	99.9	98.7	100.2	98.0	100.2
	4449	101.0	101.4	101.2	101.7	101.2	100.5	100.4	101.2	101.4	101.0	101.3	100.8	100.7	100.4	99.8	100.6
	4519	101.7	101.1	101.7	101.8	101.3	100.9	101.2	101.1	101.5	101.0	101.3	100.3	100.2	101.1	100.6	101.3
	4521	100.9	100.5	101.5	101.5	100.2	100.7	100.2	100.9	100.9	100.5	100.4	99.5	100.5	99.7	100.2	100.2
	4610	101.5	101.3	101.8	102.0	101.1	101.5	101.2	101.3	101.3	101.6	101.0	101.4	100.6	100.8	101.1	100.7
Ctrl	4579	101.4	101.1	101.3	101.8	101.1	100.7	99.5	101.0	101.0	100.9	101.1	100.5	100.8	100.9	101.4	100.9
	4604	101.3	101.3	101.1	102.2	100.8	101.2	101.0	101.2	100.9	101.2	101.2	100.4	101.0	100.6	100.7	101.2
	4608	101.2	101.2	101.2	101.8	101.0	101.2	101.1	101.1	101.0	101.0	101.1	100.9	100.7	101.0	100.9	99.7
	4612	101.5	101.2	101.4	101.8	100.8	101.1	100.4	100.8	100.9	101.0	101.0	101.1	100.9	100.7	100.6	101.3
	4619	101.3	100.3	101.2	100.8	101.1	100.5	100.4	100.5	100.5	100.9	100.6	100.0	100.3	100.6	100.4	100.6
	4620	100.7	100.9	101.3	101.8	100.3	100.2	100.5	101.0	101.0	101.4	100.9	101.9	99.4	100.6	99.8	100.6

Appendix 3. The relationship among serum or milk anti-SCSP and –SASP antibody titers and number of *S. aureus* shedding through milk during challenge period

DepVar	RegVar	Control Slope	Trt	Slope	Slopes differ p-value
Shedding_AVG	ssa_igG	-354 (1655)	SASP	-3981 (2504)	0.24
Shedding_AVG	scns_igG	3183* (1154)	SCSP	-614 (1406)	**0.04***
Shedding_AVG	ssa_igG1	-648 (899)	SASP	-1667 (6396)	0.88
Shedding_AVG	scns_igG1	-1952 (1728)	SCSP	142 (721)	0.27
Shedding_AVG	ssa_igG2	-522 (1220)	SASP	804 (3028)	0.69
Shedding_AVG	scns_igG2	441 (373)	SCSP	-892 (1163)	0.28
Shedding_AVG	ssa_igA	1341 (1316)	SASP	-4894 (3058)	0.07
Shedding_AVG	scns_igA	2103 (1353)	SCSP	1880 (2670)	0.94
Shedding_AVG	msa_igG	-2808 (3423)	SASP	-1647 (9266)	0.91
Shedding_AVG	mcns_igG	-1273 (3835)	SCSP	-1327 (2690)	0.99
Shedding_AVG	msa_igG1	-3240 (5562)	SASP	3537 (15535)	0.68
Shedding_AVG	mcns_igG1	-10613 (40703)	SCSP	555 (3653)	0.79
Shedding_AVG	msa_igG2	1413 (4388)	SASP	-354 (3679)	0.76
Shedding_AVG	mcns_igG2	1105 (1220)	SCSP	-1197 (2419)	0.4
Shedding_AVG	msa_igA	1507* (638)	SASP	-3862 (2293)	**0.03***
Shedding_AVG	mcns_igA	1171 (1409)	SCSP	1314 (2432)	0.96

Appendix 4. The relationship among anti-SCSP and –SASP antibody titers and somatic cell (SCC) during challenge period

DepVar	RegVar	Control Slope	Trt	Slope	Slopes differ p-value
log_SCC	ssa_igG	0.990 (0.741)	SASP	-1.32 (0.703)	**0.03***
log_SCC	scns_igG	0.149 (0.960)	SCSP	-0.477 (1.22)	0.69
log_SCC	ssa_igG1	-0.053 (0.406)	SASP	-1.48* (0.497)	**0.03***
log_SCC	scns_igG1	-0.591 (1.02)	SCSP	0.628* (0.312)	0.25
log_SCC	ssa_igG2	-0.411 (0.537)	SASP	0.105 (0.656)	0.54
log_SCC	scns_igG2	0.103 (0.226)	SCSP	-0.071 (0.479)	0.74
log_SCC	ssa_igA	0.663 (0.729)	SASP	0.070 (0.535)	0.51
log_SCC	scns_igA	1.30 (0.778)	SCSP	1.64 (0.896)	0.78
log_SCC	msa_igG	-0.003 (1.29)	SASP	2.36 (1.74)	0.28
log_SCC	mcns_igG	-1.39 (1.85)	SCSP	0.206 (1.09)	0.46
log_SCC	msa_igG1	-3.33* (1.13)	SASP	1.77 (1.00)	**0.001***
log_SCC	mcns_igG1	1.50 (20.6)	SCSP	1.31 (1.43)	0.99
log_SCC	msa_igG2	-0.160 (1.56)	SASP	-1.32* (0.503)	0.48
log_SCC	mcns_igG2	-0.104 (0.612)	SCSP	1.23 (0.966)	0.25
log_SCC	msa_igA	0.471 (0.275)	SASP	-0.346 (0.663)	0.26
log_SCC	mcns_igA	-0.248 (0.719)	SCSP	0.726 (1.02)	0.44

Appendix 5. The relationship between serum antibody titers at Ch0 and milk production status

DepVar	RegVar	Control Slope	Trt	Slope	Slopes differ p-value
7 – 47 DIM	ssa_igG	23.95 (50.53)	SASP	9.55 (73.66)	0.822
7 – 47 DIM	scns_igG	-20.94 (23.43)	SCSP	13.46 (18.49)	0.332
7 – 47 DIM	ssa_igG1	16.55 (6.71)	SASP	-90.36* (20.95)	0.017*
7 – 47 DIM	scns_igG1	-9.23 (23.67)	SCSP	12.34 (19.97)	0.536
7 – 47 DIM	ssa_igG2	22.59 (19.37)	SASP	30.74 (26.44)	0.82
7 – 47 DIM	scns_igG2	-3.14 (4.95)	SCSP	22.76 (18.34)	0.266
7 – 47 DIM	ssa_igA	7.66 (17.24)	SASP	-30.56 (57.72)	0.571
7 – 47 DIM	scns_igA	21.05 (86.39)	SCSP	-15.07 (40.29)	0.73
47 – 87 DIM	ssa_igG	21.95 (51.48)	SASP	14.10 (85.70)	0.942
47 – 87 DIM	scns_igG	13.18 (26.68)	SCSP	-8.92 (33.04)	0.639
47 – 87 DIM	ssa_igG1	26.95 (10.10)	SASP	-53.86 (34.58)	0.111
47 – 87 DIM	scns_igG1	-4.75 (32.14)	SCSP	5.33 (28.58)	0.83
47 – 87 DIM	ssa_igG2	31.17 (20.26)	SASP	0.424 (27.22)	0.432
47 – 87 DIM	scns_igG2	-6.74 (6.14)	SCSP	18.24 (19.01)	0.3
47 – 87 DIM	ssa_igA	6.41 (17.97)	SASP	23.76 (58.96)	0.797
47 – 87 DIM	scns_igA	-0.876 (107.23)	SCSP	-28.81 (51.67)	0.83
87 – 127 DIM	ssa_igG	6.14 (38.40)	SASP	-68.39 (52.55)	0.335
87 – 127 DIM	scns_igG	4.88 (26.40)	SCSP	5.58 (31.90)	0.988
87 – 127 DIM	ssa_igG1	24.08 (11.14)	SASP	-26.03 (29.65)	0.212
87 – 127 DIM	scns_igG1	3.86 (30.66)	SCSP	-4.57 (30.08)	0.857
87 – 127 DIM	ssa_igG2	23.40 (20.36)	SASP	-3.88 (23.55)	0.445
87 – 127 DIM	scns_igG2	-7.30 (5.06)	SCSP	19.81 (15.04)	0.186
87 – 127 DIM	ssa_igA	0.938 (14.78)	SASP	47.19 (47.74)	0.423
87 – 127 DIM	scns_igA	-28.52 (85.72)	SCSP	-69.66 (55.49)	0.714
Milk per day	ssa_igG	17.60 (36.07)	SASP	-25.50 (45.02)	0.509
Milk per day	scns_igG	10.55 (19.64)	SCSP	-18.87 (27.39)	0.447
Milk per day	ssa_igG1	21.36* (6.63)	SASP	-27.97 (17.73)	0.08
Milk per day	scns_igG1	-3.89 (24.49)	SCSP	12.24 (26.45)	0.685
Milk per day	ssa_igG2	24.76 (11.34)	SASP	14.77 (11.81)	0.584
Milk per day	scns_igG2	-5.32 (4.84)	SCSP	15.26 (16.42)	0.316
Milk per day	ssa_igA	5.15 (13.16)	SASP	19.59 (45.03)	0.778
Milk per day	scns_igA	-0.268 (83.10)	SCSP	-34.44 (55.08)	0.754
Peak milk	ssa_igG	25.07 (43.70)	SASP	-16.09 (68.96)	0.649
Peak milk	scns_igG	14.57 (20.45)	SCSP	-14.17 (25.68)	0.446
Peak milk	ssa_igG1	24.13* (7.36)	SASP	-58.86 (28.67)	0.068
Peak milk	scns_igG1	-7.52 (26.10)	SCSP	4.89 (22.07)	0.741
Peak milk	ssa_igG2	29.69 (15.82)	SASP	-8.23 (20.19)	0.236

Peak milk	scns_igG2	-5.52 (5.25)	SCSP	15.94 (20.14)	0.378
Peak milk	ssa_igA	7.67 (15.76)	SASP	-6.52 (53.51)	0.816
Peak milk	scns_igA	10.39 (89.85)	SCSP	-17.15 (41.33)	0.799

Appendix 6. Relationship between milk antibody titers at Ch0 and milk production status during time period following experimental vaccination and challenge

DepVar	RegVar	Control Slope	Trt	Slope	Slopes differ p-value
7 – 47 DIM	msa_igG	24.80 (11.84)	SASP	-79.80 (39.49)	0.127
7 – 47 DIM	mcns_igG	-156.3 (150.08)	SCSP	-48.53 (36.58)	0.536
7 – 47 DIM	msa_igG1	-9.06 (150.41)	SASP	89.18 (118.33)	0.659
7 – 47 DIM	mcns_igG1	-440.64 (461.21)	SCSP	-4.95 (35.42)	0.416
7 – 47 DIM	msa_igG2	-41.25 (16.95)	SASP	21.22 (13.45)	0.102
7 – 47 DIM	mcns_igG2	0.028 (21.47)	SCSP	19.77 (35.65)	0.668
7 – 47 DIM	msa_igA	-4.83 (8.08)	SASP	-88.70 (67.20)	0.341
7 – 47 DIM	mcns_igA	-37.70 (22.69)	SCSP	13.40 (24.57)	0.262
47 – 87 DIM	msa_igG	41.92 (10.06)	SASP	-118.56 (34.01)	**0.046***
47 – 87 DIM	mcns_igG	-176.03 (228.56)	SCSP	-28.18 (43.23)	0.57
47 – 87 DIM	msa_igG1	-75.77 (209.72)	SASP	152.11 (176.56)	0.493
47 – 87 DIM	mcns_igG1	-530.09 (590.01)	SCSP	12.89 (83.20)	0.429
47 – 87 DIM	msa_igG2	-62.42 (27.15)	SASP	24.56 (19.87)	0.123
47 – 87 DIM	mcns_igG2	-8.26 (28.03)	SCSP	2.73 (37.64)	0.83
47 – 87 DIM	msa_igA	-11.06 (12.71)	SASP	-71.10 (77.66)	0.525
47 – 87 DIM	mcns_igA	-47.85 (26.60)	SCSP	0.021 (26.73)	0.294
87 – 127 DIM	msa_igG	38.66 (12.00)	SASP	-71.77 (28.37)	0.07
87 – 127 DIM	mcns_igG	-95.28 (244.62)	SCSP	-5.00 (34.00)	0.739
87 – 127 DIM	msa_igG1	-115.84 (153.17)	SASP	138.94 (98.40)	0.297
87 – 127 DIM	mcns_igG1	-325.57 (488.97)	SCSP	197.77 (156.21)	0.383
87 – 127 DIM	msa_igG2	-52.00 (35.92)	SASP	9.45 (22.79)	0.285
87 – 127 DIM	mcns_igG2	-13.94 (25.80)	SCSP	-6.20 (63.38)	0.917
87 – 127 DIM	msa_igA	-12.40 (10.55)	SASP	-21.31 (20.82)	0.739
87 – 127 DIM	mcns_igA	-38.48 (27.61)	SCSP	-14.1 (26.04)	0.566
Milk per day	msa_igG	33.20 (9.25)	SASP	-95.65 (75.11)	0.069
Milk per day	mcns_igG	-140.43 (177.47)	SCSP	-19.17 (27.85)	0.548
Milk per day	msa_igG1	-59.29 (164.97)	SASP	76.03 (110.86)	0.566
Milk per day	mcns_igG1	-422.28 (455.18)	SCSP	-15.48 (49.13)	0.44
Milk per day	msa_igG2	-49.52 (12.94)	SASP	19.82 (8.79)	**0.047***
Milk per day	mcns_igG2	-6.45 (19.64)	SCSP	48.46 (55.31)	0.419
Milk per day	msa_igA	-8.72 (8.58)	SASP	-17.28 (14.69)	0.665
Milk per day	mcns_igA	-37.98 (19.82)	SCSP	11.23 (23.00)	0.203

Peak milk	msa_igG	37.03 (15.46)	SASP	-95.65 (75.11)	0.226
Peak milk	mcns_igG	-182.53 (160.39)	SCSP	-46.37 (42.02)	0.472
Peak milk	msa_igG1	-48.41 (175.95)	SASP	164.87 (185.74)	0.492
Peak milk	mcns_igG1	-534.10 (430.66)	SCSP	-18.85 (33.30)	0.319
Peak milk	msa_igG2	-57.39 (24.75)	SASP	-5.12 (20.09)	0.243
Peak milk	mcns_igG2	-4.75 (21.65)	SCSP	21.19 (31.49)	0.546
Peak milk	msa_igA	-8.88 (11.17)	SASP	77.08 (96.71)	0.47
Peak milk	mcns_igA	-44.44 (17.44)	SCSP	9.89 (18.81)	0.124

VITA

Caitlin Elizabeth Merrill was born in Rockville, MD on April 17th, 1992. Caitlin grew up in Englewood, FL where she graduated from Lemon Bay High School in 2010. Caitlin received her Bachelors of Science in Biology and Bachelors of Art in Communication from Florida Gulf Coast University in Fort Myers, FL in 2014. Upon graduation, Caitlin pursued an internship at Collingswood Animal Hospital. She then began her Masters in Animal Science at the University of Tennessee, Knoxville under the mentorship of Dr. Oudessa Kerro Dego, in August 2016. Beginning in August of 2018, Caitlin will pursue her Doctor of Veterinary Medicine at the University of Tennessee, Knoxville.