EVALUATING ANTIMICROBIAL THERAPY AND NON-PHARMACOLOGICAL ALTERNATIVES FOR TREATMENT AND PREVENTION OF MASTITIS CAUSED BY GRAM-POSITIVE PATHOGENS

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ABSTRACT

Treatment and management of mastitis accounts for the majority of antimicrobial usage on dairy farms in the United States today (USDA–APHIS–VS–CEAH–NAHMS, 2014). The overall objective of this dissertation is to evaluate the use of evidence-based strategies to reduce use of important antimicrobials for treatment of mastitis caused by Gram-positive pathogens. The first aim of this dissertation is to compare clinical and bacteriological outcomes of treatments of naturally occurring, non-severe clinical mastitis caused by Gram-positive pathogens. The second aim is to determine if nutritional supplementation with a commercially available postbiotic that is a fermentation product of *Saccharomyces cerevisiae* (SCFP) influences clinical and bacteriological outcomes of cows that receive an IMM challenge with *Streptococcus uberis.* The overall hypothesis of this dissertation is that evidence-based strategies can be used to reduce the usage of antimicrobials to treat clinical mastitis caused by Gram-positive pathogens on dairy farms. This dissertation begins in chapter 1 with a review of studies that evaluated the treatment of mastitis caused by Gram-positive pathogens in the United States. In chapter 2, several mechanisms to reduce antimicrobial usage are explored including shortening the duration of IMM therapy, treating with a lower class of antimicrobial, or not treating cows with clinical mastitis at all. Chapter 3 explores the susceptibility and AMR of Gram-positive bacteria identified as causing clinical mastitis on Michigan farms. Chapter 4 explores the clinical and immunological outcomes post-challenge with *Streptococcus uberis*. Together these four chapters demonstrate applied mechanisms that can be utilized to reduce antimicrobial usage on modern dairy farms.

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INTRODUCTION

The most common treatment for clinical mastitis is the use of IMM antibiotics. Mastitis directly affects the primary product produced on dairy farms, milk, and makes the milk unsaleable. As a result, mastitis is a costly disease affecting all dairy farms. The role of IMM antibiotics in the treatment of clinical mastitis is to interrupt bacterial replication which hopefully allows the immune system to respond effectively, and the infection will then cure. Intramammary antibiotics are only efficacious when the bacteria being treated is within the spectrum of activity of the antibiotic, as the primary mechanism of action for IMM antibiotics is to interrupt cell wall synthesis. However, not all mastitis infections required administration of antibiotics as mastitis infections can cure spontaneously through the animal's own immune response. Chapter 1 explores the different antimicrobial treatments and non-pharmacological alternatives currently available in North America for the treatment and prevention of clinical mastitis.

CHAPTER 1: LITERATURE REVIEW

1.1 Abstract

Clinical mastitis is a ubiquitous disease primarily caused by bacteria that affects dairy cows throughout the world. Gram-positive bacteria are responsible for approximately one-third of all clinical mastitis cases but their treatment accounts for most antimicrobial doses given to adult animals. Reducing use of critically important antimicrobials is part of judicious usage guidelines. There is a paucity of negatively controlled studies that have evaluated IMM antibiotic therapy for Gram-positive pathogens, with most studies being positively controlled and comparing products by testing a non-inferiority hypothesis. Conducting negatively controlled studies allows estimation of spontaneous cure of pathogens which is essential information for understanding the value of IMM antimicrobial therapy. While antimicrobials are the most used therapy for mastitis, the emphasis on reducing antimicrobial usage has stimulated research interest in use of alternatives such as immunomodulators. Currently there are three products that have been described as having an impact on the bovine immune system, though the mechanism of action for the nutritional products is not well defined. While clinical mastitis caused by Gram-positive pathogens will always be a disease on dairy farms, treatment protocols and recommendations will continue to evolve as the collective understanding of the best treatments continue.

Key words: dairy cow, treatment, clinical mastitis, Gram-positive

1.2 Introduction

Current State of Mastitis on Today's Dairy Farms

Mastitis is a ubiquitous and costly bacterial disease occurring in adult dairy cows, which presents in both a subclinical and clinical state and contributes to a large proportion of antimicrobial usage. Subclinical mastitis, defined by increased somatic cell count (SCC) but no clinical signs, is estimated to affect 15-30% of cows at dry-off and is associated with decreased milk production and poor milk quality (Pantoja et al., 2009; Arruda et al., 2013). Clinical mastitis is defined as the occurrence of abnormal milk which may or may not be associated with other signs of disease (Pinzón-Sánchez and Ruegg, 2011). A nationally representative survey of U.S. dairy farmers reported that clinical mastitis affected 24.8% of their cows each year (USDA-APHIS-VS-CEAH-NAHMS, 2014). Another recent evaluation of clinical mastitis on large Wisconsin dairy farms estimated the incidence of clinical mastitis 24.2 cases/ 100 cow-lactations (Gonçalves et al., 2022). Part of the reason for the ubiquitous nature of mastitis is that most IMIs are now caused by opportunistic bacterial pathogens that teats are commonly exposed to in the environment (Ruegg, 2017). Previously the most prevalent cause of mastitis were contagious pathogens such as *Streptococcus agalactiae* and *Staphylococcus aureus*, but today those pathogens are well controlled on most U.S dairy farms. Environmental pathogens are present in the housing areas of dairy cows and can overcome bovine anatomical and immune defenses resulting in IMM infection. Microbiological results of milk samples obtained from quarters affected with clinical mastitis in cows on modern dairy herds are often distributed as no microbial growth (30%), coliforms (30%), environmental Streptococci (25%), and non-aureus staphylococci (NAS; 6%), with the remaining isolates being split among *Staph aureus* and other pathogens (Ruegg, 2018). Environmental Streptococci and NASare the two largest groups of

Gram-positive pathogens that collectively represent dozens of species of opportunistic bacteria found in the cow's environment. Exposure to these environmental pathogens is ubiquitous and likely represents a permanent barrier to eliminating mastitis and thus management of the disease including treatment and prevention will remain a necessary part of dairy production.

1.3 Diversity of Gram-Positive Mastitis Pathogens

Mastitis is caused by a wide variety of pathogens with at least 137 different species having been identified as causing clinical mastitis (Watts, 1988). The environmental Gram-positive mastitis pathogens are commonly grouped as environmental streptococci and NAS. The collective scientific understanding of the diversity of pathogens that are grouped as NAS and environmental streptococci has expanded in the past two decades (Oliver et al., 1998; Jayarao et al., 1999; Condas et al., 2017). Environmental *Streptococci spp*. have traditionally been considered to include *Streptococcus uberis*, *Streptococcus dysgalactiae*, and *Enterococcus species* and have long been considered as important pathogens causing clinical and subclinical mastitis in cows which are typically exposed from environmental sources (Hogan et al., 1989; Phuektes et al., 2001; Vakkamäki et al., 2017). Among environmental streptococci, *Streptococcus uberis* was generally considered to be the most common cause of intramammary infection (IMI) (Jayarao et al., 1999; Riekerink et al., 2008) but this belief has been challenged by development of more accurate microbiological methods. Until recently, identification of species of *Streptococci* was based on phenotypic methods that have limited discriminatory ability. With the use of Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF), scientists have learned that organisms previously characterized as streptococci (based on esculin reaction) are now more accurately identified as *Lactococcus lactis* and other species (Werner et al., 2014). A recent survey of Gram-positive, catalase-negative cocci in New York reported that

these organisms were composed of *Streptococcus dysgalactiae* (33%), *Streptococcus uberis* (32%), *Lactococcus lactis* (24%), *Lactococcus garvieae* (3%), and other environmental streptococci (8%) (Scillieri Smith et al., 2020). The newfound diversity of this group of organisms has created a gap in understanding about appropriate treatment as only *Streptococcus uberis* and *Streptococcus dysgalactiae* have typically been identified when determining antibiotic treatment efficacy for CM.

The NAS group, (previously referred to as coagulase-negative staphylococci) are primarily commensal colonizers of teat skin, about 6-10% of cases of clinical mastitis are typically caused by these pathogens (Condas et al., 2017; Ruegg, 2018). As a group NAS are generally considered to be a minor mastitis pathogen however there is a high prevalence of IMI caused by NAS (Schukken et al., 2009). While 22 different NAS species have been detected as causing IMI (Watts, 1988; Wuytack et al., 2020), most IMI caused by NAS are identified as 5 species with *Staphylococcus chromogenes* being the most frequently detected followed by *Staphylococcus simulans*, *Staphylococcus xylosus*, *Staphylococcus haemolyticus*, and *Staphylococcus epidermidis* (Vanderhaeghen et al., 2014). There are considerable gaps in our understanding about the role that various NAS species may play in outcomes from clinical mastitis cases (Simojoki et al., 2011; Vanderhaeghen et al., 2014; Wuytack et al., 2020). While NAS are considered a minor mastitis pathogen there are growing concerns about the role NAS may play in antimicrobial resistance (AMR) because of the high prevalence of NAS in the teat canal they are frequently exposed to intramammary (IMM) antibiotics. Researchers have found an association between the prevalence of AMR in NAS and the systemic (but not IMM) use of antimicrobials on dairy farms (Nobrega et al., 2018). The precautionary principle relative to reducing antibiotic usage on dairy farms is warranted because of documented interspecies

transfer of NAS (Thorberg et al., 2006) and because NAS may serve as reservoirs for AMR genes (Nobrega et al., 2018). While NAS and environmental streptococci are frequently grouped and together referred to as Gram-positive it is important to remember that these two groups comprise dozens of different bacterial species that may have different susceptibilities to antibiotic therapy.

1.4 Antibiotic usage on dairy farms

Over the past several decades, use of antibiotics on dairy farms has come under increasing scrutiny due to growing concerns about development of AMR and increasing consumer perception that antibiotic usage poses a threat to human health (Wemette et al., 2021). Judicious usage guidelines encourage only necessary usage of antimicrobials following guidelines such as those set forth by the World Health Organization with an emphasis on limiting used of highest priority, critically important antimicrobials (CIA) (WHO, 2018). Antimicrobial classes are defined as highest priority, CIA when they are the sole (or one of a few) option to treat specific human diseases. The CIA designation is meant to highlight antimicrobials that should be used sparingly to prevent development of resistance. Treatment of clinical mastitis is the most common reason that antimicrobials are given to adult dairy cows and management of mastitis treatment represent an avenue to reduce use of CIA antimicrobials (Leite de Campos et al., 2021; Gonçalves et al., 2022).

In the United States there are 7 licensed IMM products (Table 1) four of which are β-lactam penicillin's (Table 1) but only 5 are currently marketed in the US. The only non β-lactam product is a lincosamide that contains pirlimycin (Pirsue®), which was withdrawn from the market in 2021 while another product (containing cloxacillin; DairyClox®) was withdrawn in 2022. Ceftiofur hydrochloride is the only third-generation cephalosporin that is FDA approved to

be used on dairy farms and it is classified by the WHO as a highest priority CIA. Ceftiofur is approved for use on dairy farms in two-IMM formulations and 3 systemically administered forms. In 2014, about 34% of USA dairy farm operations reported use of ceftiofur as their primary antimicrobial for treatment of mastitis and 32% reported using cephapirin sodium (a first generation cephalosporin) (USDA-APHIS-VS-CEAH-NAHMS, 2014). A recent survey that quantified antimicrobial usage on 40 larger farms in Wisconsin reported that ceftiofur comprised 75% of IMM antibiotics used on a large dairy farms (Leite de Campos et al., 2021). While all 5 IMM antimicrobials are beta-lactam antibiotics, ceftiofur is the only product that is classified as critically important. Research that compares the efficacy of the different IMM treatments represents an opportunity to help dairy producers ensure judicious usage of CIA by providing evidence supporting continued use of ceftiofur or by helping explore treatment protocols using one of the other four products which belong to less critically important antimicrobial classes.

In the USA, most cases of mastitis are treated symptomatically using IMM antimicrobials without knowledge of etiology (Ruegg, 2017) but in recent years selective treatment protocols based on identification of etiology have gradually been adopted and have been shown to be economically beneficial while also reducing antimicrobial usage (Lago et al., 2011; Pinzón-Sánchez et al., 2011; Vasquez et al., 2017; Fuenzalida and Ruegg, 2019ab). Selective treatment protocols are based on classifying mastitis pathogens as Gram-positive, Gram-negative, or culture negative. Selective treatment protocols use culture results to classify or group causative pathogens based on research showing that non-severe clinical mastitis caused by Gram-negative mastitis pathogens do not benefit from IMM antimicrobial therapy (Suojala et al., 2013; Vasquez et al., 2017; Fuenzalida and Ruegg, 2019a). Cases of non-severe CM caused by *Escherichia coli* experience a high rate of spontaneous cure. Spontaneous cure occurs when the immune response

of the cow is effective in responding to an IMI, resulting in bacteriological cure without intervention. Estimates of spontaneous bacteriological cure in Gram negative infections caused by *Escherichia coli* are frequently greater than 75% (Suojala et al., 2013; Fuenzalida and Ruegg, 2019c). By not treating those cases producers are able to reduce antimicrobial usage compared to non-selective treatment approaches that were traditionally used. Additional reductions in antimicrobial usage can be achieved by identification of culture-negative cases. While there is variation among farms, at least 25% of clinical mastitis cases are culture negative when detected (Schukken et al., 2013; Vasquez et al., 2016; Ruegg, 2018) meaning there is no active bacterial growth. A study comparing antibiotic treated and non-treated culture negative cases of non-severe clinical mastitis reported no difference in quarter SCC, recurrence, or daily milk yield (Fuenzalida and Ruegg, 2019c). Withholding antibiotics from culture negative non-severe cases is one way farms using selective treatment programs have reduced use of CIA (Lago et al., 2011b). The success of selective treatment protocols generates questions about re-examining treatment of Gram-positive mastitis, which are estimated to have a lower rate of spontaneous cure. However, few clinical mastitis trials conducted in North America have included a negative control group, making it difficult to estimate expectations for spontaneous cure of many of Gram-positive species.

1.5 Treatment of mastitis caused by Gram-positive pathogens

Administration of IMM antibiotics is the most common treatment for clinical mastitis in North America (USDA-APHIS-VS-CEAH-NAHMS, 2014) but a limited number of studies have been conducted to evaluate treatments (Table 2). For this review, studies were retrieved by searching three scientific databases and web platforms (PubMed, Google Scholar, and Web of Science). Studies were required to be written in English and at least 20% of enrolled cases had to

be Gram-positive pathogens resulting in clinical mastitis. Search terms included randomized, clinical, treatment, and mastitis. Additional studies were found by reviewing the references from relevant papers. Only cases that resulted from natural exposure and randomly allocated to treatments were included. Studies were required to have been conducted in North America in the previous 30 years and to evaluate antimicrobial therapies (Table 2). Thus, studies that evaluated supportive, homeopathic, or herbal treatments were not included.

In general, studies that have evaluated treatment of Gram-positive clinical mastitis have lacked consistency. Among studies retrieved for this review, only two that evaluated outcomes of treatment of Gram-positive clinical mastitis included a negative control group (Table 2). One negatively controlled trial enrolled 85 cases but 50% were either Gram-negative or contained no significant growth of bacteria when detected, thus, antimicrobial treatment would not be expected to improve outcomes. Based on limited power to detect differences in the small number of Gram-positive cases enrolled in that study, it was unsurprising that no differences were reported among antibiotic treated, frequent milk out, or no treatment experimental groups identified (Roberson et al., 2004). The other negatively controlled study that enrolled Gram-positive cases of mastitis withheld antibiotics for cows in the negative control group for only 5 days and then treated them using IMM Hetacillin potassium (Tomazi et al., 2021). In that study, clinical and bacteriological cure were assessed 14 days after enrollment but treatment of animals in the negative control group at day 5 precluded the ability to assess spontaneous bacteriological cure or other longer-term outcomes (Tomazi et al., 2021). Tomazi et. al. (2021) also assessed quarter level SCC on days 0, 3, 5, 8 and 14 post clinical mastitis onset, and reported a significant difference in quarter SCC at day 5 among the IMM antibiotic treatment groups. However, a SCC decrease would not be expected by day 5 as some cows would not yet have achieved clinical

cure and SCC in infections caused by *Streptococcus species* are slow to return to pre-clinical levels, often taking weeks (De Haas et al., 2002).

Most randomized clinical trials that evaluated treatment of clinical mastitis have been positively controlled and designed to test a non-inferiority hypothesis (Table 2). Non-inferiority studies evaluate whether the treatment of interest is at least as efficacious as a reference product (Piaggio et al., 2012). In these studies, the null hypothesis states that one treatment is inferior to the reference, while the alternative hypothesis states that the treatment of interest is not inferior to the reference product by more than a predefined margin of inferiority (usually plus or minus 15%) (Piaggio et al., 2012). Another hypothesis that is commonly tested is a superiority hypothesis where the study is designed to test whether one intervention is superior to another. Mastitis is recognized based on detection of non-pathogen specific signs of inflammation but is caused by a variety of bacterial pathogens which vary in expectations for spontaneous cure and have differing susceptibilities to approved antimicrobials. Many positively controlled trials (Table 2) have enrolled a mixture of cases that included Gram negative and culture negative cases which ranged from 4% (McDougall et al., 2019) to 68% of total enrolled cases (Wenz et al., 2005). Allocation of treatments to cases without regard for etiology will result in over-estimation of treatment effects due to high rates of spontaneous cure in culture negative and Gram-negative cases. A recent narrative review of clinical mastitis treatments highlighted that inclusion of large proportions of Gram-negative and culture negative cases in a non-inferiority trial makes it mathematically difficult to find any product non-inferior (Ruegg, 2021). The outcomes reported for these studies varied but most commonly were: bacteriological cure (using various definitions), clinical cure (assessed at different time points using different definitions), and SCC. While outcomes were not consistently defined among studies, most researchers

reported no differences between treatments. The lack of reported difference among many of the treatment trials possibly reflects the similar spectrum of activity of the antibiotics, but in the absence of negative controlled studies it is difficult to draw conclusions about appropriate treatments of these diverse pathogens.

1.6 Extended duration therapy

The labeled duration of treatment for IMM products approved in the USA is determined during the FDA approval process and infers efficacy of the products when the label directions are followed. However, extra-label guidelines for treatment allow for variation in the duration of treatment of all products (except ceftiofur) when prescribed by a veterinarian. Most IMM products have been approved for once or twice daily treatments for 1– 3 days. Only 1 IMM antibiotic (ceftiofur) has a variable duration label (that ranges from 2-8 days) but restrictions on this CIA do not allow extra label usage of this compound (Table 1). While there are no natural exposure clinical trials that have evaluated the impact of duration of treatment on outcomes of Gram-positive mastitis, there are 2 studies that used experimental challenges with *Strep uberis*. Both of these studies indicated benefits of longer duration IMM antibiotic treatment for *Streptococcus uberis* (Table 3), with each reporting at least one outcome that benefited from longer duration therapy. Oliver et al (2004), noted that longer duration therapy (5 d or 8 d) improved bacteriological cure rates as compared to 2 d of treatment. However, they reported no difference in SCC for cases that received longer duration treatment as compared to the shorter duration (Oliver et al., 2004). Another study that included a negative control group was conducted in the United Kingdom, and compared 7 different treatments (Hillerton and Kliem, 2002). No overall differences in clinical or bacteriological cure were reported in this study. However, Hillerton and Kliem (2002) noted when comparing the “aggressive” antibiotics (6

infusions of Leo Yellow every 12 h) to the negative control group the intensive IMM therapy resulted in faster clinical cure (Hillerton and Kliem, 2002). The only natural infection study in North America that was found in this literature search investigated the effect of longer duration IMM ceftiofur administration in non-severe clinical mastitis cases (Truchetti et al., 2014; Table 2). Truchetti et al. (2014) enrolled 197 cases, but many (25.9%) cases were caused by *Staph aureus* and 32% of cases were culture negative. Duration of treatment is well known to improve apparent bacteriological cure for *Staph aureus* (Barkema et al., 2006), while treatment length is not likely to benefit culture negative cases. The research by Truchetti et al (2014) also included many cases that were no growth or caused by *E. coli*, for which treatment is unlikely to be of benefit (Fuenzalida and Ruegg, 2019b). As a result, it is unclear if extended duration is appropriate for mastitis caused by *Streptococci* species as there were not enough cases enrolled to assess that individually (Truchetti et al, 2014). Other studies conducted in Europe have reported a benefit of extended duration treatment for IMIs caused by *Streptococcus uberis* (Deluyker et al., 2005; Milne et al., 2005). A review of mastitis caused by *Strep uberis* notes that *Strep uberis* strains are highly variable, with a wide range of host adapted and non-host adapted strains (Zadoks, 2007).

Based on studies that used either natural or induced infection, there is limited evidence that extended duration treatment for *Streptococcus uberis* improves clinical or bacteriological cures. The induced infection studies were limited to one strain (UT888, Table 3) and as a result it is difficult to extrapolate those results to the wide variety of *Strep uberis* strains. Research on the duration on *Strep uberis* infections found a range between 1-309 days with a median of 42 days (Zadoks et al., 2003). Based on literature included in this review, there are no studies that utilized natural infections with *Strep uberis* that had a negative control group longer than 5 days. A

previous review of treatment of clinical and subclinical *Streptococcus uberis* found bacteriological cure rates ranging between 0-95% on studies (Zadoks, 2007). In North America, there is a need for more negatively controlled research which could provide better estimates of spontaneous cure rates for *Streptococcus uberis* and help guide treatment decisions. It is especially important for this research to be conducted now, as scientists have learned that other Strep-like organisms, such as *Lactococcus lactis*, may have been previously grouped with *Strep uberis*, as they were indistinguishable in the absence of more advanced identification methods such as MALDI-TOF. Research exploring the appropriate length of treatment is important because shorter treatments use less antimicrobials and reduce costs associated with treatment (Pinzón-Sánchez et al., 2011).

1.7 Emerging Treatments

Intramammary antimicrobial therapy remains the primary treatment for non-severe clinical mastitis occurring in dairy cows in North America, however research on alternatives to antimicrobials are increasing. Antibiotics assist the immune system in achieving bacteriological clearance and efforts to stimulate the immune system to be more effective are an emerging area of research. Immunomodulation, or manipulation of the immune system, is showing promise as other more commonly used mechanisms (such as vaccination) have been less successful. Vaccination as a control mechanism for mastitis has been explored with varying degrees of success (González et al., 1989; Hogan et al., 1992; Vangroenweghe et al., 2020). Some vaccines (such as J5 core-antigen vaccines) have been shown to be effective in reducing mastitis severity in Gram-negative mastitis cases (Wilson et al., 2007; Gurjar et al., 2013) but there has been little success in vaccine development for Gram-positive pathogens (Schukken et al., 2014; Collado et al., 2018). The mammary gland has unique obstacles for developing effective vaccines. Milk is

an excellent bacterial growth medium and milk components such as fat and casein inhibit phagocyte capacity (Rainard et al., 2022). In a healthy mammary gland, there are relatively few immune cells to protect a large surface area of secretory epithelium (Rainard et al., 2022). Those physiological factors are difficult to overcome in the mammary gland. Immunization against mastitis is also hampered because the disease is caused by a wide variety of pathogens with a large diversity of surface antigens making it difficult to find a target for an universal vaccine (Sordillo, 2005; Rainard et al., 2022). Since vaccines may not be the most feasible mechanism to treat or manage mastitis there is interest in alternative mechanisms by which the bovine immune response could be modified to reduce disease severity.

An effective bovine immune response begins with innate immunity as the initial line of defense when the mammary gland is exposed to a pathogen. If the innate immune response is effective, localized mammary cell populations can facilitate pathogen recognition and stimulate the immune response and the pathogen could be neutralized before any changes to the milk or mammary tissue occur (Sordillo, 2018). An effective mammary gland immune response has a rapid onset to neutralize the bacterial pathogen followed by a timely resolution to avoid dysregulation associated with chronic mastitis (Sordillo, 2018). The inflammatory cascade usually begins when immune cells in the mammary gland detect pathogen-associated molecular patterns triggering the release of inflammatory mediators such as cytokines and oxylipids. This stimulates changes in vascular permeability leading to diapedesis of neutrophils (Sordillo, 2018). Various treatments have been explored to stimulate the immune system of dairy cattle to modulate or enhance the immune response to mastitis. There is a known nutritional link, with many vitamins and minerals playing a key role in antioxidant mechanisms for immune responses including vitamins A, C, E and selenium, copper, and zinc (Sordillo, 2016). Aside from vitamins

and minerals, there are currently two nutritional products (OmniGen® and Nutritek®) that are available on the market in the USA that have some research available about their impact on mastitis (Table 4). Both nutritional products include some yeast components, but the formulations are closely guarded trade secrets.

Yeast products have been commercially available for decades as a nutritional supplementation for dairy cattle. Supplementation with yeast products in bovines increases dry matter intake leading to improved animal performance, with a proposed mechanism of action being the altered microbial populations in the rumen (Robinson and Erasmus, 2009; Mullins et al., 2013). As research on yeast products has advanced, some companies have added micronutrients to their formulations which are thought to aid in fermentation of bacterial populations, thus improving fiber digestibility (Robinson and Erasmus, 2009). Research on Omnigen-AF (Phibro Animal Health, Illinois, USA) found that supplementation with the product during the periparturient period decreased prevalence of IMI, incidence of new IMI and decreased SCC in supplemented animals in the following lactation (Nace et al., 2014). However, as the study by Nace et al (2014) was a small study with only 40 animals (20/group) and only evaluated primiparous animals, larger studies across all lactations are needed. The mechanism of action for Omnigen-AF is thought to be stimulation of antibacterial activity of neutrophils and the expression of cell surface trafficking proteins during involution of the udder (Nace et al., 2014; Nickerson et al., 2019). Another yeast-based product currently available is a post-biotic fermentation product of *Saccharomyces cerevisiae* (SCFP) (NutriTek®; Diamond V, Iowa, USA). This product (NTK, Table 4) is a yeast byproduct that is marketed based on its ability to alter rumen microbial populations and volatile fatty acid production resulting in increased milk production and components (Poppy et al., 2012; Olagaray et al., 2019). While dairy producers

have shared anecdotal evidence of the benefits of SCFP, an observational retrospective analysis using data from 25 US dairy herds found that feeding NTK reduced the linear score SCC and clinical mastitis incidence in the herds (Ferguson et al., 2018). However, causality has not been established and the biological mechanisms by which this might occur still need to be more fully explored. While the mechanisms of action are not yet understood, yeast products represent an opportunity to potentially decrease CIA usage on farm by decreasing the incidence of IMI or reducing the duration of IMI.

The role of NTK as an immunomodulator is only beginning to be investigated but one study found that cows supplemented with NTK had improved neutrophil phagocytosis and oxidative capacity (Vailati-Riboni et al., 2021). The NTK-supplemented animals also upregulated specific genes related to immune cell antibacterial function (Vailati-Riboni et al., 2021). The researchers challenged the animals intramammarily with 2,500 CFU of *Streptococcus uberis* 0140J and collected mammary and liver biopsies, milk samples for SCC, cow clinical information, and blood samples to assess circulating immune function (Vailati-Riboni et al., 2021). Researchers reported increased phagocytotic capacity in monocytes and neutrophils in blood of NTK supplemented animals (Vailati-Riboni et al., 2021). The SCC of milk differed at several time points during the 36-hour challenge but overall did not differ. Three key genes (NOS2, TNF, and CATHL4) were upregulated, all of which have antibacterial functions indicating some sort of immunological modulation but more research is needed to further characterize the immune response and the mechanisms of action that NTK may play (Vailati-Riboni et al., 2021). Impacting the phagocytosis capacity would be one possible mechanism by which SCFP could be impacting immune response directly.

The other product that is available (Table 4) for immune stimulation is a polyethylene

glycol –conjugated bovine granulocyte colony-stimulating factor (G-CSF) (Imrestor™; Elanco, North Carolina, USA). While this product is not currently sold in the USA, it is a cytokine that is bound to polyethylene glycol and the function of the cytokine is to stimulate production and differentiation of neutrophils in the bone marrow. Researchers demonstrated that when G-CSF was injected to cows during the periparturient period and post calving there was a decrease in the incidence of clinical mastitis (Hassfurther et al., 2015; Canning et al., 2017). The clinical case definition for these two studies, however, included CMT scoring which would skew the cases towards enrollment of mild clinical cases, which might not have been detected in other studies using a more traditional scoring system. Thus it is unsurprising that other researchers have not been able to demonstrate an effect of G-CSF on clinical mastitis incidence (Zinicola et al., 2018; Van Schyndel et al., 2021). Recently a pilot study was conducted that found that G-CSF administered at dry off, instead of the periparturient period, decreased the incidence of IMI in the subsequent lactation (de Campos et al., 2022). Additional research about immunomodulators for bovine mastitis represents a possible mechanism by which usage of CIA could be reduced on dairy farms.

1.8 Conclusions

Mastitis remains the most common and costly disease on dairy farms and treatment of clinical mastitis accounts for a large percentage of CIA usage on dairy farms. Currently there are 7 licensed IMM antibiotics for use in treatment of CM, but there are few negatively controlled studies to determine the best treatment protocols for various etiologies. Choosing the appropriate duration of treatment or using a lower class of antimicrobial are two possible mechanisms by which highest priority CIA usage could be reduced. Another mechanism to reduce usage of highest priority CIA is to prevent the need for treatment of CM using immunomodulators. The

use of immunomodulators and the mechanisms of action are areas where additional research is needed.

1.9 Tables

Table 1.1. Antibiotics approved for intramammary treatment of mastitis in the United States

Active Antimicrobial[1]	**Product Name**	**Antimicrobial Class**	**Labeled Treatment**	**Indication**
Amoxicillin	Amoxi-mast®	Beta-lactam penicillin	3 doses administered 12 hours apart	*Streptococcus agalactiae* and penicillin-sensitive *Staphylococcus aureus*
Ceftiofur	Spectramastl LC®	Beta-lactam 3rd generation cephalosporin	Once/day for 2 to 8 days	Coagulase negative staphylococci (NAS), *Streptococcus dysgalactiae*, and *Escherichia coli*
Cephapirin sodium	Today®	Beta-lactam 1st generation cephalosporin	2 doses, 12 hours apart	*Streptococcus agalactiae* and *Staphylococcus aureus*
Cloxicillin sodium	Dariclox®[2]	Beta-lactam penicillin	3 doses administered 12 hours apart	*Streptococcus agalactiae* and non-penicillinase producing *Staphylococcus aureus*.
Hetacillin Potassium	PolyMast®	Beta-lactam penicillin	Once/day for 3 days	*Streptococcus agalactiae, Streptococcus dysgalactiae, Staphylococcus aureus* and *Escherichia coli*
Penicillin Procaine G	Hanford's /U.S. Vet MASTI-Clear™	Beta-lactam penicillin	3 doses administered 12 hours apart	*Streptococcus agalactiae, Streptococcus dysgalactiae* and *Streptococcus uberis.*
Pirlimycin	Pirsue®[2]	Lincosamide	Once/day for 2 to 8 days	*Staphylococcus aureus Streptococcus agalactiae, Streptococcus dysgalactiae*, and *Streptococcus uberis*

[1]All antimicrobial information from https://www.drugs.com/vet/
[2]Not currently available in the United States

Table 1.2. Natural infection trials evaluating treatment of non-severe clinical mastitis that included Gram-Positive Pathogens in North America from 1993 to 2023

Citation	Location	Cases (n)	Pathogen Distribution	Treatments	Hypothesis Tested	Outcomes Recorded
Negatively Controlled						
(Roberson et al., 2004)	NY, USA	85	Str. uberis: 29.1% Str dys.: 2.9% NAS[1]: 4.9% GN[2]: 37.9% NSG[3]: 11.7% S. aureus: 2.9% Other: 10.6%	1. IMM Amoxicillin (1 tube every 12 hours apart for 1.5 day) 2. Frequent milk out (4x/day) 3. Combination IMM Amoxicillin 1.5 days and frequent milking (4x/day) 4. No treatment	Not Stated	Clinical cure, Bacteriological cure, NIMI, CMT, Milk production
(Tomazi et al., 2021)	NY, USA	696	Str. uberis: 60.2% Str dys.: 18.8% NAS: 5.6% Other Strep. Spp.: 8.6% Entero.: 1.6% Mixed: 5.2%	1. IMM Amoxicillin (1 tube every 12 hours apart for 1.5 day) 2. IMM Amoxicillin-Extra label (1 tube/day for 5 days) 3. IMM Ceftiofur labeled (1 tube/day for 5 days) 4. No treatment for 5 days followed by 3 d IMM hetacillin potassium	Superiority	Clinical Cure, Bacteriological cure, Recurrence, Composite SCC, Test day milk production
Positively Controlled Studies						
(Guterbock et al., 1993)	USA	254	Str. uberis: 10.6% Str dys.: 7.5% Coliform: 37% Aero. Viridans: 5.5% NAS: 5.5% NSG: 24% Other: 10%	1. IMM Amoxicillin 1 tube every 12 hours apart for 1.5 day) 2. IMM Cephapirin sodium (1 tubes every 12 hours for 1 day) 3. 100 IU of Oxytocin for 3 milkings	Not Stated	Bacteriological cure, Clinical cure
(Erskine et al., 2002a)	USA	104	Coliform: 53.8% NSG: 20.2 Strep spp: 13.5% Staph spp: 8.6% Other: 3.8%	1. Systemic ceftiofur 1x/day for 5 days 2.IMM pirlimycin 1x/day for 3 d Fluids and NSAID for all cows as they were severe cases	Not Stated	Clinical cure

Table 1.2 (cont'd)

(Wenz et al., 2005)	USA	144	Str. Spp: 14.6% Other Gram +: 7.6% GN: 68.8% Mixed: 9.0	1. IMM pirlimycin 1x/d for 2 d 2. IMM pirlimycin IMM for 2 d & systemic Ceftiofur for 3 d 3. IMM cephaparin 2x/d for 3 d 4. IMM cephaparin 2x/d for 3 d & systemic Ceftiofur for 3 d	Not Stated	Recurrence, Culling, Quarter Dry
(Schukken et al., 2013)	NY, USA	296	Str. uberis: 8.1% Str dys.: 14.2 % S. aureus: 3.4% NAS: 3.0% GN: 23.6 % NSG: 27.7 % Contaminated: 7.1% Other: 12.9%	1. IMM cephapirin sodium (3 tubes, 12 hours apart) 2. IMM ceftiofur 1x/d for 5 days	Non-Inferiority	Bacteriological cure, Clinical cure
(Truchetti et al., 2014)	Canada	241	Strep spp: 19.8% S. aureus: 25.9% GN: 6.6% NSG: 32% NAS: 2.5% Other: 13.2%	1. IMM Ceftiofur 1x/day for 2 days 2. IMM Ceftiofur 1x/day for 8 days	Superiority	Bacteriological cure, Clinical cure, NIMI
(Vasquez et al., 2016)	NY, USA	588	Str. uberis: 7.8% Str dys.: 14.3 % S. aureus: 8% NAS: 1.9% GN: 19.2% NSG: 35.9 % Other: 12.9%	1. IMM Hetacillin potassium 1x/d for 3 d 2. IMM Ceftiofur 1x/d for 5 d	Non-Inferiority	Bacteriological cure, clinical cure,
(Viveros et al., 2018)	Mexico	292	Str. uberis: 5.1% Str dys.: 8.2% S. aureus: 9.2% NAS: 10.2% GN: 18.5% NSG: 38.4% Other: 10.4%	1. IMM Enrofloxacin suspension 1x/d for 3 d 2. IMM Enrofloxacin powder 1x/d for 3 d 3. IMM Ceftiofur 1x/d for 3 d 4. Enrofloxacin systemic for 1x/ d for 3 d	Not Stated	Clinical cure, bacteriological cure, composite SCC

[1]Non-aureus staphylococci (NAS) [2]Gram-Negative (GN) [3]No Significant Growth (N

Table 1.3. *Streptococcus uberis* challenge trials evaluating duration of intramammary treatments

Citation	Location	Number (n)	Strain (CFU)[1]	Length of Challenge	Treatments Used	Outcome	Benefit of Extended Duration Therapy?
(Oliver et al., 2004)	USA	37 quarters	UT888 (1500)	Treated when CM onset occurred	1. 2d IMM[2] ceftiofur 2. 5-d IMM ceftiofur 3. 8-d IMM ceftiofur	BC[3], CC[4], SCC[5]	Yes
(Almeida et al., 2003)	USA	103 quarters	UT888 (1000)	Treatment occurred by 7 days, based on severity score some cows treated earlier	1. 2d IMM pirlimycin 2. 5-d IMM pirlimycin 3. 8-d IMM pirlimycin	BC, SCC	Yes

[1]Colony Forming Unit [2]IMM [3]Bacteriological Cure [4]Clinical Cure [5]Somatic Cell Count

Table 1.4. Immunomodulators evaluated for clinical mastitis prevention or treatment

Product Name	Manufacturer	Proposed Mechanism of Action	Studies Demonstrating Mammary Effect
Nutritional			
Omnigen-AF®	Phibro Animal Health	Stimulating polymorphonuclear neutrophilic leukocytes antibacterial activity	(Nace et al., 2014; Nickerson et al., 2019)
NutriTek™	Diamond V	Activating leukocyte responses and mammary epithelial defenses	(Vailati-Riboni et al., 2021)
Cytokine			
Imrestor	Elanco	The active ingredient polyethylene glycol –conjugated bovine granulocyte colony-stimulating factor stimulates production and differentiation of neutrophils in the bone marrow	(Hassfurther et al., 2015; Canning et al., 2017; Van Schyndel et al., 2021)

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CHAPTER 2: NEGATIVELY CONTROLLED, RANDOMDIZED CLINICAL TRIAL TO EVALUATE OUTCOMES OF INTRAMAMMARY ANTIBIOTIC TREATMENTS OF NON-SEVERE CLINICAL MASTITIS IDENTIFIED AS GRAM-POSITIVE USING SELECTIVE AGAR

2.1 Abstract

The objective of this negatively controlled, randomized clinical trial was to compare microbiological and clinical outcomes of cases of Gram-positive clinical mastitis (CM) that received 3 different IMM antimicrobial treatment protocols to outcomes of cases that received no antimicrobial therapy. Cases classified as non-severe CM were enrolled when microbial growth was identified on the Gram-positive segments of a selective culture media. Eligible cases were randomly assigned to one of 3 IMM treatment groups: 3-d Hetacillin potassium (n = 69), 3-d Ceftiofur (n = 69), 8-d Ceftiofur (n = 70) or to receive no treatment (n = 32). Duplicate quarter milk samples were collected from the affected quarter at enrollment and at 14, 21, and 28 days (±3 d) after enrollment. Clinical outcomes were followed for 90 d. Of 240 cases that had growth on the Gram-positive segment of the selective media, only 107 were identified as *Streptococci* or *Staphylococci.* Statistical analysis was performed using SAS (v9.4) and for key outcomes multivariable mixed models were created. Based on laboratory evaluation of milk samples, etiologies were distributed as *Streptococcus* species (I21.7%; n = 52), *Lactococcus* species (19.2%; n = 46), NAS (16.3%; n = 39), *Staphylococcus aureus* (6.3%; n = 16), *Enterococcus* species (5.0%; n = 12) and other organisms (10%; n = 24). Thirty-five (14.5%) duplicate milk samples resulted in no significant growth in the laboratory while 16 (6.7%) were classified as contaminated. Bacteriological cure at day 21 ranged from 73 - 79% and did not vary among treatment groups. The estimated spontaneous cure rate of CM cases caused by Gram-positive

pathogens was 79% ± 11.0. Due to relatively few quarter level recurrences, recurrence was analyzed for nontreated versus a combined treatment group and there was no significant difference (P = 0.45) in risk of quarter-level recurrence of clinical mastitis among groups (17.1% ± 7.9 and 10.9% ± 2.5 for the nontreated and treated groups, respectively). Days to normal milk ranged from 5.0 – 5.8 days and was not affected by treatment. The weekly quarter $\log_{10}$SCC after enrollment for treated cows was 6.04 ± 0.06 as compared to 6.20 ± 0.14 for non-treated cows and was not affected by treatment. Of enrolled cows, 17% (n = 40) were culled or died before the end of the 90-d follow-up period, but treatment did not affect culling risk. As compared to cows that received 3 d of IMM ceftiofur, cows that received 8 d produced 2.9 kg more milk/d on average during the 90-d follow-up period, but milk yield did not differ from cows that received 3 d of hetacillin nor from cows in the negative control group. Greater milk discard in those cows resulted in minimal differences in overall saleable milk. These results demonstrate that a diverse group of organisms are identified as Gram-positive based on growth on selective media. No differences in clinical and microbiological outcomes were observed between groups treated with different active ingredients nor for the groups that received shorter durations of therapy. The relatively high spontaneous cure rate in the small negative control group included in this trial indicates that use of IMM antibiotics for treatment of Gram-positive cocci that are not in genera included on product labels should be further investigated.

Key words: dairy cow, treatment, clinical mastitis, antimicrobial, Gram-positive

2.2 Introduction

While bulk tank somatic cell counts (SCC) historically have declined, many dairy farmers continue to struggle to manage clinical mastitis (CM). In the United States, most cases of CM are treated based on clinical signs using IMM (IMM) antimicrobials (USDA–APHIS–VS–

CEAH–NAHMS. 2014; Gonçalves et al., 2022). While there are concerns about the development of AMR as a result of nonspecific use of antibiotics, few linkages between use of IMM antimicrobials and emergence of resistance have been substantiated (Erskine et al., 2002b; Oliver and Murinda, 2012; Nobrega et al., 2018). However, principles of judicious use encourage limited and specific usage of narrow-spectrum antimicrobials targeted after diagnosis of a bacterial pathogen (Weese, Page and Prescott, 2013). The emphasis on judicious usage has led to the development of culture-based selective treatment protocols for non-severe cases of CM. On-farm culture programs based on use of selective agars for phenotypic identification of bacterial etiologies are used to help guide treatment decisions for uncomplicated cases of non-severe CM (Lago and Godden, 2018). In most instances, IMM antimicrobial therapy is recommended for cows with CM caused by Gram-positive bacteria, while it is not generally recommended for cases that are caused by Gram-negative bacteria nor those that are culture negative when detected (Lago et al., 2011b; Vasquez et al., 2017; Ruegg, 2018). For example, selective treatment programs generally emphasize the need to use antibiotics to treat CM caused by *Streptococci* and some *Staphylococci* but not for treatment of non-severe CM caused by *Escherichia coli* (due to high rates of spontaneous cure), nor CM associated with chronic infections of *Staphylococcus aureus*.

Streptococci spp. and NAS(NAS) have been recognized as important causes of mastitis for decades (Hogan et al., 1989; Vakkamäki et al., 2017). Using classical microbiological techniques to identify environmental streptococci, researchers have evaluated outcomes after treatment of CM based on results of *in vitro* susceptibility (Cattell et al., 2001), after experimentally induced streptococcal infections (Oliver et al., 2004), or as a part of non-inferiority studies (Vasquez et al., 2016). Based on improved rates of bacteriological clearance

after experimental challenge, extended duration antimicrobial treatment has been frequently recommended for CM caused by *Streptococcus uberis,* (Gillespie et al., 2002; Hillerton and Kliem, 2002; Oliver et al., 2004). However, longer durations of antibiotic therapy contribute to increased costs (due to more days of milk discard and longer treatment), increased mass of antibiotics used (Pinzón-Sánchez et al., 2011), and increased risk of iatrogenic infections or infections acquired in the hospital pen (Pieper et al., 2012).

Recently, use of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry (MALDI-TOF) to identify Gram-positive, catalase-negative cocci isolated from bovine milk samples has demonstrated that *Lactococcus spp.* are a considerable proportion of organisms that had been previously classified as "environmental streptococci" (Werner et al., 2014; Scillieri Smith et al., 2020). When grown on many selective medias that are used for on-farm culture (OFC), *Lactococcus spp*. are indistinguishable from many *Streptococci spp*. The diversity of organisms that were previously considered to be environmental streptococci has highlighted our lack of understanding about antimicrobial therapy because none of the 7 FDA-approved IMM antimicrobials include efficacy data for these "other" Gram-positive pathogens such as *Lactococcus spp*. and *Enterococcus spp*. The benefit of antimicrobial therapy is the marginal difference between the spontaneous cure rate and the therapeutic cure rate, but few negatively controlled clinical trials have been performed for naturally occurring non-severe CM caused by Gram-positive bacteria. The objective of this study was to compare microbiological and clinical outcomes of cases of non-severe Gram-positive CM using selective agar. Cows were randomly assigned to receive IMM treatment of 3 d of Hetacillin potassium, 3 d of Ceftiofur hydrochloride, 8 d of Ceftiofur hydrochloride or received no antimicrobial treatment. We hypothesized that cows who received IMM antimicrobials would have improved clinical and

bacteriological outcomes as compared to cows that did not receive antibiotics as measured by somatic cell count (both at the quarter and composite level), bacteriological cure, and clinical cure. We additionally hypothesized that longer duration of antimicrobial therapy would improve clinical (milk production, time to clinical cure, somatic cell count) and bacteriological outcomes (bacteriological cure) compared to shorter-duration therapy.

2.3 Materials and Methods

Study Design

This negatively controlled, randomized clinical trial was conducted on commercial dairy farms in Michigan (n = 3) and Minnesota (n = 1). Cows were housed in free-stall barns and milked 3 times per day. Cows were a variety of breeds including Jersey, Holstein, and Holstein crosses and the farms ranged in size from 900-10,000 (Table 1). Clinical mastitis was detected by trained farm workers by observation of foremilk and udders prior to milking. All parlors in Michigan contained automated daily milk yield recording systems, while this data was not available at the Minnesota facility. The three parlors in Michigan were either herringbone or parallel parlors while the Minnesota farm used a rotary. Managers at all sites recorded health events in computerized management programs (DairyComp 305 [n = 3], Valley Agricultural Services, Tulare, CA; or PCDart [n = 1], Dairy Records Management Systems, Raleigh, NC). Enrolled farms were conveniently located to Michigan State University or the University of Minnesota. Farms were required to utilize computerized cow records, record clinical mastitis cases, and have experience utilizing culture-based treatment protocols. The study was approved by the Institutional Animal Care and Use Committee at Michigan State University (PROTO201900087).

Case Enrollment Criteria

Eligible cases of CM were enrolled between June 2019 until March 2020 when the study was prematurely ended due to the global COVID-19 pandemic. Clinical mastitis cases were considered eligible if they presented as non-severe single quarter cases in cows that were not expected to be dried off in the next 60 days and had not received any other antibiotic treatment or experienced CM in the last 30 days. Cows were followed for 90 days after enrollment. Before the study began, researchers trained farm personnel to aseptically collect milk samples following National Mastitis Council (NMC) guidelines (NMC, 1999) and to record standardized mastitis severity scores (Pinzón-Sánchez and Ruegg, 2011). Researchers ensured compliance on the weekly farm visit and reviewed farm standard operating procedures to ensure compliance for new employees. Milking technicians identified CM based on observation of foremilk. Cases were scored as: mild (abnormal milk), moderate (abnormal milk and visible inflammation of the udder), or severe (abnormal milk accompanied by systemic signs). Severe cases were not eligible and were treated according to existing herd treatment protocols. Eligible cows were moved to the hospital pen and identified for surveillance.

After identification of non-severe CM in an eligible cow, duplicate milk samples were aseptically collected from the affected quarter and one milk sample was used to perform OFC using selective media as previously described (Fuenzalida and Ruegg 2019). Briefly, trained farm workers (3 farms) or veterinary technicians (1 farm) used a sterile swab to inoculate approximately 0.1 mL of milk onto selective agars on a tri-plate (Minnesota Easy Culture System; Laboratory for Udder Health, St. Paul, MN, USA). The tri-plate contained three segments, one with MacConkey agar for Gram-negative, and the Factor™ and Focus™ with select for all Gram-positive and Streptococci specifically. After inoculation, bronopol was added

to the milk sample to preserve it for SCC analysis. The second milk sample was frozen on the farm until picked up for further microbiological testing at Michigan State University or the University of Minnesota's Laboratory for Udder Health. After 24 hours of incubation at 37°C, tri-plates were observed for microbial growth. Cows with milk samples that were culture negative (or non-significant growth, < 3 colonies), contained ≥3 types of bacteria, or resulted in Gram-negative microbial growth were not eligible for the study and were treated according to existing herd protocols. Cases that resulted in ≥3 colonies of Gram-positive growth were assigned to an experimental group by opening an envelope that randomly assigned treatment or through a random assignment via DairyComp305. Unequal randomization was used in Michigan with a target of enrolling a minimum of 81 animals per farm, with 6 negative control animals per farm and 25 animals in each antibiotic treatment group (Dumville et al., 2006), while the Minnesota herd who used DairyComp305 had a 1 in 4 randomized allocation. Farm employees were not aware which treatment would be allocated until random assignment occurred, but were not blinded to treatments.

Interventions

Quarters that were assigned to receive IMM antimicrobials were treated once daily using 1 of 3 protocols: 3 d of Hetacillin potassium (PolyMast, Boehringer Ingelheim, Duluth GA, USA), 3 d of Ceftiofur hydrochloride (Spectramast LC, Zoetis, Kalamazoo, MI, USA), or 8 d of Ceftiofur hydrochloride. Quarters assigned to the negative control group did not receive any antimicrobial therapy and milk from all enrolled cows was discarded until it returned to normal (nontreated group) or until the end of the milk withholding period after conclusion of the prescribed treatment. Clinical cure was defined when milk and the udder resumed a normal appearance for at least two consecutive milkings during the 10-d observation period. Milk was defined as

abnormal if it was: chunky, creamy, watery, blood, and if it contained clots or flakes. Based on the expectation that antimicrobial therapy would be beneficial for CM caused by Gram-positive bacteria, fewer animals were randomly assigned using unequal randomization ratios to the non-treated control group (Dumville et al., 2006). No systemic or supportive treatments were administered to any cows.

Data Collection

Cows were followed to record recurrence of CM and herd retention for 90 d after enrollment or until the cow was dried off or left the herd. Daily milk yields were collected for the three farms in Michigan. For the first 10 d after enrollment, trained farm workers administered treatments and recorded appearance of milk, the udder, and the attitude of the cow. These observations were used to determine when clinical cure occurred. Researchers visited farms weekly to collect duplicate milk samples from enrolled quarters at 14, 21, and 28 ± 3 d after enrollment. During these visits, researchers retrieved computerized health records and assessed study compliance. Study compliance was assessed by verifying the signature on trial enrollment forms provided for cow observations and antimicrobial administration. One duplicate milk sample was preserved with bronopol, refrigerated, and transported to the local commercial laboratory in Michigan (CentralStar, Grand Ledge, MI) and in Minnesota to the local commercial DHIA laboratory (Zumbrota DHIA Lab, Zumbrota, MN) for SCC analysis while the other frozen sample was used for microbiological analysis at the respective university within one week. Composite SCC information was taken from the dairy herd management system for each enrolled cow.

Laboratory Microbiological Analysis

Microbiological analysis of quarter-milk samples was performed following National Mastitis Council guidelines (NMC, 2017). In brief, 10 µL of milk was inoculated on 5% Sheep Blood

Agar (ThermoFisher Scientific, Waltham, MA) and MacConkey Agar (Udder Health Lab Minnesota, Minneapolis, MN). Samples with significant growth (≥ 3 colonies) of Gram-positive organisms were isolated, pure sub-cultures were frozen, and the sub-cultures were sent to the Laboratory for Udder Health (University of Minnesota, Minneapolis, MN) for identification using MALDI-TOF. Additionally, quarter milk samples were pooled and 100 µL was plated on Hayflick's agar (Biological Media Services, Davis, CA) and incubated in 5% CO_2 at 37°C for 7 days and observed for growth of *Mycoplasma spp.* daily.

Definitions

Clinical mastitis history was defined as the occurrence of CM (binary) in any quarter of the cow during the 55 d before the case was enrolled. Subclinical mastitis history (binary) was defined when the previous monthly DHIA SCC before the case was > 150,000 cells/mL (Lavon et al., 2011). Subclinical mastitis history was also tested in models as a continuous variable with the $\log_{10}$ composite SCC from 21 d to 55 d prior to case detection added as a covariate. While both binary and continuous variables for subclinical mastitis history were offered to the model, the variable with the best model fit was retained in the final model. For analysis, relevant covariates were offered to the model including parity (primiparous or multiparous), severity at detection (mild or moderate), stage of lactation at case detection (0-100 DIM, 101-200 DIM, >201 DIM), and for the Michigan herds by average milk production the week prior to case detection (low ≤ 35 kg, middle 35 - 45 kg, and high > 45 kg). Season of enrollment was defined as warm (June-August) or cool (September-March).

Outcomes

Bacteriological cure (BC) was evaluated using milk samples collected on day 21 and was defined as failure to isolate the same bacteria that was isolated at enrollment (0 d). New IMI (NIMI) was

diagnosed when bacteria different than the original agent were recovered at day 21. Recurrence of CM (QREC) was evaluated only for cases that achieved clinical cure and was defined as detection of abnormal milk from the enrolled quarter at least 14 days post-enrollment regardless of etiology. Culling was defined as being sold from the herd within the 90-day follow-up period (FUP). Time until clinical cure, recurrence of CM, and culling were also assessed as time to event outcomes. Individual SCC of quarter milk samples were evaluated at 14, 21, and 28 days ± 3 d. Composite cell counts were available for animals from 3 farms and was evaluated for the 3 test days following CM detection (days 14 to 110 post-enrollment). As only 3 of the 4 farms had composite SCC, subclinical mastitis history was only available for 3 of the 4 farms.

Statistical Analysis

Statistical analyses were conducted using SAS (version 9.4; SAS, 2011). The experimental unit was quarter, and analyses followed intent-to-treat principles. This study was designed as a superiority study with the overall hypothesis that outcomes of cases treated using IMM antibiotics would be superior to outcomes from cases that did not receive antimicrobial treatment. *A priori* sample size estimates were performed to determine the number of quarters to enroll to demonstrate a clinically relevant treatment outcome based on specific groups of Gram-positive pathogens (*Streptococcus spp*., *Lactococcus* and *Enterococcus spp.,* NAS and others and a non-treated control). Based on expectations of the distribution of pathogens and using estimates (Pinzón-Sánchez et al., 2011; Truchetti et al., 2014) for the most limiting dichotomous outcome for pathogen-specific analysis (bacteriological clearance), enrollment of 190 quarters per experimental group ($\alpha = 0.05$; $\beta = 0.80$; difference of 10%) was planned, with a target to enroll 200 quarters per group to account for loss during follow-up. Sample size estimates for the negative control group were based on assumptions from available literature of the benefit of

antimicrobial therapy for treating Streptococcal infections (Oliver et al., 2004; Pinzón-Sánchez et al., 2011). Based on these assumptions ($\alpha = 0.05$; $\beta = 0.80$; difference of 12%) an enrollment of 45 quarters was planned, with a goal of enrolling 50 quarters to account for cow lost to follow up. A smaller non-treated control group was enrolled using unequal randomization because of previous literature documenting the benefits of antimicrobial therapy (Oliver et al., 2004; Dumville et al., 2006; Truchetti et al., 2014). Enrollment in the study ended prematurely due to prolonged research restrictions imposed by the COVID-19 global pandemic. During this period a mid-study assessment was performed to determine an acceptable sample size to compare overall experimental outcomes (rather than pathogen-specific outcomes). A post-hoc sample size analysis was performed for bacteriological cure, quarter recurrence, and days to normal milk using SAS. Number needed to treat (NNT) or number needed to harm was calculated for selected outcomes to describe the practical impact of the 3-d or 8-d treatments compared to the negative control (Altman and Andersen, 1999). The NNT is an epidemiological method used to estimate the likelihood that a treatment (when compared to the control) will result in an improved outcome. As NNT increases more animals need to be treated to result in 1 animal with the improved outcome.

Descriptive statistics of farm characteristics were assessed for categorical outcomes using Fisher's exact analysis (PROC FREQ). Descriptive statistics of cow-level variables were analyzed using Fisher's Exact tests and performed using PROC FREQ and continuous variables were analyzed using an ANOVA with PROC GLM to test for uniformity of the experimental groups.

For all outcomes, possible confounding variables were offered for univariate analysis. Possible confounding variables included: parity, stage of lactation, season of enrollment,

subclinical mastitis history (categorical) or last test day SCC (continuous), severity at detection, and average milk production prior to onset and their interactions. Explanatory covariates with a *P*-value of <0.25 were offered for multivariable models in the analysis of BC, QREC, QSCC, and daily milk production. In all multivariable models, farm was included as a random effect. For time-to-event outcomes, explanatory covariates were offered to the model using the ASSESS statement in PROC PHREG. As the effect of IMM antibiotic treatment was a primary interest for all outcomes, outcomes of a combined treatment group (3-d IMM Hetacillin, 3-d IMM Ceftiofur, 8-d IMM Ceftiofur) were also compared to the negative control group.

To evaluate the effect of experimental group on bacteriological cure, a multivariable logistic regression model was constructed using PROC GLIMMIX and explanatory variables were offered to the model. Experimental group was forced in all models and stepwise regression analysis was used to evaluate risk factors. Model fit was assessed using Akaike's information criterion (AIC) from PROC GLIMMIX. Each variable offered to the model was evaluated by manual elimination until a model was chosen based on the goodness-of-fit. Farm was included in the model as a random effect. Adjusted probabilities of outcomes were generated using LSMEANS and ODDS RATIO option of PROC GLIMMIX. If variables were missing, the case was not included in that analysis.

New IMIs (NIMI) were analyzed using a multivariable binary regression model in PROC GLIMMIX with farm included as a random effect. Univariate relationships between NIMI and the categorical explanatory variables were tested using chi squared or Fisher's exact test in PROC FREQ. Only explanatory variables with a $P < 0.25$ were offered to the multivariable model and of the categorical variables, only season of enrollment met the criteria. After a stepwise regression and evaluating model fit using AIC, from PROC GLIMMIX the only

variable that remained was the experimental group.

To evaluate the effect of experimental group on days to clinical cure, QREC, and time to culling, time-to-event analysis was performed using PROC LIFETEST, where time was the number of days until the defined event occurred. Censoring occurred when a case left the study such as being dried off or culled during the FUP. Cases that failed to achieve the outcome of interest by the end of the FUP were right-censored. Survival curves were calculated using the Kaplan-Meier method. Equality was tested over strata using log-rank and Wilcoxon tests. PROC PHREG was used to estimate the hazard ratio after adjusting for covariates including stage of lactation (categorical), clinical mastitis history, mastitis severity at onset, and parity group (categorical). The ASSESS statement was used to evaluate the proportional hazards assumption. Time-to-Event figures were modeled using SigmaPlot v. 12.5 (Systat, California, United States).

The effect of experimental group on quarter SCC (QSCC) was analyzed using Gaussian models with PROC GLIMMIX. The best model fit was the combined model and included the combined experimental groups of treated and nontreated cases, time, subclinical mastitis history, an interaction of experimental group and time, and farm as a random effect. Differences in the means were assessed using the DIFFS and LINES options of PROC GLIMMIX.

The monthly composite SCC was analyzed using PROC MIXED. The explanatory variables that met the inclusion criteria were clinical mastitis history and QREC (binary). The final model included experimental group, time, QREC, time, and farm as a random effect. Differences in the means were assessed using the DIFF and PDIFF options of PROC MIXED.

For the effect of experimental group on daily milk production on the three Michigan farms during the 90-d FUP, a repeated measures ANOVA was constructed using PROC MIXED. Variables were analyzed using an autoregressive covariance structure. The final model for the

effect of experimental group on daily milk production included experimental group, stage of lactation, parity, clinical mastitis history, milk production at case onset (categorical) and quarter recurrence. Multiple comparisons were performed using Tukey tests, generated using ADJUST= Tukey statement in the LSMEANS option of PROC MIXED and assessed using the DIFF statement.

2.4 Results

Characteristics of Herds and Cases of Clinical Mastitis

Cows were enrolled from 3 farms located in Michigan and 1 farm located in Minnesota (Table 1). Two farms milked Holsteins, and 1 farm each milked Holstein by Jersey crossbreds or Jerseys. The farms ranged in size with the largest farm located in Minnesota with 10,418 milking cows and the smallest containing 921 milking cows. The distribution of parities was associated with farm (P <0.001). The monthly incidence of CM (P = 0.003) and severity of enrolled cases (P = 0.001) varied among farms (Table 1), with Farm 3 enrolling a higher percent of moderate cases than the other farms.

Among all farms, 256 cases of clinical mastitis were enrolled between June 2019 and March 2020, but 16 cases from 1 farm were removed due to protocol noncompliance (Table 2), resulting in 240 cases enrolled in the study. Enrolled cases were identified based on growth of $\geq$3 colony forming units on the Gram-positive segments of the tri-plate used in the OFC system. Of duplicate milk samples tested in university labs, no bacteria were grown from 15% of samples, and 7% were either contaminated or missing (Table 3). Among culture-positive duplicate milk samples with growth (n = 189) that were definitively identified in university laboratories using MALDI-TOF (Table 3), etiologies included a diverse group of *Streptococci spp*. (22%), *Lactococci spp*. (19%), NAS (16%), other organisms (10%), *Staph. aureus* (7%), and

Enterococci spp. (5%). The distribution of pathogens did not vary among experimental group (*P* = 0.24) but did vary among farms (*P* = 0.001).

During the FUP, 22 quarters from 1 farm were voluntarily dried off and were distributed among cows assigned to the negative control group (n = 5), 3 d Hetacillin potassium (n = 9), 3 d ceftiofur hydrochloride (n = 2), 8 d ceftiofur hydrochloride (n = 6). This was a farm management decision, based on a combination of factors including persistently high SCC, continued abnormal milk past 10 days, and recurrent clinical mastitis. As there were so few quarters dried off voluntarily, this outcome was analyzed for differences between the combined antibiotic treated group and negative control. Average duration until quarters were dried off was 73.4 ± 7.3 d and 82.6 ± 2.1 d for all quarters assigned to negative control and combined antibiotic group, respectively, but did not differ as assessed using Log-Rank (*P*= 0.08) or Wilcoxon (*P* = 0.06) tests. The distribution of severity of CM, season of enrollment, parity group, stage of lactation at enrollment and subclinical mastitis history did not vary among experimental groups (*P* > 0.06; Table 4).

Bacteriological Cure at Day 21 by Experimental Group

Bacteriological cure was assessed for 142 cases that had results of microbiological analysis at enrollment and day 21. Bacteriological cure was achieved in 76.1% of cases by day 21. Results of the univariate relationships with bacteriological cure are available in Table 5. The final logistic regression model for the effect of experimental group (3 d IMM Hetacillin, 3 d IMM Ceftiofur, 8 d IMM Ceftiofur, or negative control) on BC included only experimental group (Table 6). Experimental group had no statistical impact on the odds of BC at day 21 (*P* = 0.95).

Bacteriological cure was also assessed using a subset (n = 95, as one farm did not have monthly SCC data) of animals including subclinical mastitis history (continuous). This final

logistic regression model for the effect of experimental group (3 d IMM Hetacillin, 3 d IMM Ceftiofur, 8 d IMM Ceftiofur, or negative control) on BC included previous test day SCC, parity group, and experimental group (forced). Experimental group still had no statistical impact on the odds of BC ($P = 0.77$). The odds of primiparous cows experiencing BC by day 21 were 11.7 (95% C.I. 1.2, 113.6) times greater than for multiparous cows, but the limited number of primiparous animals that experienced mastitis resulted in a wide confidence interval for this estimate ($P = 0.03$). Each 1 log unit increase in the $\log_{10}$SCC at the last test prior to the case decreased the odds of BC by 1.78 ($P < 0.001$, C.I. -2.75, -0.81). The BC model on the subset was also run combining all cases that received antibiotics and compared to cases in the negative control group. When a model was explored comparing grouped antibiotic-treated quarters versus the negative control, the same final variables remained important in the model. Experimental group had no statistical impact on the odds of BC ($P = 0.71$). In the combined model, the odds of primiparous cows experiencing BC by day 21 were 12.4 times greater than for multiparous cows ($P = 0.03$; C.I. 1.25, 124.4). Each 1 log unit increase in the $\log_{10}$SCC at the last test prior to the case decreased the odds of BC by 1.68 ($P < 0.001$, C.I. -2.61, -0.79). The power to detect differences in the probability of BC for the combined subset model assessing treated versus negative control was 6.5%. If the true probability of BC was 89%, 357 cases would be needed per group to reject the null hypothesis with a power of 0.8 that there was no difference between cases that received antibiotics versus non-treated control. Based on NNT analysis, the number of additional Gram-positive cases that would need to be treated to result in 1 additional BC at day 21 was 20 quarters for 3-d IMM hetacillin potassium, 100 quarters for 3-d IMM ceftiofur, and 11 quarters for 8-d IMM ceftiofur.

New Intramammary Infections

New intramammary infections were assessed at day 21 using 150 cases from four farms and 13% (20 cases) experienced a NIMI. Of enrolled cases, NIMI occurred in 11.3%, 15.2%, 10.0%, and 17.0% of cases in the no treatment, 3 d IMM hetacillin, 3 d ceftiofur, and 8 d ceftiofur groups, respectively. Of categorical explanatory variables offered, only season and experimental group (forced) met inclusion criteria for the multivariable model (data not shown). The final model included only the random effect of farm and the forced effect of experimental group which had no statistical impact on the odds of a NIMI ($P = 0.35$). When NIMI was evaluated using the combined antibiotic treated group, the final model included only the random effect of farm and the forced effect of the combined experimental group which had no statistical impact on the odds of a NIMI ($P = 0.13$).

Quarter-Level Recurrence

Quarter-level recurrence during the 90-d FUP was assessed for 129 cases from 3 farms (Farms 1, 2, 4). Farm 3 did not have any recurrent mastitis cases. Due to the small number of recurrences (n = 17), this outcome was modeled only using the combined antibiotic treatment compared to the negative control group. Of explanatory variables, only subclinical mastitis history before case detection met inclusion criteria for multivariable modeling (Table 6). Cows with a history of an increased SCC before the case were 2.5 (95% C.I, 1.2, 5.4) times as likely to experience a recurrence as those without such a history. Experimental group did not influence the probability of QREC ($P = 0.47$) with a LSM for QREC of 17.1 ± 0.09% and 10.9 ± 0.03% for the nontreated and treated groups, respectively. The power to detect differences in the probability of QREC for the combined treatment group versus negative control was 0.11. If the true probability of QREC were 17.1 for the negative control and 10.9 for cows that received antibiotics, 1,717 cases would

be needed in each experimental group to reject the null hypothesis with a power of 0.80. The number needed to harm was also calculated and if the probability was 17.1% of a QREC for the control group and 10.9% for the animals in the treated group, for every 16 quarters not treated, one additional cow would experience a QREC.

Days to Clinical Cure

Days to clinical cure were evaluated for 10 days post-enrollment for 213 cases from 4 farms. Cases (n= 27) were excluded due to increased severity (2 cases in each of the experimental groups that received antibiotics and 3 cases in the non-treated control), dried quarter (4 cases for control, and 6 cases for 3 d IMM hetacillin), premature culling (1 case for 3 d IMM hetacillin), and missing data observations (4 cases for IMM hetacillin, 2 cases for 3 d IMM ceftiofur and 1 case for 8 d IMM ceftiofur). Estimates for the days to clinical cure were 4.96 ± 0.43 (negative control), 5.54 ±0.34 (3 d hetacillin potassium), 5.64 ± 0.32 (3 d ceftiofur hydrochloride), and 5.76 ± 0.36 (8 d Ceftiofur hydrochloride). When assessed using PROC PHREG, the probability of clinical cure within 10 days was not associated with experimental group ($P = 0.63$), parity ($P = 0.88$), season ($P = 0.33$), or subclinical mastitis history ($P = 0.68$) but was associated with severity of CM ($P = 0.02$) and farm ($P = 0.04$). For all experimental groups, by day 5 after detection of the CM, most cases had experienced clinical cure (Figure 1a). The probability of clinical cure was also assessed using PROC LIFETEST, and the survival curves did not vary among experimental groups as measured by the Log-rank test ($P = 0.54$) or Wilcoxon test ($P = 0.65$).

Days to clinical cure was also estimated using PROC LIFETEST for the combined treated group (5.6 ± 0.19) versus control (4.96 ± 0.43). The probability of clinical cure did not vary based on treatment ($P = 0.31$), season ($P = 0.22$), farm ($P = 0.10$), or subclinical mastitis

history (P = 0.55) but was associated with severity of CM (P = 0.04). As compared to moderate cases, cases that were mild were 1.6 (95% C.I: 1.03, 2.63) times more likely to achieve clinical cure within 10 days. The probability of clinical cure did not vary among experimental groups (data not shown) as measured by the Log-rank test (P= 0.36) and Wilcoxon test (P = 0.40).

Days to Culling

Culling and death during the 90-d FUP were evaluated for all cows until they were culled or died; cows that were removed from the study for other reasons were not assessed in this analysis. During the 90 d FUP, 4 cows from 2 farms died and they distributed as negative control (n=1), 3 d Hetacillin potassium (n = 1), and 3 d Ceftiofur hydrochloride (n = 2). During the FUP 15% of cows (n = 36) were culled and they were distributed among negative control (n = 4), 3 d Hetacillin potassium (n = 11), 3 d Ceftiofur hydrochloride (n = 11), and 8 d Ceftiofur hydrochloride (n = 10) groups. The days until cows were culled were 81.5 ± 4.5, 82.4 d ± 2.7, 79.9 ± 3.2, 81.3 ± 2.9 d for non-treated control, 3 d Hetacillin potassium, 3 d Ceftiofur hydrochloride, 8 d ceftiofur hydrochloride, respectively (Figure 1b). The probability of remaining in the herd was not affected by season (P = 0.45), stage of lactation (P = 0.82), clinical mastitis history (P = 0.44), severity score at detection (P = 0.71), parity group (P = 0.79), or experimental group (P = 0.95).

Days until culling was also analyzed as the combined treatment group versus the negative control. The probability of remaining in the herd was not affected by season (P = 0.83), stage of lactation (P = 0.79), clinical mastitis history (P = 0.49), severity score at detection (P = 0.58), parity group (P = 0.63), or experimental group (P = 0.95). The average duration until a cow was culled was 81.5 ± 4.5 and 82.3 ± 1.7 d for cows in the control versus treated groups.

Weekly Quarter SCC

Quarter SCC of milk samples collected on days 0, 14, 21, and 28 were available from 207 cases from 4 farms. As the univariate relationship showed no effect of treatment, experimental groups were grouped into treated versus control (Figure 2). The log_{10}SCC for the treated animals was 6.04 ± 0.06 as compared to 6.20 ± 0.14 for the non-treated control cases. The model included experimental group (P = 0.28), interaction of experimental group by week (P = 0.12), and subclinical mastitis history (P = 0.13). The observed power of the model comparing treated and non-treated animals for QSCC was 93.5%.

Monthly Composite SCC

Monthly DHIA somatic cell counts were evaluated using herd records for 3 test days (between 14 - 110 d) after the case was enrolled. Monthly composite SCC data was available for 204 cases of clinical mastitis from 3 farms. The final model included month (P = 0.01), experimental group (P = 0.31), and an interaction of experimental group and month (P = 0.18). Using this model, LSM for monthly composite log_{10}SCC were 5.07 ± 0.1, 5.31 ± 0.08, 5.16 ± 0.08, 5.19 ± 0.07 for quarters enrolled in negative control, 3 d Hetacillin potassium, 3 d ceftiofur hydrochloride, and 8 d ceftiofur hydrochloride, respectively. The observed power in this model was 55.5%.

Monthly DHIA somatic cell counts were also analyzed using the combined approach in a model that included month (P = 0.01), combined treatment group (P = 0.22), and an interaction of treatment group and month (P = 0.22). Using this model, the estimate for the monthly composite cell count for the treated animals was 5.2 ± 0.4 log_{10} cells/mL while the negative control group estimate was 5.1 ± 0.1 log_{10} cells/mL. Month was significant with the first test estimate (5.25 ± 0.1 log_{10} cells/mL) being greater (P = 0.004) than the following test day (5.08 ± 0.1 log_{10} cells/mL).

Daily Milk Production

Daily milk yield data was available from 89 cases from 3 farms. During the FUP, daily milk yield (Figure 4) was analyzed using a model that included experimental group ($P < 0.001$), parity ($P < 0.001$), stage of lactation at case onset ($P < 0.001$), milk production at case onset ($P < 0.001$), quarter recurrence ($P < 0.001$) and previous history of clinical mastitis in the lactation ($P = 0.01$). Across the 90-d FUP, as compared to cows that received 3 d of ceftiofur (31.6 ± 0.91 kg/d) or 3 d of hetacillin (31.9 ± 0.86 kg/d), milk yield in the 90-d FUP was greater for cows that received 8 d IMM ceftiofur (34.5± 0.76 kg/d) but did not differ from daily milk yield of cows in the non-treated group (33.4 ± 1.09 kg/d). When comparing least square means the difference between the 8-d IMM ceftiofur group and both 3-d IMM groups was statistically significant ($P = 0.001$). However, there was no statistically significant difference between the milk yield of the negative control cows and cows in any of the IMM-treated groups. As compared to milk yield of multiparous cows (34.5 ± 0.8 kg/d), primiparous cows produced 3 kg/d less milk (31.1 ± 0.92 kg/d; $P = 0.001$). Cows that had a history of clinical mastitis before the enrolled case produced 31.7 ± 1.08 kg/d milk as compared to 34.0 ± 0.69 kg/d for healthy cows ($P = 0.01$). Cows that had CM in later stages of lactation produced less milk ($P <0.001$), with means of 35.7 ± 0.78 kg/d (<100 DIM), 34.1 ± 0.81 kg/d (101-202 DIM), and 28.8 ± 1.12 kg/d (>201 DIM) at case onset.

Milk production was also analyzed using the combined approach of comparing the antibiotic treatment groups versus the negative control group. In this model, the treatment was not significant ($P = 0.52$) with cows that received antibiotic treatment producing 33.7 kg/d ± 1.18 and cows in the non-treated group producing 34.2 kg/d ±0.87. Similar to the other model, parity ($P < 0.001$), stage of lactation at case onset ($P < 0.001$), milk production at case onset ($P <$

0.001), and quarter recurrence ($P < 0.001$) were significant; in contrast, subclinical mastitis history was not significant ($P = 0.23$).

2.5 Discussion

The herds enrolled in this study used management and milking practices that are typical for larger midwestern dairy farms (Leite de Campos et al., 2021) and results of this study should be applicable to similar herds. When enrolling farms in the study, clinical mastitis records were reviewed to identify herds that were experiencing sufficient cases of clinical mastitis cases caused by Gram-positive pathogens to enroll cases in a timely manner, thus explaining the greater herd-level incidence of clinical mastitis that was observed in these herds as compared to other studies (Gonçalves et al., 2022). There were a small number of cases on 2 farms that were caused by *Staphylococcus aureus* but the distribution of this refractory pathogen did not vary among experimental groups.

Based on samples submitted to diagnostic laboratories, NAS and environmental streptococci are together responsible for approximately one third of all cases of CM but etiology has typically been determined based on classical microbiological testing (Oliveira et al., 2013; Hertl et al., 2014; Ruegg, 2018). In recent years, increased use of MALDI-TOF has demonstrated a broader diversity of Gram-positive organisms (Nonnemann et al., 2019) and efficacy of approved IMM antibiotics for many of these pathogens is lacking. Consistent with observations of Scillieri-Smith et al (2020), almost 20% of enrolled cases in some herds were caused by *Lactococcus* species. In the U.S., there are 7 approved IMM antimicrobials, most of which are labeled as effective against various *Streptococci* and *Staphylococci* (Ruegg, 2021) but their efficacy against other genera, such as *Lactococcus species*, has not been documented. Our cases were enrolled based on growth on the Gram-positive segment of a tri-plate. Results of

duplicate samples that were frozen, cultured in our laboratory and identified using MALDI-TOF indicated a large diversity of results. The use of duplicate milk samples was interesting because about 15% of the duplicate samples cultured in our university laboratories resulted in an outcome of no significant bacterial growth. While there are several potential reasons for this discrepancy, one possibility is that cases with fewer colonies of bacteria may have been in the process of achieving spontaneous cure and the freeze-thaw cycle may have reduced numbers to below the detection limit. Another possibility is the difference in inoculation volume; the on-farm culture system contains about 0.1 mL of milk, whereas university laboratories used calibrated loops containing 0.01 mL. While it is possible that some of the pathogens in the duplicate samples did not survive the freeze-thaw process, this outcome is more likely when samples contained fewer number of bacteria. The objective of this research was to evaluate treatment of cases identified as Gram-positive based on growth on tri-plates, and the use of duplicate milk samples may have contributed to some discrepancy through contamination of samples. The lack of knowledge about expectations for spontaneous cure of CM caused by *Lactococcus spp*. and other non-typical isolates requires further research.

The herds enrolled in this study had already adopted culture-guided selective treatment programs and were aware of opportunities to reduce antimicrobial usage by limiting treatment to cases with growth on Gram-positive agars (Lago, et al 2011). Selective treatment protocols were originally developed based on knowledge that most cases of non-severe CM caused by Gram-negative bacteria (Suojala et al., 2013; Fuenzalida and Ruegg, 2019a) and those that are culture negative when detected (Fuenzalida and Ruegg, 2019b) do not benefit from IMM antimicrobial therapy. Selective treatment protocols that restrict antibiotic usage to cases that demonstrate growth on the Gram-positive segment of selective agar have been shown to reduce antimicrobial

usage by about 50% (Lago et al., 2011b) without adversely affecting animal health. Antimicrobial treatment of cases that result in growth of Gram-positive bacteria is usually encouraged based on low expectations of spontaneous cure of cases that are presumed to be caused by *Staphylococci* or *Streptococci* that are susceptible to approved IMM antimicrobials (Ruegg, 2018). Intramammary administration of ceftiofur was used as one of our treatments because it is the most commonly administered antibiotic for the treatment of CM in the United States (USDA–APHIS–VS–CEAH–NAHMS, 2014; de Campos et al., 2021). However, this product is a 3rd generation cephalosporin and has been categorized by the WHO as a highest priority critically important antimicrobial (CIA) (WHO, 2018). Judicious use guidelines dictate that CIA should only be used when medically necessary and in the absence of alternative therapies (US Food and Drug Administration, 2012). A recent meta-analysis of CM caused by NAS and Streptococcus-like-organisms (SLO) reported no difference in BC based on use of CIA versus lower classified IMM antibiotics (Nobrega et al., 2020). In our study we explored several mechanisms to reduce usage of CIA by investigating a shorter duration of treatment as well as usage of a different antimicrobial or no antibiotics at all.

Surprisingly, our results suggest that most outcomes of cases that received longer duration IMM treatment using ceftiofur did not vary statistically from outcomes of cases that received no treatment or from cases that received 3-d of IMM treatment using either ceftiofur or hetacillin. The diversity of etiologies enrolled in this study may have contributed to this outcome. Previous researchers have examined the benefit of extended duration therapy using experimentally induced *Streptococcus uberis* cases (rather than natural infections) (Almeida et al., 2003) and have not included cases caused by other less understood catalase negative Gram-positive cocci. Many researchers have performed positively controlled clinical trials that have

evaluated results without discriminating between cases caused by Gram-negative or Gram-positive bacteria (Wenz et al., 2005; Schukken et al., 2013; Truchetti et al., 2014). Inclusion of cases caused by Gram-negative pathogens or those that are culture negative results in larger numbers of enrolled cases but will inflate spontaneous cure rates and bias positively controlled studies toward findings of non-inferiority. Observed rates of BC of cases enrolled in positively controlled trials are the result of both spontaneous cure due to effective immune responses as well as additional therapeutic effects of treatments. Inclusion of a negative control group allows separation of the spontaneous cure rate from the additive effect of therapy. We evaluated BC on day 21 and did not identify an effect of treatment in either of the models. The absence of a difference in BC between antimicrobials is unsurprising as both ceftiofur and hetacillin are β-lactam antimicrobials that have similar spectrums of activity against Gram-positive pathogens. Previous researchers performed a positively controlled non-inferiority study that compared outcomes of treatment with IMM Hetacillin potassium (3 d IMM) to outcomes of treatment using ceftiofur hydrochloride (5 d IMM) (Vasquez et al., 2016). While 588 CM cases were enrolled in this study, many cases were enrolled with non-significant growth (38% of enrolled cases) or were pathogens (such as yeast and Prototheca) that are outside of the spectrum of activity of the treatments. Similar to our results, those researchers reported a bacteriological cure of 73% and 68% for Gram-positive cases that received IMM ceftiofur or hetacillin, respectively (Vasquez et al., 2016). Similar results were reported for BC of Gram-positive pathogens in another study that compared IMM amoxicillin and IMM ceftiofur (Tomazi et al., 2021).

One result of our study was the importance of many previously identified cow level risk factors including parity, mastitis severity and subclinical mastitis history on the likelihood of BC (Pinzón-Sánchez and Ruegg, 2011). As reported by others (Vasquez et al., 2016; Fuenzalida and

Ruegg, 2019b), primiparous cows had much greater odds of BC. Subclinical mastitis history was another risk factor that influenced BC. Cows with a history of greater SCC before CM were less likely to achieve BC, which is possibly a reflection of the duration of the infection which may be influenced by pathogen specific virulence factors (Tassi et al., 2013).

Producers should review SCC history, case severity, and parity to help make informed decisions about treatment of individual animals. While BC is a desirable outcome, it is not strongly associated with many clinical outcomes such as days to clinical cure, milk production, or herd retention (Ruegg, 2021); thus it is important to assess clinical outcomes separately. Both BC and NIMI were assessed at a single time point, while other clinical outcomes involve repeated observations over multiple time periods. It is important to differentiate between a NIMI and QREC. New intramammary infection was when a pathogen other than the pathogen isolated at enrollment was detected while a QREC was a recurrence of clinical mastitis. In this study, there were relatively few QREC and there was no effect of treatment. This is possibly because these farms culled and voluntarily dried the quarter of many of the enrolled cases, though there was no effect of treatment on any of the BC models run.

Administration of IMM antibiotics is expected to reduce bacterial numbers and hasten the immune response in achieving BC (Bradley and Green, 2009). Few researchers have included non-treated control cases when evaluating treatment of Gram-positive bovine mastitis. Tomazi et al. (2021) included a negative control group that remained untreated for only 5 days. They compared outcomes among cases that received IMM amoxicillin (3 infusions, every 12 hours), extra-label administration of IMM amoxicillin (1x/day for 5 days), IMM ceftiofur (5 infusions, 1x/day), to outcomes of a delayed treatment group that remained untreated for 5 days but then received 3 IMM treatments (1x/day) using hetacillin potassium (Tomazi et al., 2021). The

authors noted that the linear SCS for cases in the delayed treatment group was greater at day 5 as compared to cows that received IMM antibiotics at day 1. However, at day 5, while many cows would have achieved clinical cure, expectations for quarter level SCC reduction are limited (Ruegg, 2021). We followed cases for a longer duration and found no difference in QSCC up to 28 days nor in the DHIA composite SCC. Bacteriological cure must precede reductions in SCC and time to BC is likely shorter when antimicrobials are used. After BC has been achieved, researchers have demonstrated that the SCC will gradually decline, as reported in studies enrolling cases caused by Gram-negative bacteria (Fuenzalida and Ruegg, 2019b) as well as for cases that were culture-negative at enrollment (Fuenzalida and Ruegg, 2019a). While SCC is a practical outcome, immediate decline in cell count should not be expected, regardless of treatment. This was shown in our combined treated versus negative control model, where composite SCC was greater at the first test post-enrollment and had declined by the next test date. Many of our results were consistent with results reported by Tomazi et al (2021). Both studies compared two β-lactam antibiotics and reported no difference in BC, risk of recurrence of CM, clinical cure, or survival to 90 days. Together these results support use of short-duration IMM treatment, using approved IMM narrow spectrum antimicrobials. Decreasing treatment length is an important mechanism that can help reduce unnecessary usage of antimicrobials.

Clinical cure is an outcome that is often assessed by producers but should be used with caution because inflammation resolves with or without treatment for most cases by day 5-7 (Pinzón-Sánchez and Ruegg, 2011; Oliveira et al., 2013; Fuenzalida and Ruegg, 2019a). In our study, there was no difference in clinical cure among experimental groups. One should note that milk from cows that do not receive antibiotics can be sold after the appearance returns to normal, as there is no antibiotic withholding time. In the United States, shorter duration IMM antibiotic

treatments will return animals to producing saleable milk more quickly with fewer days of discarded milk. When making treatment decisions for cows experiencing CM caused by Gram-positive pathogens, using a short duration of ceftiofur hydrochloride may reduce costs without negatively impacting animal health.

Based on our original sample size calculations and recommendations to use IMM antibiotics for the treatment of Gram-positive mastitis we included a smaller group of animals in the non-treated group (Almeida et al., 2003; Roberson et al., 2004; Tomazi et al., 2021). Based on previous literature, we estimated that the benefit of IMM antibiotic treatment would be greater than we observed. While we initially planned to enroll more cases and perform pathogen specific analyses, data collection was prematurely terminated by months-long research and travel restrictions imposed by the global COVID-19 pandemic. While our sample size was not sufficient for pathogen specific analyses, we did achieve a sufficient sample size to identify biologically important outcomes, especially relative to duration of treatment. To estimate the practical impact of our results we calculated the number needed to treat or harm for several key outcomes. Bacteriological cure was our most limiting outcome, and three separate models were analyzed on all cases, and then on cases with SCC data available, and in all models, treatment was not significant. For BC, in our most limited model (n = 95), antibiotic therapy of Gram-positive cases would result in an additional BC as compared to non-treatment, but 11 (8-d ceftiofur), 20 (3-d hetacillin) or 100 (3-d ceftiofur) cases needed to be treated to achieve 1 additional BC. Similarly, antibiotic treatment of 16 Gram-positive cases would prevent 1 quarter-level recurrence (as compared to no-treatment). These values indicate some value of antibiotic therapy for treatment of similar cases as compared to no treatment and should be considered relative to individual farm goals. Additional negatively controlled studies are needed to better

define pathogen and cow characteristics that can be used to refine treatment protocols. Although we enrolled fewer cases than planned, our sample size is comparable to other published studies (Truchetti et al., 2014; Cortinhas et al., 2016) and our conclusions can be applied to cases that are treated based on growth of Gram-positive bacteria on selective media. While we ended up enrolling 240 cases of Gram-positive clinical mastitis, there were 189 cases that had significant growth of an isolate in the university labs and 111 cases that completed the FUP. It is a limitation that the longer duration outcomes frequently have a smaller number of cases included in the analysis, but this reflects the adverse outcomes that happen to cows experiencing clinical mastitis. Of the 240 cases enrolled, 15% of the enrolled cases were culled before 90 days. Larger studies would be useful for evaluating pathogen-specific results, especially those of cases caused by *Lactococci spp.* and other emerging pathogens.

Enrolling larger numbers of cases in pathogen-specific studies is time-consuming because mastitis is caused by a variety of etiologies that are indistinguishable by clinical presentation. On a 1,000-cow dairy farm we would anticipate that about 25% of lactating cows would experience a first clinical mastitis case during a lactation (Gonçalves et al., 2022). Based on typical distribution of etiologies, of those 250 cases, approximately 75 would be caused by Gram-positive pathogens in a year. While there are studies that demonstrated a benefit of extended therapy for *Streptococcus uberis* (Oliver et al., 2004), for producers making selective treatment decisions by differentiating only at the Gram level (Gram-positive or -negative) using selective medias (Lago et al., 2011; USDA–APHIS–VS–CEAH–NAHMS, 2014), these results (BC, QREC, QSCC) suggest that utilizing a longer treatment period would not be beneficial.

In our study, cases that received 8-d IMM ceftiofur and cases that received no treatment had the greatest daily milk production in the 90 d FUP, but there was no difference between

them. These contradictory results were puzzling but were not substantiated when milk yield was compared between treated and non-treated animals. Most previous studies of milk yield after treatment have evaluated monthly DHI test day data. Tomazi et al (2021) reported greater test day milk yields for cows that received 3 IMM treatments 12 hours apart as compared to 5-day IMM ceftiofur treatment. In our study, using daily milk weights, we did not observe a difference in milk yield between cows that received 8-d of IMM ceftiofur and cows that received no treatment. However, we did see greater daily milk yield in cows that received 8 d IMM ceftiofur as compared to cows that received either of the 3 d IMM therapies, but neither differed from milk yield observed in the cows that did not receive IMM antibiotics. When the results were analyzed using the combined approach with a combined antibiotic treatment group versus the negative control there was no effect of treatment on milk yield.

In our study, extended duration IMM ceftiofur hydrochloride resulted in the greatest milk production but it did not differ from no treatment. An economic calculation of this benefit must account for the fact that the total amount of discarded milk for this treatment was 11 days, while the shorter duration protocol required milk discard for only 6 days. If milk price were $20/ hundred weight (cwt) and the milk production were the estimated 34.5 kg/day for a cow over the course of 79 days (after accounting for 11 days of milk discard) the cow would produce 2725.5 kg of saleable milk. If the same cow was assigned to the short 3 d treatment and produced the estimated 31.6 kg/day over the course of 84 cays the cow would produce 2,654.4 kg of saleable milk. When converted (1 cwt = 45.36 kg and 1 cwt= $20) the value of the milk produced by the extended duration is $1,201.72 and the shorter duration is $1,170.37. The net increase in milk production is valued at $31.35 before accounting for the additional direct costs of labor and antibiotics. This quick economic analysis during a 90-d window indicates that caution should be

used when making the decision for extended duration therapy. The additional direct costs of labor and antibiotics are unlikely to bring a cost benefit under these study conditions. Future research could continue to look more longitudinally to evaluate milk production over time if there are different stages of production where extended duration therapy is potentially beneficial. There were only a limited number of cases included in the analysis (n = 89) so future research should continue to explore potential pathogen effects that may be related to these results.

Our results demonstrate that several important characteristics of cows should be considered before making treatment decisions which is consistent with previous literature (Pinzón-Sánchez and Ruegg, 2011). Cows with a history of increased SCC at the last test date before the CM case produced less milk and were less likely to achieve bacteriological cure. Primiparous animals were more likely to achieve bacteriological cure. Given these insights, reviewing the history of a cow might be one mechanism to reduce antimicrobial usage. For example, treating (especially extended duration) a multiparous cow with a chronic infection (high cell count prior) in late lactation is unlikely to maximize the economics of treatment because regardless of treatment most cows will achieve clinical cure within 5 days.

2.6 Conclusion

Mastitis caused by Gram-positive pathogens on 4 Midwestern dairy farms was caused by a diverse group of pathogens, many of which are not listed on product labels of IMM antibiotics and for which treatment efficacy is unknown. Among these diverse pathogens, BC rates were high and did not vary based on the duration of treatment. Several of our results support that reviewing a cow's history before making treatment decisions may help reduce antibiotic usage, as mild cases of CM in primiparous animals without a history of subclinical mastitis were more likely to achieve bacteriological cure and had a lower SCC during the 90-d FUP. Overall, there

was no difference in SCC, BC, clinical cure, or retention in the herd based on duration of IMM antimicrobial therapy or based on choice of antimicrobial. While larger studies are needed, these results indicate that longer-duration treatments may have minimal benefit for many of the organisms identified as Gram positive using OFC. Mastitis cases caused by some Gram-positive bacterial species likely benefit from treatment; however, based on this research, for non-severe clinical mastitis cases a shorter duration 3-d treatment appears to be effective for most clinical and bacteriological outcomes.

2.7 Acknowledgements

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2.8. Tables and Figures

Table 2.1. Characteristics of commercial dairy farms in Michigan and Minnesota that enrolled cases of non-severe clinical mastitis (CM) caused by Gram-positive bacteria in a randomized clinical trial (RCT) June 2019-March 2020

Variable	Farm 1		Farm 2		Farm 3		Farm 4		*P*-value	All Farms	
	%	No.	%	No.	%	No.	%	No.		%	No.
State		MI		MI		MI		MN			
Breed		HolsteinX[1]		Holstein		Holstein		Jersey			
Lactating Cows		3,526		921		5,924		10,418			20,789
Average 305 mature equivalent Milk (kg)		11,841		12,059		13,231		8,236			
Parity distribution									<0.001		
Parity 1	38.5	1,357	30.1	277	39.9	2,361	37.4	3,896		37.9	7,891
Parity 2	28.4	1,003	25.9	239	29.3	1,734	24.1	2,507		26.4	5,483
Parity 3+	33.1	1,166	43.9	405	30.9	1,829	38.5	4,015		35.7	7,415
Incidence of Clinical Mastitis[2]		0.052		0.065		0.037		0.059	0.003		
Total Cases of Clinical Mastitis[3]		657		227		744		3,624	0.19		4,942
Total Cases Enrolled[4]	33.8	81	18.8	45	9.2	22	38.3	92	0.03		240
Severity of Enrolled Case									<0.001		
Mild	97.5	79	77.8	35	63.6	14	73.9	68		81.7	196
Moderate	2.5	2	22.2	10	36.4	8	26.4	24		18.3	44

[1]Holstein Crossbred Cattle.
[2]The monthly incidence of CM was calculated as the number of new cases of CM divided by the number of lactating cows at risk per month. Cases of CM that occurred >14 days from the previous case were considered to be new cases.
[3]Total Cases of CM Between June 2019-March 2020.
[4]Number of single-quarter CM cases identified as Gram-positive using on-farm culture enrolled in the RCT

Table 2.2. Description of non-severe cases of clinical mastitis enrolled in a randomized clinical trial on 4 dairy farms during June 2019-March 2020 based on growth on Gram-positive selective culture media

Cases (n)	Farm 1	Farm 2	Farm 3	Farm 4	Total
Enrolled[1]	45	81	22	108	256
No treatment	3	25	8	22	58
3-d Hetacillin potassium	16	25	6	35	82
3-d Ceftiofur hydrochloride	12	25	6	26	69
8-d Ceftiofur hydrochloride	14	6	2	25	47
Removed for protocol[2] noncompliance	0	0	0	16	
Retained in Analysis	45	81	22	92	240
Cases with incomplete 90 d follow up:					
Cow died	0	2	0	2	4
Cow culled	2	22	3	9	36
Dried off at end of lactation	3	9	1	5	18
Quarter dried	0	0	0	21	21
Clinical cure failure at 10 days[3]	4	0	0	2	6
Case severity increase	2	0	5	2	9
Total cases with incomplete follow-up	11	33	9	41	94
Total number of cases completed 90 d follow up					146

[1]Total number of animals enrolled by farm.
[2]Animals that were not included in the analysis due to protocol non-compliance.
[3]Animals that failed to return to normal milk after 10-day observation period and were treated with additional antimicrobials in consultation with herd veterinarian.

Table 2.3. Results from university laboratory analysis using MALDI-TOF of isolates from duplicate milk samples collected from 240 cases of non-severe clinical mastitis enrolled in a RCT based on identification as Gram-positive organisms using selective agar from June 2019-March 2020

Organism	3-d Hetacillin Potassium	3-d Ceftiofur hydrochloride	8-d Ceftiofur hydrochloride	No treatment	Total	%[2]
Streptococcal-Like-Organisms						
Enterococcus						5.0%
Enterococcus aquimarinus	1	0	0	0	1	
Enterococcus faecalis	0	1	0	0	1	
Enterococcus saccarolyticus	3	2	3	2	10	
Lactococcus						19.2%
Lactococcus garvieae	4	4	7	3	18	
Lactococcus lactis	14	6	7	1	28	
Streptococcus						21.7%
Streptococcus dysgalactiae	7	13	7	5	32	
Streptococcus uberis	4	4	1	0	9	
Streptococcus species	3	2	4	2	11	
Non-Aureus Staphylococci						16.3%
Staphylococcus chromogenes	2	8	8	2	20	
Staphylococcus epidermidis	0	0	1	0	1	
Staphylococcus haemolyticus	1	1	1	0	3	
Staphylococcus simulans	1	1	3	1	6	
Staphylococcus species	1	2	2	3	8	
Staphylococcus xylosus/saprophyticus	0	0	1	0	1	
Staphylococcus aureus						6.7%
Staphylococcus aureus	3	7	3	3	16	
Other						10.0%
Bacillus species	2	3	2	0	7	
Corynebacterium species	1	0	0	0	1	

Table 2.3 (cont'd)

Organism	3-d Hetacillin Potassium	3-d Ceftiofur hydrochloride	8-d Ceftiofur hydrochloride	No treatment	Total	%[2]
Escherichia coli	0	0	1	0	1	
Paenibacillus species	1	0	0	0	1	
Pseudarthrobacter species	0	0	1	0	1	
Pseudomonas species	1	1	1	0	3	
Trueparella pyrogenes	0	1	0	1	2	
2 causative pathogens	3	1	1	0	5	
Yeast	1	1	1	0	3	
No Significant Growth in duplicate sample[1]	12	6	11	6	35	14.6%
Contaminated and missing	4	5	4	3	16	6.7%
Total	69	69	70	32	240	

[1]At enrollment duplicate quarter milk samples were collected. One sample was used for on-farm culture and the sample was enrolled based on those results. The second sample was frozen until transported to the research lab where the culture results had MALDI-TOF performed.

[2]Percent of total enrolled cases

Table 2.4. Description of clinical mastitis (CM) (n = 240) at enrollment in a RCT by experimental group from June 2019-March 2020

	Negative Control		3-d IMM hetacillin		3-d IMM ceftiofur		8-d IMM ceftiofur		*P*-Value	Total
Factor	%	No	%	No	%	No	%	No		No.
Severity of CM									0.24	
Mild	78.1	25	82.6	57	75.4	52	88.5	62		196
Moderate	21.9	7	17.4	12	24.6	17	11.4	8		44
Season of Enrollment									0.06	
Warm (Jun-Aug)	71.9	23	43.5	30	49.3	35	48.6	34		122
Cool (Sep.-March)	28.1	9	56.5	39	50.7	34	51.4	36		118
Parity Group									0.38	
1	12.5	4	14.5	10	24.6	17	18.6	13		44
2	87.5	28	85.5	59	75.4	52	81.4	57		196
Stage of lactation									0.58	
0-100 (early)	53.1	17	55.1	38	50.7	35	48.5	34		124
101-200 (mid)	31.3	10	27.5	19	39.1	27	30.0	21		77
300+ (late)	15.6	5	17.4	12	10.1	7	21.4	15		39
Subclinical Mastitis History[1]									0.34	
No	58.3	14	48.0	24	65.4	34	60.4	29		101
Yes	41.7	10	52.0	26	34.6	18	39.4	19		73
Total		32		69		69		70		240

[1]Subclinical Mastitis History was defined as a test day in the preceding 55 d above 150,000 cells/mL, 66 animals did not have a test day in the preceding 55 d.

Table 2.5. Univariate associations between selected risk factors and quarter-level bacteriological cure (BC) at 21 days after enrollment in a RCT from June 2019-March 2020

Bacteriological Cure (n = 142)			
Predictor	%	No.	*P*-Value
Experimental Group			0.98
Negative Control	78.6	11/14	
3 d IMM Hetacillin	76.1	35/46	
3 d IMM Ceftiofur	78.1	32/41	
8 d IMM Ceftiofur	73.2	30/41	
Severity of CM			0.25
Mild	74.0	91/123	
Moderate	89.4	17/19	
Season of Enrollment			0.93
Warm (Jun.-Aug.)	76.5	39/51	
Cool (Sep.-Mar.)	75.8	69/91	
Parity			0.17
Primiparous	80.8	21/26	
Multiparous	75.0	87/116	
Stage of Lactation			0.48
0-100 (early)	74.3	55/74	
101-200 (mid)	82.2	37/45	
300+ (late)	69.6	16/23	

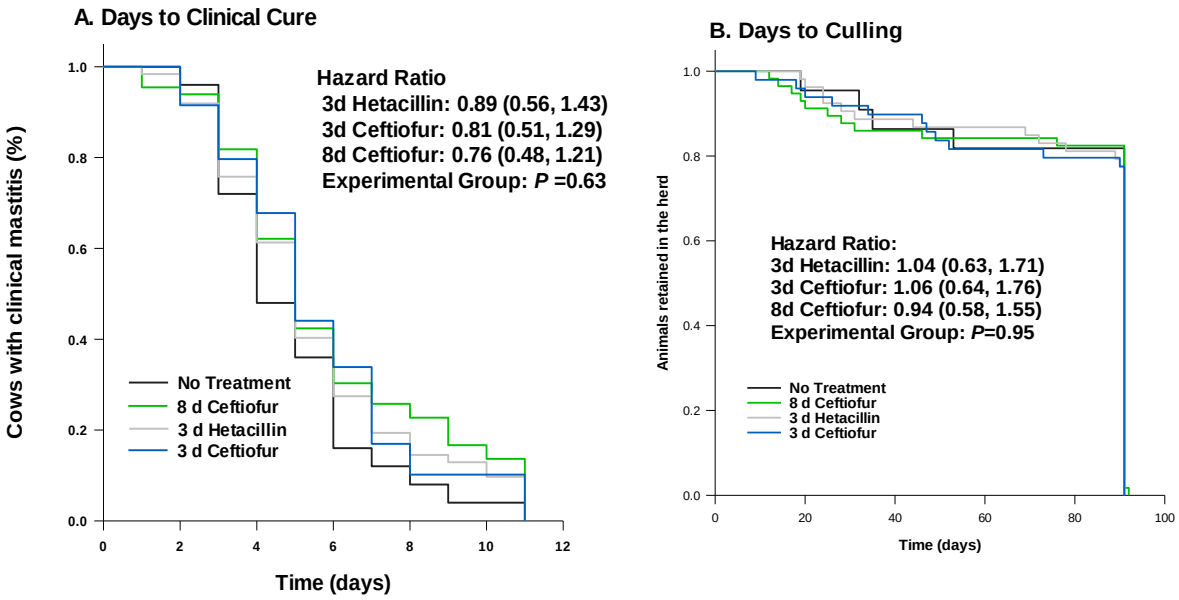
Table 2.6. Final logistic regression models of effects of experimental treatment group on bacteriological cure at 21 days after enrollment and quarter recurrence within 90 days after enrollment from June 2019-March 2020

Predictor	n	LSM	SEM	Odds Ratio (95% C.I.)	*P*-Value
Bacteriological Cure	142				
Experimental Group					0.95
Negative Control (n = 14)	11	0.79	0.11	Referent	
3 d IMM hetacillin (n = 46)	35	0.76	0.06	0.87 (0.2, 3.6)	
3 d IMM ceftiofur (n = 41)	32	0.78	0.06	0.97 (0.2, 4.3)	
8 d IMM ceftiofur (n = 41)	30	0.73	0.07	0.74 (0.17, 3.2)	
Quarter Recurrence (n = 129)	129				
Experimental Group					0.47
Negative Control (n = 3)	3	0.17	0.09	Referent	
Combined Antibiotic (n = 14)	14	0.11	0.03	1.67 (0.40, 6.98)	
Intercept of Last Test Day		0.91	0.39	2.48 (1.15, 5.37)	0.02

Table 2.7. Univariate associations between selected risk factors and quarter recurrence (QREC) after enrollment in a RCT from June 2019-March 2020

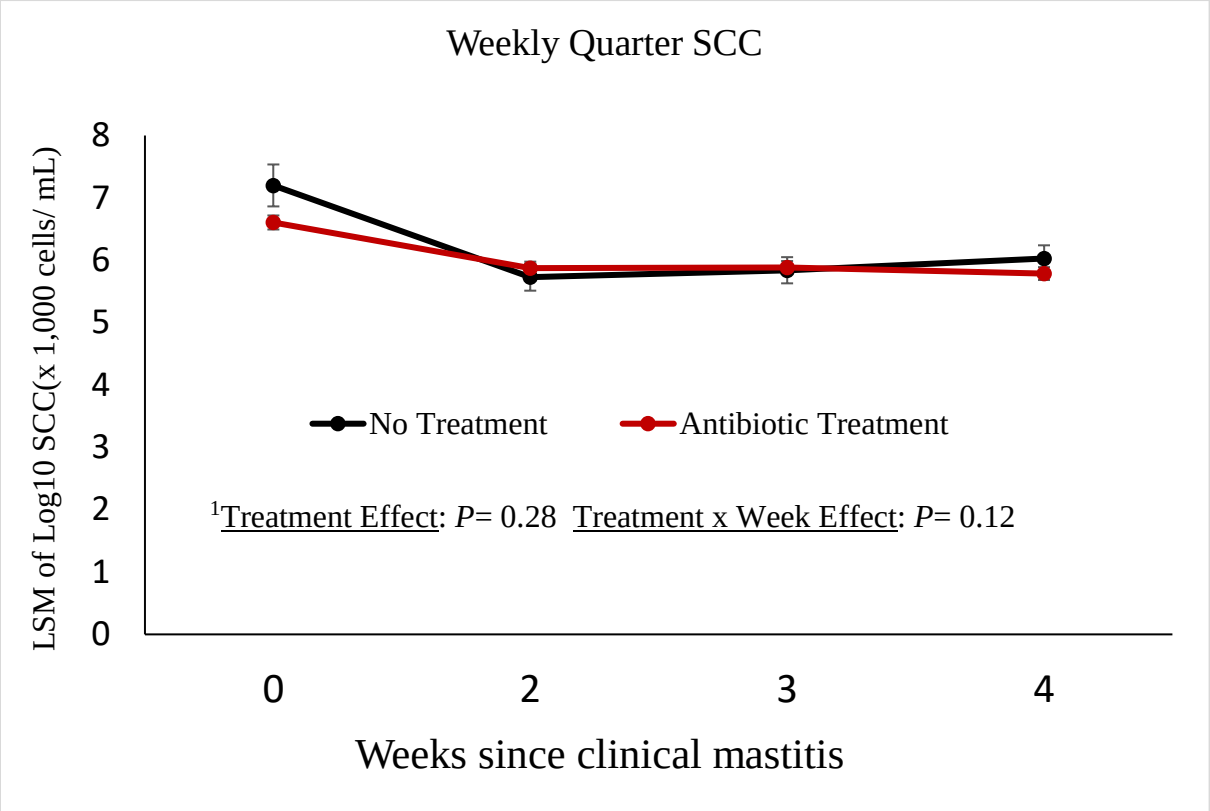
	Quarter Recurrence (n=129)		
Predictor	%	No.	*P*-Value
Experimental Group			0.69
Combined Antibiotic Treatment	12.5	14/112	
Negative Control	17.7	3/17	
Severity of CM			0.29
Mild	13.5	16/115	
Moderate	4.6	1/14	
Season of Enrollment			0.60
Warm (Jun.-Aug.)	15.1	11/73	
Cool (Sep.-Mar.)	10.7	6/56	
Parity			0.52
Primiparous	16.7	4/24	
Multiparous	12.4	13/105	
Stage of Lactation			0.79
0-100 (early)	16.0	8/50	
101-200 (mid)	11.1	6/54	
300+ (late)	12.0	3/25	
SCC history (Continuous)			0.02

Figure 2.1. Unadjusted survival plots[1] of Gram-positive cases treated with antibiotics or no treatment describing the probability of clinical cure and culling for a randomized clinical trial between June 2019-March 2020



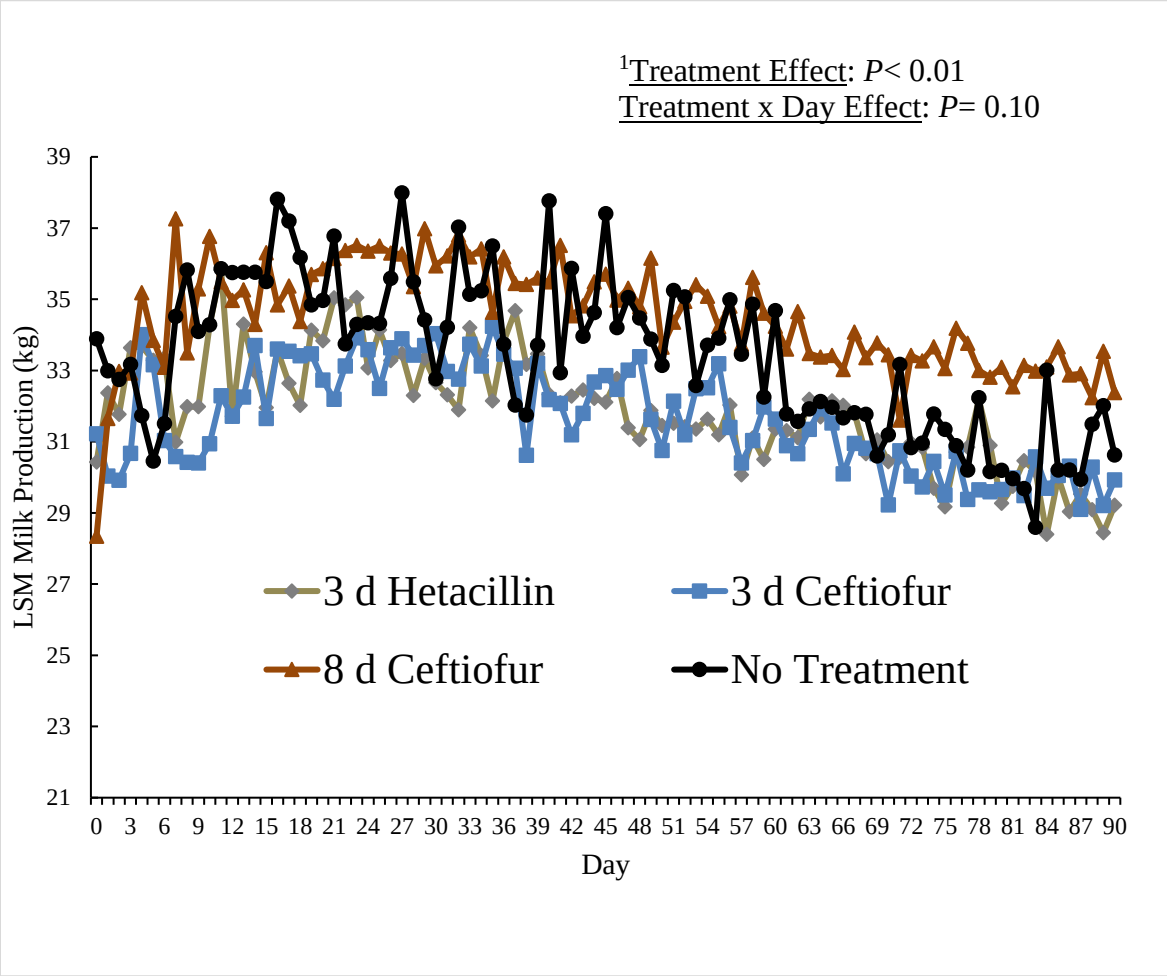
[1]Figure 2.1. is the unadjusted survival plots describing the probability of A) clinical cure (n = 212) and B) days to culling (n = 240) by experimental group. Time is the number of days until the event occurred. Hazard ratios are comparing the antibiotic treatment to the negative control and values in the parenthesis represent the 95% confidence interval.

Figure 2.2. Estimates for weekly quarter SCC from cows post-enrollment in a randomized clinical trial between June 2019-March 2020



[1]Least Square Means of weekly quarter SCC, the estimate for antibiotic treatment was 6.04 ± 0.06 and the estimate for the non-treated control was 6.20 ± 0.14. Negative control (n=25) and combined IMM treatments (n=182). Repeated measures analysis that included the effect of treatment, time, and subclinical mastitis history (*P*=0.13).

Figure 2.3. Estimates of daily milk production following Gram-positive clinical mastitis onset from Michigan farms (n= 3) enrolled in a randomized clinical trial between June 2019-March 2020



[1]The estimates of daily milk yield by treatment group were 33.4±1.09 kg/d, 31.9 ± 0.86 kg/d, 31.6 ± 0.91 kg/d, and 34.5 ± 0.76 kg/d for cows in the negative control group, 3 d IMM hetacillin potassium, 3 d IMM ceftiofur, 8 d IMM ceftiofur, respectively. There is no difference in milk yield for cows that received 3d IMM ceftiofur or 3d IMM hetacillin (P = 0.96), the 8d IMM ceftiofur and negative control (P = 0.59), or between the 3d IMM products and the negative control (P > 0.39). The 8 d ceftiofur product was between both of the 3 d IMM treatments (P < 0.001).

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CHAPTER 3: COMPARISON OF MINIMUM INHIBITORY CONCENTRATIONS OF NON-AUREUS STAPHYLOCOCCI, ENTEROCOCCI, LACTOCOCCI, AND STREPTOCOCCI TO SELECTED ANTIMICROBIALS

3.1 Abstract

The objective of this study was to compare minimum inhibitory concentrations of antimicrobials included in a commercial broth microdilution panel among Gram-positive pathogens that caused non-severe clinical mastitis on three Michigan dairy farms. Duplicate quarter milk samples were collected from eligible quarters of cases enrolled in a randomized clinical trial based on growth on Gram-positive selective agars. One duplicate milk sample was cultured in a university laboratory and identified using MALDI-TOF. The causative pathogen was identified and grouped by genus as Enterococcus species (n = 11), Lactococcus species (n = 44), Non-aureus Staphylococcus (n = 39), or Streptococcus species (n = 25). Minimum inhibitory concentrations (MIC) were determined using the mastitis panel of a commercial broth microdilution test. *In vitro* susceptibility was determined using an approved guideline and included breakpoints for mastitis pathogens or (when not available) breakpoints determined using other species. Most isolates were inhibited at or below breakpoints that demonstrate *in vitro* susceptibility. The proportion of susceptible isolates varied among pathogens for pirlimycin, penicillin, and tetracycline. The greatest proportions of resistant isolates were observed for pirlimycin, tetracycline, and sulfadimethoxine. Survival analysis was performed to evaluate differences in MIC among pathogen groups. Minimum inhibitory concentrations varied among pathogens for ceftiofur, cephalothin, erythromycin, penicillin, pirlimycin, and tetracycline. However, nearly all of the isolates were susceptible to ceftiofur and cephalothin, indicating that pathogen differences in MIC are not likely clinically relevant. While differences in *in vitro* susceptibility were

observed for some antimicrobials (including penicillin and pirlimycin), most isolates were susceptible to the most commonly used cephalosporin based IMM treatments and did not vary among pathogens.

3.2 Introduction

Prevention and treatment of mastitis are the primary reasons for administration of antimicrobials to adult dairy cows (USDA, 2014; Leite de Campos et al., 2021). Use of antimicrobials in food-producing animals is increasingly scrutinized because of growing fears of antimicrobial resistance (AMR) with an emphasis on reducing usage of critically important antimicrobials (CIA) that belong to classes that are important for treatment of human diseases (US Food and Drug Administration, 2012). Phenotypic susceptibility testing is one tool used to measure occurrence of AMR among bacterial pathogens. While there is sparse evidence that AMR is driven by IMM (IMM) administration of antimicrobials (Oliver and Murinda, 2012) there is limited evidence that systemic antibiotic treatments of non-aureus staphylococci (NAS) may contribute to AMR (Nobrega et al., 2018). One mechanism to reduce usage of CIA is to use narrow spectrum antibiotics when possible (US Food and Drug Administration, 2012). While results of phenotypic susceptibility testing are not strongly predictive of clinical outcomes (Constable and Morin, 2002; Hoe and Ruegg, 2005; Apparao et al., 2009), it is important that veterinarians recommend treatments with appropriate spectrum of activity for pathogens causing clinical mastitis (CM). Most IMM antimicrobials available in the U.S. were developed to treat mastitis caused by *Staphylococci aureus* and some *Streptococci spp*. and have not been evaluated for efficacy against other NAS, *Enterococcus spp*. and *Lactococcus spp.*, which are increasingly isolated from mastitic cows on modern dairy farms (Scillieri Smith et al., 2020). The objective of this study was to compare the MIC of Gram-positive pathogens that caused non-severe CM on

Michigan dairy farms. We hypothesized that Streptococci, Enterococci, Lactococci, and non-aureus staphylococci (NAS) would have different MIC and susceptibilities to the tested antimicrobials.

3.3 Materials and Methods

Cows with non-severe CM were enrolled in a randomized clinical trial conducted on 3 Michigan dairy farms and 1 Minnesota farm during June 2019-March 2020. The clinical trial was approved by the Institutional Animal Care and Use Committee at Michigan State University (PROTO201900087). Bacteria isolated in the MSU udder health laboratory from cases enrolled in MI were cryopreserved at -80°C and were eligible for this analysis. Of 148 eligible cases, 20 were not included because fewer than 10 isolates were recovered [*Staph aureus* (n = 4), *Trueperella pyogenes* (n = 2), *Bacillus species* (n = 3), *Corynebacterium spp.* (n = 1), *Pseudoarthrobacter spp.* (n = 1), *Paenibacillus species* (n=1), *Pseudomonas spp.* (n = 3)], the milk sample contained two pathogens (n = 3), or *Yeast spp.* (n = 2). Of 128 remaining isolates, 1 did not survive cryopreservation and an additional 19 cases were not included because the duplicate milk sample was contaminated (n = 6), had no-significant growth (n = 11), or was missing (n = 2). The isolates were grouped by genus as NAS (n = 28), *Streptococci species* (Strep; n = 25), *Lactococci species* (Lact; n = 44), and *Enterococci species* (Enter; n = 11; Table 1). Minimum inhibitory concentrations (MIC) were determined using the mastitis panel from a commercial broth microdilution test (Sensititre Vet Mastitis CMV1AMAF, Thermo Fisher Scientific) following guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2018). Pure subcultures were grown from the frozen isolates and broth microdilution was performed according to manufacturer's instructions. Quality control was performed in accordance with CLSI using *S. aureus* (ATCC 29213) and *Staphylococcus epidermidis* (ATCC 51625),

Lactococcus cremoris (ATCC 19257), *Lactococcus garvieae* (ATCC 43921). The control strains susceptibility results were compliant with the quality control ranges.

Antimicrobial resistance was defined as the ability of the bacterial isolate to demonstrate growth at the defined concentration of the antimicrobial above the breakpoint (CLSI, 2018). Minimum inhibitory concentration (MIC) was defined as the least concentration of an antimicrobial that inhibited visible growth, while MIC_{50} and MIC_{90} were defined as the antimicrobial concentration where 50% and 90% respectively of the isolates were inhibited. Breakpoints for susceptibility were as defined in the CLSI guidelines (CLSI, 2020). Bovine mastitis specific interpretative criteria were available for ceftiofur, penicillin and novobiocin, and pirlimycin (CLSI, 2020). The interpretative criteria for ampicillin, cephalothin, erythromycin, oxacillin, penicillin, and tetracycline were based on human criteria (CLSI, 2020) which is consistent with previous literature (Ruegg et al., 2015; Cameron et al., 2016). Current CLSI guidelines (2020) do not have a recommendation for breakpoints for sulfadimethoxine for any bovine mastitis pathogens or human pathogen so to be consistent with previous literature the susceptibility breakpoint was defined as $\leq$128 μg/mL (Ruegg et al., 2015). As *Lactococci spp.* do not have specific breakpoints listed in CLSI, breakpoints for *Streptococci spp.* were used (Table 2). Based on the breakpoints, the susceptibility (binary outcome) of each pathogen to each antimicrobial was determined (Table 2). Based on observed variation among pathogen groups in susceptibility to ampicillin, penicillin, pirlimycin, sulfadimethoxine, and tetracycline, susceptibility of selected antimicrobials was modeled using PROC GLIMMIX to test that hypothesis that susceptibility varied between pathogen groups. Enterococci were not included in the ampicillin and penicillin susceptibility models as all 11 isolates demonstrated susceptibility (Table 2). Susceptibility was not modeled for ceftiofur, cephalothin, erythromycin, oxacillin, and

penicillin and novobiocin because >80% of all the isolates across all pathogen groups were susceptible. For susceptibility models, univariate relationships among selected potentially confounding variables and susceptibility were assessed and variables that met inclusion criteria (P <0.20) were offered to multivariate models. Variables considered included season (warm or cool), days in milk (DIM) category (0-100 DIM, 101-200 DIM, >201 DIM), parity (primiparous or multiparous), clinical mastitis history (defined as occurrence of CM in any quarter of the cow during the 55 d before the case was enrolled) and subclinical mastitis history (defined as previous monthly DHIA SCC before the case > 150,000 cells/mL).

To evaluate differences in MIC among pathogen groups, survival analysis was performed using PROC LIFETEST in SAS (v. 9.4). The antimicrobial concentration was used as "time" while the event was defined as inhibition of bacterial growth (Table 3). Isolates that grew at the greatest concentration of antimicrobial included in the commercial panel were censored. Kaplan-Meier survival curves were produced for each of the antimicrobials and pathogen groups. The Log-Rank and Wilcoxon tests were used to evaluate differences in survival curves among pathogen groups with the null hypothesis that there were no differences in survival of the strata (pathogen groups). The Log-Rank test is more sensitive to equality of strata in survival at the higher antimicrobial concentrations while the Wilcoxon test is more sensitive to equality of strata at lower antimicrobial concentrations.

3.4 Results and Discussion

Descriptive characteristics of cows enrolled in this study have been previously reported (Chapter 2). The pathogens included a diverse group of Gram-positive cocci. *Lactococcus spp.* were most prevalent, with half of the cases caused by *Lactococcus spp.* or *Enterococcus spp.* (Table 1). Traditionally, Gram-positive, catalase negative cocci were diagnosed as *Streptococcus spp*.

(Smith et al., 1985). However, use of MALDI-TOF (Scillieri Smith et al., 2020) and other improved laboratory methods (Fortin et al., 2003) have led to more precise diagnoses of mastitis pathogens and that has been proposed as one reason that more *Lactococci spp*. are being identified from milk samples obtained from cows with mastitis (Werner et al., 2014). No available IMM antimicrobials that are currently marketed in the U.S. have labeled efficacy for *Lactococci* or *Enterococci* thus more data about potential differences in *in vitro* susceptibility of these pathogens is useful. The 108 isolates that met inclusion criteria for this study were tested against 10 class representative antibiotics that are included in a commercially available MIC panel used for mastitis. Of these antibiotics, 4 are currently marketed in the US as IMM treatments for clinical mastitis (ampicillin, ceftiofur, cephalothin, penicillin), 1 is available as an IMM dry cow product (penicillin-novobiocin), 2 are approved for IMM treatment but no longer marketed in the US (pirlimycin and erythromycin), 1 (tetracycline) is labeled for systemic administration in dairy cows but not labeled for treatment of mastitis and 1 (sulfadimethoxine) is labeled for treatment of only pneumonia and footrot in dairy cows (no extra label usage of this product is allowed). Oxacillin is included in the panel as a potential marker for methicillin resistance (Table 2).

Susceptibility to antimicrobials included in the commercial panel ranged from 6.8% (susceptibility of *Lactococcus* to penicillin) to 100% (most pathogen groups for both cephalosporins and the combination of penicillin and novobiocin; Table 2). All isolates demonstrated *in vitro* susceptibility to oxacillin and the majority were susceptible to erythromycin (Table 2). Susceptibility of NAS to commercially available IMM products ranged from 79% (penicillin) to >90% (ceftiofur, cephapirin, combination of penicillin and novobiocin) but were 57% for sulfadimethoxine and 72% for tetracycline (Table 2). Susceptibility of *Strep*. to

IMM products were 44%, 80%, and 100% for penicillin, ampicillin, and ceftiofur, cephapirin, and the combination of penicillin and novobiocin, respectively (Table 2). However, the minority of *Streptococci* were susceptible to sulfadimethoxine or tetracycline (Table 2). Only 7% of *Lactococci* demonstrated *in vitro* susceptibility to penicillin, while the majority were susceptible to ampicillin and all were susceptible to ceftiofur, cephapirin and the combination of penicillin and novobiocin (Table 2). All *Enterococci* were susceptible to all commercially available IMM antibiotics, but few were susceptible to tetracycline or sulfadimethoxine (Table 2).

Susceptibility to ampicillin was not associated with pathogen (Lact, NAS or Strep) and no potentially confounding variables met inclusion criteria for that model, ($P = 0.165$). Susceptibility to penicillin varied among pathogen groups ($P < 0.001$) and was influenced by parity ($P = 0.04$). As compared to NAS, the odds of susceptibility to penicillin were less for Lact. (OR = 0.014, 95% CI [0.002, 0.094]) and Strep (OR = 0.067, 95% CI [0.011, 0.39]). For penicillin, isolates obtained from cases that occurred in primiparous animals were less likely to be susceptible than multiparous animals (OR = 0.25, 95% CI [0.06, 0.96]). Susceptibility to tetracycline varied among pathogens ($P = 0.02$) and was influenced by occurrence of subclinical mastitis prior to clinical case ($P = 0.04$). When SCC >150,000 cells/mL preceding the CM case, the odds of susceptibility to tetracycline significantly decreased (OR = 0.30, 95% CI [0.10, 0.92]). Susceptibility to sulfadimethoxine was not associated with pathogen group ($P = 0.12$) and no potential confounder met inclusion criteria for the model. Susceptibility to pirlimycin varied among pathogen groups ($P = 0.01$). As compared to NAS, Lact. were less likely to be susceptible to pirlimycin (OR = 0.11, 95% CI [0.01, 0.89]). Overall, the Lactococci group had the lowest rate of susceptibility to pirlimycin with 40.9% not inhibited at the greatest tested concentration (Table 2). Previous research in Wisconsin grouped *Lactococci spp.* with the Strep-like-organisms

and found that 71% of the isolates were susceptible to pirlimycin (Ruegg et al., 2015). Additional work from Canada found similar rates (59.4%) of resistance to pirlimycin among Lactococci isolates from mastitis pathogens (Cameron et al., 2016). It has been previously proposed that *Lactococcus garvieae* are intrinsically resistant to clindamycin, and pirlimycin is a derivative of clindamycin (Elliott and Facklam, 1996). In our study, 88% (n = 16) of the *Lactococcus garvieae* isolates were resistant to pirlimycin while only 11.5% (n = 3) of the *Lactococcus lactis* isolates were resistant. Future research should evaluate AMR genes among the Lactococci species identified to look for possible interspecies transfer between *Lactococcus garvieae* and *Lactococcus lactis*.

For cephalothin, oxacillin, penicillin novobiocin, and pirlimycin the MIC_{50} was the same for all four pathogen groups (Table 2). For penicillin, each pathogen group had a different MIC_{50}. For ampicillin, the MIC_{50} was the least tested concentration (0.12 μg/mL) for all of the pathogen groups except Lact (0.25 μg/mL, Table 2). For ceftiofur, the MIC_{50} was the least tested concentration (0.5 μg/mL) for all pathogen groups except for NAS (1 μg/mL, Table 2). The MIC_{90} could not be calculated for tetracycline or sulfadimethoxine (for all pathogens), pirlimycin (Strep. or Lact.), or erythromycin (Strep.) because greater than 10% of these isolates were not inhibited at the greatest concentration included in the MIC panel (Table 2).

Associations between pathogen group and MIC were evaluated for all 10 antimicrobials using survival curves. While all isolates were inhibited below the susceptibility breakpoint for cephalothin, differences in MIC were observed among pathogens (Log-Rank and Wilcoxon, $P < 0.001$). The survival analysis for ceftiofur found variations in MIC for the different pathogen groups, (Log-Rank and Wilcoxon, $P \leq 0.001$). Tetracycline, penicillin, and pirlimycin had significant variations in MIC (Log-Rank and Wilcoxon, $P \leq 0.009$). Of antimicrobials tested,

only oxacillin, the combination of penicillin and novobiocin, and sulfadimethoxine had no variation in MIC when assessed using Log-Rank ($P > 0.119$) and Wilcoxon ($P > 0.13$, Table 2) tests.

No differences among pathogen groups in the equality of strata at the higher antimicrobial concentrations were observed for ampicillin (Figure 1A; Log-Rank, $P = 0.32$) but there was a difference at the lower concentrations based on the Wilcoxon test ($P = 0.03$). As compared to the other pathogen groups (Fig 1a), Lactococci were not inhibited until greater antimicrobial concentrations. Of five IMM products used for treatment of CM in the US, one of the product's active antimicrobials is an aminopenicillin (Hetacillin; PolyMast®, Boehringer Ingelheim). In the mammary gland, hetacillin potassium is converted back into ampicillin and acetone (Lindquist et al., 2015). For NAS, results were remarkably similar to previous results for ampicillin (Pol and Ruegg, 2007a), and in both their study and ours found >80% of NAS were susceptible at the lowest tested concentration (0.12 μg/mL). Overall, except for Lactococci most Gram-positive pathogens were inhibited at the least tested concentration of ampicillin (Table 2).

Inhibition of bacterial growth by pirlimycin varied among pathogen groups (Figure 1B). The MIC varied among pathogen groups (Log-Rank, $P = 0.002$; Wilcoxon, $P \leq 0.001$) with *Lactococci* having the greatest percentage of isolates not inhibited at the greatest tested concentration (Fig 1B). However, the MIC_{50} was the least tested concentration for all isolates (Fig 1B). As compared to previous research in Canada (> 2 μg/ml, Cameron et al, 2016), the MIC_{50} for Lactococci enrolled in our study was less, In contrast, the MIC_{50} for *Streptococci* isolated from Canadian mastitis cases (Cameron et al., 2016) was remarkably similar to our results.

There was no difference among pathogens in MIC for sulfadimethoxine (Figure 1C);

however, many isolates were not inhibited at the greatest tested concentration (Table 2). Similar to sulfadimethoxine, a large percentage of isolates were not inhibited at the greatest tested concentration of tetracycline (Table 2). Both antimicrobials are approved in the U.S. to treat respiratory disease in dairy cattle and can be used to treat other bacterial diseases systemically but are not available as IMM formulations. These two antimicrobials are also used widely in food animal species. Tetracyclines comprise the largest percentage (66%) of the total mass of medically important antimicrobials sold annually in the U.S. (FDA, 2020). Sulfonamides are also a large percentage (5%) of the total medically important antimicrobials sold in the U.S. Both tetracyclines and sulfonamides are primarily used in animal feed for other livestock production and cannot be fed to dairy cows. It is also important to note that the 1 sulfonamide that is approved for use in dairy cows is labeled only for treatment of pneumonia and foot rot and cannot be used to treat mastitis in dairy cows. While the majority of CM cases in the United States are treated with IMM Ceftiofur (USDA–APHIS–VS–CEAH–NAHMS, 2014), IMM administration accounts for <1% of the total mass of all medically important antimicrobials sold in 2020 (FDA, 2020). Previous research has reported high rates of resistance to sulfadimethoxine and tetracycline in clinical mastitis pathogens (Pol and Ruegg, 2007a; Ruegg et al., 2015; Cameron et al., 2016),. Research on trends in antimicrobial susceptibility from 1994-2000 in Michigan found that 54.8% of *Strep uberis* and 39.8% of *Strep dysgalactiae* isolates were susceptible to tetracycline (Erskine et al., 2002b). The CM isolates in this study collected two decades later confirm that tetracycline resistance remains common in streptococci from CM isolates.

There were several encouraging results from this study. Oxacillin is included in the commercial panel as an initial screen for possible resistance to methicillin which is then

confirmed by identification of *mecA* or *mecC* genes. The *mecA* or *mecC* genes are frequently associated with AMR and have been found in the NAS group (Frey et al., 2013). Notably, all isolates in our study exhibited phenotypic susceptibility to oxacillin (Table 2). Similarly, all but one of the isolates were susceptible to the combination of penicillin and novobiocin (Table 2). Based on defined breakpoints, antimicrobial susceptibility results can be classified as susceptible, intermediate, or resistant, where intermediate implies that clinically efficacy can be achieved if the antimicrobial reaches therapeutic concentration (CLSI, 2020). Except for 2 NAS isolates that were classified as intermediate, all of our isolates were considered as susceptible to ceftiofur (Table 2) which is similar to previous studies (Ruegg et al., 2015; Cameron et al., 2016). Ceftiofur is the only third generation cephalosporin licensed for IMM administration which is a CIA and it is also the most commonly used IMM product (US Food and Drug Administration, 2012; USDA–APHIS–VS–CEAH–NAHMS, 2014). Intramammary ceftiofur is available in the U.S as both a dry cow and lactating cow formula as well as in 3 systemically administered formulations. Our study is consistent with previous research (Ruegg et al., 2015; Cameron et al., 2016) that reported high rates of *in vitro* susceptibility of Gram-positive mastitis pathogens to ceftiofur, with identical rates of susceptibility observed from 1994-2000 in Streptococci (Erskine et al., 2002b).

For both the cephalosporins (ceftiofur and cephalothin) the overall rates of resistance were low, though the MIC distribution differed among Gram-positive pathogens (Table 2, curves not shown). The MIC distribution patterns for the MIC_{50} and MIC_{90} are similar to previous literature for most of the Gram-positive pathogens, with the majority of the isolates being inhibited at the lowest tested concentration (Pol and Ruegg, 2007b; Cameron et al., 2016). For cephalothin, the *Lactococci* have a greater MIC_{90} than other Gram-positive pathogens, which is

consistent with previous literature (Pol and Ruegg, 2007b; Ruegg et al., 2015; Cameron et al., 2016). The CLSI guidelines have interpretative criteria for CM isolates for only three antimicrobials (ceftiofur, penicillin novobiocin, and pirlimycin) and only for a few pathogens. For the antimicrobial combinations and pathogens not listed, CLSI recommends using human MIC breakpoints. While comparing the non-specific breakpoints to groups is the best available approach, it limits our ability to understand the role these different distributions of MIC may have biologically, especially for cephalothin where we are limited to a breakpoint from humans.

Interestingly, our results demonstrated variation (Log-Rank, $P = 0.0012$) in MIC of penicillin among the Gram-positive pathogens with 80% of NAS being inhibited at the least tested concentration in contrast to only 7% of Lactococci (Table 2, curves not shown). The IMM formulation for penicillin is not commonly used to treat mastitis in the United States (only 0.8% of operations reported using it; USDA, 2014). Previous research using laboratory submissions in Michigan conducted from 1994-2000 reported that 94.5% of *Streptococcus dysgalactiae* and *Streptococcus uberis* were susceptible to penicillin (Erskine et al., 2002b). In our study, only 44% of Streptococci and 6% of Lactococci were susceptible to penicillin. Our results were more similar to recent Canadian research that reported 22% of *Strep. uberis* and 0% of *Lactococcus spp*. were susceptible to penicillin (Cameron et al., 2016). In 2010, Swiss researchers demonstrated a shift towards *in vitro* resistance to penicillin in *Strep uberis* (Haenni et al., 2010). Haenni et al (2010) highlighted that *Strep uberis* has a reservoir of penicillin binding protein mutations and that these genes could possibly be transmitted to other species. Future research to explore possible AMR gene transmission from *Strep uberis* would be interesting. It is important to note that there are no breakpoints for resistance of mastitis pathogens to penicillin, so these results are limited to the available human breakpoints.

Overall, our results indicate that there are some differences in the MIC among Gram-positive mastitis pathogens. It has been well documented that *in vitro* susceptibility testing is not strongly predictive of clinical outcomes, which is likely a reflection of the complex interaction of bacterial pathogenesis, bovine immune response, and pharmacology of available mastitis treatments (Constable and Morin, 2002; Hoe and Ruegg, 2005; Apparao et al., 2009). On modern larger dairy farms, many are culturing CM cases using on-farm culture programs which differentiate bacterial growth simply into "Gram-positive", "Gram-negative" or "Contaminated." When making treatment decisions for CM, among approved IMM products the greatest proportion of isolates were susceptible to first and third generation cephalosporins and we found no differences among those antimicrobials. Future research should look for possible AMR genes in *Lactococci* species, which have different MIC and susceptibility for many of the antimicrobials tested. Ultimately, while differences in *in vitro* susceptibility were observed for some antimicrobials, overall susceptibility to the most commonly used cephalosporin based IMM treatments did not vary among pathogens.

Notes:
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3.5 Tables and Figures

Table 3.1. Identification of organisms using MALDI-TOF on duplicate milk samples from non-severe cases of clinical mastitis that grew on Gram-positive selective agars occurring in cows on 3 Michigan dairy farms from June 2019- March 2020

Etiology	Number	Percent (%)
Non-Aureus Staph	28	25.9
Staphylococcus chromogenes	18	16.7
Staphylococcus simulans	5	4.6
Staphylococcus haemolyticus	2	1.8
Staphylococcus species	2	1.8
Staphylococcus epidermidis	1	0.9
Streptococci	25	23.1
Streptococcus dysgalactiae	16	14.8
Streptococcus uberis	5	4.6
Streptococcus species	4	3.7
Lactococci	44	40.7
Lactococcus lactis	26	24.1
Lactococcus garvieae	18	16.7
Enterococci	11	10.2
Enterococcus saccarolyticus	9	8.3
Enterococcus faecalis	1	0.9
Enterococcus aquimarinus	1	0.9
Total	108	100%

Table 3.2. Distribution of Minimum Inhibitory Concentrations (MIC) from clinical mastitis collected from Michigan dairy farms between June 2019- March 2020

Drug	Etiology	B.P[1]	Number	Sus.[2]	Percent of Isolates at each indicated MIC (μg/ml)								N.I[3].
				%	0.12	0.25	0.5	1	2	4	8	16	%
Ampicillin†	NAS	≤ 0.12[4]	28	82.1	**82.1**	0.0	3.6	7.1	3.6	0.0	0	-	0.0
	Strepto.	≤0.25[4]	25	80.0	**64.0**	16.0	16.0	0.0	4.0	0.0	0.0	-	0.0
	Lacto.	≤0.25	44	63.7	45.5	**18.2**	34.1	2.3	0.0	0.0	0.0	-	0.0
	Entero.	≤8.00[4]	11	100.0	**81.8**	9.1	9.1	0.0	0.0	0.0	0.0	-	0.0
Ceftiofur*†	NAS	≤2.00	28	92.9	-	-	25.0	**39.3**	26.6	7.1	0.0	-	0.0
	Strepto.	≤2.00	25	100.0	-	-	**76.0**	24.0	0.0	0.0	0.0	-	0.0
	Lacto.	≤2.00	44	100.0	-	-	**93.2**	4.6	2.3	0.0	0.0	-	0.0
	Entero.	≤2.00[5]	11	100.0	-	-	**63.6**	27.3	9.1	0.0	0.0	-	0.0
Cephalothin*†	NAS	≤8.00[4]	28	100.0	-	-	-	-	**100**	0.0	0.0	0.0	0.0
	Strepto.	≤8.00[4]	25	100.0	-	-	-	-	**84.0**	8.0	8.0	0.0	0.0
	Lacto.	≤8.00	44	100.0	-	-	-	-	**61.4**	11.4	27.3	0.0	0.0
	Entero.	≤8.00[5]	11	100.0	-	-	-	-	**81.8**	18.2	0.0	0.0	0.0
Erythromycin*†	NAS	≤0.50[4]	28	89.2	-	32.1	**57.1**	3.6	0.0	0.0	-	-	7.1
	Strepto.	≤0.25[4]	25	80.0	-	**80.0**	0	4.0	4.0	0.0	-	-	12.0
	Lacto.	≤0.25	44	97.7	-	**97.7**	2.3	0.0	0.0	0.0	-	-	0.0
	Entero.	≤0.50[4]	11	100.0	-	**100**	0.0	0.0	0.0	0.0	-	-	
Oxacillin	NAS	≤2.00[4]	28	100.0	-	-	-	-	**100**	0.0	-	-	0.0
	Strepto.	≤2.00[4]	25	100.0	-	-	-	-	**100**	0.0	-	-	0.0
	Lacto.	≤2.00	44	100.0	-	-	-	-	**100**	0.0	-	-	0.0
	Entero.	≤2.00[5]	11		-	-	-	-	**100**	0.0	-	-	0.0
Penicillin*†	NAS	≤0.12[4]	28	78.6	**78.6**	7.1	0.0	7.1	0.00	-	-	-	7.1
	Strepto.	≤0.12[4]	25	44.0	44.0	**36.0**	16.0	4.0	0.00	-	-	-	0.0
	Lacto.	≤0.12	44	6.8	6.8	34.1	**31.8**	25.0	2.3	-	-	-	0.0
	Entero.	≤8.00[4]	11	100.0	**54.6**	9.1	27.3	9.1	0.00	-	-	-	0.0
Penicillin novobiocin[7]	NAS	≤1/2[6]	28	96.4	-	-	-	-	**96.4**	0.0	0.0	0.0	3.6
	Strepto.	≤1/2	25	100.0	-	-	-	-	**100**	0.0	0.0	0.0	0.0
	Lacto.	≤1/2	44	100.0	-	-	-	-	**100**	0.0	0.0	0.0	0.0

Table 3.2 (cont'd)

	Entero.	≤1/2[5]	11	100.0	-	-	-	-	**100**	0.0	0.0	0.0	0.0
Pirlimycin*†	NAS	≤2.00[6]	28	92.9	-	-	**85.7**	3.6	3.6	3.6	-	-	3.6
	Strepto.	≤2.00	25	84.0	-	-	**76.0**	4.0	4.0	0.0	-	-	16.0
	Lacto.	≤2.00	44	59.1	-	-	**52.3**	0.0	6.8	2.3	-	-	38.6
	Entero.	≤2.00[5]	11	90.6	-	-	**63.3**	18.2	9.1	9.1	-	-	0.0
Tetracycline*†	NAS	≤4.00[4]	28	71.5	-	-	-	**64.3**	3.6	3.6	3.6	-	25.0
	Strepto.	≤2.00[4]	25	8.0	-	-	-	8.0	0.0	16.0	0.0	-	76.0
	Lacto.	≤2.00	44	34.2	-	-	-	29.6	4.6	2.3	4.6	-	59.1
	Entero	≤4.00[4]	11	45.0	-	-	-	45.0	0.0	0.0	0.0	-	55.0
Sulfadimethoxine[8]	NAS	≤128[9]	28	57.2	-	-	-	-	39.3	3.6	**14.3**	0.0	39.3
	Strep	≤128[9]	25	24.0	-	-	-	-	12.0	12.0	0.0	0.0	76.0
	Lacto.	≤128[9]	44	38.6	-	-	-	-	31.8	2.3	4.6	2.3	59.1
	Entero	≤128[9]	11	36.4	-	-	-	-	36.6	0.0	0.0	0.0	63.6

[1]B.P indicates the breakpoint at which an isolate is considered susceptible according to CLSI Vet01S-Ed5. As there is no B.P for *Lactococcus spp.* in CLSI Vet01S-Ed5, the breakpoint for Streptococci was used.

[2]Percent of susceptible isolates based on the breakpoints in CLSI Vet01S-Ed5.

[3]Isolates that were not inhibited at the highest concentration of the antimicrobial tested.

[4]No breakpoint for bovine mastitis organism exists in CLSI, interpretative criteria are from humans.

[5]No breakpoint for mastitis pathogens for enterococci, the B.P is from Streptococci was used.

[6]No B.P available for mastitis pathogens for NAS, the B.P from *Staph aureus* for mastitis was used.

[7]Tested concentrations of Penicillin Novobiocin were 1/2, 2/4, 4/8, 8/16 they are represented in the table by the concentration in ug/ml of Novobiocin.

[8]Tested concentrations of Sulfadimethoxine were 32, 64, 128, and 256 which are represented in the table at the 2, 4, 8, and 16 ug/ml respectively.

[9]The current CLSI guidelines do not have recommendations for sulfadimethoxine for any human or bovine mastitis pathogen to be consistent with previous literature susceptibility was defined as ≤128 µg/mL.

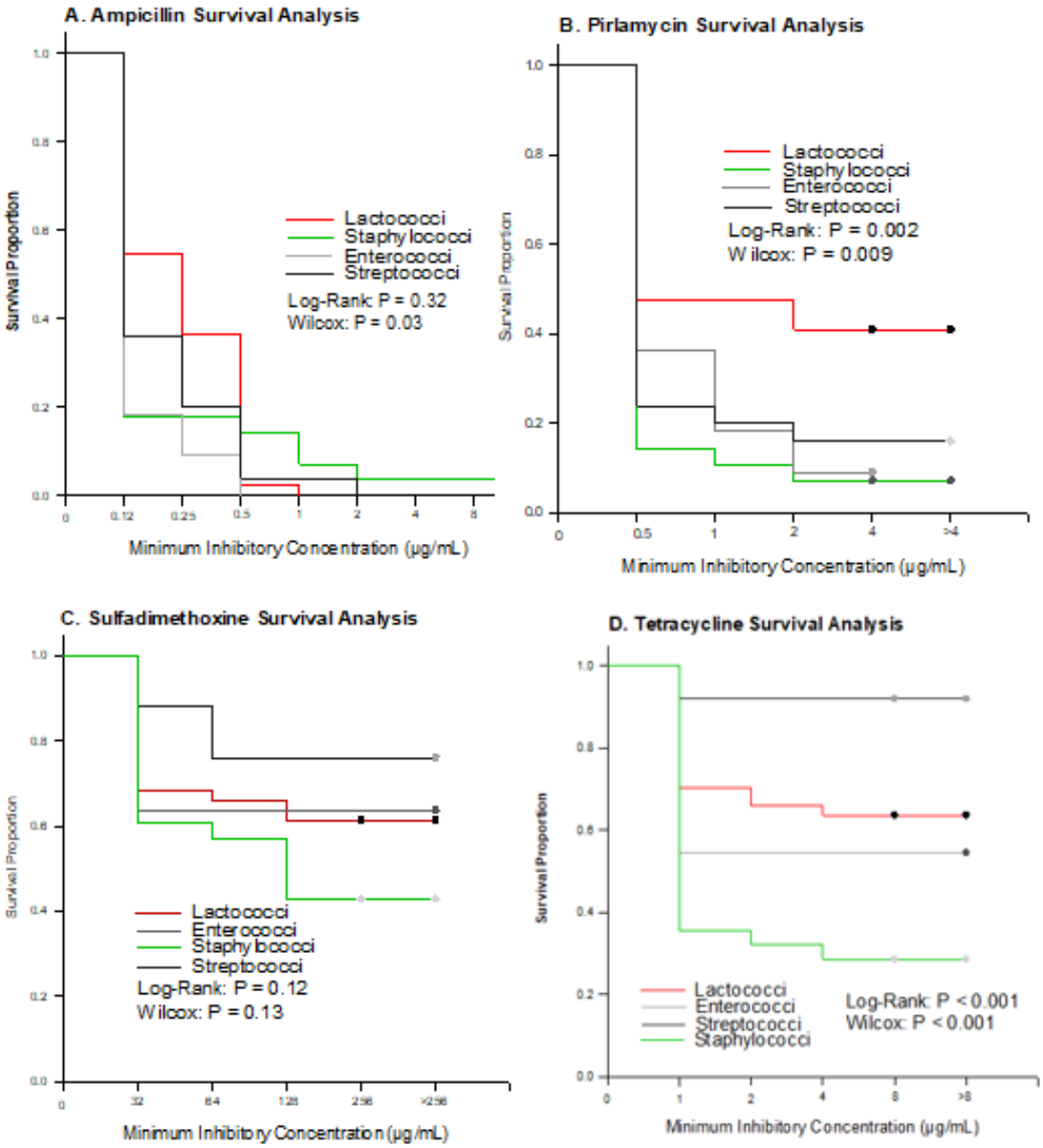
Bold indicates MIC_{50} while underlined MIC_{90}.

- Indicates values not assessed for the indicated antimicrobial.

*Log-Rank test for equality of strata (pathogen) at higher minimum inhibitory concentration was significant $P < 0.002$.

†Wilcoxon test for equality of strata (pathogen) at lower minimum inhibitory concentration was significant $P < 0.009$.

Figure 3.1. Survival analysis for minimum inhibitory concentrations from isolates[1] (n=108) identified as causing clinical mastitis between June 2019-March 2020 by pathogen group for ampicillin, pirlimycin, sulfadimethoxine and tetracycline



[1]Kaplan-Meier plots showing survival proportion of 108 isolates stratified by Lactococci (n = 44), Enterococci (n = 11), Staphylococci (n = 28) and Streptococci (n = 25). Log-Rank test for equality of strata was used at high MIC while the Wilcoxon test for equality of strata was used at the lower MIC.

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CHAPTER 4: IMPACT OF DIETARY SUPPLEMENTATION WITH *SACCHAROMYCES CEREVISAE* FERMENTATION PRODUCT ON CLINICAL AND IMMUNOLOGICAL OUTCOMES IN DAIRY COWS DURING CHALLENGE WITH *STREPTOCOCCUS UBERIS*

4.1 Abstract

Streptococcus uberis is a common Gram-positive mastitis pathogen for which efforts to make vaccines have been largely unsuccessful and current treatment protocols rely on antibiotics. *Saccharomyces cerevisiae* fermentation products (SCFP) have been shown to positively impact cattle during immunologically stressful times and decrease linear somatic cell count. The objective of this randomized study was to evaluate the effect of oral supplementation with SCFP on clinical, immunological, and production outcomes in response to an experimental IMM challenge with *Streptococcus uberis* 0140J. Healthy cows (n=42; parities 1-5; >120 DIM) were enrolled if their pre-trial test day SCC was <200,000 cells/mL and they had no history of clinical mastitis in the preceding 60 d. Cows were blocked based on BLV status, parity, milk yield, and days in milk then randomized to treatment (SCFP, control diet) within each of 4 weekly enrollment periods (cohorts). Cows were fed 19 g/d SCFP on the total mixed ration for 45 d. During that period, quarter milk samples were collected weekly to evaluate SCC and presence of IMM infection. Cows that did not develop subclinical mastitis during the pre-sampling period (n = 37) were challenged with approximately 2,000 cfu of *S. uberis* 0140J in one rear quarter. Quarter milk samples were collected daily (0 – 7 d) during the challenge week. Additional milk samples were collected on day 0 and 3 after IMM challenge and used for flow cytometric analysis. Statistical analysis was performed with SAS v. 9.4 and linear mixed models were created for each outcome of interest. Supplementation with SCFP did not have an overall

effect on dry matter intake, milk production, or milk components during the pre-challenge, challenge, or post-challenge periods ($P > 0.19$). There were no significant effects of SCFP on the number of *Strep uberis* O140J (cfu/mL) or SCC (log cells/mL) in milk samples. The mean time until onset of clinical mastitis was 3.88 ± 0.42 d for cows in the control group versus 4.84 ± 0.44 days for cows that received SCFP ($P = 0.33$). The mean days to bacteriological cure was 25.9 ± 3.5 for cows in the control group and 22.5 ± 3.1 for cows that received SCFP ($P = 0.50$). Pre-challenge, SCFP tended to impact the myeloid cell population ($P = 0.07$) by increasing the milk CD14-positive cell population in multiparous cows ($P = 0.03$). For all lymphoid markers evaluated in this study, supplementation with SCFP did not impact the mammary immune cell populations. This study suggests that SCFP may impact myeloid cell populations in milk, but we did not observe an impact of SCFP on clinical outcomes measured in this study.

4.2 Introduction

Mastitis is caused by bacteria that overcome innate immune defenses to establish an IMI thus triggering an adaptive immune response, which coincides with the clinical presentations of disease (Sordillo, 2018). Mastitis is a ubiquitous and costly disease with about 25% of cows experiencing a clinical mastitis case each year (USDA–APHIS–VS–CEAH–NAHMS, 2014). Treatment and prevention of mastitis accounts for the majority of antibiotics used on dairy farms (USDA–APHIS–VS–CEAH–NAHMS, 2014; Leite de Campos et al., 2021). Globally, there are growing concerns about AMR and considerable interest in alternatives to antibiotics for the management of mastitis. *Streptococcus uberis* is among the most common causes of clinical mastitis (CM) (Petrovski et al., 2009; Vasquez et al., 2016; Cheng et al., 2019). *Strep uberis* is primarily an environmental pathogen and can be found in soil and bedding (Zadoks et al., 2005), however transmission from cow-to-cow is possible (Phuektes et al., 2001; Zadoks et al., 2001).

As *Strep uberis* has large strain variations, efforts to make effective vaccines have had limited success (Leigh et al., 1999; Jones et al., 2004) and as a result, *Strep uberis* has even been proposed as a permanent barrier to mastitis prevention (Leigh, 1999).

One mechanism to reduce the need for antibiotics to control *Strep uberis* would be to stimulate the cow's immune system through dietary supplementation. Fermentation products derived from *Saccharomyces cerevisiae* (SCFP) are commercially available. These feed supplements are produced by fermentation of yeast with other compounds such as vitamins, nucleotides, and oligosaccharides (Hristov et al., 2010). Researchers have reported improved milk yields and dry matter intake from SCFP supplementation (Hristov et al., 2010; Poppy et al., 2012; Olagaray et al., 2019). More recently, research has focused on health effects of SCFP supplementation during immunologically stressful periods. Beneficial health effects have been reported during calf respiratory challenge (Mahmoud et al., 2020), the transition period of dairy cows (Knoblock et al., 2019), and in feed restricted cows (Coleman et al., 2023). Previous research on SCFP supplementation and mastitis found that supplementation reduced linear somatic cell scores (Ferguson et al., 2018). A recent subclinical mastitis challenge study found that SCFP supplementation reduced rectal temperature and activated cellular mechanisms associated with cytoprotection (Vailati-Riboni et al., 2021). Further research is needed to evaluate the potential impact of SCFP supplementation, and to define potential biological mechanisms.

The objective of this study was to evaluate the effect of feeding dairy cows SCFP on clinical and production outcomes after an experimental IMM challenge with *Streptococcus uberis*. A secondary objective was to characterize the mammary immune cell populations after supplementation with SCFP and during IMM challenge. We hypothesized that dietary

supplementation with SCFP would improve clinical outcomes and productivity after challenge with *Strep. uberis*.

4.3 Materials and Methods

Study Design and Enrollment Criteria

This study was designed as a randomized complete block to evaluate the impact of feeding SCFP (Nutritek®, Diamond V) on production and immune outcomes of dairy cows after IMM challenge with *Strep uberis*. Cows at the Dairy Cattle Teaching and Research Center at Michigan State University were eligible if they were healthy, had four functional teats and were >120 DIM at the beginning of the trial. Cows that were diagnosed with clinical or subclinical mastitis (2 previous DHI tests $\geq$200,000 cells/mL) or any other major health event during the previous 60 days were not eligible. Cows that were seropositive for bovine leukemia virus (BLV) were enrolled only if a blood count demonstrated < 10,000 lymphocytes/mL at enrollment. To manage data collection,10-12 cows were enrolled sequentially in 4 weekly cohorts from May until June. Cows (n = 42) were blocked based on BLV status, parity, milk yield, and days in milk and randomly assigned to a treatment within block. All cows were fed a basal diet and cows assigned to the treatment group received 19 g/cow per day SCFP top-dressed on the basal diet while cows assigned to the control group received 19 g/cow per day of ground corn. Cows were housed in tie stalls that contained mattresses bedded with sawdust. Cows were milked three times per day and animal health records were recorded in computerized records (Dairy Comp 305, Valley Agricultural Software). All researchers involved in mastitis challenge and sample collection were blinded to treatment during the study and analysis periods.

The study was designed with 4 phases (Figure 1) where phase 1 was acclimatization to basal diet and screening. The pre-challenge period, (phase 2), was a 45-d feeding trial, where

quarter milk samples were collected once weekly and evaluated for SCC and for IMI. During the challenge period, (phase 3), 1 randomly assigned rear quarter of all cows was infused with approximately 2,000 cfu of *Streptococcus uberis* 0140J and monitored for 7 d (cohorts 1 & 2) or 5 days (cohorts 3 & 4). During the post challenge period (phase 4), the challenged quarters were treated for 8 days with 1 tube (125 mg) of IMM Ceftiofur hydrochloride per day (labeled treatment using SpectramastLC, Zoetis) and quarter milk samples were collected weekly for SCC and bacteriology. Additional IMM antibiotic treatments with the same products were administered to some cows after this period, when necessary to achieve bacteriological clearance. All milk samples used for culture were collected aseptically following National Mastitis Council (NMC) guidelines (NMC, 2017). This study was approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University (PROTO 2021-00019). In consultation with IACUC, after the first two cohorts were completed, the non-treated period of cows was shortened to 5 (rather than 7 d).

Data Collection

During phase 1 (acclimatization), quarter milk samples were aseptically collected from all four quarters and cultured to identify IMI to identify subclinical mastitis infections (based on IMI and increased quarter SCC). During phase 2), quarter milk samples were aseptically collected for culture and SCC screening once weekly. When cows developed IMI during phase 2, they were removed from the trial. During the week immediately preceding IMM challenge, quarter milk samples were collected on d -3 and d 0 immediately prior to IMM infusion of *Strep uberis* 0140J. After the morning milking was completed on day 0, cows (n = 37) were challenged with approximately 2,000 cfu of *Strep. uberis* 0140J suspended in 2 mL of PBS (1,000 cfu/mL) in the pre-assigned rear quarter. During phase 3, quarter milk samples were aseptically collected

each day from the challenged quarter and evaluated for SCC and IMI. Foremilk and mammary gland swelling was observed one time per day. During phase 3, rectal temperatures were measured once per day in the morning, while ruminal and vaginal temperatures were measured continuously. After 7 days of challenge quarter milk samples were aseptically collected for culture and SCC screening once weekly. During all phases cows were body condition scored and weighed once weekly by trained investigators and dry matter intake and milk yield were collected daily

Definitions

Clinical mastitis cases were scored based on severity. Mild cases of CM were defined as solely abnormal milk, moderate cases had evidence of mammary gland swelling or inflammation while severe cases had evidence of systemic signs of illness such as fever, depression, or anorexia (Pinzón-Sánchez and Ruegg, 2011). Fever was defined as a rectal temperature of $\geq$39.7 ^{o}C. Cows with signs of systemic infection including fever and anorexia or others were examined by the herd veterinarian.

Intramammary Bacterial Challenge

All IMM infusions with *Strep uberis* 0140J were performed by 1 researcher (PLR). After milking was completed, teat ends were thoroughly cleaned using gauze soaked in 70% ethanol and administered in a 2 mL suspension into a rear quarter using a teat cannula (Jorgensen Laboratories Inc., Loveland CO). After infusion, teats were dipped (Bovadine®, DeLaval). The methods to prepare the inoculum has been described previously (Swartz et al., 2021). In brief colonies of *Strep uberis* 0140J were grown overnight at 37^{o}C from stock culture (Swartz et al., 2021). From the incubated plates, two colonies were transferred to 25 mL of Todd-Hewitt broth and placed in a shaking incubator 37^{o}C at 200 rpm for 8 hours to reach stationary growth. The

incubated broth was centrifuged at 2,500 *x g* for 20 minutes at 4°C before the pellet was resuspended in sterile PBS to achieve approximately 1 x 10^4 cfu/ml. The concentration of the challenge inoculum was determined by drop-plating 10 µL of 1: 10 dilutions in quadruplicate on 5% Sheep Blood Agar (ThermoFisher Scientific, Waltham, MA). The quadruplicate serial dilutions were counted after 24 hours of incubation at 37°C to confirm the challenge inoculum concentration.

Bacterial Enumeration and SCC

Microbiological culturing of quarter-milk samples was performed following National Mastitis Council guidelines (NMC, 2017). In brief, 10 µL of milk was inoculated on 5% Sheep Blood Agar (ThermoFisher Scientific, Waltham, MA) and incubated for 24 h at 37.0 °C. During phase 2, samples with significant growth (≥ 3 colonies) were isolated and sent to the Michigan State Veterinary Diagnostic Laboratory for confirmation using MALDI-TOF. Quarter milk samples with significant bacterial growth and SCC ≥100,000 cells/mL were removed from the trial (n = 5) during phases 1 and 2. During phase 3, bacterial infection was identified as Gram-positive, catalase negative before being speciated as *Strep uberis* 0140J using the API 20 Strep kit (Biomeriex, Durham, NC). Bacterial enumeration was performed on milk collected from the challenged quarter daily during phase 3 and eight serial 10-fold dilutions were made in sterile Falcon tubes (Thermo Fisher Scientific, Waltham, MA). For each dilution four 10 µL replicates were inoculated onto the surface of 5% sheep blood agar (ThermoFisher Scientific, Waltham, MA). The serially diluted plates were incubated for 24 h at 37.0 °C and counted at the appropriate dilution. Bacterial counts are expressed at colony forming units (cfu) per milliliter of milk.

To determine the SCC of quarter milk, foremilk samples were collected and bronopol

was added. Samples were refrigerated and sent to the local commercial DHI laboratory (CentralStar, Grand Ledge, MI). Quarter SCC was log-transformed for analysis.

Vaginal temperature was monitored hourly using a data logger (iButton DS1922L, Thermochron, Baulkham Hills, NSW, Australia) attached to a blank controlled internal drug releasing device (CIDR). The data loggers were inserted at -1 d and utilized for the duration of phase 3. The vaginal temperatures were averaged by day. Udder surface temperature was determined using a thermal imaging camera (Fluke TiR1, Everett, WA) immediately before each morning milking. The thermal imaging camera was positioned 1 meter behind the udder with the tail held to the side to ensure that the entire rear udder was included. The camera software recorded the portion of the rear udder with the hottest surface area. Two images were obtained daily, and the average of the hottest surface area was used for analysis. The THI of the barn housing the cows was recorded each day using temperature and humidity sensing thermometer (Govee Wi-Fi Thermometer, Hong Kong).

Flow Cytometric Data Collection and Analysis

Quarter milk for flow cytometric analysis (250 mL) was collected at day 0 and 3 during phase 3. On day 0, 250 mL of milk were collected before IMM challenge in sterile Nalgene bottles. On day 3 of phase 3, 100 mL of milk was collected from the challenged quarter (n = 37). Milk was immediately cooled and transported to the laboratory where it was centrifuged at 2000 x *g* for 40 minutes at 4C. The cream line was removed, cells were washed with 250 mL of cold PBS and centrifuged at 400 x *g* for 10 minutes at 4 ^{o}C. Cells were suspended in 10 mL of cold PBS and strained through a 40 μm cell strainer into a 50 mL Falcon tube. The 250 mL bottle was washed twice using cold PBS and washed PBS then filtered. The cells were centrifuged at 400 x *g* and supernatant was removed by flicking. The cells were then resuspended in 1 mL of cold

PBS and counted using a hemocytometer. Based on the cell count, the cell pellet for each well was then adjusted to 2.0 x 10^5 and placed in a 96 well plate. Florescence minus one (FMO) and single stained control wells were also created. All the cells were centrifuged and resuspended in 100 µL of the multicolor antibodies (Table 1) or the appropriate FMO before being incubated for 20 minutes at room temperature in the dark. The cells were then washed in 100 µL of flow cytometry staining buffer (FACS buffer, PBS with 5% Fetal Bovine serum) and centrifuged for 5 minutes at 300 x *g* at 4 ºC. The wash step was repeated three times. The cells were fixed using 100 µL of 2% Formalin for 30 minutes at room temperature in the dark. The cells were washed twice with FACS buffer by centrifugation for 5 minutes at 300 x *g* at 4 ºC. The fixed cells were refrigerated overnight, and the next morning ran on the Cytek Aurora (Cytek Biosciences, Fremont, California). Analysis was performed using FCS Express (De Novo Software, Pasadena, CA) and following a conservative gating strategy (Figure 2).

Outcomes

Cow-level outcomes included dry matter intake (DMI), body condition score, and milk yield. During phase 3, vaginal and udder surface temperature were recorded. Quarter level outcomes included SCC and results of microbiological culture. Time to onset of clinical signs was defined as the number of days until abnormal milk occurred. Time to bacteriological cure was defined as the number of days until no *Strep uberis* were cultured from milk collected from the challenged quarter.

Statistical Analysis

Statistical analysis was performed using SAS v. 9.4. The baseline model used to analyze the data was as follows:

$$Y_{ijkl} = \mu + T_i + D_j + (T_i \, x \, D_j) + P_k + B_l + (T_i \, x \, B_l) + R_B + R_C + e_{ijkl}$$

Where Y_{ijkl} = the dependent variable, μ = the mean, T_i = fixed effect of treatment, D_j = fixed effect of time, $T_i \ x \ D_j$= interaction of treatment and time, P_k = the effect of parity (1 or 2), B_l = BLV status, $(T_i \ x \ B_l)$ = interaction of treatment and BLV status, R_B= the random effect of block, R_C = the random effect of cow, and e_{ijkl}= residual error. The three-way interaction of treatment, BLV status, and time was also assessed but was not significant and was removed from all the models.

Sample size calculations were performed using Proc Power (SAS 9.4) based on 80% power to detect a change in SCS of 1.5 (or 35,000 cells/mL) based conservatively on the results reported by Vailati-Riboni et al. (2020). The power analysis indicated that 34 cows were required. To account for possible exclusions 42 cows were enrolled in the study.

During phase 3, an additional covariate was added to the baseline model for mean THI. The resulting model looked like:

$$Y_{ijkl} = \mu + T_i + D_j + (T_i \ x \ D_j) + P_k + B_l + (T_i \ x \ B_l) + H_w + R_B + R_C + e_{ijkl}$$

Where Y_{ijkl} = the dependent variable, μ = the mean, T_i = fixed effect of treatment, D_j = fixed effect of time, $T_i \ x \ D_j$= interaction of treatment and time, P_k = the effect of parity (1 or 2), B_l = BLV status, $(T_i \ x \ B_l)$ = interaction of treatment and BLV status, H_w = mean THI of phase 3, R_B= the random effect of block, R_C = the random effect of cow, and e_{ijkl}= residual error. As surface temperature is known to be largely affected by ambient THI (Velasco-Bolaños et al., 2021) for models evaluating udder surface temperature instead of H_w which was a weekly mean, a daily mean THI (H_d) for the two hours immediately preceding the infrared thermography images were offered to each model.

During phase 4, an additional covariate was added to the baseline model. The phase 4 model was as follows:

$$Y_{ijkl} = \mu + T_i + D_j + D(A_n) + (T_i\, x\, D_j) + P_k + B_l + (T_i\ x\ B_l) + H_w + R_B + R_C + e_{ijkl}$$

Where Y_{ijkl} = the dependent variable, μ = the mean, T_i = fixed effect of treatment, D_j = fixed effect of time, $D(A_n)$ = use of IMM antibiotics (yes, no) nested by week, $T_i\ x\ D_j$ = interaction of treatment and time, P_k = the effect of parity (1 or 2), B_l = BLV status, $(T_i\ x\ B_l)$ = interaction of treatment and BLV status, H_w = mean THI of phase 3, R_B = the random effect of block, R_C = the random effect of cow, and e_{ijkl} = residual error. All variables were assessed for all two-way interactions with treatment, and tested for a three-way interaction between BLV status, treatment, and time. Explanatory covariates and their interactions with a $P \leq 0.10$ were retained in models. For the outcomes evaluated based on time to event, explanatory covariates were offered to the model using the ASSESS statement in PROC PHREG.

To evaluate the effect of treatment group on dry matter intake, milk production, milk components, and body condition scores, a mixed model was created using PROC MIXED in SAS (v. 9.4). The models included fixed effects of treatment and time, all relevant explanatory covariates, and the interaction between treatment and time. Block and cohort were included in all models as random effects. Differences between means were assessed using the DIFF and PDIFF option of PROC MIXED. For weekly averages of milk yield and DMI, were used in each model and results were analyzed by week during phases 2 and 4 and daily during phase 3.

Quarter somatic cell count (QSCC) was modeled in phase 2, phase 3, and phase 4 using PROC MIXED. The final phase 2 and phase 4 models included data from all four quarters while the phase 3 model only included the challenged quarter. While all possible covariates and their interactions were offered to the models, the final models for QSCC in all phases included the fixed effects of treatment, time, and the interaction of treatment of time and the random effects of cow, cohort, and block. Differences were assessed using the DIFF and PDIFF option.

The effect of SCFP on vaginal temperature during phase 3 was modeled using PROC GLIMMIX. The final model included the fixed effects of treatment, time, parity, and the interaction of treatment and time and included the random effects of cow, cohort, and block. Tukey adjusted differences were assessed using the SLICEDIFF option.

The effects of SCFP on udder surface temperature in phase 3 was modeled using PROC MIXED. The final model included the fixed effects of treatment, time, BLV status, daily mean THI, and the interaction of treatment and time and included the random effects of cow, cohort, and block. Means were adjusted using Tukey and assessed using the DIFF and PDIFF option. To compare the mean udder surface temperature and mean vaginal temperature and ANOVA was performed using PROC GLM.

To evaluate the effect of treatment on days to bacteriological cure during phase 4, a time-to-event analysis was performed using PROC LIFETEST, where time was the number of days until bacteriological cure occurred. Censoring occurred when a cow was removed from the study (n =2). The Kaplan-Meier method was used to calculate the survival curves by treatment. Equality over strata was assessed using log-rank and Wilcoxon tests. PROC PHREG was used to estimate the hazard ratio after adjusting for covariates including parity, BLV status, and antibiotics (binary) where the time-to-event outcomes occurred during follow up. The ASSESS statement was used to evaluate the proportional hazards assumption. The RANDOM statement was used to account for the random effects of block and cohort. All time-to-Event figures were modeled using SigmaPlot v 12.5 (Systat, California, United States).

To evaluate the effect of SCFP on to time to clinical mastitis onset, during phase 3, a time-to-event analysis was performed using PROC LIFETEST, where time was the number of days until clinical mastitis occurred. Time to clinical mastitis onset was modeled from day 1

through day 5 after challenge for all cohorts. Cows who did not achieve clinical mastitis within 5 days were right censored. PROC PHREG was used to estimate the hazard ratio after adjusting for covariates including parity and BLV status.

To evaluate the effect of treatment with SCFP on mammary immune cell populations, cell populations were modeled prior to and during challenge with *Strep uberis*. During phase 2, after 45 days of supplementation with SCFP, mammary immune cell populations were analyzed using linear mixed models that included effects of treatment, BLV status, median THI, and two-way interactions with treatment. During phase 3, mammary immune cell populations after challenge with *Strep uberis* were analyzed using repeated measures mixed model including the same fixed effects as the pre-challenge. All models included cow, block, and cohort as random effects.

4.4 Results

Study Population

Of mid-lactation cows (n = 42) enrolled in phase 1, 5 were removed before challenge based on development of clinical (n = 1) or sub-clinical mastitis (n = 4). Of cows (n = 37) that received IMM challenge, 19 were randomly assigned to received SCFP while 18 were assigned to the control group (Table 2). There were no differences in DIM, milk yield, parity of BLV status between groups prior to challenge (Table 2). The average number of *Streptococcus uberis* in the IMM challenge dose for each of the 4 cohorts were 3.1, 3.2, 3.9, 3.2 $\log_{10}$ cfu/mL for cohort 1, 2, 3, and 4 respectively. During phase 3, one cow was severely lame and removed from the study on day 7 and a second cow was removed on day 27 after being humanely euthanized due to a broken leg. During phase 4 the challenged quarter of a cow assigned to the SCFP spontaneously dried up. Following the intent to treat principles, all data from these cows (n = 3)

was included until their removal. Towards the end of phase 3, the body temperature of 3 cows (n = 2 control and 1 SCFP) exceeded 39.7 C and the cows were given oral electrolytes. One of these cows was also given flunixin meglumine on day 6 and her temperature data were not included in analysis.

Dry Matter Intake and Milk Production

Cows were fed the basal diet at the MSU dairy farm, which was a typical midwestern diet comprised of 31% corn silage, 50% concentrate mix, 12% haylage, and 7% whole cottonseed. The diet was 52.3% ± 2.7 dry matter on an as fed basis. Treatment did not affect body condition score or body weight during the study ($P > 0.34$; Table 3). Dry matter intake was not affected by supplementation with SCFP during any phase ($P > 0.18$; Table 3). During the pre-challenge period DMI was affected by the interaction of BLV with treatment where supplementation with SCFP increased DMI in BLV positive cows 1.3 kg ± 0.6 ($P = 0.02$). This corresponded with an increase in milk production during phase 2 where SCFP increased production of milk in BLV positive cows (2.3 kg ± 0.9) compared to control cows ($P = 0.02$), although treatment did not affect overall milk yield. Milk components were not affected by treatment ($P > 0.09$; Table 3). However, there was an interaction between BLV status and treatment ($P = 0.003$) in the phase 2 where SCFP increased milk protein percent by 0.12% ± 0.06 in BLV positive cows ($P = 0.03$).

Strep uberis Colony Counts

Of 37 cows that were infused with *Strep uberis* in a rear quarter, colonies of *S. uberis* were recovered on days 1 -7 from quarter milk of all except one cow. The culture negative quarter was enrolled in cohort 1 in the SCFP treatment group and showed signs of clinical mastitis six days after challenge but remained culture negative. In the final model for number of *Strep uberis* (cfu/mL) in milk from the challenged quarter (Figure 3) there was no difference

between treatments (P = 0. 11) but number of colonies was affected by time (P < 0.001). During phase 3, the treatment by day interaction was not significant (P = 0.66), but on day 4 the SCFP supplemented cows had fewer *Strep uberis* (P = 0.07) recovered from milk samples as compared to milk obtained from control cows.

Quarter Somatic Cell Count

The effect of SCFP on SCC of quarter milk samples was analyzed during phases 2, 3 and 4 (Figure 4). During phase 2, treatment did not affect SCC (P = 0.65), but time was significant (P < 0.001). In phase 2, day -24 had a lower SCC of 3.2 ± 0.14 than the other days tested whose estimates ranging from 3.4-3.8, but there was no difference between treatments (Figure 4a). During phase 3, neither treatment nor the interaction of treatment by day were significant (P > 0.51), but SCC increased with time (P < 0.001) which reflected progression of infection (Figure 4b). In phase 4, supplementation with SCFP did not affect SCC (P = 0.35), but there was a tendency for SCC to decrease with time (P = 0.07), however overall treatment by time (P = 0.13) was not significant. In the post challenge period (Figure 4c), there was a significant difference in the SCC at day 39 between SCFP and control cows (P = 0.049) and the following week there was a tendency at day 46 (P = 0.098).

Time to Clinical Mastitis Onset

The onset of CM included the occurrence of CM during the 5 d after challenge. The mean time to clinical mastitis was 3.88 ± 0.42 days and 4.84 ± 0.44 days for control and SCFP supplemented cows (Log-Rank P = 0.23; Wilcoxon P = 0.16, Figure 5). Time to onset of clinical mastitis was also modeled using a proportional hazards model and it was not affected by treatment (P = 0.33), parity (P = 0.64), BLV infection (P = 0.75), or median THI (P = 0.36).

Time to Bacteriological Cure

Time to bacteriological cure was modeled from 14-53 days after IMM challenge with *Strep uberis*. The mean days to bacteriological cure was 25.9 ± 3.5 for control cows and 22.5 ± 3.1 for SCFP supplemented cows but there was no effect of treatment on this outcome (Log-Rank $P = 0.41$, Wilcoxon methods $P = 0.43$; Figure 6). Time to bacteriological cure was also modeled using a proportional hazards model and it was not affected by parity ($P = 0.83$), BLV infection ($P = 0.43$), or treatment ($P = 0.50$).

Vaginal Temperature

The effect of treatment on vaginal temperature was evaluated for 5 days during phase 3 after IMM challenge with *Strep uberis* (Figure 7). There was no difference in vaginal temperature based on treatment ($P = 0.29$), but body temperature increased with time ($P < 0.001$), and treatment by day was not significant ($P = 0.25$).

Udder Surface Temperature

The effect of treatment on udder surface temperature was modeled during phase 3. Supplementation with SCFP did not affect udder surface temperature ($P = 0.92$), but temperature increased with time ($P = 0.03$). There was no treatment by day interaction ($P = 0.46$) but mean THI immediately preceding thermal imaging was correlated with the surface temperature and as mean THI increased so did udder surface temperature ($P = 0.01$). Udder surface temperature was not affected by BLV status ($P = 0.10$). Both udder surface temperature and vaginal temperature were analyzed as repeated measures analysis and the mean udder surface temperatures was 3 degrees cooler than the vaginal temperature (vaginal 39.0°C, udder surface 35.9 °C, $P = 0.001$).

Flow Cytometry

Supplementation with SCFP for 45 days tended ($P = 0.07$) to increase the number of

CD172α positive cells. There was a significant interaction between parity and treatment (P = 0.03). Of the CD172α positive cells in milk, supplementation with SCFP decreased the number of CD16 positive myeloid cells *(P* = 0.006). When co-expression of both CD14 and CD16 monocytes was evaluated to separate monocytes into classical, intermediate, and non-classical monocytes there was a tendency to decrease the non-classical monocytes population (Table 5, P = 0.06). T-cells (CD172α negative/ CD3 positive) comprised < 10 percent of total cells in milk and their population was not affected by SCFP supplementation (P = 0.19). Among T-cells in milk, CD8 positive cells were about one-third of the cells, and their expression was not affected by SCFP (P = 0.75). Natural Killer (NK) T cells comprised less than 12% of the CD3 positive T cells and treatment did not affect their population (P = 0.31).

During phase 3, after an IMM challenge with *Strep uberis*, supplementation with SCFP did not significantly impact the milk immune cell population for CD172α, classical, intermediate, and non-classical monocytes (P > 0.32, Table 6). After challenge with *S. uberis*, non-classical monocytes increased from 2.28% ± 0.98 to 11.6% ± 1.12 (P < 0.001) and classical monocytes decreased from 48.8% ± 3.7 to 26.9% ± 4.2 of all myeloid cells. During challenge, the percentage of classical monocytes drops from 48.8% ± 3.7 at day 0, to 26.9% ± 4.2 (P = 0.005) by day 3 of infection. Natural Killer cells were not affected by treatment during challenge (P = 0.41) nor was the lymphoid cell population (P > 0.29, Table 6). Unlike the myeloid cell population, the CD3 positive T cells remained constant during challenge with the immune cell population at day 0 comprising 6.3% ± 4.1 and three days after challenge 5.9% ± 4.3.

4.5 Discussion

We enrolled mid-lactation cows to reduce possible confounding by the effects of common transition cow diseases as we were interested in exploring the effect of SCFP

supplementation on responses to IMM infection. We used an IMM challenge to ensure that cows were exposed to a standard inoculum of a common pathogen and to allow us to closely monitor the response to IMI. *Streptococcus uberis* was chosen because it is a common mastitis pathogen (Petrovski et al., 2009; Vasquez et al., 2016). The 0140J strain was chosen as it is a well characterized strain of *S. uberis* that is frequently used in challenge trials (Ward et al., 2009). We found no impact of supplementation with SCFP on milk production, milk components, DMI, body weight, or body condition score (Table 3). Previous reports found that SCFP increased DMI and milk yield compared to control cow, we did not find this in our study (Zaworski et al., 2014; Olagaray et al., 2019). However, increased in milk yield and components was primarily seen in transition cows (Zaworski et al., 2014; Olagaray et al., 2019) and other researchers who enrolled mid-lactation cows also reported no increase in milk yield or components based on supplementation with SCFP (Vailati-Riboni et al., 2021). Our results included a novel finding of the impact of SCFP on BLV positive animals improving DMI, milk yield, and milk protein concentration during phase 2. Michigan researchers have reported a negative association between prevalence of BLV and herd-level milk production (Erskine et al., 2012). At cow-level, BLV positive cows have been reported to produce about 132 kg less milk per lactation than BLV negative cows (Norby et al., 2016). In Michigan, about 86% of dairy herds contain infected cows with average in-herd prevalence of about 34% (range of 0-78.1% of cows; (Norby et al., 2016). Our experimental group contained only 9 BLV positive cows so our results should be viewed as preliminary, however, the potential impact of SCFP on production of BLV positive cows is an area for future research.

After IMM challenge with *Strep uberis* the challenge bacteria were recovered from milk samples of all except 1 of the cows. The culture negative cow did develop visibly abnormal milk

(and increased SCC) six days after challenge. This outcome was not unexpected because during an effective immune response, the immune system phagocytizes bacteria and thus the milk may have contained bacteria that were below the laboratory detection limit. Somatic cell count increased as the number of *Strep uberis* colonies in milk increased. Consistent with previous research we did not find an effect of SCFP supplementation on SCC during phase 2 (Acharya et al., 2017). Somatic cell count enumerates the number of leukocytes (as well as a few other cells) in the milk, which function as a part of the immune response with the intention of elimination of potential pathogens (Ruegg and Pantoja, 2013). Previous researchers have reported decreased SCC after supplementation with SCFP (Ferguson et al., 2018), this effect was not seen across the cows in this study. Ferguson et al (2018) performed a retrospective analysis of 25 herds after feeding SCFP and found a decreased composite DHIA cell count. Perhaps, the reason we did not see this effect is because the cell counts in this study were collected at the quarter level following a challenge with specifically *S. uberis* 0140J, compared to a natural exposure with multiple pathogen types. Perhaps SCFP is more protective against other pathogens in a natural infection environment compared to a challenge, which is an area for future research.

The cows in the study were enrolled in four cohorts of 10 cows, and after enrolling the first two cohorts it became apparent that many cows were still infected with *S. uberis* after receiving the initial IMM antibiotic treatment during phase 4. This was the first study to evaluate the effect SCFP supplementation on time to bacteriological cure in mid-lactation dairy cattle. A review of the literature found the mean time to bacteriological cure in natural infection studies was 20 days (Zadoks et al., 2003; McDougall et al., 2004). McDougall et al., (2004) reported a mean duration of infection of 24.5 days for *S. uberis*, after natural infection and treatment using penicillin (McDougall et al., 2004). While there is variation in duration of infection among

strains of *S. uberis* more than 50% of nontreated sub-clinical natural infections have been reported to persist for at least 42 days, and many infections with *S. uberis* become chronic (Zadoks et al., 2003). Time to bacteriological cure was not impacted by parity which was interesting because previous work in New Zealand had found that first lactation cows had a shorter duration of infection than second lactation cows (McDougall et al., 2004). Perhaps differences in parity were not seen because the New Zealand study sampled 503 cows on five farms, and the collected *S. uberis* were of several different strains. Another difference between these studies is treatment, where the cows were treated with penicillin in McDougall et al (2004), while in our study the cows were treated with ceftiofur.

Streptococcus uberis is able to attach and integrate into mammary epithelial cells as well as bovine macrophages (Tamilselvam et al., 2006). A genome analysis of *Strep uberis* 0140J found *Strep uberis* adhesion molecules (SUAM) and lactoferrin binding proteins aid the colonization of the mammary gland (Ward et al., 2009). The healthy mammary gland is protected by lymphocytes and macrophages although some neutrophils are present (Sordillo, 2018). When IMI occurs, cytokines and oxylipids stimulate diapedesis. Neutrophils rapidly migrate from the blood to the site of the infection in the mammary gland (Sordillo, 2018). To measure this immune response, we used a flow cytometry panel that examined lymphocytes and myeloid cells in milk.

One proposed mechanism by which SCFP could impact the immune system is through innate immune system training which means the immune cells exist in an “trained” or primed state which enables more rapid responses to pathogenic bacteria. Immune training is primarily thought to occur in the myeloid cells (monocytes and macrophages). Yeast cell wall components such as β-glucans are able to train the immune system in humans and rodents (Quintin et al.,

2012; Saeed et al., 2014) and could possibly have similar function in bovines. We chose CD172α as a marker of all myeloid cells and included CD14 and CD16 to further identify classical, intermediate, and non-classical monocytes. Monocytes are cells that originate in the bone marrow and circulate in peripheral blood and eventually migrate to tissues to replenish the macrophage or dendritic cell populations (Hussen et al., 2013; Sordillo, 2018). Bovine monocytes isolated from blood have been shown to have distinct functional capabilities, with classical monocytes comprising the largest percentage and having the most phagocytotic capability, while intermediate monocytes produced the most inflammatory cytokines (Hussen et al., 2013). Bovine non-classical monocytes have the lowest phagocytic capacity and their function *in vivo* requires more research (Hussen et al., 2013). Previous researchers demonstrated that increased concentrations of classical and intermediate bovine monocytes in blood during the prepartum period are predictive of postpartum diseases including mastitis (Pomeroy et al., 2017). The authors theorize that individual monocyte populations and subsets may play a role in disease susceptibility but more research is needed (Pomeroy et al., 2017).

We found that supplementation with SCFP tended to increase the percentage of CD172α positive cells in the mammary gland primarily through an increase in CD14 positive cells in multiparous cows. There was a corresponding tendency for a decrease in non-classical monocytes (CD172α positive/ CD16 positive /CD14 negative) cells. Non-classical monocytes in the blood have the lowest phagocytic capacity, so decreasing their prevalence in the udder and increasing other populations could possibly improve immune response to infection, but more research is needed to understand the role of non-classical monocytes in milk (Hussen et al., 2013). Previous research using mid-lactation dairy cows that received SCFP found an increase in circulating blood monocytes 36 hours after challenge with *S. uberis* 0140J but they did not find a

difference in the oxidative burst capacity (Vailati-Riboni et al., 2021). Our research builds on this previous work, as these circulating blood monocytes could possibly migrate to the udder where they would further differentiate. Future research could look at proliferation or differentiation of the monocytes with additional antibodies. It is important to note that the oxidative burst capacity of these blood monocytes was not changed in vitro for mid-lactation dairy cattle (Vailati-Riboni et al., 2021) and is unknown in milk. Previous researchers have evaluated the effect of SCFP supplementation on respiratory disease in calves and reported decreased respiratory monocyte respiratory burst activity (Mahmoud et al., 2020). Importantly though, these differences in monocytes were not seen during the period of challenge with *Strep uberis*. So, while the potential impact of SCFP supplementation on the myeloid cell population is a potential mechanism by which SCFP may impact the immune system, the clinical relevance is unknown. While we observed a decrease in the non-classical monocyte population, the clinical outcomes that we observed were not affected, thus we were unable to demonstrate biological relevance of the increase in the cell population. Perhaps biological relevance was not seen because a challenge model was used, which utilizes a relatively large number of colonies infused into the udder compared with natural infection where only a few colonies would overwhelm the innate immune responses to cause infection.

We also measured T-lymphocytes using markers for all T-cells (CD3 positive) and cytotoxic T cells (CD8 positive). CD8 positive cells can be either cytotoxic where they act as scavengers in the mammary gland to remove damaged secretory cells or as suppressors where they modulate the immune response (Sordillo et al., 1997). T-lymphocytes have not been previous evaluated relative to response after supplementation with SCFP and IMM challenge but have been evaluated in calves (Mahmoud et al., 2020). In calves, oral supplementation with

SCFP did not influence circulating T-lymphocytes nor affect CD4 or CD8 cell proliferation (Mahmoud et al., 2020). Natural Killer cells (identified as CD335 positive) were targeted because NK cells are innate immune cells that have cytotoxic activity that is independent of the major histocompatibility complex and are therefore thought of as a potential first line of defense against bacterial infections (Boysen and Storset, 2009; Sordillo, 2018). Natural Killer cells have been shown to be active in the mammary gland against both Gram-negative and Gram-positive pathogens (Storset et al., 2004; Sipka et al., 2016; Sordillo, 2018). Previous in vitro research found that yeast culture was able to activate NK cells isolated from bovine blood (Jensen et al., 2008). Supplementation with SCFP did not impact the NK cell population (CD335 positive) in the mammary gland, which was consistent with previous research in calves where SCFP did not impact circulating NK cells (Mahmoud et al., 2020). Future research could look at the activation status of the NK cells in the udder or respiratory system, to confirm if the in vitro results seen by Jensen (2008) are seen in vivo. The mechanism of SCFP on the immune system is still an area where more studies are needed, however our research is consistent with other studies that SCFP acts on a myeloid cell population and not lymphoid cells, but the clinical biological relevance of this impact is still unclear.

4.6 Conclusions

In summary, supplementation with SCFP did not impact milk yield, DMI, SCC, number of *Strep uberis* colonies, or time to bacteriological cure in mid-lactation dairy cattle after challenge. This research supports that after infection with *Strep uberis*, the mammary gland requires a long duration to achieve bacteriological cure regardless of SCFP supplementation. Supplementation with SCFP did tend to increase the percentage of myeloid cells (CD172α) and decrease non-classical monocytes indicating a possible innate immune system training to

enhance protection of the mammary gland. Future studies should be aimed at further characterizing the impact of SCFP on differentiated monocyte cell populations such as macrophages and dendritic cells to further understand the role that SCFP may play in immune training.

4.7 Acknowledgements

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4.8 Tables and Figures

Table 4.1 Antibodies used for Flow Cytometric Analysis in Bovine Milk

Antibody	**Target Population**	**Fluorochrome**	**Dilution**	**Clone**	**Source**[1]
CD3	All T-Lymphocytes	AF350	1 in 50	PC3/188A	Novus Biologicals
CD8	CD8+ Lymphocytes	AF594	1 in 20	CBB/1595	Novus Biologicals
CD14	Monocytes	Pacific Blue	1 in 20	CC-G33	Bio-Rad
CD16	Monocytes	PE	1 in 25	KD1	Bio-Rad
CD45	All nucleated cells	FITC	1 in 20	CC1	Bio-Rad
CD172a	All Myeloid Cells	RPE-CY5	1 in 100	CC149	Bio-Rad
CD335	NK Cells	AF647	1 in 50	AKS1	Bio-Rad
Zombie NIR	Live/Dead Marker	NIR	1 in 5000	100	Biolegend

[1]Novus Biologicals (Centennial, Colorado), Bio-Rad (Hercules, CA), Biolegend (San Diego, CA)

Table 4.2 Study population of cows enrolled in a randomized trial to assess the impact of *Saccharomyces cerevisiae* fermentation product (SCFP) on production in mid-lactation dairy cows

Variable	Control (± SD) n = 18	SCFP (± SD) n = 19	*P* - Value
Days in Milk	178.3 (± 22.2) d	181.8 d (± 14.7) d	0.58
Milk yield	41.1 (± 7.9) kg	40.8 (± 8.6) kg	0.91
Parity	1.8 (± 1.1) lactations	1.6 (± 0.8) lactations	0.65
BLV Status (Binary)			0.27
BLV Positive	27.8 % (n = 5)	21.1 % (n = 4)	

Table 4.3. The effects of a yeast fermentation product on dry matter intake, milk production, and body weight prior to (phase 2; -45 - 0 d), during (phase 3; 1- 7 d), and after (phase 4; 7 - 53 d) an intramammary challenge (n = 37) with *Streptococcus uberis* 0140J

Item[2]	Treatment[1]		SEM	*P*-values		
	CON	SCFP		Trt	Time	Trt × Time
DMI, kg/d						
Phase 2[3]	25.3	25.8	0.75	0.16	<0.01	0.74
Phase 3	23.1	23.0	1.29	0.92	<0.01	0.18
Phase 4	23.8	23.6	0.62	0.73	<0.01	0.59
Milk yield, kg/d						
Phase 2[3]	38.3	39.0	0.52	0.23	<0.01	0.92
Phase 3	33.2	34.3	1.94	0.27	<0.01	0.30
Phase 4	34.3	34.7	1.53	0.63	<0.01	0.41
Milk fat, %						
Phase 2	3.75	3.66	0.266	0.57	<0.01	0.23
Phase 3	4.05	4.00	0.170	0.76	-	-
Phase 4	4.08	4.14	0.230	0.79	0.45	0.50
Milk fat, kg/d						
Phase 2	1.44	1.41	0.055	0.59	0.07	0.66
Phase 3[4]	1.21	1.22	0.090	0.88	-	-
Phase 4	1.25	1.26	0.114	0.72	<0.01	0.09
Milk protein, %						
Phase 2[3]	3.12	3.15	0.044	0.44	0.29	0.71
Phase 3	3.27	3.14	0.078	0.19	-	-
Phase 4	3.25	3.14	0.111	0.28	<0.01	0.80
Milk protein, kg/d						
Phase 2	1.21	1.21	0.041	0.94	<0.01	0.69
Phase 3	1.00	0.99	0.084	0.81	-	-
Phase 4	1.02	0.98	0.085	0.39	<0.01	0.19
MUN, mg/dL						
Phase 2	12.8	12.2	0.55	0.24	<0.01	0.42
Phase 3	13.3	13.1	0.61	0.77	-	-
Phase 4[5]	13.9	13.7	0.79	0.71	<0.01	0.97
ΔBW, kg/d						
Phase 2	0.42	0.41	0.131	0.96	0.48	0.62
Phase 3	-1.24	-1.50	0.950	0.65	-	-
Phase 4	0.74	0.88	0.199	0.99	<0.01	0.34
ΔBCS, point/wk						
Phase 2	0.02	0.02	0.023	0.91	0.03	0.71
Phase 3	0.02	0.02	0.025	0.95	-	-
Phase 4	0.05	0.04	0.074	0.84	0.05	0.94

Table 4.3 (cont'd)

[1]CON = 19 g of corn grain, SCFP = 19 g of yeast fermentation supplement (Diamond V, Cedar Rapids, IA).

[2]DMI = dry matter intake, MUN = milk urea nitrogen, ΔBW = change in bodyweight, BCS = body condition score (1-5 scale)

[3]BLV × treatment interaction ($P \leq 0.05$)

[4] THI × treatment interaction ($P \leq 0.05$)

[5]Final CFU × treatment interaction ($P \leq 0.05$)

Table 4.4. Comparison of vaginal and udder surface temperature of dairy cows (n= 37) during phase 3 (d 0- 5) after challenge with *Streptococcus uberis* 0140J

	Vaginal Temperature[1]			Udder Surface Temperature[2]		
Day relative to challenge	Control	SCFP	*P*- Value	Control	SCFP	*P*- Value
0	38.6 ± 0.2	38.5 ± 0.2	0.62	35.5 ± 0.3	35.7 ± 0.3	0.99
1	38.7 ± 0.2	38.8 ± 0.2	0.87	36.2 ± 0.3	35.9 ± 0.3	0.99
2	39.0 ± 0.2	39.0 ± 0.2	0.85	35.5 ±0.3	35.9 ± 0.3	0.98
3	39.2 ± 0.2	39.1 ± 0.2	0.46	35.4 ± 0.3	35.4 ± 0.3	1.00
4	39.3 ± 0.2	39.0 ± 0.2	0.04	36.3 ± 0 .3	36.3 ± 0.3	1.00
5	39.1 ± 0.2	39.1 ± 0.2	1.00	36.6 ± 0.3	36.5 ± 0.3	1.00

[1]The final model for vaginal temperature included the fixed effects of treatment ($P = 0.34$), time ($P < 0.001$), the interaction of treatment and time ($P = 0.25$), lactation ($P = 0.03$) with the random effects of cohort and block.
[2]The final model for udder surface temperature included the fixed effects of treatment ($P = 0.92$), time ($P = 0.03$) and the interaction of treatment and time ($P = 0.46$), BLV status ($P = 0.10$), and daily mean THI ($P = 0.01$) with the random effects of cohort and block.

Table 4.5. The effect of 45 d of supplementation with post biotic yeast fermentation product (SCFP) on milk immune cell profile (n = 37)

Item[1]	Treatment %		SEM	Trt
	CON	SCFP		
Myeloid Cell Markers				
CD172a[2]*	19.5	26.7	2.6	0.07
CD14 positive[3]	32.7	43.0	5.3	0.24
Lactation 1	40.2	26.2	7.4	0.24
Lactation 2**	25.7	59.8	7.4	0.03
CD16 positive[2]**	7.0	4.9	0.9	0.01
Classical Monocytes[3]	46.2	52.7	5.4	0.45
Intermediate Monocytes[2]	8.8	8.6	1.3	0.86
Non-Classical Monocytes*	3.6	1.5	0.6	0.06
NK+ Cells	1.0	0.66	0.3	0.33
Lymphoid Cells Markers				
CD3 positive[4]	6.8	5.7	4.4	0.19
CD8 positive	38.0	36.8	13.4	0.75
NK T-Cells[5]	11.1	7.7	2.3	0.31

[1]Effect of SCFP on populations; $^{**}P \leq 0.05$, $^{*}P \leq 0.10$)
[2]Immune cell population affected by a covariate with median THI covariate ($P \leq 0.05$)
[3]Immune cell population affected by lactation × treatment interaction ($P \leq 0.05$)
[4]Immune cell population affected by lactation covariate ($0.05 \leq P \leq 0.10$)
[5]Immune cell population affected by median THI covariate ($0.05 \leq P \leq 0.10$)

Table 4.6. The effects of supplementation with SCFP (n = 37) on milk immune cell populations after 3 d of challenge with *Strep uberis* 0140J

Item[1]	Treatment %		SEM %	*P*- value		
	CON	SCFP		Trt	Time	Trt × Time
Myeloid Cell Markers						
CD172a[23]	37.0	40.9	4.1	<0.01	<0.01	0.63
CD14 positive	29.7	29.3	3.3	0.86	0.02	0.81
CD16 positive	7.9	6.0	1.3	0.11	0.15	0.99
Classical Monocytes	36.9	38.9	3.7	0.68	0.01	0.67
Intermediate Monocytes[236]	8.1	7.2	1.3	0.46	0.55	0.44
Non-Classical Monocytes[6]	7.3	6.5	1.0	0.56	<0.01	0.31
NK positive Cells[6]	0.8	0.6	0.2	0.30	0.19	0.41
Lymphoid Cells Markers						
CD3 positive[45]	5.7	6.6	4.2	0.49	0.81	0.29
CD8 positive	32.3	29.2	11.6	0.07	0.08	0.71
NK T-Cells	9.5	4.9	2.2	0.47	0.76	0.35

[1]Effect of mastitis challenge on cell population; $^{**}P \leq 0.05$, $^{*}P \leq 0.10$)

[2]Immune cell population significantly affected by BLV infection status ($P \leq 0.05$)

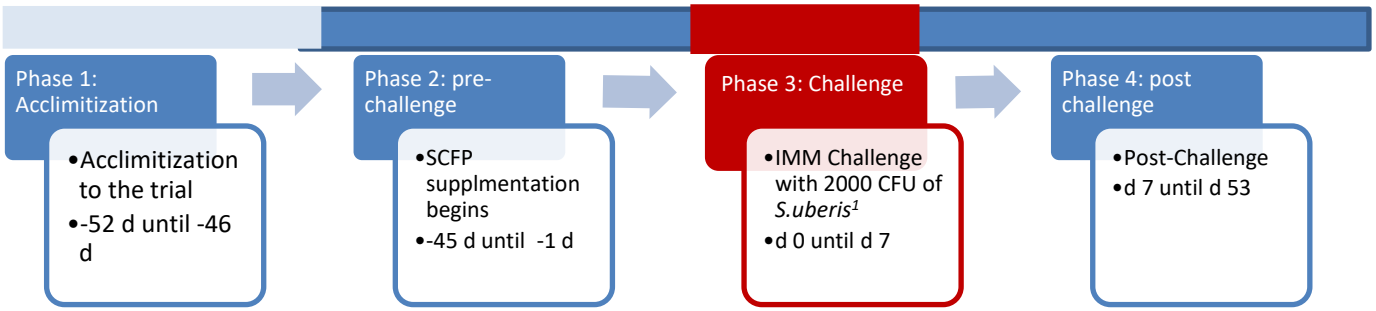
[3]Immune cell population significantly affected by an interaction between BLV × treatment ($P \leq 0.05$)

[4]Immune cell profile significantly affected by parity ($P \leq 0.05$)

[5]Immune cell profile affected by lactation × treatment interaction ($0.05 \leq P \leq 0.10$)

[6]Immune cell profile was affected by mean THI covariate ($0.05 \leq P \leq 0.10$)

Figure 4.1. Study design for the randomized complete block design



[1]Study design for the randomized complete block design to evaluate the supplementation of SCFP on mammary immune response after intramammary challenge with Strep uberis 0140J. Day 0 represents the day of challenge is 2000 cfu of *Strep. uberis* 0140J. Cows were sampled weekly until day 53 post challenge.

Figure 4.2. Flow Cytometry Gating Strategy

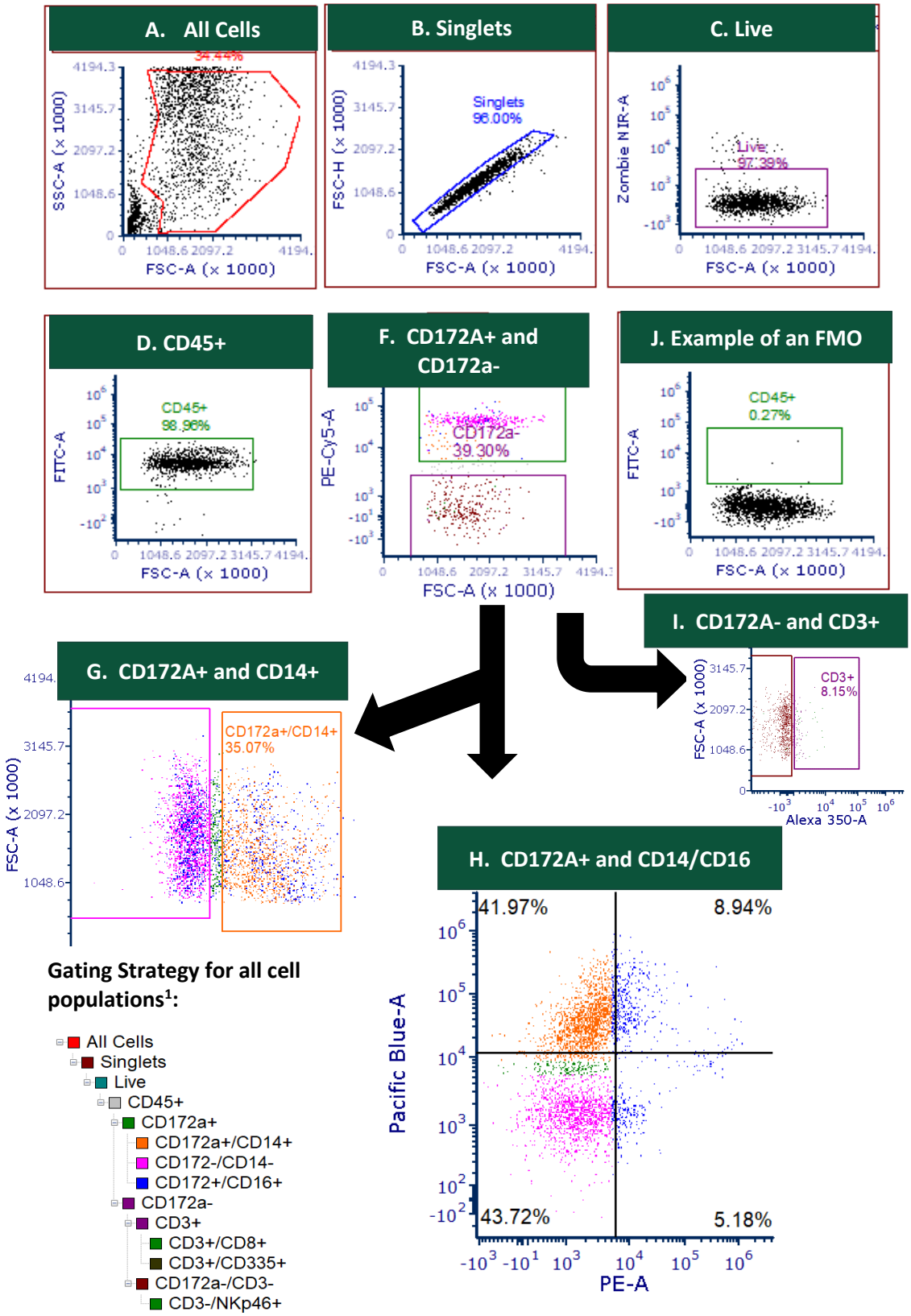
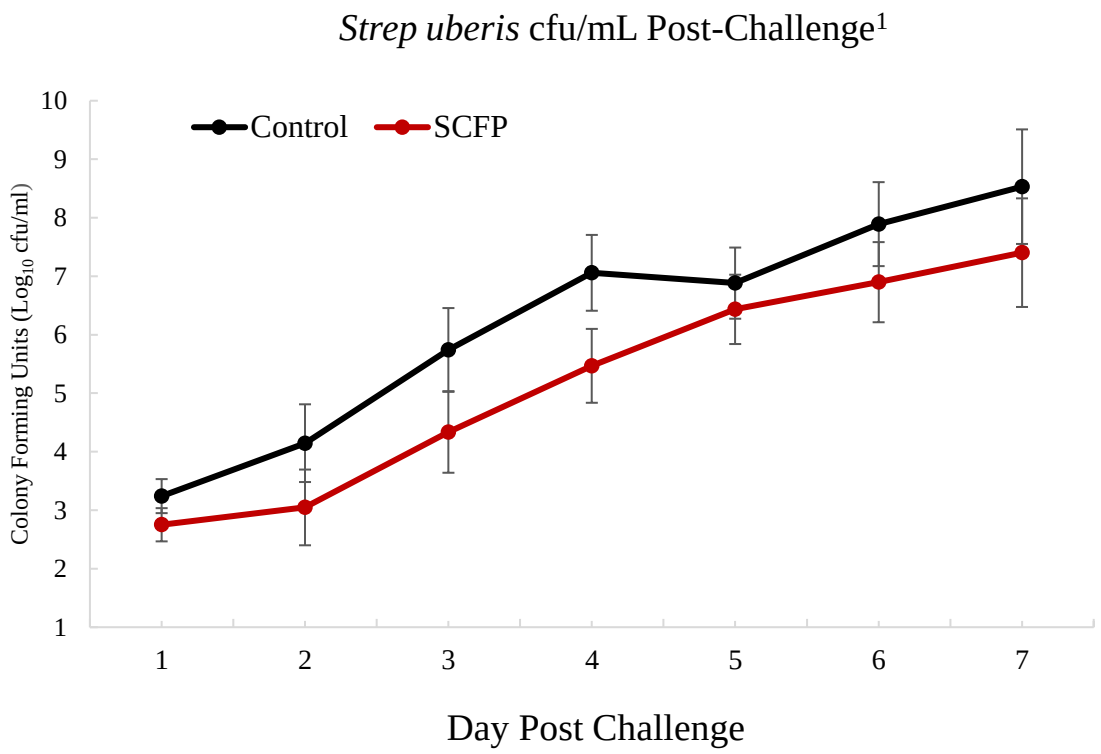


Figure 4.2. (cont'd)
[1]Depicts the gating strategy used for the mammary immune cells isolated from milk before and after challenge with *Strep uberis* 0140J. Single (**B**), live (**C**), nucleated cells (**D**) were characterized based on their expression of myeloid and lymphoid cell markets. **E**). The gating strategy used CD172α to identify CD14+ and CD16+ cells. **G**). Depicts a representative example of the CD14+ population gating. **H**). depicts the co-expression and gating strategy used to classify cells at classical (CD172α+/CD14+/CD16-), intermediate (CD172α+/CD14+/CD16+), or non-classical (CD172α+/CD14-/CD16+). **I**.) is a representative of the gating done on the lymphoid populations CD45+/CD172α-. **J**.) Is a representative example of how FMO controls were used to set the gating strategy.

Figure 4.3. Bacterial colonies of *Strep uberis* 0140J recovered following intramammary challenge of cows (n = 37) after supplementation with a post biotic yeast fermentation product or control



[1]There was no significant difference between the control and SCFP (P = 0.11) and there was not significant interaction of treatment and time (P = 0.66) supplemented cows but at day 4 there is a tendency for SCFP supplementation to decrease bacterial cfu (P = 0.07).

Figure 4.4. Quarter Somatic Cell Count from cows (n = 37) prior to, during and after challenge with *Strep uberis* 0140J

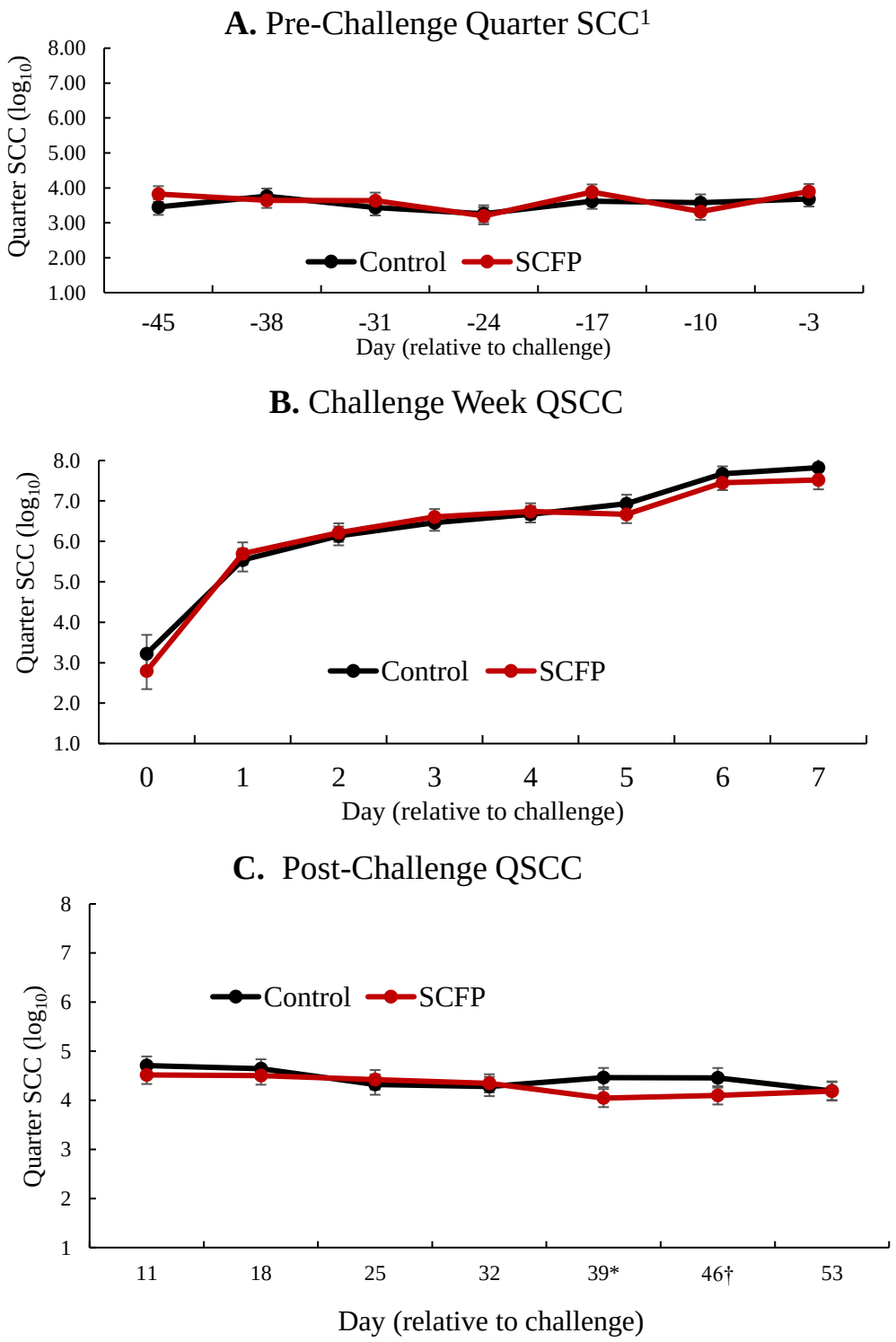
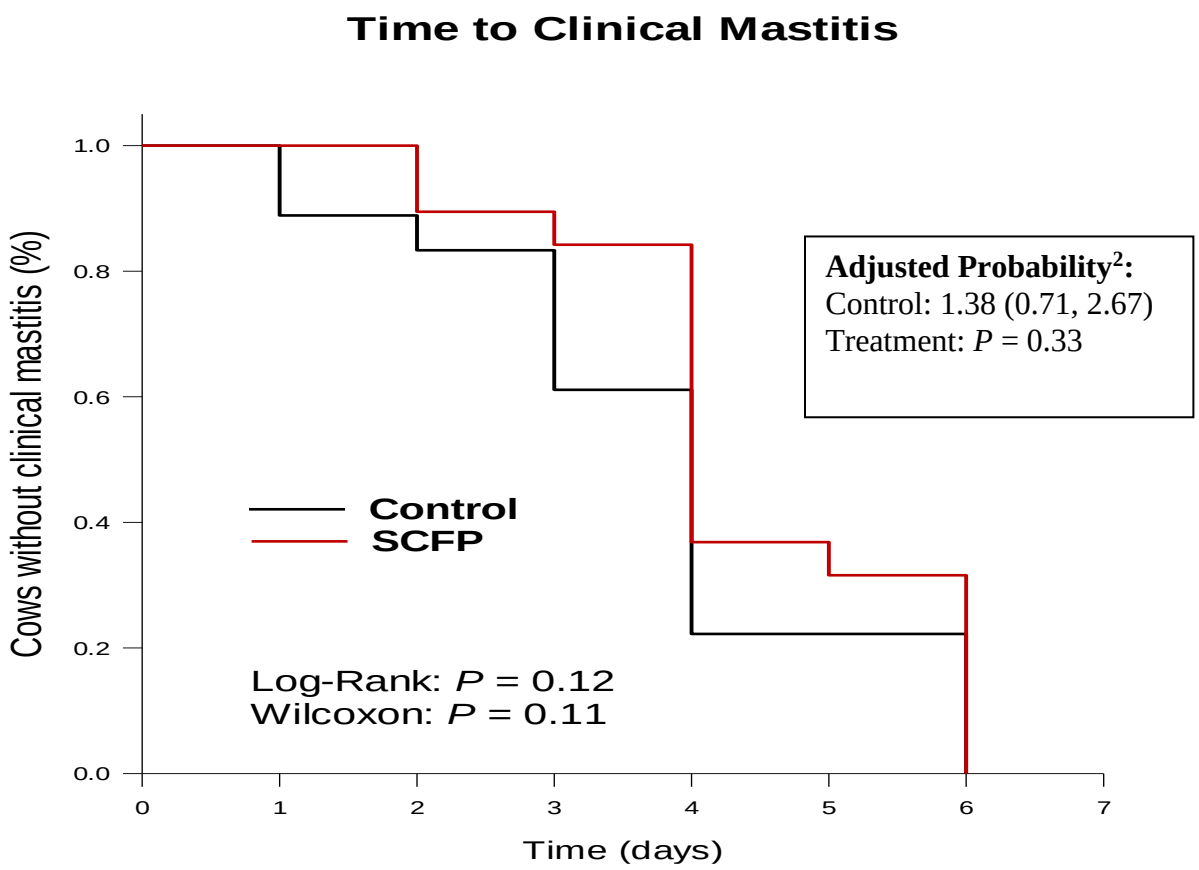


Figure 4.4. (cont'd)
[1]Quarter somatic cell count during the pre-challenge, challenge, and post challenge period. The pre-challenge (A, n = 37) and post-challenge period (C, n= 35) included all four quarters individually while the challenge week includes only the challenged quarter (B). Time was significant in the models for pre-challenge and challenge week. Treatment was not significant in any model, but there was a tendency for the interaction of treatment x time in the pre-challenge period. There were some significant treatment differences at some weeks in the post challenge period represented with *$P < 0.05$ and †$P < 0.10$.

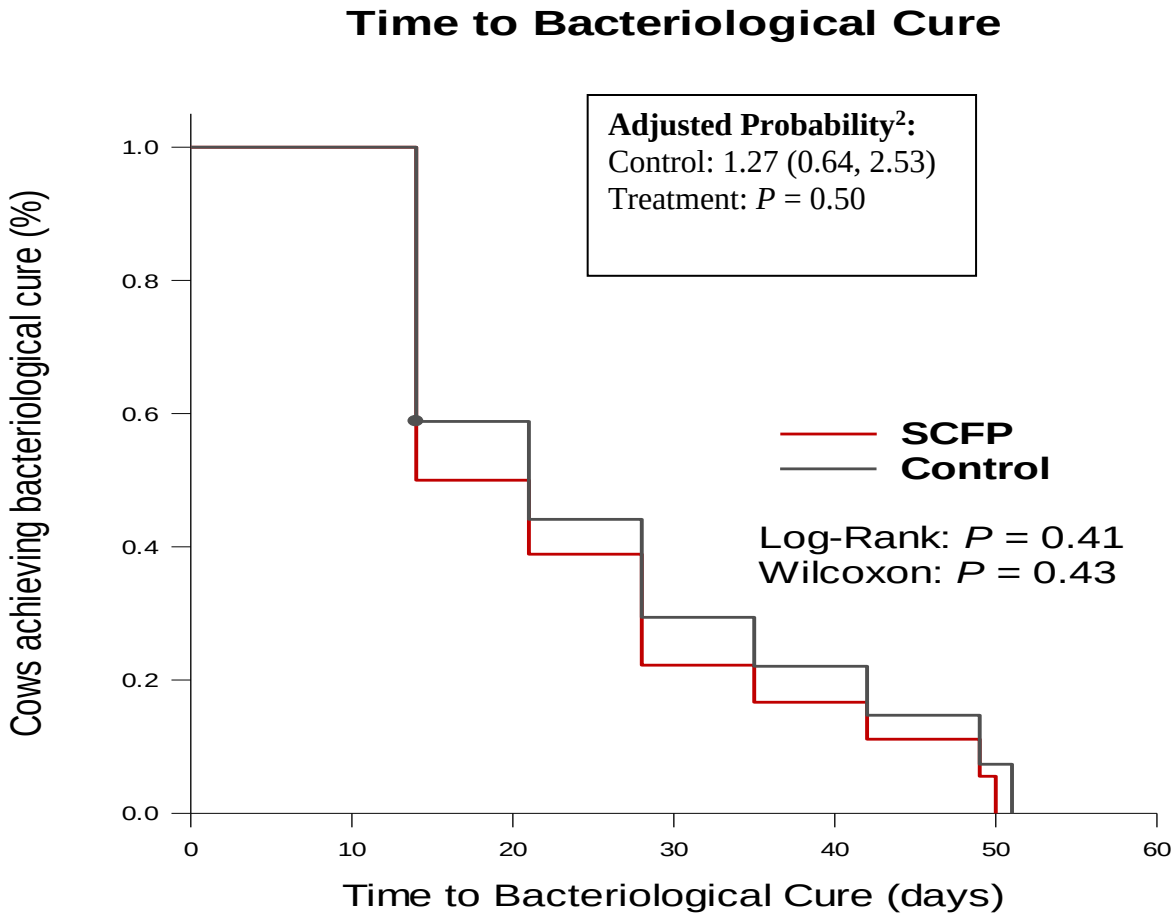
Figure 4.5. Time to clinical mastitis onset[1] by treatment for cows (n = 37) challenged intramammarily with Strep uberis 0140J



[1]The unadjusted survival plot describes the time until clinical mastitis onset. Differences in the survival curves by treatment were evaluated using Log-Rank and Wilcoxon. Time is the number of days until clinical mastitis occurred.

[2]The adjusted survival plots were also created using PROC PHREG and the hazard ratio compares the control and SCFP supplemented cows and parenthesis represent the 95% confidence interval.

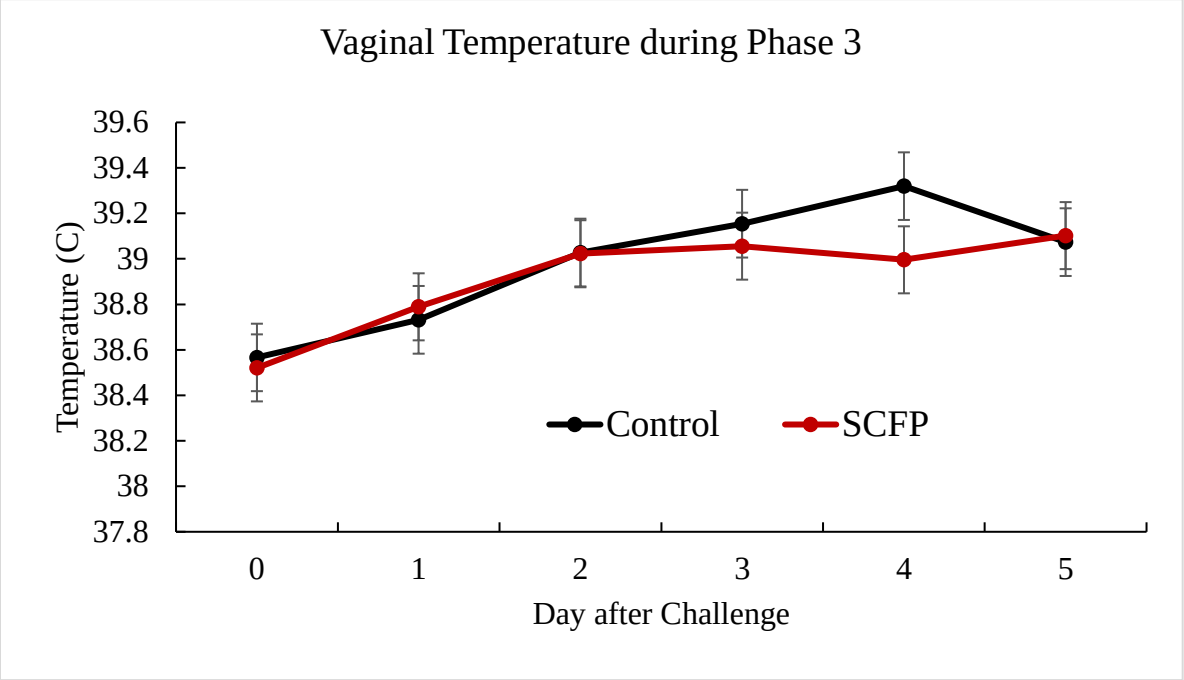
Figure 4.6. Time to Bacteriological Cure[1] by treatment for cows (n = 37) challenged intramammarily with *Strep uberis* 0140J



[1]The unadjusted survival plot of time to bacteriological cure, treatment differences were evaluated using Log-Rank and Wilcoxon. Time is the number of days until bacteriological cure occurred.

[2]The adjusted survival plots were also created using PROC PHREG and the hazard ratio compares the control and SCFP supplemented cows and parenthesis represent the 95% confidence interval.

Figure 4.7. Body Temperature[1] of cows (n = 37) after challenge with *S. uberis* 0140J



[1]Depicts the daily mean vaginal temperature during phase 3 after cows (n = 37) were challenged with *Strep. uberis* 0140J. Nutritional supplementation with SCFP did not affect temperature (P = 0.29) during the 5 days following challenge. As challenge progressed vaginal temperature increased ($P < 0.001$) but there was no significant interaction of treatment and day (P = 0.25)

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SUMMARY

This dissertation explored several mechanisms to reduce the use of IMM antimicrobials on dairy farms in the United States. While there is scarce evidence that the use of IMM antimicrobials is leading directly to the development of resistance in mastitis pathogens, judicious use principles dictate that the responsible use of antibiotics is the collective responsibilities of dairy farmers, veterinarians, and researchers. The overall objective of this dissertation was to evaluate the use of evidence-based strategies to reduce use of important antimicrobials for treatment of mastitis caused by Gram-positive pathogens. In Chapter 2, a randomized clinical trial was conducted with the aim of comparing clinical and bacteriological outcomes in a negatively controlled randomized clinical trial. Chapter 2 found no difference for most of the clinical and bacteriological outcomes between any of the treatments. The results of chapter 2 indicate that using a shorter duration or lower class of drug are potentially valid mechanisms to reduce the use of critically important antibiotics in the treatment of clinical mastitis.

Chapter 3 explored antimicrobial susceptibility and the minimum inhibitory concentrations of bacterial isolates identified as causing clinical mastitis on Michigan dairy farms. There were several promising results demonstrating and absence of the development of AMR on these farms. These isolates were universally susceptible to oxacillin indicating they likely do not possess the *mecA* genes. These isolates were also universally susceptible to both a first and third generation cephalosporin *in vitro*, which were identical results to studies conducted in Michigan more than 30 years ago.

In chapter 4, the nutritional supplementation with a commercially available postbiotic fermentation production of *Saccharomyces cerevisiae* was found to increase the myeloid cell population in the mammary gland but this did not have a clinical benefit during challenge with

Streptococcus uberis.

The overall hypothesis of this dissertation was that evidence-based strategies can be used to reduce the usage of antimicrobials to treat clinical mastitis caused by Gram-positive pathogens on dairy farms and the work contained in these chapters demonstrates many promising areas to reduce antibiotic usage. To future students, mammary gland immunobiology still has a lot left to be discovered. Future researchers should focus on pathogen specific susceptibilities of mastitis pathogens to IMM antibiotics and new developing new mechanisms to manipulate the bovine immune system.