PROMOTING MAMMARY HEALTH IN DAIRY COWS THROUGH USE OF SOMATIC CELL COUNTS AND EXPLORATION OF THE MILK MICROBIOTA

by

JENNA REED WILLIAMSON

(Under the Direction of Valerie Ryman)

ABSTRACT

Mastitis continues to impact mammary health and milk production in dairy cows. Antibiotic usage is a necessary strategy to protect animal health, however, changes in consumer demand and regulation necessitates reduced antibiotic usage and alternatives to antibiotics (ATA). Our objective was to: a) investigate elements that may contribute to antibiotic success, including utilization of somatic cell counts (SCC), to refine treatment recommendations, and b) evaluate biological microbiomes, including milk microbiota, to identify protective populations. Initial studies herein focused on identifying and testing a SCC threshold value for antibiotic treatment associated with antibiotic success. Results demonstrated that using SCC alone without determining infecting pathogen or cow-related factors is not successful as a treatment strategy. In investigation of an ATA, studies explored the microbiota of rumen, fecal, and milk samples and the potential for essential oil derivatives, such as d-Limonene, to support rumen metabolism and healthy, protective microbiota.

INDEX WORDS: Mastitis, Somatic cell count, Antibiotic treatment, Bacteriological cure, Intramammary infection, Microbiome, Essential oil

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B.S., The University of Georgia, 2020

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2022

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May 2022

DEDICATION

First, I would like to dedicate this work to my best friend and most loyal companion, Simba. While he still has not learned how to read, he has provided his indomitable spirit throughout my college career in times when I needed it most. Whether it be through backyard zoomies, teeth chomping, button pressing, or dryer sheet rolling, he always knows how to make me smile after a difficult day. I aspire to be more like Simba, to be as forgiving, to appreciate the small pleasures, to listen with intent, to slow down and breathe it all in, and most importantly, love and be loved. I will never forget the support, protection, and friendship he has provided me.

Second, to the more wild and fierce member of my family, Weezy. He never fails to make every day a new adventure. Nothing is better than using the excuse "there was a chipmunk in my house" for being late to meetings. I will always remember the cuddles he smothered me in when I was stressed, the reminders to take a break by stepping on my laptop, and the morning wake up calls for food when I was still asleep. I hope to retain his independent and powerful nature.

Chiefly, to my parents and sister. While being able to read is a plus, it is the unwavering support they have provided me throughout my entire life that sets them apart. To dad, who never forgets to remind me how proud he is of me, even when I change my life plans on him in a split second. Thank you for making me who I am today. To mom, my home away from home. Thank you for always being available for me, even if it means hour(s) long phone calls every other day. And to my sister, my inspiration, who tackles all

of the above for many people, and looks fabulous while doing it. Thank you for showing me new perspectives when I could only see one way and helping to keep me on the right path.

ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Valerie Ryman for being an outstanding mentor and giving me the opportunity to explore my passions. I have learned so much from you the past few years, and now feel more prepared for my future and more confident in my abilities to excel in my field.

I would like to thank Dr. Todd Callaway for leading me on the path of graduate school. You believed in my potential and helped me act on it when I was unsure of my plans. Your willingness to serve on my committee as well as assist me in on my projects will always be appreciated.

A special thanks to Dr. Jeferson Lourenco, a member of my committee who I wish I could have begun to learn from sooner. I will never forget all the time you dedicated to helping me and guidance you provided. To Dr. Emmanuel Rollin, for agreeing to be a part of my committee and providing suggestions to improve both my research and writing skills. Thank you for always challenging me to look at things from a different perspective.

I would also like to acknowledge any graduate students and undergraduate researchers who helped assist me on my projects. Additionally, the UGA dairy farm staff, who put up with all my projects and trusted me to lead. To Taylor Krause and Osman Koyun, who advised me in all things laboratory techniques and never let me forget it is okay to mess up or not get the results expected, as that is what science is all about! I will always cherish the positive attitudes you both bring to a room. Christina Welch, who always came to the rescue whenever I was in need, even if it meant driving to a dairy farm

hours away in the early morning. I am so glad that graduate school brought us together, you have taught me so much and I will always admire you. To all the friends I made in graduate school, you made me feel at home. I will never forget all our laughs and get-togethers and hope to continue them in the future.

Lastly, I would like to acknowledge Jeff Balco, my backbone throughout my graduate career. I am forever grateful for the care and support you have provided for me the last few years. I can't wait to see what our future has in store for us, hopefully a lot of goats and a lot of grass.

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CHAPTER 1

INTRODUCTION

Mastitis, defined as inflammation of the mammary gland, is one of the most prevalent diseases in dairy production. Though mastitis can be a result of physical or chemical trauma, it is most commonly a result of intramammary infections (IMI) caused by pathogenic organisms, such as bacteria. Mastitis is a costly disease due to decreased milk production, discarded milk, and other factors including antibiotic therapy costs. Reports suggest antibiotic treatment accounts for almost 30% of direct costs for mastitis (Rollin et al., 2015). Although antibiotic usage can be expensive to producers and is heavily scrutinized by consumers, effective therapy is crucial to ensure the productivity and health of the dairy herd. In order for producers to maintain the effectiveness of antibiotic treatment, proper confirmation of IMI must occur to ensure prudent antibiotic usage.

The standard diagnosis of IMI is obtained via growth of a bacterial culture, which confirms the presence of pathogens in a milk sample. However, emphasis has been placed on using the somatic cell count (SCC) of milk to diagnose cases of IMI, especially on-farm, as producers have desired a more immediate indication of potential illness. The SCC (i.e., the total number of host cells per mL of milk) becomes elevated during an infection as cells, especially white blood cells, are directed towards the mammary gland to fight off bacteria. An elevated SCC (>200,000 cells/mL), which is usually associated with decreased milk production, is considered to be abnormal and a good indicator of IMI (National Mastitis Council, 2016). Not all cows with elevated SCC have an IMI, as other sources of

stress such as calving can also increase SCC (Alhussien & Dang, 2018), which limits the use of SCC as an IMI indicator. Moreover, SCC will remain elevated after an infection has been cleared by the cow's immune defenses. Nonetheless, all of the dynamics that have significant impact on milk SCC in the presence of IMI (Dohoo & Meek, 1982; Harmon, 1994) including the type of mastitis case (i.e., clinical vs subclinical), infecting pathogen, stage of lactation, cow age, and stressors, have not been fully investigated as a whole (Kirkeby et al., 2020), though previous research does provide insight into the associations of SCC with IMI outcome, especially when antibiotic therapy is considered for intervention.

Studies show that when SCC levels are lower, antibiotic therapy for IMI tends to be more effective, indicative of an antibiotic:pathogen ratio effect. As SCC rises above 800,000 cells/mL, there is a 50% decrease in antibiotic cure rates (Bradley & Green, 2009). Additionally, a 5-fold difference was noted in the pre-treatment SCC between mastitis quarters that were treated with antibiotic therapy and failed to cure, compared to quarters that were treated and successfully cured (Nickerson et al., 2018). Utilizing SCC in treatment guidelines may be effective for increasing antibiotic cure rates, but more studies should be done to examine the practicality of SCC diagnosis. Therefore, the purpose of this research was to evaluate several elements which contribute to antibiotic success or failure, including SCC, and the association of SCC with antibiotic success of IMI treatment. Additionally, investigation of an unexplored niche, whether variation in SCC at the time of IMI could be related to resident microbiota in the milk/mammary gland, and the potential of manipulating the microbiota using alternative to antibiotics to promote mammary health in the dairy cow.

CHAPTER 2

LITERATURE REVIEW

Diagnosis and Treatment of Mastitis

Identification of Mastitis

Mastitis in cattle can be described in two ways: clinical or subclinical. Clinical mastitis (CM) cases show signs and symptoms including changes in the mammary gland, such as redness, heat, and swelling, and abnormalities in the milk including clots, flakes, and blood. Subclinical mastitis (SM) does not cause any visible changes in milk or the mammary gland but can still have many underlying consequences to the well-being of the animal such as changes in milk composition, reduced milk production, and increased SCC (Batavani & Sakka, 2007). While the proportionate mortality of mastitis remains lower (16.5%) in comparison to other reasons for cow death such as lameness or injury (20%), calving difficulty (17%), and unknown reasons (20%) (McConnel et al., 2008; USDA, 2007), mastitis can cause other detrimental effects to the animal and the operation, especially when considering economic productivity.

Past studies have demonstrated that mastitis remains the most expensive disease within the dairy industry in comparison to other diseases such as lameness and milk fever (Kossaibati & Esslemont, 1997). Researchers estimated the full cost of an individual CM case within the first month of lactation to be $444. Direct costs (e.g., veterinary services, additional labor, discarded milk, death loss, and therapeutics) accounted for $128 of this expense, with therapeutics accounting for almost 30% of this amount (Rollin et al., 2015).

Similarly, Heikkilä et al., (2012) found that veterinary services and therapeutics accounted for 31% of total costs of CM, while other studies estimated treatment-associated costs to represent 27.9% of total costs (Bar et al., 2008).

Indirect costs of mastitis are much more difficult to calculate as they are not 'visible' costs, and require accurate decision-making by the farmer to prevent long term economic loss (Hagnestam-Nielsen & Østergaard, 2009). Premature culling and replacement loss, future milk production loss, and future reproductive loss are all considered indirect consequences of mastitis, with past studies finding decreased milk production to be the largest expense due to IMI (Liang et al., 2017; Seegers et al., 2003). Rollin et al. (2015) found that within the first 30 days of lactation, almost 71% of the total cost of CM ($444) was attributed to indirect costs like decreased milk yield and future reproductive loss. It is estimated that short-term milk yield loss due to CM ranges from 0 to 100 kg of milk, while monthly yield loss ranged from 40 to 160 kg of milk (Seegers et al., 2003). Moreover, in a previous study, there was an increased risk of culling in cows that have experienced CM, with the highest risk attributed to cases occurring in early lactation and the dry period (Seegers et al., 2003). Aside from a premature reduction in herd size, the more cows that are culled due to diseases such as mastitis the greater the economic loss to the dairy farm.

Though costs are great as a result of CM, CM only represents a small portion of the disease in most herds (6.8%-25%), while SM has a much larger prevalence, ranging from 56.1%-73.1% (Mbindyo et al., 2020; Mdegela et al., 2009; Miles & Huson, 2021). It is normal to have 15-40 SM cases for every one CM case (Nickerson & Ryman, 2019). Due to the nature of the mammary gland and milk appearing normal during SM, it can be more

difficult to diagnose and resolve, representing a significant drain on a herd that is often not acknowledged or recognized as impacting the bottom line. As an example, SM is estimated to cause a constant production loss at approximately −91 kg per lactation for primiparous cows, and −181 kg per lactation for multiparous cows (Ruegg, 2017).

Adding to the challenges that SM presents, there have been many inconsistencies in how SM cases are diagnosed. The measuring of milk SCC is a standard method that indicates if inflammation is present within the mammary gland (Satu Pyörälä, 2003). The SCC thresholds for defining mastitis have changed several times throughout the years, as researchers have struggled to arrive at a consensus on the definition of "normal" milk (Ruegg, 2017). Several papers have used different methods of diagnosis of SM, such as having a SCC > 500,000 cells/mL (Batavani & Sakka, 2007), or other methods like cows that have recorded a SCC > 250,000 cells/mL twice in the past two months or recorded a SCC > 400,000 cells/mL once (Deluyker et al., 2005). However, not all cows with high SCC levels have bacterial IMI. Researchers have diagnosed SM using the California mastitis test, considering cows to be positive for mastitis if they had readings of (1+, 2+, 3+), which has been considered an accurate tool to diagnose IMI in cows due to its high specificity (86.2%) and sensitivity (88.5%) (Iraguha et al., 2017; Mbindyo et al., 2020). Similarly, microbiological culturing to identify the presence of bacteria is considered an accurate measure for diagnosis of SM, with researchers defining a cow to be positive when a pathogen is isolated from a milk sample (Mdegela et al., 2009). According to National Mastitis Council Guidelines, the diagnosis of SM is based on the SCC, with milk from clinically healthy cows almost always being < 200,000 cells/mL, and any higher SCC in conjunction with bacterial culture indicating IMI (2016).

Treatment of Mastitis

Antibiotics are used on dairy farms for both prevention and treatment of disease. Management practices associated with antibiotic use can vary from herd to herd, depending on individual goals of the producer. The use of antibiotics is a principal practice for the control and treatment of mastitis, and the most common use of antibiotics in dairy cattle (Grave et al., 1999). In a study examining overall antibiotic usage on dairy farms by Sawant et al, (2005), antibiotics were used to treat mastitis in 5% of heifers, 8% of cows in the dry period, and 14% of lactating cows in a single year. However, the basis for treatment of an infected quarter is variable between herds. In a survey examining the reasoning for antibiotic treatment of mastitis on farms, 66% of responders 'sometimes' cultured milk from the infected quarter or used another on-farm test for mastitis; although most producers based the decision for treatment upon visible signs rather than culture results (Raymond et al., 2006).

Antibiotic-mediated cures can be defined as clinical or bacteriological. A clinical cure is defined as no persisting symptoms during and post-therapy, but is not considered a reliable indicator of antibiotic success (Ruegg, 2021). Bacteriological cure is defined as having occurred when the pathogen that was isolated pre-treatment is not isolated from post-treatment samples. Consecutive samples are necessary due to the nature of some bacteria, like *Staphylococcus aureus*, that shed from infected mammary glands in cycles (Sears et al., 1990). While producers may consider antibiotic treatment successful due to clinical cure, it is possible the pathogen remains in the mammary gland in a dormant state, thereby mis-diagnosing the cow. Evaluation of SCC at the quarter level should be done for at least 21 days, as a gradual decline to a "healthy" SCC level should occur when

bacteriological cure is achieved (Ruegg, 2021). As treatment success is beginning to become more defined, the factors that affect antibiotic success need to be more thoroughly investigated.

Factors that Affect Antibiotic-Mediated Versus Spontaneous Cure

In order to promote judicious antibiotic usage, all factors that may influence the success of antibiotic treatment for mastitis need to be evaluated, including the possibility of a spontaneous cure. The success of antibiotic treatment varies based on several elements that will be discussed throughout this review: the type of infection, the pathogen causing the infection, and the individual cow SCC. Other factors such as management practices, lactation stage, and cow age may have a more significant impact on SCC and will also be discussed.

The Role of Infection Type on Antibiotic Treatment Success

The type of infection (i.e., clinical vs subclinical) can substantially impact the rate of success of any antibiotic regimen. Many studies have evaluated the efficacy of antibiotic treatment on cases of CM. In a study evaluating CM in large dairy herds by Oliveira et al. (2013), treatment cure was determined by assessment of bacterial cultures pre-treatment and post-treatment, with bacteria identified pre-treatment being culture negative on post-treatment samples. Results showed an overall treatment cure of 64.6% but differed depending on pathogen, with gram-negative pathogens having a much higher cure rate (75%) than gram positive pathogens (50.8%) (Oliveira et al., 2013). A more recent study that defined bacteriological cure in the same way as the previous study found similar results; an overall cure rate of 73.3% with gram-negative pathogens having the highest cure rate (Schmenger & Krömker, 2020). These studies demonstrate that for most CM cases

antibiotic therapy is valuable and improves animal performance. Although, it is important to remember that CM and SM need to be critically evaluated separately in terms of mastitis control and management as they differ in terms of frequency, severity, and duration.

The rationale for treatment of SM infections has proven to be more difficult to rationalize. While CM poses an urgent threat to mammary gland function and cow health, SM does not (Erskine et al., 2003), which has led to many debates on the value of antibiotic treatment of SM. Avoiding SM issues on farms may not be economically feasible if controlling pathogens and reducing bulk milk SCC by treating SM can save producers money (StRose et al., 2003). For example, treatment of contagious *Staphylococcus* and *Streptococcus* species that cause SM is advised (S Pyörälä, 2009) to reduce the number of CM infections and spread of contagious pathogens (StRose et al., 2003). On the other hand, past studies determined that the cure rates in SM cases that were treated vs those not treated were 75% and 68%, respectively (Wilson et al., 1999), a small difference in cure rates when considering the treatment costs. Using antibiotics to treat SM in which spontaneous cure rates are high has proven to be uneconomical, but if any existing IMI were not cleared from the mammary gland before the next lactation, it is possible that SM will be higher next lactation (Gruet et al., 2001) or the IMI will become chronic.

As indicated, duration of infection could impact cure rates after clinical presentation of mastitis without clarity as to whether this clinical presentation is evidence of a new IMI or could in fact be a result of a chronic infection with a clinical flare-up. Studies have shown that 64% of cows that have had two clinical cases of IMI within a lactation will have a third case before the end of that lactation (Nickerson & Ryman, 2019). If IMI persists for several months and is caused by the same pathogen or a recurrent IMI,

it is deemed chronic (Cheng & Han, 2020). The industry standard cure rate of antibiotic treatment normally settles around 50%, but drops to 35% in chronic infections (Barkema et al., 2006; Nickerson & Ryman, 2019). Most SM infections develop into chronic infections: having elevated SCC over 4-week intervals by the time they are diagnosed (Cobirka et al., 2020; StRose et al., 2003), but other studies may not agree this period is long enough to deem an infection to be chronic. Although, Erskine et al. (2003) agrees that antibiotic treatment of SM has a poor prognosis as many of these cases are chronic, therefore therapy would not be cost-effective. Researchers have concurred that control of chronic IMI should rely more heavily on prevention of new infections than antibiotic therapy (Gruet et al., 2001), but still include antibiotic intervention and bacteriological cure (N Wente et al., 2020).

The Role of Individual Pathogens on Antibiotic Treatment Success

The success of antibiotics varies based on the infecting pathogen due to differing physiology, intracellular structures, and presence of antimicrobial resistance genes of mammary gland bacteria, (e.g., penicillin-resistant vs penicillin-susceptible). Mastitis-causing pathogens have been described in past studies by several means: gram-negative vs gram-positive, major vs minor, and environmental vs contagious. Gram-negative bacteria, or the coliforms, such as *Escherichia coli*, are more resistant to antibiotic drugs that cannot penetrate the outer membrane of the bacteria. Whereas gram-positive bacteria (staphylococci, streptococci, coryneforms) do not have an impenetrable outer membrane. Researchers have reported a wide range of cure rates (38-100%) for gram-negative pathogens, possibly due to gram-negative pathogen *Klebsiella pneumoniae* having lower cure rates than *E. coli* (74% vs 98%) (Fuenzalida & Ruegg, 2019; Oliveira et al., 2013).

Klebsiella spp. are more resistant to treatment than *E. coli*, with some treatment labels having efficacy statements for *E. coli* but not *Klebsiella* spp. (Ruegg, 2021). However, the use of antibiotics to treat gram-negative pathogens like *E. coli* is up for debate due to the high rate of spontaneous cures (55.2-90%) (Ruegg, 2021; Smith et al., 1985). Some gram-positive pathogens such as *Staph. aureus* have a spontaneous cure rate close to 0%, making the value of antibiotic treatment much more significant (Ruegg, 2021). Studies have reported bacteriological cure rates varying from 50.8-63.5% for gram-positive pathogens, with higher cure rates attributed to *Streptococcus* spp. in comparison to *Staph. aureus* (Cattell et al., 2001; Oliveira et al., 2013; Schmenger & Krömker, 2020); differences in *Streptococcus* spp. and *Staphylococcus* spp. will be discussed in further detail in the next classification of pathogens. Gram-negative bacteria and gram-positive bacteria can be split into another mastitis pathogen classification, major vs minor pathogens, that also demonstrate noteworthy differences in treatment outcome.

Mastitis caused by major pathogens, *Staph. aureus, E. coli*, and environmental streptococci (*Streptococcus dysgalactiae, Streptococcus uberis)*, have been documented as causing the greatest loss in milk yield and reproductive efficiency (Dalanezi et al., 2020; Heikkilä et al., 2018). *Staph. aureus* is one of the most prevalent mastitis pathogens and is frequently isolated from cases of CM and SM, with milk production loss almost equal in both cases (Heikkilä et al., 2018). Treatment success of mastitis caused by *Staph. aureus* varies significantly with cure rates ranging from 38%-52% (Sol et al., 2000). Control of *Staph. aureus* has been a significant challenge to farms due to the limited antibiotic efficacy, but if the right quarters are chosen for treatment i.e., have greater potential for cure, antibiotic therapy can be justified. The cure rates of mastitis caused by *E. coli* in the

context of antibiotic therapy is less relevant due to the nature of the bacteria which does not respond to the most common broad-spectrum antibiotics. Most published results do not promote the use of antimicrobials for gram-negative bacteria species, especially when antimicrobials may increase selection pressure of animals producing bacteria that are resistant (Suojala et al., 2013). Lastly, environmental streptococci have shown to be the most common pathogen responsible for CM, and when regarding severe mastitis cases, *Strep. uberis* is detected just as frequently as *E. coli* and more than *Staph. aureus* (Oliveira et al., 2013; Schmenger & Krömker, 2020). In a study evaluating streptococcal cure rates, 10 out of 15 (66.7%) streptococci infections that were treated successfully cured, whereas only 6 out of 17 (35.3%) infections that were not treated cured spontaneously (Shephard et al., 2000). Similarly, researchers have found that environmental streptococci have a treatment cure rate of 52.8%, but when broken down into specific species in a later study, *Strep. dysgalactiae* had a cure rate of 82.9% and *Strep. uberis* had a lower cure rate of 73.9% (Oliveira et al., 2013; Schmenger & Krömker, 2020). Mastitis caused by contagious *Streptococcus agalactiae* will be investigated further when discussing environmental vs contagious pathogens.

Several pathogens are classified as minor mastitis pathogens, such as non-aureus staphylococci (NAS) and coryneforms (i.e. *Corynebacterium bovis*). The duration and chronicity of these minor pathogens may be similar to those of major pathogens, but IMI by NAS and *C. bovis* are usually less severe. In a study evaluating different types of NAS, 80.5% of NAS mastitis cases indicated persistence of the same infection for at least 10 months (Gillespie et al., 2009). Similarly, NAS had a 90-d recurrence rate of 25.7% in comparison to *E. coli* (26.9%), however NAS only caused 4.4% of severe infections while

30.5% were caused by *E. coli* (Oliveira et al., 2013; Schmenger & Krömker, 2020). As discussed, minor pathogens may occur just as frequently or more than the major pathogens, but the likelihood of antibiotic success is much greater. When examining antibiotic success across five different treatment products at both short-duration and long-duration treatment times, it was determined that NAS had the highest cure rate (85.7%), followed by environmental streptococci (36.4%), and then *Staph. aureus* (25%) (Nickerson et al., 2018). While minor pathogens may have higher cure rates than the major pathogens, recent studies concluded that the losses associated with minor pathogens have been vastly underestimated, with NAS resulting in 5.7% loss of the 305-d milk yield in comparison to 10.6% loss caused by *E. coli*. (Heikkilä et al., 2018). It is pertinent that producers know what pathogen is causing mastitis in each individual cow to effectively treat the IMI, as antibiotic success varies with each pathogen.

Mastitis-causing pathogens can be further delineated into environmental and contagious pathogens. Management practices have a large impact in determining which pathogen is causing IMI. *Staph. aureus* and *Strep. agalactiae* are both contagious pathogens that spread from cow to cow, and presence is highly influenced by improper management inside the milking parlor. Dirty towels and hands, as well as the milking machine itself, can all act as reservoirs. However, the prevalence of IMI caused by *Strep. agalactiae* and *Staph. aureus* has declined with advances in milking machines and elimination of improper milking techniques (Ruegg, 2017). As contagious pathogen-caused IMI occurrence has dwindled over the years, gram-negative pathogens and environmental streptococci have become the prevalent diagnosis of mastitis studies (Oliveira et al., 2013; Ruegg, 2017; Schmenger & Krömker, 2020). Environmental

pathogens that come from the cow's environment, such as *Strep. uberis* and *Strep. dysgalactiae,* NAS, and coliforms, are influenced by management of soil, bedding, and other factors outside the parlor. While originally described as environmental, there have been recent debates that *Strep. dysgalactiae* and *Strep. uberis* may occur as both contagious and environmental pathogens (Nicole Wente & Krömker, 2020; Zadoks et al., 2003). In either classification, contagious or environmental, prevention is the key to controlling these pathogens, as treatment is costly and not always successful. A clean environment, proper milking parlor hygiene, and appropriate milking machine function is required to reduce the risks of mastitis and lower bulk tank SCC.

SCC and Treatment Outcome

The somatic cells present in normal bovine milk generally include macrophages, lymphocytes, neutrophils, and epithelial cells. While neutrophils make up 1%-11% of total SCC in a healthy gland, this number may increase up to 90% in a quarter with an IMI (Satu Pyörälä, 2003; Ruegg, 2003). This increase is due to the inflammatory response to invading bacteria, attracting neutrophils into the gland to engulf the pathogen. Many cows maintain SCC values that are less than 100,000 cells/mL, although a strong indicator of mastitis is if the SCC exceeds 200,000 cells/ml (National Mastitis Council, 2016). Results from recent studies showed that a higher SCC value is correlated with increasing milk yield losses, concluding that SCC levels should be used in treatment and culling decisions (Hadrich et al., 2018). Producers will make most decisions for mastitis treatment based on expected losses to revenue, therefore early determination of antibiotic treatment outcome would be valued.

Several investigations have examined treatment and respective cure rates based on SCC levels. Models have shown that the probability of bacteriologic cure after treatment decreased from 40% for cows with a pre-treatment SCC of <200,000 cells/mL to 27% for cows with a pre-treatment SCC of 200,000 cells/mL to 800,000 cells/mL (Bradley & Green, 2009), indicating pre-treatment SCC is a useful predictor of treatment outcome. In a preliminary study, infected quarters that cured with antibiotic therapy had a lower average SCC (587,000 cells/mL) on day 0 of treatment, while quarters that failed to cure had a much higher SCC average (2,994,000 cells/mL) on day 0 of treatment, suggesting that an established SCC threshold may aid in estimating treatment success (Nickerson et al., 2018). Further, Sol et al. (2000) found that *Staph. aureus* infected cows with a lower pre-treatment SCC had higher bacteriological cure rates in comparison to cows with a higher pre-treatment SCC, although averages were not given. Similarly, in a review of antibiotic success of *Staph. aureus* by Barkema et al. (2006), almost all studies reported that if pre-treatment SCC was lower, bacteriological cure rate was higher.

Evaluation of the efficacy of mastitis treatment on cattle with high SCC on DHI reports (400,000 cells/mL) showed some interesting results (Timms & Schultz, 1984). Cows were separated into a high SCC group or a clinical symptom group (Figure 1), with no significant decrease in SCC post-treatment within the high SCC group (Timms & Schultz, 1984). The bacteriological cure rate was low (23.3%), suggesting that treating cows with high SCC values may only be desirable in cases occurring in young cows within early lactation. Shephard et al. (2000) found similar results by examining cows with SCC above 500,000 cells/mL, with treatment offering almost no financial gain to producers due to low cure rates. Although, it is important to remember that high SCC cows may have

been chronically infected and not shown clinical signs of mastitis at time of treatment, thus explaining the lack of change in SCC over time.

The impact of SCC on cure rates, no matter the cause, is directly affected by factors which influence either basal SCC or changes in SCC. An explanation for why a higher pre-treatment SCC has an adverse effect on antibiotic treatment success could be due to an influx of cells when immune function is insufficient in immunologically stressed animals, conversely, the duration and severity of IMI may also be relevant (Bradley & Green, 2009). The identity of the infecting pathogen plays a key role in the duration and severity of IMI as discussed previously, and pathogenicity can have its own contribution to SCC that will impact treatment success.

Impact of SCC on Cure Rates

Infecting Pathogen

Past studies have reported that quarters free of IMI have a SCC ranging from 113,000 to 251,000 cells/mL, while quarters harboring bacteria had a SCC range of 190,000 to 519,000 cells/mL (Dohoo & Meek, 1982). However, when the bacteria were separated into major and minor pathogens, quarters infected with major pathogens produced, on average, a SCC > 600,000 cells/mL compared to that of minor pathogens with a SCC range of 100,000 to 300,000 cells/mL. The major pathogens (*Staph. aureus*, environmental streptococci, coliforms) cause the greatest SCC increase, while the minor pathogens (*C. bovis*, NAS) only moderately increase the SCC (Harmon, 1994). A more recent study examined both major and minor pathogens, as well as other uncommon mastitis pathogens (gram-negative and gram-positive species, mycoplasmas, *Candida tropicalis, Prototheca sp*.) in several herds. In herd 1, major pathogens had an average SCC

of 327,000 cells/mL, minor pathogens averaged at 172,000 cells/mL, and other pathogens averaged at 497,000 cells/mL, while herd 2 had an average SCC of 373,000 cells/mL, 230,000 cells/mL, and 295,000 cells/mL, respectively (Kirkeby et al., 2020). Major pathogens cause a notably higher SCC than minor pathogens, while the less common pathogens can have variable SCC, and need to be investigated more thoroughly.

Other researchers have focused on individual bacterial species rather than grouping into major and minor pathogens and have found similar results. Coagulase-negative staphylococci have proven to be associated with lower SCC at the time of treatment in both quarters that cure and quarters that fail to cure in comparison with *Staph. aureus* and environmental streptococci, with quarters that failed to cure having pre-treatment SCC at least 2-3 times greater than quarters that successfully cured (Nickerson et al., 2018). Not only does coagulase-negative staphylococci cause less severe IMI than other mastitis pathogens, but are also associated with lower SCC, possibly explaining why the chances of antibiotic cure are much higher in comparison to other pathogens. In a study evaluating all microorganisms (including fungi and algae) the average SCC value was lowest for IMI caused by cornyeforms, NAS, and mold, and was highest for IMI caused by gram-negative pathogens and *Streptococcus* spp. (Wilson et al., 1997). Yeasts, algae, and *Staph. aureus* had average SCC between the lowest and highest groups, however *Staph. aureus* had the largest range of SCC values, from 191,000 cells/mL to 9,433,000 cells/mL. Over 75% of the IMI in this study were caused by *Streptococcus* spp., *Staph. aureus*, and NAS (Wilson et al., 1997), although it must be reiterated that more recent studies have found reductions in IMI caused by *Staph. aureus* and increases in the prevalence of gram-negative pathogens. While NAS and gram-negative pathogens are similar in terms of frequency,

gram-negative pathogens are associated with higher SCC values that may reflect upon the severity of these infections in comparison to infections by NAS. Importantly, SCC is significantly altered by the individual pathogen causing mastitis and pathogen identity should be considered when interpreting SCC in relation to mastitis detection. There are also different economic impacts to the producer depending on the microorganism at play, confirming it is crucial to know what organism is causing IMI in order to reduce herd SCC levels, increase mastitis cure rates, and decrease costs associated with mastitis.

Lactation Number/Age

The age of the cow and SCC are positively correlated, and both risk factors for antibiotic cure. The probability of a cure using antibiotic treatment has been noted to decrease with an increase in both SCC and cow age (Sol et al., 1994). As the age of the cow increases, SCC tends to increase, possibly due to the increased number of infected quarters over time and the development of tissue damage from chronic infections (Alhussien & Dang, 2018). Correspondingly, the parity of the cow is also a risk factor for antibiotic cure and will also be discussed in terms of association with SCC.

Barkema et al. (1998) evaluated the incidence of CM in several herds. First-calf heifers had the lowest incidence rate, whereas cows that calved eight times had the highest incidence rate, with rates increasing linearly as parity increased. Similarly, Deluyker et al. (2005) found a significant interaction between SCC levels pre- and post-treatment and calving parity. Higher SCC levels prior to treatment resulted in larger differences in SCC post-treatment between parities (Deluyker et al., 2005). This was suggested to be impacted by cure rates decreasing with cow age, and that the magnitude in SCC reduction that can be achieved is less in older cows than younger cows, in agreement with a more recent

review by Alhussien & Dang (2018) on factors influencing SCC. Issues may arise with older cows being prone to more IMI because their SCC typically remains higher overall, potentially increasing the probability of developing chronic infections (Alhussien & Dang, 2018). Likewise, previous work shows that when cows were separated into groups by age and IMI status (Figure 2), milk from uninfected quarters displays little change in SCC as the number of lactations increase/as the cow ages, however there is a significant increase in SCC when the quarter is infected with either minor or major pathogens (Harmon, 1994; Eberhart et al., 1979). This suggests that while parity/age has an impact on SCC, the inclusion of minor and major pathogens can significantly change the extensiveness of that impact.

Not only has SCC been evaluated across lactations or parities, but within individual lactations as well. Immediately following parturition, SCC levels are usually elevated due to changes coinciding with calving and colostrum production or even dysfunctions as a result of stresses but decreases to a normal level a few days after calving in a healthy mammary quarter (Alhussien & Dang, 2018). It has been shown that SCC remains high in infected quarters after these first few days, thus indicating that SCC should be useful in detecting new IMI early in lactation (Dohoo & Meek, 1982; Satu Pyörälä, 2003). When looking at lactation curves based on SCC, SCC is high right after parturition, decreases to a minimum value around 50 days in milk, and slowly increases again towards the end of lactation (de Haas et al., 2002). The effect of CM on the lactation curve and SCC was large, but differed based upon the causative pathogen and the parity of the cow; multiparous cows had higher SCC at each day in milk than did first lactation heifers (de Haas et al., 2002), providing more evidence that older cows have higher SCC overall. The SCC was much

lower in lactations without a case of both CM and SM in comparison to all lactations and lactations without a case of CM, and the difference between the SCC of each lactation curve was much smaller in primiparous than multiparous cows (de Haas et al., 2002). This data explains that SCC may have a larger effect on CM than SM, but the size of that effect depends on the infecting pathogen and associated SCC, suggesting that test-day SCC may be useful in indicating IMI in cows with different parities and inclusion of pathogen characteristics could help develop current mastitis control programs.

Relationship of SCC with Mammary and/or Milk Microbiota

Characterization of IMI causing bacteria is an essential component influencing the disease's treatment outcome. Classically, bacteriological culture has been used to assess the causative agent but has proven to have its limitations, with reports of at least 20-30% of milk samples from CM having no growth after 48 hours (Taponen et al., 2009). To overcome this, DNA-based, culture-independent methods are now used to identify microbial species/pathogens present. While the mammary gland of healthy cows was previously considered to be a sterile environment, more recent investigations using DNA-based molecular methods have discovered the mammary gland has a natural population of microbes (Addis et al., 2016). DNA sequencing of bacteria isolated from healthy quarters identified bacteria in milk; a) known to cause IMI, b) bacteria that are not known to cause IMI, c) bacteria that are currently unknown pathogens, as well as d) an anaerobic bacterial community (Oikonomou et al., 2012), confirming the existence of a resident natural community of microbes in the mammary gland.

While some progress has been made in assessment of the native milk microbiome, little research has been published investigating the possible associations between the

microbiome and milk quality metrics. A study by Rodrigues et al. (2017) focused on the relationship between bulk tank milk microbiome and SCC, with gram-negative pathogens and *Streptococcus* increasing in relative abundance with an increase in SCC, consistent with their role as mastitis pathogens. A high abundance of bacterial family *Ruminococcaceae* was reported in all 4 SCC quartiles (=54,000 cells/mL; 120,000 cells/mL; 170,000 cells/mL; 450,000 cells/mL) (Rodrigues et al., 2017). Ruminococci are fiber-degrading bacteria that are typically associated with the rumen and hindgut, indicating a need for further investigation of the role of this bacterial family in the milk microbiome of healthy cows and cows with IMI. It is possible that bulk tank bacteria may originate from outside the gland, such as milk from dirty cows or a dirty milking machine, however similar results of shifting pathogen abundance were also seen when evaluating individual cow milk samples. Significant differences were found between the milk microbial populations of healthy cows vs IMI cows (SCC<200,000 cells/mL vs SCC>200,000 cells/mL), indicating that changes of milk microbial communities is related to the health status of the cow (Scarsella et al., 2021). With evidence that the milk microbiome changes depending on the health condition of the mammary gland, these instances highlight the potential enormity of the milk microbiota's impact on dairy cow health. More thorough investigation of the milk microbiome and use of SCC to determine different health statuses of the mammary gland could help promote judicious antibiotic usage by modifying the milk microbiome to prevent common mastitis pathogens. Research of the milk microbiome and SCC associated with both healthy and infected quarters will assist in identifying when it is appropriate to administer antibiotic therapy, ultimately

increasing cure rates and allowing producers to make culling decisions with increased confidence.

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Table 2.1. Cow quarter somatic cell (SCC) profiles for 3-wk monitoring period; N = 74. (Adapted from Timms & Schultz, 1984).

		% Quarter cell counts < 400,000 cells/mL	
Group	Treatment day	1 wk post treatment	2 wk post treatment
High somatic cells	7.0	9.5	10.0
Clinical	1.9	32.7[a]	40.0[a]

[a]Different from treatment day (P<0.01).

Table 2.2. Mean somatic cell count (SCC) by cow infection status and age (Adapted from Eberhart et al., 1979; Harmon, 1994).

	Infection Status ($x10^3$/mL)			
Age (yr)	All cows (3130)	None	Minor Pathogens	Major Pathogens
2	232	126	190	614
3	314	149	218	661
4	390	148	233	753
5	564	180	308	977
6	544	194	322	880
7	654	251	320	986
>7	868	113	519	1207

CHAPTER 3

ASSOCIATION OF PRE-TREATMENT SOMATIC CELL COUNTS WITH BACTERIOLOGICAL CURE FOLLOWING DIAGNOSIS OF INTRAMAMMARY INFECTION

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[1]Williamson, J. R., E. Rollin, T. R. Callaway, V. E. Ryman. To be submitted to *Research in Veterinary Science*.

Abstract

Antibiotic administration is crucial to ensure the health and productivity of dairy cattle. Mastitis is a disease that is typically a result of an intramammary infection (IMI), and antibiotic regimens are implemented to aide in curing IMI. Diagnosis is usually by detection of elevated milk somatic cell counts (SCC) and/or presence of culturable pathogens in the milk. Antibiotic treatment success is associated with the SCC at the time of treatment, though this correlation is still poorly understood. The objective of this project was to evaluate pre-treatment SCC and its association with IMI cure incidence following a standard antibiotic treatment. We hypothesized that pre-treatment SCC would be significantly lower in cases where the IMI ultimately cured compared to cases where the IMI failed to cure. Aseptic milk samples were collected from lactating cow quarters experiencing clinical or subclinical mastitis (n=52). Clinical mastitis was diagnosed by a trained milking technician and subclinical mastitis was diagnosed at the quarter level as a SCC > 200,000 cells/mL and bacterial growth present in milk at time of treatment. After collection of the day 0 (D0) milk samples, the SCC was enumerated, and the milk sample cultured. Intramammary antibiotic therapy SPECTRAMAST® LC was administered once/day for 5 days. Post-treatment samples were collected 14 d (D14) and 28 d (D28) later. A bacteriological cure was confirmed when both the D14 and D28 samples were free of culturable pathogens. The overall cure rate was 46.2%. Interestingly, the cure rates of antibiotic therapy decreased as pre-treatment SCC increased. Quarters that experienced bacteriological cure demonstrated a lower pre-treatment SCC (507,041 cells/mL ± 127.86 SEM, P = 0.01) compared to cows that did not cure, which had high pre-treatment SCC (1,640,392 cells/mL ± 333.28 SEM). Quarters that failed to cure had higher values 28 days

post-treatment in comparison to quarters that cured (P<0.001). Future studies should investigate whether we can develop unique SCC-dependent mastitis treatment protocols which increase mastitis cure rates and enhance overall mammary health.

Key words: somatic cell count, mastitis, antibiotic treatment, cure, intramammary

Abbreviation key: IMI = intramammary infection, **SCC** = somatic cell count, **CM =** clinical mastitis, **SM** = subclinical mastitis, **DIM** = days in milk, **MSA** = mannitol salt agar, **NAS** = non-aureus staphylococci

Introduction

Mastitis, a disease defined as inflammation of the mammary gland, is costly to dairy producers because of decreased milk production, discarded milk, and need for therapeutics. Although mastitis can occur from physical or chemical trauma to the mammary gland, it is most commonly caused by infectious pathogens, such as bacteria that cause intramammary infection (IMI). Antibiotics are often used to treat these bacterial infections and can account for almost 30% of direct costs for mastitis (Rollin et al., 2015). While treatment may be costly, effective antibiotic therapy is imperative to ensure the health and productivity of the dairy herd.

Mastitis may be diagnosed early by visual inspection of abnormal milk, detection of elevated milk somatic cell counts (SCC), and/or growth of culturable pathogens in milk. The SCC represents the total number of cells per mL of milk, predominately white blood cells. Once pathogens are detected in the mammary gland by resident mammary and immune cells, an influx of white blood cells into the mammary gland results in a precipitous increase in SCC (Ezzat Alnakip et al., 2014). Interestingly, past studies have demonstrated that there is a 50% decrease in cure rates following antibiotic administration when the SCC $\geq$ 800,000 cells/mL (Bradley & Green, 2009). Moreover, SCC levels prior to treatment were associated with success or failure of the antibiotic regimen, with SCC of quarters that failed to cure after treatment being 5-fold greater than those that successfully cured (Nickerson et al., 2018). This suggested that utilization of SCC for treatment guidelines could increase antibiotic cure rates and be a viable managerial strategy for dairy producers.

Utilization of SCC as a decision-making tool for treatment may be impacted by several factors, which include but are not limited to, the type of infection (i.e., clinical or

subclinical), chronicity of infection, the specific pathogen, lactation number, and days in milk (DIM) (Kirkeby et al., 2020). In order to promote judicious usage of antibiotics by use of pre-treatment SCC values, all factors that may influence SCC and antibiotic cure rates need to be evaluated.

Our present objective was to identify differences in pre-treatment SCC levels and their association with bacteriological cure following a standard antibiotic regimen. We hypothesized that the SCC of cured quarters following antibiotic treatment would be significantly lower than quarters that did not cure. The overall goal of this work is to enhance the cure rates of a standard antibiotic regimen, SPECTRAMAST® LC, for mastitis by developing SCC-based treatment guidelines, potentially reducing costs associated with mastitis and ensuring prudent usage of antibiotics.

Materials and methods

All procedures involving live animals were verified and approved by the University of Georgia's Office of Animal Care and Use (AUP #A2020 06-029-Y2-A1). The dairy cattle used in this study were located at the University of Georgia Teaching Dairy in Winterville, GA (33°54'32.9"N 83°14'50.9"W).

Animal selection and diagnoses

Milk from mammary quarters of lactating Holstein and Jersey cows (n = 52) presenting with clinical mastitis (n = 14) or subclinical mastitis (n = 38) were aseptically collected at the UGA Teaching Dairy. Subclinical mastitis quarters were identified by increased SCC at the monthly test date (> 200,000 cells/mL) and presence of bacteria growth (see below). Intramammary treatment with 10 mL of lactating cow antibiotic therapy (SPECTRAMAST® LC; Zoetis, Parsippany-Troy Hills, NJ, USA) was infused

into the infected quarter once/day for a total of 5 days. Samples were refrigerated during transport to the UGA Mastitis Lab on wet ice. Milk SCC of the sample was determined using a DeLaval Direct Cell Counter (DeLaval, Tumba, Sweden). Milk samples were cultured on trypticase soy agar plates with 5% sheep blood and incubated at 37°C. Samples were also plated on mannitol salt agar (MSA) and MacConkey agar plates, to aide in presumptive identification.

After 24- and 48-hr of incubation, plates were examined prior to further diagnostic tests where necessary(Hogan et al., 1999). If samples did not display bacterial growth on D0 after incubation or displayed any other pathogen growth not of bacterial origin, they were removed from the study. Samples were collected 14 (D14) and 28 days (D28) post-antibiotic treatment. Bacterial cultures and SCC enumeration were performed on each day as described above, and the success or failure of the antibiotic was determined. A quarter was officially labeled as "Cured" if milk from both D14 and D28 were free of bacterial growth and labeled "Failed to Cure" if bacterial growth was detected in milk on one or both days.

Statistical analysis

A two-sample t-test was performed to compare pre-treatment SCC of quarters that cured vs. pre-treatment SCC of quarters that failed to cure. The SCC over time of quarters that either cured or failed to cure were compared using 2-way ANOVA. Cure rates were calculated regardless of infectious agent, as well as calculated between pathogens. Correlations were tested on overall cure rates based on SCC range. A chi-square test for trend was utilized to analyze cure rates by DIM and cure rates by lactation number. The significance of cure rates in chronic vs new, clinical vs subclinical, and major pathogens

vs minor pathogens was assessed with a Fisher's exact test. Two-sample t-tests were used to identify a relationship between pathogen-specific pre-treatment SCC and the outcome of treatment. The relationship between the outcome of treatment and pre-treatment SCC dependent on the type of mastitis case (i.e., clinical vs subclinical), days in milk (DIM), lactation number, and chronicity was also evaluated using 2-way ANOVA. A SCC threshold value for treatment was predetermined based on overall SCC levels irrespective of pathogen. Differences were considered significant at $P \leq 0.05$ using Tukey's pairwise comparisons.

Results and discussion

SCC and cure rates

The D0 pre-treatment SCC of quarters that were cured by D28 was significantly lower (507,041 cells/mL ± 127.86 SEM) than quarters that failed to cure (1,640,392 cells/mL ± 333.28 SEM) (P=0.01; Figure 1). In quarters that cured, SCC was lower on both D14 and D28, in contrast to a decline on D14 and then increase by D28 in quarters that failed to cure (Figure 2). Previous investigation of SCC prior-to and post- antibiotic treatment found similar results, with no evidence of SCC returning to high pre-treatment levels by D28 in quarters that successfully cured (Deluyker et al., 2005).

Quarters that failed to cure had significantly higher cell counts on D28 ($P<0.01$) than quarters that successfully cured. Deluyker et al. (2005) also reported decreases in SCC by D28 in quarters that cured with a 2-day antibiotic treatment, but not in quarters that failed to cure. However, Deluyker et al. (2005) also reported decreases in SCC by D28 in quarters that cured as well as quarters that failed to cure when the antibiotic treatment period was 8 days. In contrast, the present study utilized a treatment period of 5 days,

whereas some research suggests a period of 8 days may be more effective, especially in quarters infected with major pathogens, such as *Staphylococcus aureus* (Barlow et al., 2013). However, the costs associated with a longer duration of antibiotic therapy must be considered. A blanket policy of longer duration of antibiotic therapy may not be beneficial for all IMI or pathogens. Previous evidence demonstrates that in order to minimize losses associated with treatment and promote responsible antibiotic usage, short-duration antibiotic therapy should be implemented when etiology is unknown; however, if the mastitis case is culture-negative or caused by *Escherichia coli*, it is unlikely that cases will benefit from antibiotic treatment (Ruegg, 2020).

The overall cure rate of enrolled cases in the present study was 46.2%. Results showed that as pre-treatment SCC increased, the cure rates following a 5d antibiotic therapy significantly decreased ($P=0.0074$, $r=0.9661$, $r^2=0.9334$; Figure 3). This is in agreement with past models by Bradley and Green (2009) which demonstrated the probability of bacteriological cure post-treatment decreased by 13% when pre-treatment SCC was above 200,000 cells/mL. In the present study, we found a 33.00% decrease in bacteriological cure rates when the pre-treatment SCC was > 200,000 cells/mL (Figure 4). When the SCC was > 500,000 cells/mL, the bacteriological cure rate dropped to 0.00%. Surprisingly, the bacteriological cure rate increased again to 46.15% when evaluating SCC between 1 and 3 million cells/mL. It is unclear why no infections were cured by antibiotic treatment when SCC levels were mildly elevated (200,001 to 500,000 cells/mL). The dichotomy in results identified in our study compared to Bradley and Green (2009) (13% vs 33 % decrease) are most likely related to the differences in the inclusion and exclusion criteria for each study as well as the differences in sample size. The current study included

subclinical cases and cows with multiple quarters infected, whereas Bradley and Green (2009) did not. While the diverse inclusion criteria of the current study render results challenging to interpret, the enrolled quarters and cows represent realistic populations in dairy herds. Moreover, the current study focused on a singular antibiotic regimen, whereas Bradley and Green (2009) investigated 3 antibiotic regimens available in the UK. The variations in antibiotic treatment regimen chosen are also impactful. Milne et al. (2005) found that only 49% of mastitis cases of *Streptococcus uberis* were successfully cured following a single initial treatment. However, when the quarters that failed to cure were given an extended treatment (3 d), 55% of the persistent cases successfully responded, suggesting prolonged administration of antibiotics would improve cure rates. In contrast, minor pathogens, such as NAS, may cure with a short treatment (2-3 d) regimen, resulting in decreased administration of antibiotics and reduced costs attributed to the case of mastitis.

Similarly to the current study, a bacteriological cure rate for mastitis was 52% in the study by Sol et al. (2000), with the highest bacteriological cure rate attributed to quarters with low logSCC (LnSCC = 4.07-4.69) prior to treatment. Although, post-treatment samples were only taken 14 d after the last treatment. The present study identified many quarters with no pathogen growth at D14 post-treatment, which could thus be considered cured, but still demonstrated elevated SCC values. Many of these samples still had bacterial growth at D28, indicating that if only a single post-antibiotic treatment sample was collected, the bacteriological cure rate would have been overestimated. While evidence from the current study suggests SCC has the potential for use as an indicator of treatment success, misclassification of persistent infections is possible, meaning it is possible that no

pathogen growth was detected at D14 or D28, but could instead be cultured weeks later. Some studies have evaluated samples post-treatment up to 36 d, and found that 28 of 82 mastitis quarters remained clinical by 36 d (Roberson et al., 2004). Additionally, California Mastitis Test scores remained elevated for an extended period, with only 42% of infected quarters returning to a 'trace' score at 36 d (Roberson et al., 2004). Timms & Schultz (1984) found similar results, by D14 40% of clinical cases returned to a SCC < 400,000 cells/mL. In the present study, 60.0% of infected quarters had SCC < 400,000 cells/mL by D14, though this number decreased to 56.0% by D28. This decline is mostly due to quarters that failed to cure, with 39.3% of quarters having SCC < 400,000 cells/mL at D14 but decreasing to 28.6% by D28. If these quarters were deemed "cured" at D14 but remained infected, the cow would continue to contribute to elevated bulk milk SCC values and potentially go unnoticed as subclinical until monthly test-day SCC was taken. Our results demonstrate the importance of taking multiple post-antibiotic treatment samples, including both SCC and microbiology, and it is suggested that future studies include an additional post-treatment sample potentially up to 42 d post-antibiotic treatment. Practically, in herds that culture milk samples from clinical mastitis or subclinical mastitis quarters, it may be prudent to collect repeated samples following antibiotic therapy to ensure that antibiotic therapy was effective.

SCC and infecting pathogen

Quarters were grouped into major and minor pathogen infections, gram-negative infections, and dual infections (having two infecting pathogens in one quarter) based on the culture results from the pre-antibiotic treatment diagnosis. Major pathogens included *Staph. aureus* and environmental streptococci, whereas minor pathogens included all NAS

species. All gram-negative infections were presumptively identified to be *E. coli*. Minor pathogen infections (44.2%) were the most common, though followed closely by major (42.3%), dual (7.7%), and gram-negative species (5.8%; Figure 5). *Staph. aureus* is one of the most prevalent mastitis pathogens and environmental streptococci are the most common pathogen responsible for cases of clinical mastitis (Heikkilä et al., 2018; Oliveira et al., 2013; Schmenger & Krömker, 2020). However, it is common for minor mastitis pathogens to have a higher prevalence than other pathogens, with coagulase-negative staphylococci (a type of NAS) previously described as making up 42.8% of isolates, major mastitis pathogens (37.9%), and gram-negative species (0.7%) (Mbindyo et al., 2020).

Pathogen-specific differences in SCC values prior to therapy have been found to be significant or trending (Nickerson et al., 2018). In the present study, while the pre-antibiotic treatment SCC values of all pathogens that failed to cure were numerically higher than the pre-treatment SCC values that successfully cured, there were no differences of SCC between quarters that cured vs. failed to cure within any infecting pathogen group (Figure 6). One limiting factor for interpretation of this data is the limited sample size and distribution of data. Nonetheless, major pathogens had numerically higher SCC values in both quarters that cured and failed to cure in comparison to minor pathogens, agreeing with past studies by Dohoo & Meek (1982) and Harmon (1994). Additionally, gram-negative pathogens in quarters that failed to cure resulted in the highest SCC values in comparison to all other groups, similarly reported by Wilson et al. (1997).

Antibiotic efficacy varies depending on pathogen (S Pyörälä, 2009), and in the present study the pathogen-specific cure rates for minor pathogen infections most successfully cured (60.9%), followed by major pathogens (36.4%), gram-negative

pathogens (33.3%), and dual pathogen infections (25.0%; Figure 7). Additionally, a Fisher's exact test showed the cure rates of major vs minor pathogens was close to trending (P=0.139). While pre-treatment SCC values were not different between any infecting pathogens, there were noticeable impacts of pathogens on antibiotic cure rates. Other researchers have published data showing similar results, with NAS infections having the highest cure rate, followed by major gram-positive mastitis pathogens (Nickerson et al., 2018). However, past studies demonstrated that gram-negative pathogens had the highest cure rate in comparison to gram-positive pathogens and other pathogens (Oliveira et al., 2013; Schmenger & Krömker, 2020). While some antibiotic treatments have efficacy statements for gram-negative pathogens (e.g., *E. coli*), the use of antibiotics to treat these pathogens has not been promoted in past studies by Suojala et al. (2013) and may not be effective, because the spontaneous cure rate of gram-negative pathogens ranges from 55.2-90% (Ruegg, 2021; Smith et al., 1985). Disagreements with the current study is likely due to our specific IMI pathogen distribution, with gram-negative pathogens responsible only for 6% of all infections.

SCC and type of mastitis

Clinical mastitis (CM) presents as visible changes in the mammary gland and/or the milk, but subclinical mastitis (SM) is not visible with the naked eye, rather is identified by a rise in SCC above 200,000 cells/mL or presence of pathogens in the milk (Bradley & Green, 2009). While antibiotic therapy for CM has proven to be effective in most cases, the basis for treatment of SM is still debated, due to variations in what is considered a 'normal' milk SCC value (Ruegg, 2017; Schmenger & Krömker, 2020). However, if SM cases are untreated, they can become chronic. In the current study, the average D0 pre-

treatment SCC in CM that was cured was similar to SM quarters that were cured (521,250 cells/mL ± 301.45 SEM vs. 504,200 cells/mL ± 137.56 SEM respectively). The average D0 SCC of CM that failed to cure was significantly greater than all other groups ($P<0.01$; Figure 8). It is not without possibility that clinical SCC values could be impacted by the characteristics of samples, including clots, flakes, etc. (Malek dos Reis et al., 2013) resulting in artificially low or artificially high SCC.

A low antibiotic cure rate (28.57%) was seen in CM for the current study that has not been previously reported before, in contrast, SM demonstrated an antibiotic cure rate of 52.6% ($P=0.209$). These differences in cure rates between CM and SM, may be explained by a substantially greater major pathogen load (83.3% were major pathogens) in the CM cows that which impacted cure rates compared with SM cows (38.9% were major pathogens) (Oliveira et al., 2013). Nonetheless, this data presents evidence that rather than considering only an overall SCC threshold value, distinct guidelines relative to the type of mastitis (e.g., CM or SM) at time of diagnosis need to be investigated. Further studies focusing on SM cases diagnosed to the causative pathogen may be revealing as major pathogens are less responsive to antibiotic treatment. Though sample size is smaller, our study in fact did show that 35.0% of SM were caused by major pathogens and successfully cured, while 38.9% of SM were caused by major pathogens and failed to cure.

SCC and DIM

There are associations between SCC values and DIM (Alhussien & Dang, 2018; de Haas et al., 2002), however these studies did not focus investigations on SCC prior to therapy. In the current study, enrolled quarters were broken down into groups to represent early-, mid-, and late-lactation, respectively: 0-100 DIM, 101-200 DIM, and 201+ DIM.

Quarters in early lactation (0-100) that failed to cure had D0 pre-treatment SCC values that were numerically higher than the pre-treatment SCC values of quarters that successfully cured (Figure 9). After parturition, SCC may be elevated in the absence of infection due to metabolic, physiologic, and immunologic stresses (Alhussien & Dang, 2018; de Haas et al., 2002). However, SCC of infected quarters will continue to demonstrate elevated values, confirming that pre-treatment SCC can be effective as a treatment guideline for cows during early lactation (Alhussien & Dang, 2018; Satu Pyörälä, 2003). Mastitis during early lactation can have profoundly negative consequences on milk production in the current and subsequent lactations. De Vliegher et al. (2005a, 2005b) demonstrated that a one unit increase of the natural log-transformed SCC was associated with a decrease of 0.13 kg/d of milk yield later in the first lactation and a 26% increase in culling risk during the first lactation. Additionally, a one unit increase of the natural log-transformed SCC also resulted in an increase of natural log-transformed test-day SCC by 0.22 unit, with the probability of having test day SCC > 200,000 cells/mL during the whole first lactation increasing when the natural-log transformed SCC increased during early lactation (De Vliegher et al., 2004).

Antibiotic cure rates based on DIM demonstrated that mid-lactation cows had the highest cure rate (58.3%), followed by early-lactation cows (45.0%) and late-lactation cows (40.0%), with a trending decrease in cure rates (P=0.101). Mid-lactation cows are normally not as metabolically or immunologically stressed in comparison to early lactation cows, especially following peak production occurring around 60-90 DIM, which may explain the greater rate of antibiotic cure during mid-lactation. A recent study demonstrated the bacteriological cure rate of CM was highest in late-lactation cows, and was only greater for cows in early-lactation when the sample was culture-negative but showed clinical signs,

and was evaluated based on a clinical cure (Schmenger & Krömker, 2020). Our data suggests further investigation is required to understand how to address mastitis during stressful periods. At a very basic level, our data indicates that promoting mammary health during the dry period and early lactation period and preventing development of mastitis, is in the best interest of the cow and producers.

SCC and lactation number

Quarters were categorized into 1st, 2nd, and 3+ lactations in order to evaluate any associations between pre-treatment SCC and lactation number. There was no significant difference in quarters that cured vs those that failed to cure in 1st or 2nd lactations, however the quarters in 3+ lactation that failed to cure had significantly higher cell counts than did all other groups ($P<0.05$; Figure 10). As the age of the cow increases, SCC increases, and the magnitude in SCC reduction that can be achieved after antibiotic treatment is greater in younger cows than in older cows (Alhussien & Dang, 2018; Deluyker et al., 2005). While the current study did not find significant differences in SCC in quarters that cured based upon lactations, older cows (3+) did have higher SCC values in quarters that failed to cure ($P<0.01$). Additionally, data showed that the cure rates of antibiotic therapy increased as lactation number increased, with cows in 1st lactation having a cure rate of 40.0%, 2nd lactation cows having a cure rate of 41.2%, and cows in 3+ lactations having a cure rate of 70.0% ($P=0.155$). Our results contrast with studies by Sol et al. (1994) and Deluyker et al. (2005), that found bacteriological cure rates decreased with cow age. Differences may be due to having no cows in our 3+ lactation group over their 5th lactation, whereas older cows (6+lactations) may have a more significant impact on SCC values.

SCC and chronicity

Individual cow health history was available using herd management software, allowing quarters to be separated based on new vs chronic infections. Mastitis cases were deemed as chronic if they met all of the following criteria: 1) within the same lactation, mastitis was detected after 90 days from the first diagnosis, 2) the infection was in the same quarter as previous infection, 3) the infection was caused by the same pathogen. Any infection in the same quarter by the same bacteria in subsequent lactations was also classified as chronic. Quarters that did not meet these criteria were deemed new infections. Results showed the average D0 pre-treatment SCC in new quarters that cured was numerically lower than chronic quarters that cured (377,166 cells/mL ± 123.99 SEM vs. 896,666 cells/mL ± 280.28 SEM respectively) (Figure 11). Additionally, new quarters that failed to cure had on average significantly higher SCC values at D0 pre-treatment in comparison to new quarters that cured ($P<0.01$). As chronic quarters have had a persistent IMI for several months, it is expected that SCC values would remain elevated as the mammary gland continuously has an influx of cells to fight off the infection. The average D0 pre-treatment SCC in quarters that failed to cure between the two groups had little variation (1,351,250 cells/mL ± 519.08 SEM vs 1,688,583 cells/mL ± 370.98 SEM).

The duration of infection can impact cure rates of mastitis, with chronic infections dropping the industry standard cure rate of mastitis from 50% to 35% (Barkema et al., 2006; Nickerson & Ryman, 2019).We demonstrated that the antibiotic cure rate for chronic infections was 60.0%, near the industry standard for antibiotic cure rates of mastitis, while new infections had a lower antibiotic cure rate of 42.9% ($P=0.483$). This conflicts with a previous review deeming chronic infections to have a low probability of cure (35%) (Barkema et al., 2006). Conflict may be due to differing definitions of what is considered

'chronic', as Barkema et al. (2006) defined chronic as an IMI persisting for more than 4 weeks, with no information on subsequent lactations available. *Staph. aureus* chronic infections have shown a lower bacteriological cure rate (21.9%), but the definition for chronicity was unavailable (Linder et al., 2013). In the current study, *Staph. aureus* made up 80% of chronic infections, and the antibiotic cure rate of chronic *Staph. aureus* infections was 50.0%. Repeated sampling of the quarters deemed chronic but cured would have been beneficial to confirm that infections were successfully cleared, as some infections experience cyclic shedding of pathogens (Sears et al., 1990).

Conclusion

This study presents associations between pre-treatment SCC values and bacteriological cure rates following antibiotic therapy for mastitis. The present data supports that quarters with lower SCC prior to antibiotic treatment of mastitis are more likely to successfully cure than quarters with high SCC prior to treatment. Additionally, the type of mastitis at the time of infection, the pathogen causing the infection, lactation number, DIM, and infection chronicity should be considered when making future treatment decisions. Further investigation of associations between pre-treatment SCC and the antibiotic regimen chosen, and the duration of antibiotic treatment is warranted. Enhancing cure rates of antibiotics could reduce costs associated with mastitis and promote judicious antibiotic usage as it becomes increasingly demanded by consumers.

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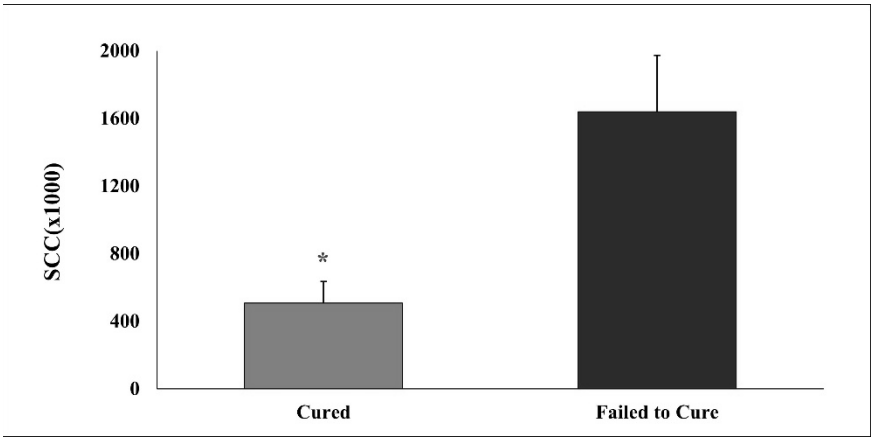

Figure 3.1. Pre-treatment (D0) somatic cell count (cells/mL) of mastitis quarters of dairy cattle that cured or failed to cure following 5-day intramammary antibiotic therapy (n=52; *P=0.01). Error bars indicate standard error.

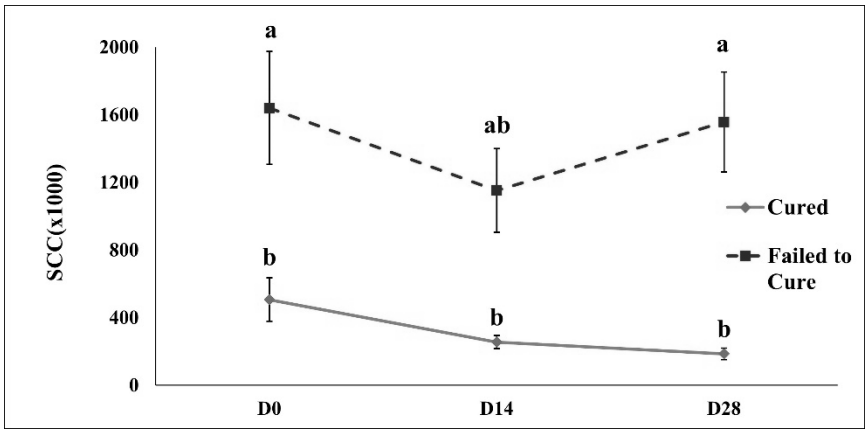

Figure 3.2. Temporal change in somatic cell count (cells/mL) of mastitis quarters that cured or failed to cure following 5-day intramammary antibiotic therapy (n=52). Error bars indicate standard error. [ab]Letters indicate significance (P<0.05) using Tukey's pairwise comparisons.

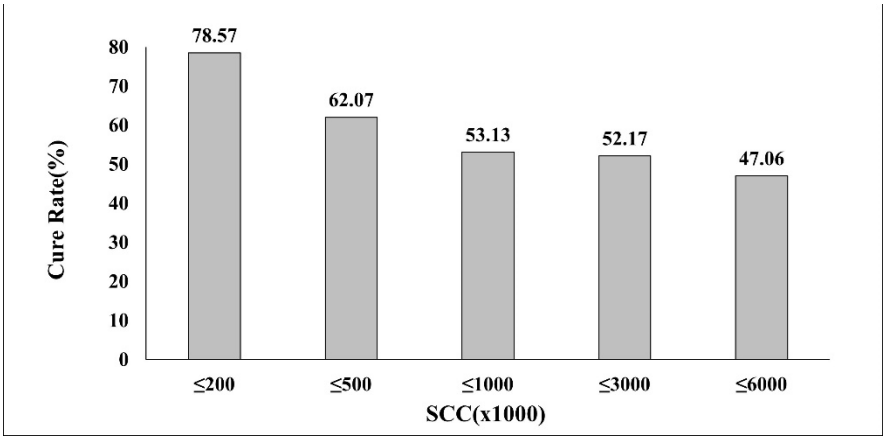

Figure 3.3. 5-day intramammary antibiotic treatment cure rates by changes in somatic cell count values (cells/mL) prior to treatment (n=52). A correlation test demonstrated that antibiotic cure rates decreased as somatic cell count prior to treatment increased (P=0.0074, r=0.9961, r^2=0.9334).

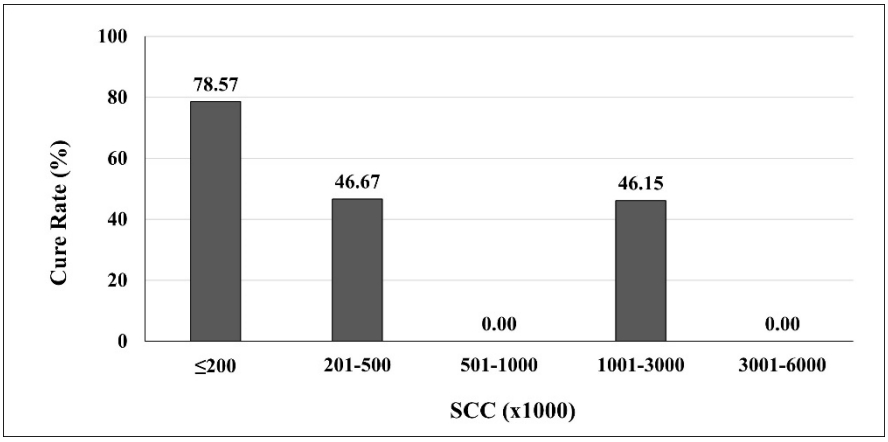

Figure 3.4. 5-day intramammary antibiotic treatment cure rates by changes in range of somatic cell count values (cells/mL) prior to treatment (n=52).

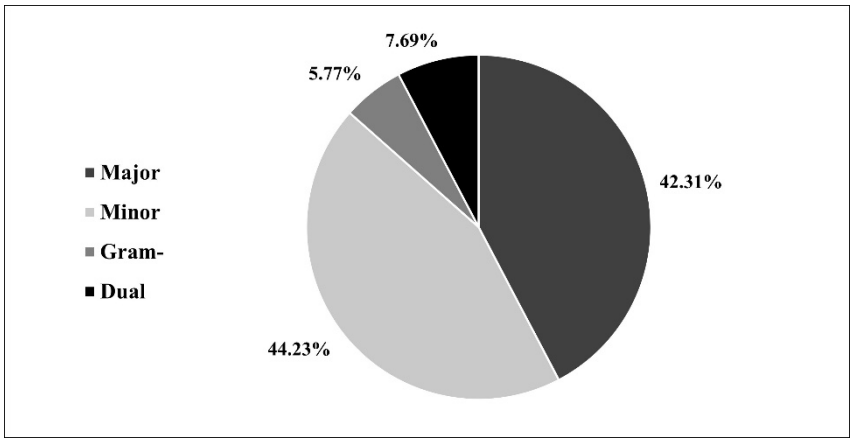

Figure 3.5. Distribution of mastitis pathogens in dairy cattle (n=52). Major pathogens included *Staphylococcus aureus* and environmental streptococci, minor pathogens included all non-aureus staphylococci, gram-negative pathogens included *Escherichia coli*, and dual infections were defined as having two pathogens present of any type: major, minor, or gram-negative.

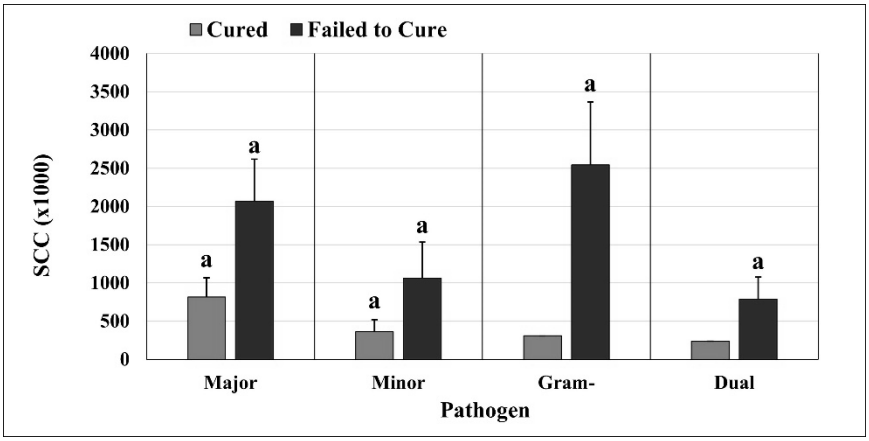

Figure 3.6. Pre-treatment (D0) somatic cell count (cells/mL) of mastitis quarters of dairy cattle that cured or failed to cure following 5-day intramammary antibiotic therapy based on infecting pathogen (n=52). Major pathogens included *Staphylococcus aureus* and environmental streptococci, minor pathogens included all non-aureus staphylococci, gram-negative pathogens included *Escherichia coli*, and dual infections were defined as having two pathogens present of any type: major, minor, or gram-negative. Error bars indicate standard error. None of the means within each group were significantly different ($P \geq 0.05$) according to Tukey's pairwise comparison.

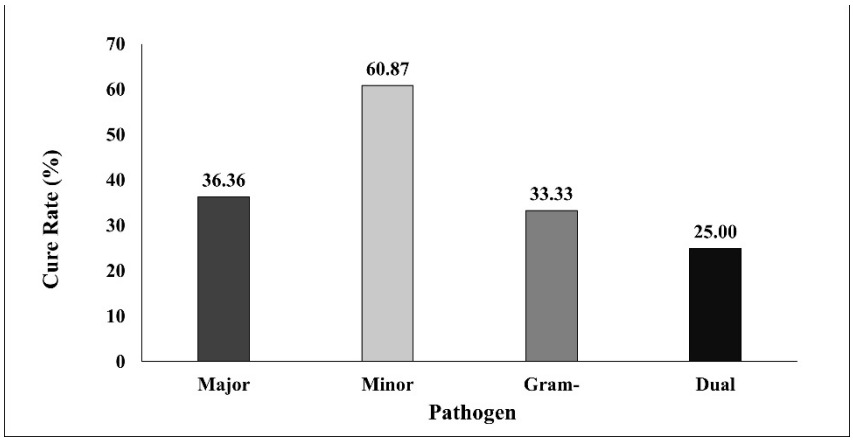

Figure 3.7. 5-day intramammary antibiotic treatment cure rates based on infecting pathogen isolated (n=52). Major pathogens included *Staphylococcus aureus* and environmental streptococci, minor pathogens included all non-aureus staphylococci, gram-negative pathogens included *Escherichia coli*, and dual infections were defined as having two pathogens present of any type: major, minor, or gram-negative.

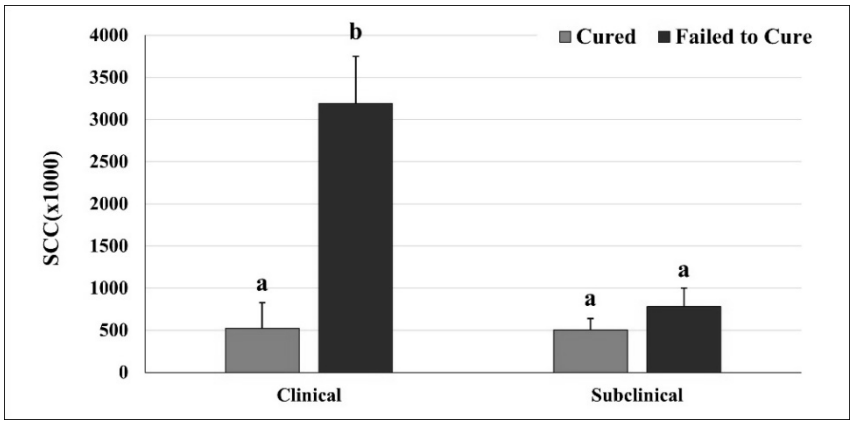

Figure 3.8. Pre-treatment (D0) somatic cell count (cells/mL) of mastitis quarters of dairy cattle that cured or failed to cure following 5-day intramammary antibiotic therapy in clinical vs subclinical mastitis (n=52). Error bars indicate standard error. [ab]Letters indicate significance ($P<0.05$) using Tukey's pairwise comparisons.

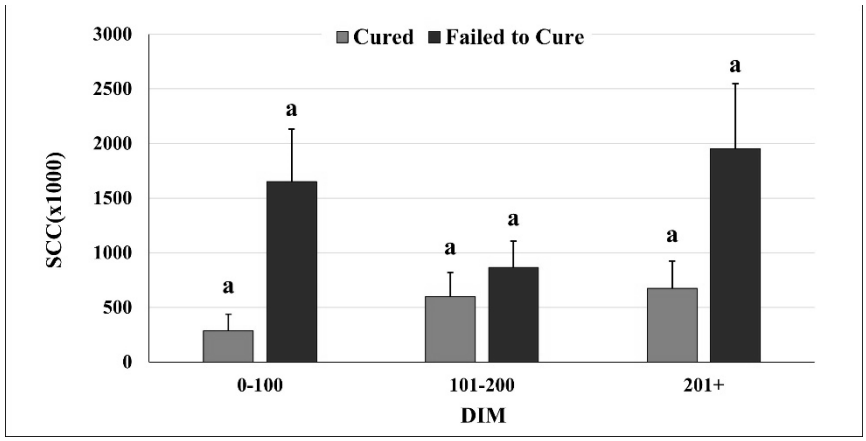

Figure 3.9. Pre-treatment (D0) somatic cell count (cells/mL) of mastitis quarters of dairy cattle that cured or failed to cure following 5-day intramammary antibiotic therapy based on days in milk (n=52). Error bars indicate standard error. None of the means within each group were significantly different ($P \geq 0.05$) according to Tukey's pairwise comparison.

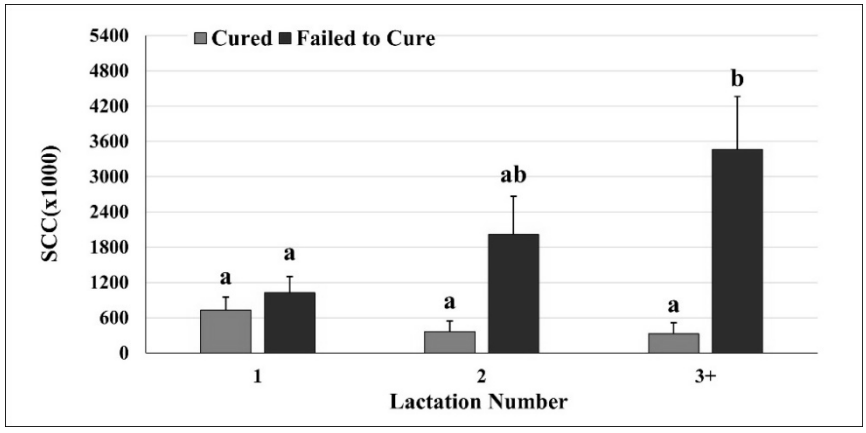

Figure 3.10. Pre-treatment (D0) somatic cell count (cells/mL) of mastitis quarters of dairy cattle that cured or failed to cure following 5-day intramammary antibiotic therapy based on lactation number (n=52). Error bars indicate standard error. [ab]Letters indicate significance ($P<0.05$) using Tukey's pairwise comparisons.

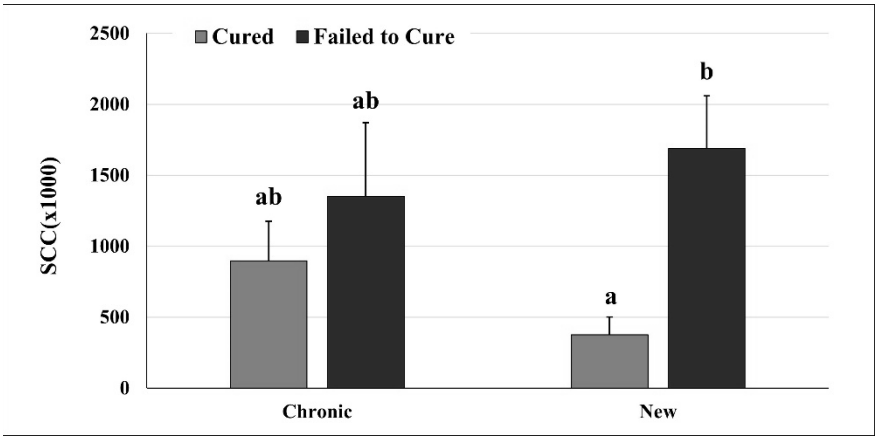

Figure 3.11. Pre-treatment (D0) somatic cell count (cells/mL) of mastitis quarters of dairy cattle that cured or failed to cure following 5-day intramammary antibiotic therapy in chronic vs new infections (n=52). Error bars indicate standard error. [ab]Letters indicate significance ($P<0.05$) using Tukey's pairwise comparisons.

CHAPTER 4

INVESTIGATION OF A SOMATIC CELL COUNT THRESHOLD FOR ANTIBIOTIC TREATMENT OF MASTITIS

[1]Williamson, J. R., E. Rollin, T. R. Callaway, V. E. Ryman. To be submitted to *Research in Veterinary Science*.

Abstract

Consumer demand for reduction of antibiotic usage in animal production is a growing issue, putting increasing pressure on dairy producers in the treatment of mastitis. However, antibiotic therapy for mastitis is necessary in many cases as numerous infections will not cure without. Even though antibiotic therapy may be necessary, cure is not guaranteed and is heavily impacted by various factors, including pre-treatment milk somatic cell count (SCC). Our study found that in quarters that cured after antibiotic therapy, the SCC was significantly lower than in quarters that failed to cure. At the conclusion of this initial study, we posited that the SCC features identified could be used in the development of a threshold for antibiotic treatment. Therefore, the objective of this study was to evaluate the bacteriological cure rate in two sample populations, quarters treated vs. not treated based on a SCC threshold value. We hypothesized that the cure rate of quarters enrolled for treatment would be increased in comparison to quarters treated in our previous study irrespective of pre-treatment SCC. Quarter milk samples from cows presenting with clinical or subclinical mastitis were collected prior to enrollment and antibiotic treatment (D0). Milk SCC was immediately enumerated, and animals were treated or not based on a pre-established threshold value. Milk samples were subsequently cultured to determine presence and type of bacteria. Samples were collected 14 d (D14) and 28 d (D28) later in both treated and untreated groups. A bacteriological cure was confirmed when both the D14 and D28 samples were free of bacteria. Quarters in the treated group had an overall cure rate of 60.7%, in comparison (P=0.2472) to the overall cure rate from the previous study investigating the association between pre-treatment SCC and antibiotic cure rates (57.1%). The spontaneous cure rate of quarters in the untreated group was 23.5%. Major

and minor pathogens had more successful cure rates in the treated group when compared to gram-negative pathogens, while the opposite was seen in the untreated group. Results demonstrate that pre-treatment SCC may not be used on its own for a treatment protocol, and that culturing prior to treatment may be a more important factor when determining treatment outcome.

Key words: somatic cell count, mastitis, antibiotic treatment, cure, intramammary, pre-treatment

Abbreviation key: SCC = somatic cell count, **CM =** clinical mastitis, **SM** = subclinical mastitis, **DIM** = days in milk**, MSA** = mannitol salt agar, **NAS** = non-aureus staphylococci

Introduction

Mastitis, defined as inflammation of the mammary gland, is commonly caused by bacterial infection, and is the top reason for antibiotic usage on dairies (Pol & Ruegg, 2007). Mastitis is the most widespread disease in dairy production and causes considerable economic losses due to reduced milk yield, discarded milk, and other factors including antibiotic treatment costs (Abebe et al., 2016). Although it can be costly and there is increasing scrutiny of antibiotic usage in production from consumers, effective antibiotic therapy is crucial to ensure the success and well-being of the dairy herd. The basis for antibiotic treatment of an infected quarter is variable between herds, and treatment success can vary with the antibiotic regimen chosen (e.g., narrow vs broad spectrum), the duration of therapy (3- vs 8-day), and other cow-related factors such as the cultured pathogen (McDougall et al., 2019; Ruegg, 2021). Even in the face of incredible developments in the field of mastitis control and treatment, the success of therapy is still subpar in many cases.

In recent years, a shift in mindset has turned to reserving antibiotic treatment only for cases of mastitis that will successfully cure (Royster & Wagner, 2015). Utilizing antibiotics in cases where success is more likely results in reduced antibiotic usage and increased economic returns. Antibiotic treatment consideration can be based on many factors, including the spectrum of activity, duration of treatment, the efficacy and intrinsic resistance of the antibiotic dependent on the infecting pathogen, individual cow-factors, and the chance of spontaneous cure (Ruegg, 2018, 2021). Many of these criteria require methodical record keeping practices by producers, and not all have on-farm culture methods to determine the infecting pathogen, let alone the ability to evaluate antibiotic susceptibility. Even so, success of antibiotic therapy can be poor. In a survey examining

the reasoning of antibiotic treatment for mastitis on farms, 66% of responders 'sometimes' cultured milk from the infected quarter or used another on-farm test for mastitis; although most producers based the decision for treatment upon visible signs rather than culture results (Raymond et al., 2006). When considering mastitis cases that do not have visible signs, defined as subclinical mastitis (SM), the most common detection of infection is through use of somatic cell counts (SCC) (Adkins & Middleton, 2018).

The SCC values have been evaluated for use in treatment protocols of mastitis, with a SCC of 200,000 cells/mL or greater often used as a threshold to diagnose infected quarters. An elevation in SCC at a basic level indicates inflammation in the gland, in many cases as a result of bacterial infection. However, this diagnosis and subsequent initiation of antibiotic therapy does not mean the infected quarter will successfully cure with antibiotic treatment. Previous work demonstrated that SCC prior to treatment has the potential to be a benchmark for deciding if an infected quarter should be treated with antibiotics (Nickerson et al., 2018). We previously conducted a study to further investigate whether SCC was associated with bacteriological cure after antibiotic therapy. Williamson et al. (2022) reported that pre-treatment SCC in quarters that cured after antibiotic administration the was significantly lower (507,041 cells/mL ± 127.86 SEM) than the SCC of quarters that failed to cure (1,640,392 cells/mL ± 333.28 SEM). The overall cure rate was 46.2% when quarters were treated without selection based on pre-treatment SCC (Williamson et al., 2022). Therefore, based on previous findings, hypothesized that the cure rate of quarters enrolled for treatment would be increased in comparison to quarters treated in our previous study irrespective of pre-treatment SCC.

Materials and methods

All procedures involving live animals were verified and approved by the University of Georgia's Office of Animal Care and Use (AUP #A2020 06-029-Y2-A1). The dairy cattle used in this study were located at the University of Georgia Teaching Dairy in Winterville, GA (33°54'32.9"N 83°14'50.9"W).

Animal selection and diagnoses

A threshold value for antibiotic therapy was established based on findings from Williamson et al. (2022). The average SCC of quarters that successfully cured was 507,041 cells/mL with a standard deviation of 626,383 cells/mL. The threshold SCC value for the current study was set at the average plus 1 standard deviation (507,041 + 626,383) resulting in a threshold of 1,133,425 cells/mL). Milk from mammary quarters of lactating Holstein and Jersey cows (n = 45) presenting with clinical mastitis (CM) were aseptically collected at the UGA Teaching Dairy (D0). The SCC was immediately taken using a Delaval Direct Cell Counter (DeLaval; Tumba, Sweden) and animals were only treated if their SCC was ≤ the threshold value established in the previous study (1,133,000 cells/mL). Subclinical mastitis (SM) quarters were identified by increased SCC at the monthly test date and presence of bacteria growth. On the day of enrollment, an aseptic sample was collected from the subclinical quarter and the SCC was immediately taken. Following this, the quarter was treated or not based on the threshold value. For treated quarters (n = 28), intramammary treatment with lactating cow antibiotic therapy (SPECTRAMAST® LC; Zoetis, Parsippany-Troy Hills, NJ, USA) was infused into the infected quarter once/day for a total of 5 days. Samples were refrigerated and transported to the UGA Mastitis Lab on ice for culturing on trypticase soy agar plates with 5% sheep blood. Samples were also

plated on mannitol salt agar (MSA) and MacConkey agar plates to aide in presumptive identification of mastitis-causing pathogens.

At 24- and 48-hours, plates were examined, and further testing was conducted based on Hogan et al. (1999) to enhance confidence in presumptive identification. If samples did not display bacterial growth on D0 or displayed any other pathogen growth not of bacterial origin, the quarter was removed from the study (n=15). Samples were taken 14 days (D14) and 28 days (D28) post-treatment, SCC was determined, and the same culturing procedures were followed. A quarter was officially labeled as "Cured" if milk from both D14 and D28 were free of bacterial growth and labeled "Failed to Cure" if bacterial growth was detected in milk on either day or on both days.

Any CM or SM quarters that had a SCC > 1,133,000 cells/mL at the time of diagnoses were not treated (n = 17). Quarters were still sampled 14 d and 28 d after diagnosis to evaluate the percent of spontaneous cure, as well as follow the change in SCC. The same procedures were followed in terms of SCC and culturing as was done for the treated group. A quarter was officially labeled as "Cured" if milk from both D14 and D28 were free of bacterial growth and labeled "Failed to Cure" if bacterial growth was detected in milk on either day or on both days. Similarly, quarters had to display bacterial growth on D0 in order to be enrolled in the study.

Statistical analysis

Cure rates were calculated for each group (treated vs. not treated) and evaluated using a Fisher's exact test. SCC at D0 was evaluated using a two-sample t-test. The SCC overtime (D0, D14, D28) of quarters that either cured or failed to cure for each group was compared using 2-way ANOVA. Two-sample t-tests were used to identify a relationship

between pathogen-specific pre-treatment SCC and the outcome of treatment within each group. Cure rates between groups for the major mastitis pathogens were evaluated using Fisher's exact test. The relationship between the outcome of treatment and the treated vs untreated group dependent on the type of mastitis case (i.e., clinical vs subclinical), days in milk (DIM), lactation number, and chronicity was also evaluated using 2-way ANOVA. A Fisher's exact test was run between the antibiotic cure rate in the current study and the previous study by Williamson et al. (2022). Differences were considered statistically significant at $P \leq 0.05$.

Results and discussion

SCC and cure rates of treated vs untreated cows

In the treated group, the D0 pre-treatment SCC average of quarters that cured was numerically lower (286,882 cells/mL ± 55.47) than quarters that failed to cure (421,363 cells/mL ± 71.20). Quarters that cured had numerically lower SCC values on D14 and D28 compared to D0, in contrast to an increase on D14 and D28 in the quarters that failed to cure (Figure 1a). Not only had SCC increased by D28 in quarters that failed to cure, but values were higher than at pre-treatment. This was a different temporal dynamic than what was found by Williamson et al. (2022), with a numerical decline on D14 and then an increase by D28. Additionally, quarters that failed to cure had on average significantly higher SCC values on D14 ($P < 0.01$) and D28 ($P = 0.05$) in comparison quarters that successfully cured. In a study by Milner et al. (1997), researchers found that 14 d after antibiotic treatment, the SCC of infected quarters treated prior to clinical signs was half that of the quarters of cows that were not given treatment until clinical signs appeared. This is similar to the current study, with both treated and untreated quarters that cured having a

decline in SCC by D14, however the untreated quarters that cured had SCC values 2.5x greater than treated quarters that cured, although these comparisons are more difficult to evaluate since the previous study did eventually apply antibiotic treatment protocols. The percent decrease in SCC of quarters that cured between D0 and D14 was 36.1%, while the percent increase in SCC of quarters that failed to cure between D0 and D14 was 48.5%. Previous research demonstrated similar results, the percent decrease in SCC of quarters that cured between D0 and D14 was 71.7%, while the percent increase in SCC of quarters that failed to cure between D0 and D14 was 41.3% (Salat et al., 2008). Differences in the percent decrease in quarters that cured may be due to selection criteria of animals, with Salat et al. (2008) focusing investigation primarily on SM quarters. Moreover, given the restricted threshold for enrollment in our current study, SCC were relatively low during infection compared to many cases of mastitis, limiting the magnitude of decrease others may report. For example, Bannerman et al. (2004) found SCC in *Staph. aureus* and *E. coli*-challenged quarters to be 44.9 x 10^6 cells/mL and 32.1 x 10^6 cells/mL, respectively, which are substantially higher than our threshold value for treatment. However, in both cases when infection continued to persist, SCC values remained elevated.

In the untreated group, the average D0 pre-treatment SCC values of quarters that spontaneously cured vs those that did not cure were similar (3,656,750 cells/mL ± 381.09 vs 3,296,538 cells/mL ± 360.96, respectively). The quarters that spontaneously cured showed significant decreases in SCC on both D14 ($P<0.01$) and D28 ($P<0.01$) in comparison to D0 pre-treatment values (Figure 1b). In quarters that did not spontaneously cure, there was a numerical decline in SCC on D14 and a significant decrease by D28 ($P<0.01$), but the decrease did not result in SCC values below 1 million cells/mL. The

percent decrease in SCC of quarters that spontaneously cured between D0 and D14 was 87.5%, while the percent decrease of quarters that did not spontaneously cure between D0 and D14 was only 38.5%. Bacteriological cure, whether with antibiotic treatment or spontaneous, results in an overall decrease of SCC, in agreement with a previous study by Salat et al. (2008).

Of the infected quarters in the treated group, the bacteriological cure rate was 60.7% (Figure 2). We expected from the previous study by Williamson et al. (2022) to see a cure rate of at least 57.1%. Our cure rate surpassed our expectations for the antibiotic cure rate by 3.6 percentage points, however cure rates were not significantly different (P=0.02472). In contrast the bacteriological cure rate (spontaneous cure) for those that were not treated was 23.5%, lower than the antibiotic cure rate (P=0.1001). When a SCC threshold value was set for treatment at SCC > 1,000,000 cells/mL in a previous study, the spontaneous cure rate was found to be 25.3% and antibiotic cure rate to be 30.1% (Lavon et al., 2021). However, in Lavon et al. (2021) the SCC had recovered to <250,000 cells/mL, whereas in the current study, when quarters that spontaneously cured demonstrated a decline in SCC by D28, this decline did not result in SCC values back to 'normal' (SCC < 200,000 cells/mL). After intramammary infection, the SCC may remain elevated about 200,000 cells/mL for several weeks before fully recovering (S Pyörälä, 1988). Moreover, the antibiotic cure rates in the current study were higher than the spontaneous cure rate, in disagreement with a study by Lavon et al (2021) where results demonstrated no significant differences between antibiotic cure rates and spontaneous cure rates at various SCC threshold levels. Researchers noted that only 10.6% of cows were treated with antibiotics, whereas in the current study, 62.2% of cows were treated with antibiotics. Additionally,

bacteriology was not done; researchers confirmed quarters as a 'cure' using three monthly SCC tests, which may account for differences in results. Lavon et al. (2021) recognized that antibiotic treatment without bacteriology caused more harm relative to the cow's recovery. When treatment is not applied following bacterial identification, and is applied by different dairy workers without knowledge if proper antibiotic protocol is followed, the cure rates of antibiotic therapy for mastitis may suffer (Lavon et al., 2021). When using electrical conductivity as an early indicator for mastitis, treatment was administered when SCC was first identified to be elevated (2,000,000 cells/mL), resulting in faster achievement of cure in comparison to cows that were not treated until SCC > 12,000,000 cells/mL (Milner et al., 1997). Achieving a 100% cure rate by this method of early detection saved more milk per cow, shortened the antibiotic withdrawal period, and used less antibiotics in total, saving approximately 25% of costs when compared to cows that were not treated until later (Milner et al., 1997), however mastitis was induced using infusion of *Streptococcus uberis* or *Staphylococcus aureus* so elevation of SCC was expected and monitored. Even so, careful monitoring for early detection of cases, especially those that are subclinical, and treatment prior to severe elevation of SCC can improve antibiotic cure rates and reduce negative consequences associated with mastitis.

SCC and infecting pathogen of treated vs untreated cows

For both the treated and untreated groups, quarters were separated into major pathogen infections, minor pathogen infections, gram-negative infections, and dual infections (having two infecting pathogens in one quarter) as done in the prior study (Williamson et al., 2022). Major pathogens included *Staph. aureus* and environmental streptococci, whereas minor pathogens included all NAS species. The distribution of

pathogens between the two groups is shown (Figure 3), with minor pathogens making up most of the treated group, while major pathogens made up the majority of the untreated group. This was not unexpected as minor pathogens are associated with lower SCC in comparison to major pathogens and gram-negative pathogens (dos Reis et al., 2011). There are several risks to not treating major pathogens, including severe milk yield loss and damage to reproductive efficiency (Dalanezi et al., 2020; Heikkilä et al., 2018). For these reasons, some herds choose to remove cows infected with major pathogens, such as *Staph. aureus*, immediately rather than risk infecting herd mates or negatively impact production levels and milk quality (Arnold & Bewley, 2011). While SCC is usually lower in minor pathogens, it is important to treat these infections as milk yield losses are also associated with minor pathogens (Heikkilä et al., 2018).

Within the treated group, D0 pre-treatment SCC averages were calculated for each pathogen that cured with treatment vs those that failed to cure (Figure 4a). Minor pathogens that successfully cured with treatment had significantly lower pre-treatment SCC values (160,909 cells/mL ± 52.85) than minor pathogens that did not cure with treatment (444,500 cells/mL ± 100.07, $P<0.05$). Additionally, the minor pathogens that cured with treatment had numerically lower SCC values prior to treatment than all other groups, in corroboration with Harmon (1994) stating that the major pathogens (*Staph. aureus*, coliforms, environmental streptococci) cause the greatest increase in SCC, while minor pathogens (NAS) only moderately increase SCC. Interestingly, major pathogens had numerically higher D0 pre-treatment SCC values in quarters that cured (464,250 cells/mL ± 50.34) when compared to major quarters that failed to cure (287,500 cells/mL ± 53.50). Though infections were low in number, Gram-negative pathogens demonstrated a similar pattern.

Within the untreated group, major pathogens and gram-negative pathogens that spontaneously cured also had numerically higher D0 SCC values compared to quarters that did not spontaneously cure (Figure 4b). Dual infections were also too low to fully investigate in the context of this study, though it is interesting, but not unexpected, that SCC was robust during infection.

The treated group demonstrated high cure rates for both major and minor pathogens (66.7% and 57.9%, respectively) in comparison to the untreated group (Figure 5). Additionally, a Fisher's exact test showed the cure rate of major pathogens that cured with antibiotic treatment was significantly higher than those that spontaneously cured (P=0.0357). This was a higher cure rate for major mastitis pathogens than previously demonstrated, with *Staph. aureus* treatment success varying significantly with reported cure rates from 38%-52% (Sol et al., 2000). While higher cure rates of gram-positive pathogens are normally attributed to *Streptococcus* spp. (52.8%-66.7%) in comparison to *Staph. aureus* (Oliveira et al., 2013; Shephard et al., 2000), 75% of major pathogens that successfully cured within the current study were of *Staph. aureus* origin. In contrast, major pathogens had low cure rates (10.00%) when left untreated which is in line with previous reports demonstrating spontaneous cure rates of major pathogens, like enviornmental streptococci, at 35.3% (Shephard et al., 2000) and *Staph. aureus*, close to 0% (Ruegg, 2021). The other three of the four mastitis cases that spontaneously cured were of gram-negative origin. The higher cure rate of gram-negative infections is not unexpected as gram-negative infections tend to demonstrate elevated incidence of spontaneous cure (Ruegg, 2021).

Results in the present study illustrate that gram-negative infections have a greater percent of spontaneous cure and are less likely to successfully cure when treated with antibiotics, and that the opposite is seen with major mastitis pathogens such as *Streptococcus uberis* and minor mastitis pathogens, including NAS (Ruegg, 2021). The present study and past literature together illustrate the benefit of treatment of these types of pathogens. Data from the current study provides evidence that use of antibiotic therapy is most beneficial for mastitis cases caused by pathogens with a low rate of spontaneous cure and a high bacteriological cure rate, such as minor mastitis pathogens. Additionally, 15 quarters had to be removed from the treated group and 11 from the untreated group that were culture negative on D0 or displayed growth not of bacterial origin, indicating the importance of knowledge of etiology for determining if antibiotic therapy is needed, or if the antibiotic regimen chosen has a spectrum of activity that is appropriate for use in specific cases of mastitis (Ruegg, 2021).

SCC and type of mastitis of treated vs untreated cows

The type of mastitis at the time of diagnosis was considered for both groups, either clinical (CM) or subclinical (SM). Within the treated group, the average D0 pre-treatment SCC of SM quarters that cured (246,000 cells/mL ± 56.27) was numerically lower than CM quarters that cured (593,500 cells/mL ± 52.68; Figure 6a). Additionally, pre-treatment SCC of SM quarters that failed to cure (381,888 cells/mL ± 84.84) was also numerically lower than CM quarters that failed to cure (599,000 cells/mL ± 95.00). Not only did SM demonstrate lower SCC values in both quarters that cured and quarters that failed to cure but also had a higher overall cure rate than CM infections (62.5% vs 50.0%, respectively). In a study investigating treatment of subclinical cows based on SCC threshold values,

researchers concluded that antibiotics are not suitable for treatment of cows with SCC < 500,000 cells/mL and no clinical symptoms (Lavon et al., 2021). The current study does not agree with this conclusion and finds treatment of SM by use of SCC threshold to be a viable option should producers understand the chances of success or failure. Possible explanations for differential conclusions between the present study and Lavon et al (2021) included but are not limited to: 1) differences in SM definition, 2) recovery vs inflammation vs bacteriological cure, and 3) antibiotic regimens evaluated. In the current study, SM was defined using SCC > 200,000 cells/mL and confirmation of pathogen presence using bacteriological culture, whereas Lavon et al (2021) defined SM as first elevation of SCC without culturing for confirmation. Additionally, Lavon et al (2021) defined recovery from inflammation as a decrease in SCC on all three-monthly test dates for both spontaneous recovery and antibiotic recovery without use of culture, whereas the present study used culturing after a 28d period to confirm both spontaneous and antibiotic cure. Moreover, Lavon et al (2021) only stated that multiple antibiotic protocols 'may' have been used, as treatments were administered in herds according to herd manager and veterinarian decisions, whereas the current study strictly used one antibiotic regimen (SPECTRAMAST® LC) for a set period (5d).

In the treated group, 85.7% of quarters were SM infections, whereas only 23.5% of the untreated group was made up of SM. It was not unsurprising, though unfortunate, that the majority of CM cases were untreated. All cows in our study presented with abnormal milk, typically clot and flakes, which are largely composed of white blood cells as a result of the inflammatory response. The white blood cells can greatly increase SCC (Zigo et al., 2021), thus, having drastically elevated SCC beyond the SCC threshold is not unanticipated

and illustrates the limitations of implementing a universal SCC threshold for all infections. This may be a possible explanation for our distribution of SM and CM between the treated and untreated group, with most all CM infections that were sampled having SCC levels above the threshold value. However, while the D0 SCC values of CM were high in the untreated group, the SM quarters that failed to cure in the untreated group had higher SCC values than CM in both quarters that spontaneously cured and failed to cure (Figure 6b). The spontaneous cure rate of CM infections was 30.8%, while SM had a spontaneous cure rate of 0%, further reiterating the importance of treating SM in an effort to prevent sustained, increased SCC in current and subsequent lactations or potentially from becoming chronic (Gruet et al., 2001). This is conflicting with data from Lavon et al. (2021), who found the spontaneous cure rate of cows with SM to be around 25-30%. Dissimilarities may be due to the previous study only investigating SM and using a decrease in SCC on three monthly test days to confirm a spontaneous recovery, instead of bacteriological cure. Additionally, it is unclear how the pathogens differed between the present study and Lavon et al. (2021) which could certainly impact cure rates.

SCC and DIM of treated vs untreated cows

Quarters enrolled into the treated group were broken down to represent early-, mid-, and late-lactation: 0-100 DIM, 101-200 DIM, and 201+ DIM. The average D0 pre-treatment SCC for quarters within the early lactation group was similar for quarters that cured and those that failed to cure (417,125 cells/mL ± 91.73 vs 478,167 cells/mL ± 108.51, respectively) (Figure 7a), which may be explained by fresh cows experiencing fluctuations in SCC due to stressors such as calving (Alhussien & Dang, 2018), as it is possible that high SCC during the first week of lactation is not due to mastitis pathogens (Santman-

Berends et al., 2012). Moreover, cows in the period of peak milk production (60-90 days) may be experiencing a negative energy balance, when dry matter intake and nutrient supply is lower than the nutrient requirements for milk production and body reserves are utilized for support (Esposito et al., 2014). During this period, host defense systems may be altered and is thought to be a main reason for a high prevalence of infections during this time, such as mastitis (Esposito et al., 2014; Kimura et al., 2002). High SCC values at pre-treatment may have been attributable to being a fresh cow in addition to infection, with cows <35 DIM (n=7) having an average D0 pre-treatment SCC of 381,714 cells/mL ± 105.77, or cows in peak production (n=4) with an average D0 pre-treatment SCC of 460,750 cells/mL ± 112.17. Within the untreated group, the average D0 SCC for early lactation cows that spontaneously cured was similar to quarters that did not cure (3,472,333 cells/mL ± 385.09 vs 3,736,000 cells/mL ± 727.71, respectively), with the SCC of quarters that did not cure being numerically higher than cows in mid- and late- lactation (Figure 7b). Previous studies demonstrated that the highest proportion of elevated SCC occurred during the first month after calving (Frössling et al., 2017). Moreover, SCC values of cows in early lactation may remain elevated after calving in quarters that become infected (Satu Pyörälä, 2003). The average D0 pre-treatment SCC for quarters that cured was lowest for cows in mid lactation (93,000 cells/mL ± 22.04), and highest for those in early lactation (417,125 cells/mL ± 91.73), similarly seen by Alhussien & Dang (2018). Mid lactation cows tend to have lower SCC, even in the absence of infection as they are less metabolically and immunologically stressed than early lactation cows, so it is unsurprising that they demonstrated the lowest SCC at time of diagnosis. On the other hand, it is important to point out that less infections were detected during mid lactation, which does skew interpretation of our data, but again,

unsurprising as this is reported in various pieces of literature (Abebe et al., 2016; Moosavi et al., 2014). More evidence of this dynamic was found after evaluating our untreated group, which had few cows in mid lactation, meaning a higher population of early lactation and late lactation cows were contributing to SCC values above the threshold established. The high SCC values associated with early lactation cows cannot be ignored, as mastitis in early lactation can result in negative consequences such as decreases in milk yield and an increased culling hazard (De Vliegher et al., 2005a; De Vliegher, et al., 2005b). Additionally, high SCC in early lactation may contribute to sustained elevated SCC in the future, with the potential of developing mastitis again (Santman-Berends et al., 2012). Moreover, as lactation progresses, the risk of exposure to pathogens that cause mastitis increases (Breen et al., 2009; Ruegg, 2003), thus early prevention is critical. Overall, differences in SCC between cured and failed to cure across lactation were unremarkable, which could be a result of our low SCC threshold.

Within the treated group, late lactation cows demonstrated the lowest cure rate (50.0%), followed by early lactation (57.1%), and mid lactation (100%). Likewise, Lavon et al (2021) found cows with more than 200 DIM showing significantly lower recovery rates compared to those with lower DIM. The untreated group also demonstrated that late lactation cows had a low chance of spontaneous cure (12.5%), but mid lactation cows did not have any cows that spontaneously cured (0%) unlike results seen with antibiotic treatment cure. Cows in early lactation had a spontaneous cure rate of 42.9%. While early lactation cows had a better chance of spontaneous cure than mid- and late- lactation cows, cure rates were higher when early lactation cows were treated with antibiotics (57.1%).

These results illustrate the importance of treating infections early in lactation, and the chance for mid-lactation cows to more likely successfully respond to antibiotic therapy.

SCC and lactation number of treated vs untreated cows

Quarters were grouped into 1st, 2nd, and 3+ lactations to evaluate any associations between success of antibiotic treatment and lactation number. There were no significant differences in D0 pre-treatment SCC between lactation groups when treated (Figure 8a). Quarters that cured from 3+ lactation cows had numerically higher D0 pre-treatment SCC (422,000.00 cells/mL ± 124.361) values than all other groups. In the untreated group, D0 SCC of quarters that spontaneously cured was highest for 2nd lactation cows (4,202,000 cells/mL ± 5.30), 1st lactation cows (3,636,000 cells/mL ± 0.00), and lowest for 3+ lactation cows (2,585,000 cells/mL ± 0.00), however numbers were too small to make any inferences about quarters that spontaneously cured in this group (Figure 8b). Although, quarters that did not spontaneously cured demonstrated similar D0 SCC values across all groups. Higher parity has been correlated with a greater incidence of elevated SCC and increased risk of CM (Frössling et al., 2017). The correlation between higher parity and elevated SCC may be explained by the magnitude in SCC reduction that can be achieved post-treatment of mastitis in younger cows compared to older cows (Alhussien & Dang, 2018; Deluyker et al., 2005). Additionally, as the age of the cow increases, SCC tends to increase even in the absence of infections, possibly due to the increased number of infected quarters over time and the development of tissue damage from recurrent infections (Alhussien & Dang, 2018). Although, issues such as decreased milk production and development of chronic infections may arise with older cows in the future if SCC remains elevated.

Quarters that failed to cure from cows in their 1st lactation had numerically higher pre-treatment D0 SCC values (493,500 cells/mL ± 259.50) than all other groups. A previous study identified first-parity cows with a high SCC on the first test day after calving as continuing to have higher SCC throughout the study period compared to first-parity cows with a low SCC at the first test day after calving (Santman-Berends et al., 2012). In the current study, results demonstrated that overtime, SCC of quarters that cured in 1st lactation cows decreased to 199,333 cells/mL by D28, while the SCC of quarters that failed to cure in 1st lactation cows increased to 1,771,500 cells/mL by D28. The high SCC associated with primiparous cows may be due to the high incidence rate of mastitis in primiparous cows in comparison to multiparous cows when considering the first week to month of lactation, which can lead to severe consequences to the productive life of the animal (Frössling et al., 2017; Naqvi et al., 2018; Nyman et al., 2007; Olde Riekerink et al., 2007; Persson Waller et al., 2020). The reason for the high incidence rate of primiparous cows in the current study requires further investigation, but prepartum antibiotic treatment can be implemented as a short-term solution to assist in control of heifer mastitis under the direct supervision of a veterinarian, although this is not universally recommended as there is not always long-lasting positive effects on SCC and milk yield (De Vliegher et al., 2012).

It is important to note that in the current study, 1st lactation cows had the highest success of antibiotic treatment, with a cure rate of 81.8%. Cows in their 2nd lactation had a cure rate of 50.00%, followed by 3+ lactation cows at 45.5%. Analogous results were demonstrated by Lavon et al (2021), with primiparous cows and second-lactation cows showing significantly higher recovery rates when compared to older cows. In the current study, 3+ lactation cows also had the lowest chance of spontaneous cure within the

untreated group (16.7%), followed by 1st lactation cows (20.0%), and 2nd lactation cows (33.3%). This data reiterates previous conclusions that older cows have less chance of antibiotic treatment success in comparison to younger cows (Deluyker et al., 2005; Sol et al., 1994), even when SCC values are lower. Primiparous cows with mastitis are more likely to successfully cure with antibiotic treatment to prevent negative consequences to the productive life of the cow, including the development of chronic infections or premature culling (Bludau et al., 2014). Of course, it should be noted that the causative pathogen could impact these findings and should be considered, further reiterating the complex interactions and confounding factors when developing mastitis treatment plans.

SCC and chronicity of treated vs untreated cows

Using farm management software, the individual cow health history was collected for all quarters enrolled into the treated group. Mastitis cases were deemed as chronic using the definition previously defined in the study establishing the SCC threshold value (Williamson et al., 2022). Briefly, cases were considered chronic if they met all of the following criteria: 1) within the same lactation, mastitis is detected after 90 days, 2) the infection is in the same quarter as previous infection, 3) the infection is caused by the same pathogen, and 4) any infection that occurred in the same quarter by the same bacteria in following lactations was also classified as chronic. Quarters that did not meet these criteria were defined as new infections. Quarters that were chronic and successfully cured (101,000 cells/mL ± 0.00) had on average lower SCC values at D0 pre-treatment in comparison to new infections (298,500 cells/mL ± 59.59; Figure 9a), but the number of chronic infections within the treated group was small. Additionally, the untreated group had D0 SCC values that were relatively similar for both chronic and new infections that spontaneously cured

or did not cure (Figure 9b). While it has been previously reported that chronic mastitis is associated with more moderate to high SCC in comparison to new mastitis (Martins et al., 2020; Rainard et al., 2018), the difference in results is likely due to sample size.

As expected, new infections had a higher success of antibiotic treatment in comparison to chronic infections (61.5% vs 50.0%). Although, the chronic infections still demonstrated a higher antibiotic cure rate than ranges of 21.9-35% previously reported (Barkema et al., 2006; Linder et al., 2013). Within the untreated group, spontaneous cure rates were 0% (chronic) and 23.5% (new), though sample size is too small to make overarching conclusions. A larger sample size with a more equal distribution of chronic and new infections is needed before any inferences can be made about these results.

Interestingly, all of the chronic infections within the treated group were also identified as a SM case. It is not uncommon for most SM cases to develop into chronic infections, or for chronic infections to persist as SM. Past research suggests antibiotic treatment of chronic SM is not cost-effective due to poor prognosis (Cobirka et al., 2020; Erskine et al., 2003; StRose et al., 2003). However, the current study shows potential for treatment of chronic SM cases when SCC is under 1 million cells/mL. The seemingly subclinical nature and associated low SCC values of these chronic infections allow them to continuously slip through the cracks, although they still contribute to elevated bulk tank SCC and likely a decline in milk production. Antibiotic treatment of these infections may be more urgent and cost-effective than previously illustrated (Erskine et al., 2003), as losses associated with a decline in milk production and elevated bulk tank SCC may be greater in comparison (StRose et al., 2003). Furthermore, data demonstrates potential for increasing chronic SM antibiotic cure rates by use of SCC. While prevention of chronic infections is

critical to their control, antibiotic intervention including focus on bacteriological cure is still warranted (Gruet et al., 2001; Wente et al., 2020). Additional examination of these specific cases of mastitis is needed to determine if we can improve the judicious use of antibiotics by the method used in this paper. Specifically, evaluating any positive economic impact, such as improved milk yield or productive life, of treating chronic cases under 1 million cells/mL would be beneficial, as well as different durations of antibiotic therapy and the antibiotic regimen chosen.

Conclusion

We sought to identify if antibiotic cure rates of mastitis could be improved by using a SCC threshold-based approach for treatment. While the overall cure rate of treated quarters was higher in the current study (compared to the cure rate in Williamson et al. (2022) (60.7%, 46.2%, respectively), limiting factors of utilizing a pre-treatment SCC threshold value are evident. Further examination of a SCC threshold with an increased sample size is warranted to determine the practicality of using pre-treatment SCC values as a treatment basis fully. At the present time, current and previous literature suggest that decision-making strategies for mastitis include consideration of mammary health history (reflected by SCC and/or culture results) and causative pathogen. With additional investigation, including SCC in this conversation may be viable, contributing to more judicious use of antibiotics in dairy cattle.

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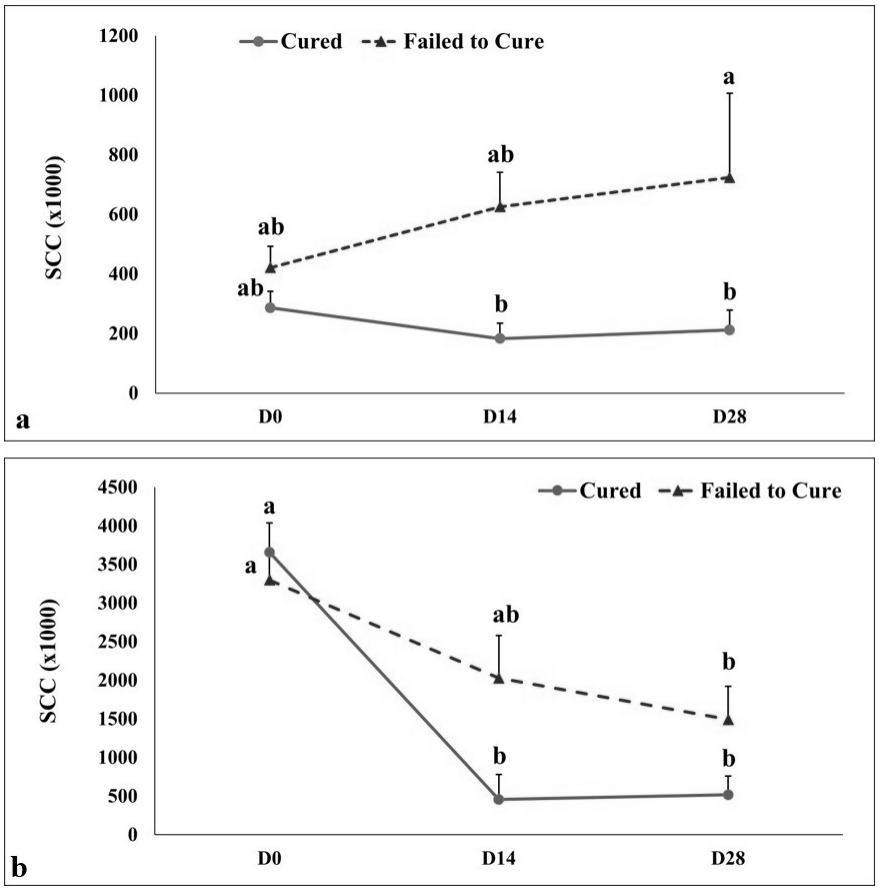

Figure 4.1. Temporal change in somatic cell count (cells/mL) of mastitis quarters that cured or failed to cure in the **a**) treated group (n=28) and **b**) untreated group (n=17). Error bars indicate the standard error. [ab]Letters indicate significance ($P<0.05$) using Tukey's pairwise comparisons.

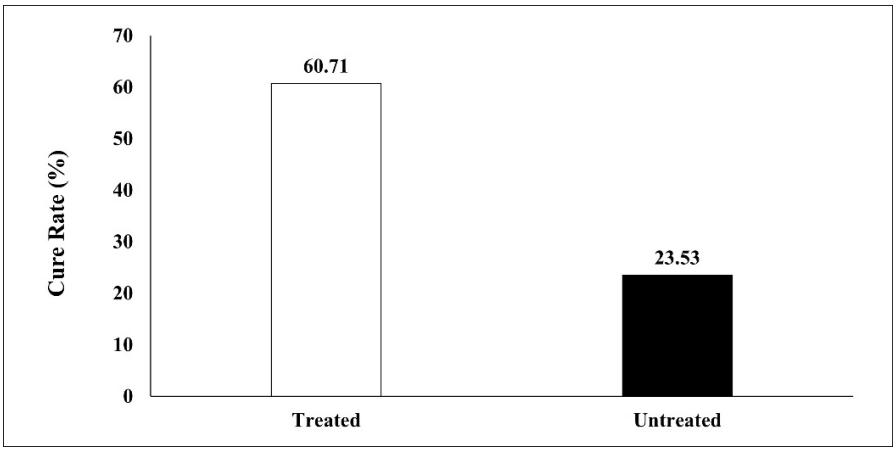

Figure 4.2. Intramammary antibiotic treatment cure rate and spontaneous cure rate of the treated (n=28) and the untreated (n=17) groups, respectively (P=0.1001). Differences in cure rates were trending based on a Fisher's exact test.

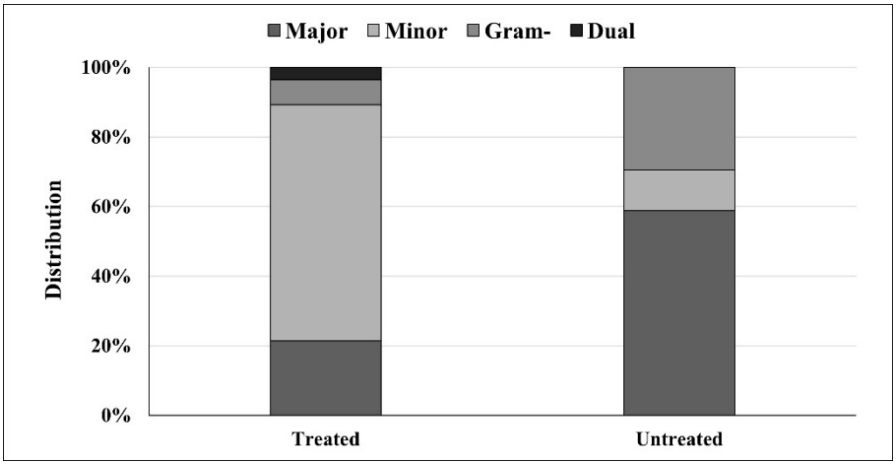

Figure 4.3. Distribution of mastitis pathogens in the treated group vs the untreated group (n=45). Major pathogens included *Staphylococcus aureus* and environmental streptococci, minor pathogens included all non-aureus staphylococci, gram-negative pathogens included *Escherichia coli*, and dual infections were defined as having two pathogens present of any type: major, minor, or gram-negative.

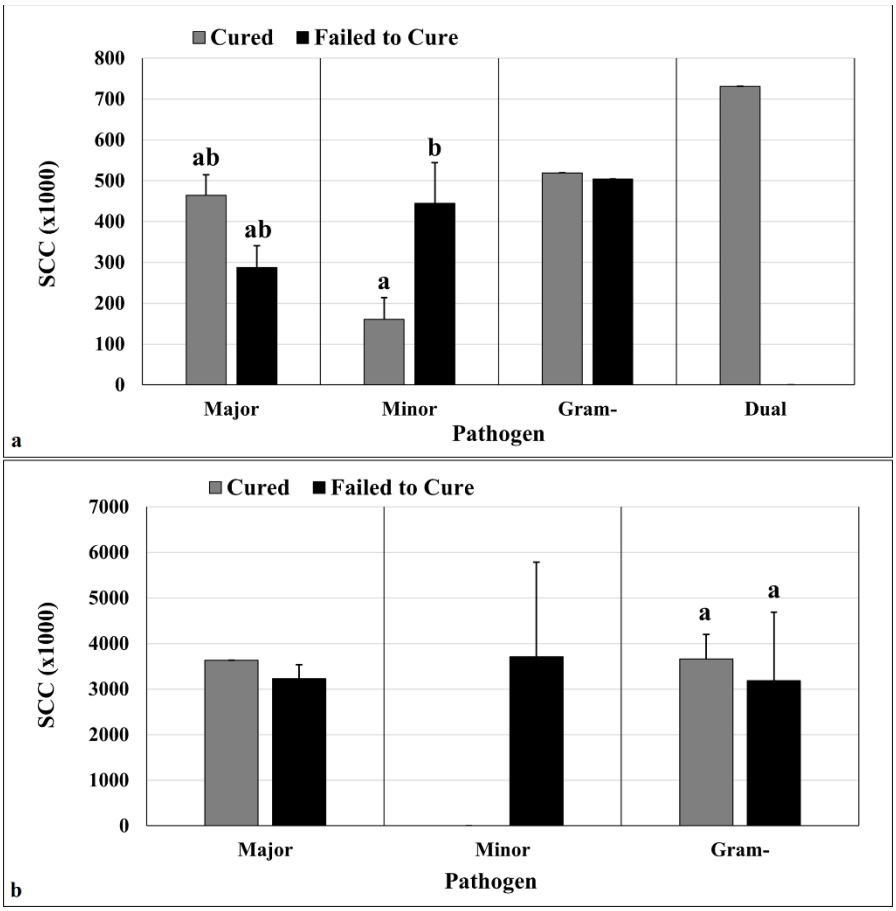

Figure 4.4. Day 0 somatic cell count (cells/mL) of mastitis quarters that cured or failed to cure based on pathogen identity in the **a**) treated group (n=28) and **b**) untreated group (n=17). Major pathogens included *Staphylococcus aureus* and environmental streptococci, minor pathogens included all non-aureus staphylococci, gram-negative pathogens included *Escherichia coli*, and dual infections were defined as having two pathogens present of any type: major, minor, or gram-negative. Error bars indicate the standard error. [ab]Letters indicate significance ($P<0.05$) using Tukey's pairwise comparisons.

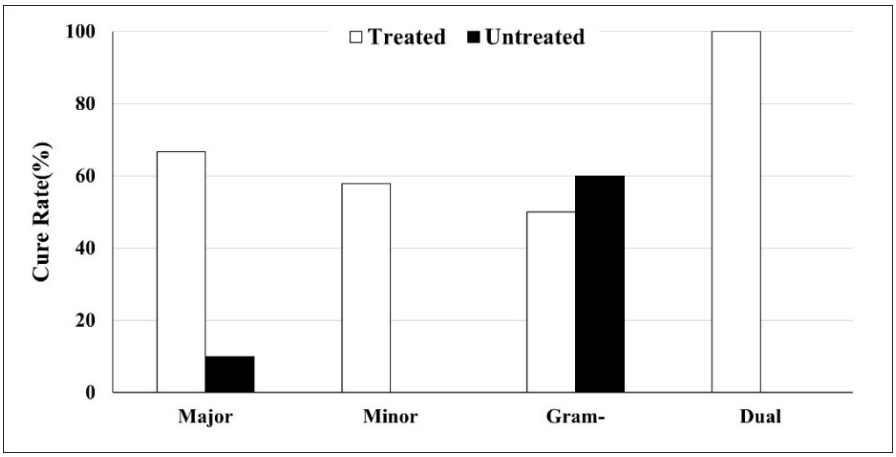

Figure 4.5. Cure rates of antibiotic treatment and spontaneous cures in the treated (n=28) vs the untreated (n=17) groups, respectively, based on pathogen identity. Major pathogens included *Staphylococcus aureus* and environmental streptococci, minor pathogens included all non-aureus staphylococci, gram-negative pathogens included *Escherichia coli*, and dual infections were defined as having two pathogens present of any type: major, minor, or gram-negative.

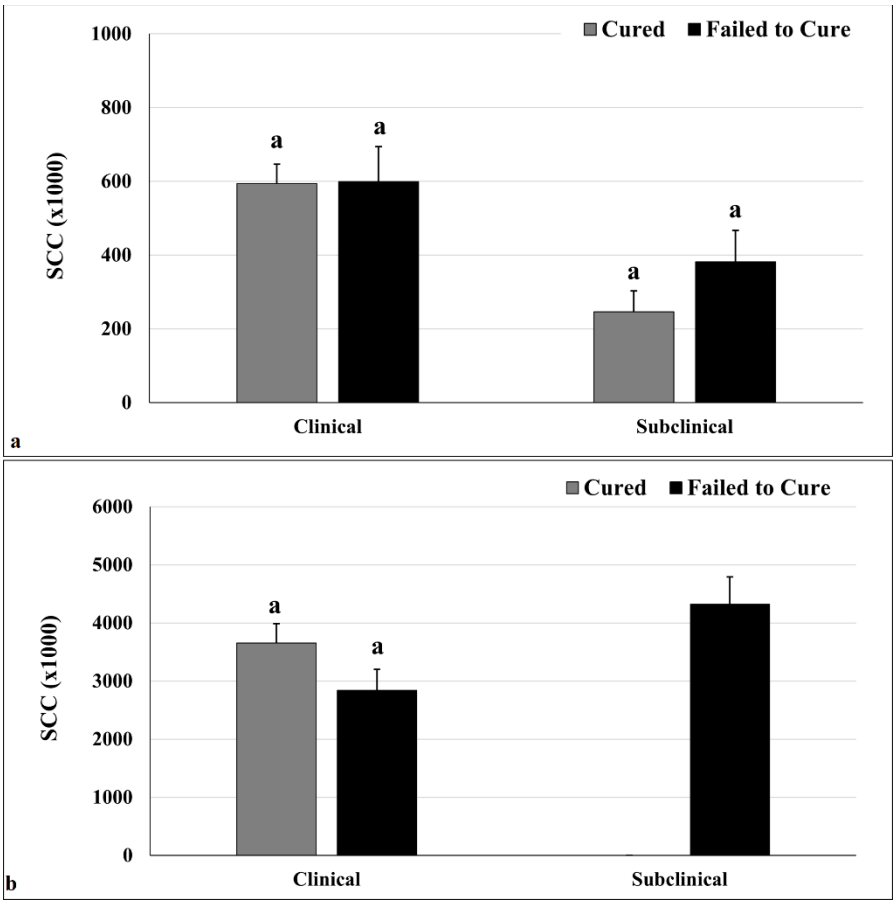

Figure 4.6. Day 0 somatic cell count (cells/mL) of mastitis quarters that cured or failed to cure based on mastitis case in the **a**) treated group (n=28) and **b**) untreated group (n=17). Error bars indicate the standard error. None of the means within each group were significantly different ($P \geq 0.05$) according to Tukey's pairwise comparison.

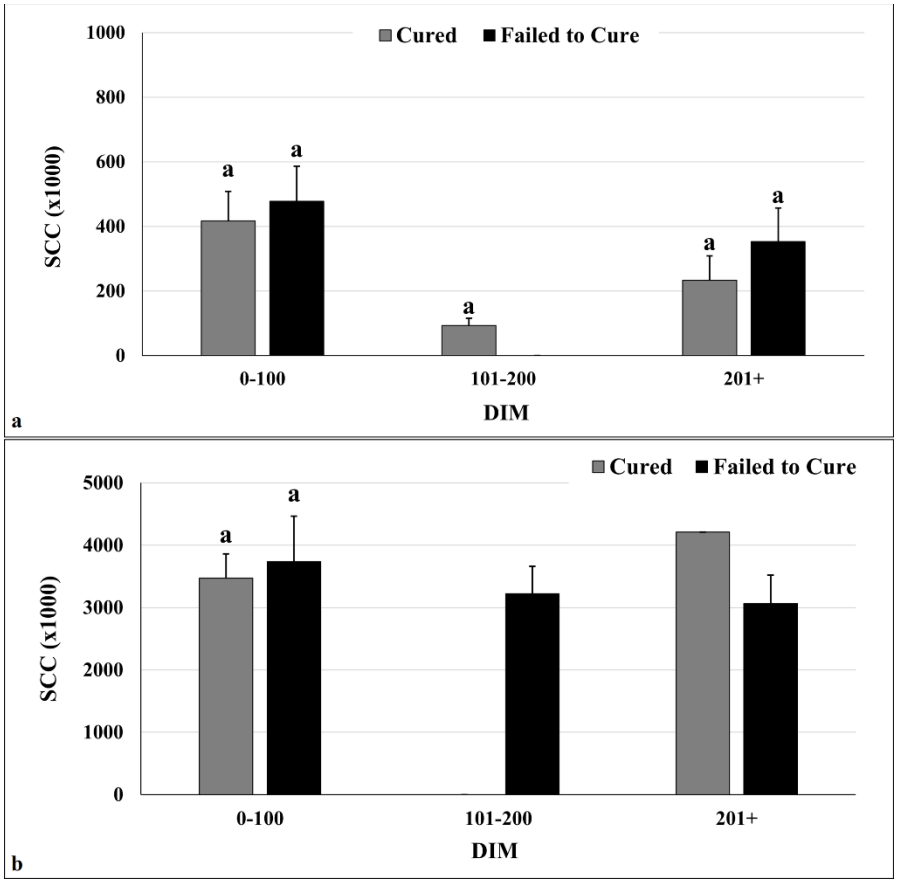

Figure 4.7. Day 0 somatic cell count (cells/mL) of mastitis quarters that cured or failed to cure based on days in milk (DIM) in the **a**) treated group (n=28) and **b**) untreated group (n=17). Error bars indicate the standard error. None of the means within each group were significantly different ($P \geq 0.05$) according to Tukey's pairwise comparison.

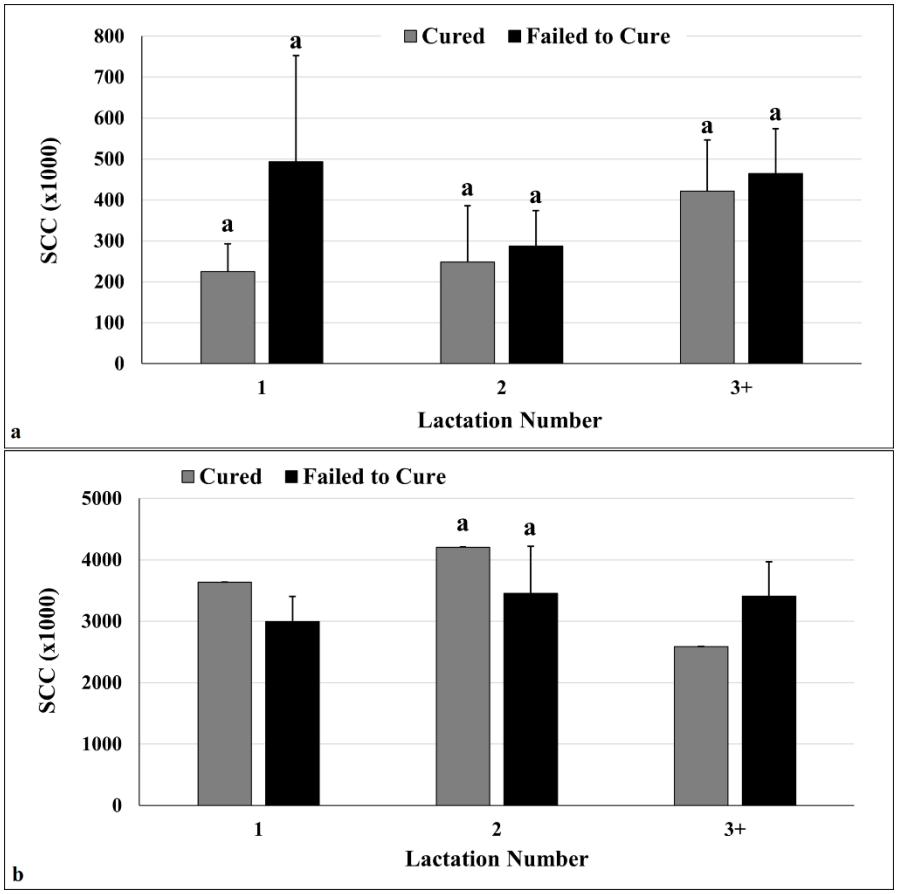

Figure 4.8. Day 0 somatic cell count (cells/mL) of mastitis quarters that cured or failed to cure based on lactation number in the **a**) treated group (n=28) and **b**) untreated group (n=17). Error bars indicate the standard error. None of the means within each group were significantly different ($P \geq 0.05$) according to Tukey's pairwise comparison.

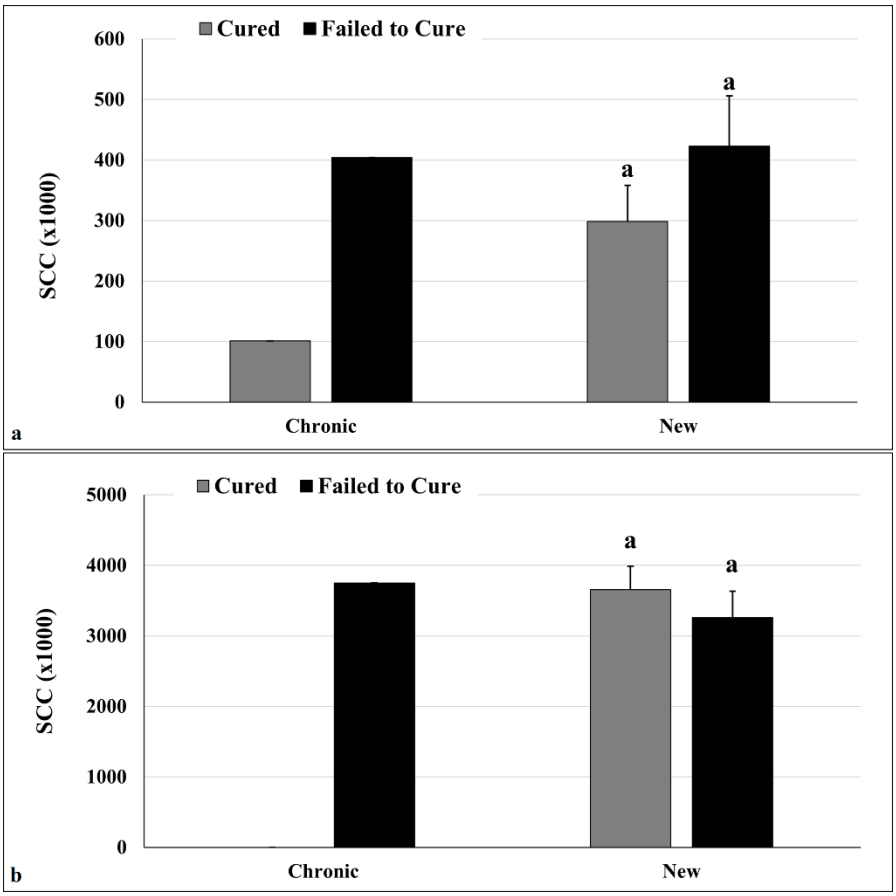

Figure 4.9. Day 0 somatic cell count (cells/mL) of mastitis quarters that cured or failed to cure based on chronicity in the **a**) treated group (n=28) and **b**) untreated group (n=17). Error bars indicate the standard error. None of the means within each group were significantly different ($P \geq 0.05$) according to Tukey's pairwise comparison.

CHAPTER 5

CHARACTERIZATION OF RUMEN, FECAL, AND MILK MICROBIOTA IN DAIRY COWS

[1]Williamson, J. R., T. R. Callaway, J. M. Lourenco, V. E. Ryman. To be submitted to *Frontiers in Microbiology.*

Abstract

Investigations of the microbiome have shown potential for improvement of feed efficiency and reduction of production costs through targeting rumen and fecal microbiota, however little progress has been made in manipulation of the gut microbiomes in dairy cattle for improvement of milk yield and milk quality. Even more neglected, the milk/udder microbiome, which is critically important to dairy production in comparison to gut microbiomes. Understanding the milk/udder microbiome may provide insight into how local microbiota correlate with milk yield and milk quality. The objective of this study was to characterize any similarities between rumen, fecal, and milk microbiota, and to investigate associations between milk microbiota and somatic cell count (SCC), milk yield, and mammary gland infection status. A total of 51 mid-lactation, multiparous Holstein dairy cattle were chosen for sampling of ruminal, fecal, and milk contents, which were then processed for microbial DNA extraction and sequencing. Cows were categorized based on low, medium, and high SCC, bacterial infection status, and low, medium, and high milk yield. Beta diversity indicated that ruminal, fecal, and milk populations were significantly distinct from one another ($P < 0.001$). Additionally, the Shannon index demonstrated that ruminal populations were more diverse in comparison to fecal and milk populations, and milk microbiota was the least diverse of the three sample types ($P < 0.001$). While diversity indices did not differ with milk yield, milk microbiome samples from cows with low SCC demonstrated a more evenly distributed microbiome in comparison to cows with high SCC values ($P = 0.053$). Ultimately, more investigation should be done between the gastrointestinal tract and the milk/mammary gland that could potentially be manipulated for improvement of mammary health and milk quality.

Key words: microbiome, rumen, fecal, milk, dairy cattle, somatic cell count, mastitis, milk yield, intramammary

Abbreviation key: GIT = gastrointestinal tract, **IMI** = intramammary infection, **SCC** = somatic cell count, **MSA** = mannitol salt agar, **NAS** = non-aureus staphylococci

Introduction

Investigation of the microbiome allows us to understand which microbes live in or on animals, and the two most studied in cattle are the ruminal and fecal microbiomes. Cattle rely on their rumen for fermentation of feedstuffs into nutrients and energy (Matthews et al., 2019), which is the largest compartment of the gastrointestinal tract (GIT) and is home to an extensive microbial population that degrades feedstuffs via fermentation. Ruminal fermentation is performed by bacteria, archaea, protozoa, and fungi, which can be targeted through diet supplementation to reduce production costs, promote feed efficiency, and improve animal health (Lourenco et al., 2020). There is a clear association between rumen and fecal microbiota and feed efficiency in cattle, which translates into reduced feed costs as the animal maintains a more adequate body weight but consumes less feed (Lourenco et al., 2020; Schären et al., 2018; Welch et al., 2020). Additionally, microbiome populations are also correlated with and/or contribute to health and disease, such as metabolic disorders, cow fertility, GIT dysfunctions, and inflammation (Matthews et al., 2019; O'Hara et al., 2020; Xu et al., 2021). However, little is still known about how milk yield and milk quality can be impacted by manipulation of the gut microbiome in dairy cattle. Aside from limited assessments of feed efficiency and milk production variables such as lactation stage and milk composition (Schären et al., 2018; M.-Y. Xue et al., 2022), much of the dairy cattle microbiome research has focused on mitigating GIT inflammation or reducing greenhouse gas emissions (John et al., 2022; Sanz-Fernandez et al., 2020). Few studies have evaluated the associations between the rumen microbiome and milk yield or quality (Buitenhuis et al., 2019; Hagey et al., 2019; M. Xue et al., 2018). Even fewer studies have investigated the fecal microbiome of dairy cattle, however they have suggested potential differences in

the rumen and fecal populations that may explain dissimilarities between low-producing vs. high-producing dairy cattle (Mu et al., 2019).

For decades, milk was assumed to be sterile unless contaminated or infected, such as intramammary infections (IMI) by mastitis pathogens. However, with the advent of metagenomics it has become clear that even when the mammary gland appears to be free from culturable pathogens, milk has its own microbiome with the vast majority not associated with mastitis (Addis et al., 2016; Derakhshani et al., 2018). Understanding the milk/udder microbiome composition may reveal how these microbial communities impact milk yield (low vs high) and milk quality. Milk quality can be measured through a variety of assessments, however the most common parameter utilized is somatic cell counts (SCC). The SCC of milk is directly related to its quality as past studies show that when SCC values are high, typically associated with IMI, the composition of milk is negatively impacted (Ballou et al., 1995). A healthy mammary gland should have a SCC < 200,000 cells/mL, while IMI typically causes SCC values to rise above 500,000 cells/mL, potentially resulting in the need for antibiotic treatment to recover mammary health. Identification of a milk microbiome that is associated with high milk yield and low SCC, a desirable pair of traits in the dairy industry, and one that would confer protection against infectious mammary pathogens would be pivotal in minimizing antibiotic usage on dairy farms. The milk microbiome may be essential for a variety of reasons, including food safety, mammary health, and economic efficiency, to name a few.

The objective of this study was to identify any associations between rumen, fecal, and milk microbial populations, and to investigate any relationships between milk SCC, milk yield, and IMI status with milk/udder microbial communities. We hypothesized that

associations between rumen, fecal, and milk microbiomes of dairy cattle would be discovered, with microbial populations that are present across all three sample types. Distinct populations of the milk microbiome are to be expected, as milk is considered an aerobic environment with different nutrients available for growth in comparison to the anaerobic GIT environment. Clear links could address hypotheses on how rumen or fecal microbiomes informs, establishes, or impacts the milk population, and thus mammary health. There are several possibilities of biological pathways/methods of transfer in establishing the milk microbiome suggested in previous literature, including: a) an entero-mammary pathway where bacteria may be translocated from the GIT to the mammary gland (Chen Ma et al., 2018; Rodríguez, 2014), b) the milk or mammary gland is inoculated with feces or other environmental sources, or c) the oral cavity of the calf contributes to milk microbiota formation of the lactating dam. The study herein is an initial step in this investigation.

Materials and methods

All procedures involving live animals were verified and approved by the University of Georgia's Office of Animal Care and Use (AUP #A2021 07-029-Y1-A0). The dairy cattle used in this study were located at the University of Georgia Teaching Dairy in Winterville, GA (33°54'32.9"N 83°14'50.9"W).

Animal selection and collection

A total of 51 Holstein dairy cattle were selected from the UGA Teaching Dairy. Animals chosen were clinically healthy, in their 2nd or greater lactation (multiparous), and within 30-305 days in milk. Cows outside these requirements may be more metabolically stressed and experiencing greater immunological challenges that could influence the

microbiome (Alhussien & Dang, 2018; de Haas et al., 2002), therefore were not included in the study. Cows were housed in a free stall environment using sand bedding that is rebed every 1-2 weeks and milked twice daily. Daily milk yield was collected for each cow from the day previous to sample collection. Nutrition information is available in Supplementary Tables 1 and 2.

Rumen fluid was collected from all cows by esophageal tubing following procedures described by Lourenco et al. (2019). Approximately 300 mL of ruminal fluid was collected from each animal by esophageal tubing using a weighted perforated metal probe and an electric vacuum pump. Immediately after, a subsample of 40 mL was transferred to a sterile 50 mL conical vial and placed immediately on ice. Samples were transported to a -20°C freezer for long-term storage until deoxyribonucleic acid (DNA) extraction could be performed approximately three months later.

Fecal samples were collected by digital palpation from all cows as described previously by Lourenco et al. (2020). Cows were rectally palpated to collect fecal material that was then transferred to a sterile 50 mL conical vial and placed immediately on ice. Samples were transported to a -20°C freezer for long-term storage as described above.

Milk from mammary quarters of all cows were aseptically collected into sterile 50 mL conical vials. Each teat of the mammary gland was wiped to remove dirt and debris and 3-5 streams of milk were stripped from each quarter. Each teat was then dipped in a germicidal dip containing 1% iodine. After 30 seconds, the germicidal dip was wiped completely from the teats and teat ends, and teat ends were then scrubbed vigorously with a cotton ball soaked in 70% isopropyl alcohol. Approximately 12 mL of milk from each

quarter was taken to create a composite sample. Samples were vortexed and immediately placed on ice for transport to a -20°C freezer for long-term storage as described above.

Somatic cell count enumeration and culturing of milk samples

In addition to the milk samples collected in 50 mL conical vials, another composite sample was collected into 15 mL sterile tubes and immediately placed on ice. These tubes were transported to the UGA Mastitis Lab the same day as collection for SCC enumeration and culturing. The additional sample was taken to avoid contamination of the prior sample for DNA extraction, as contamination of samples with low biomass can dominate the majority of sequences observed in a microbiome analysis (D. Kim et al., 2017; Salter et al., 2014). The SCC of the composite sample was determined using a DeLaval Direct Cell Counter (DeLaval; Tumba, Sweden) and samples were cultured on trypticase soy agar plates containing 5% sheep blood. After 24- and 48-hr of incubation at 37°C, plates were examined for pathogen presence. If pathogen growth was detected, further diagnostic tests were done to determine the infecting pathogen according to standard identification procedures (Hogan et al., 1999). The absence/presence of pathogen growth and SCC was recorded for all samples.

DNA extraction of milk contents

A 1 mL aliquot of milk then underwent DNA extraction and sequencing. This procedure uses 1000 µL of sample placed in 2-mL PowerBead Tubes (QIAGEN, Venlo, the Netherlands), which are heated in a water bath and then homogenized using a QIAGEN vortex adapter (QIAGEN, Venlo, the Netherlands) to disrupt the cells. The samples were then centrifuged at 10,000 x g for 1 min to remove debris and PowerBeads from the supernatant before Solution IRS (QIAGEN, Venlo, the Netherlands) was used to remove

all inhibitors from the sample. Using an MB Spin Column and a series of specialized buffers according to manufacturer's specifications (QIAamp BiOstic Bacteremia DNA Kit; QIAGEN, Venlo, the Netherlands), genomic DNA was eluted and purified. The concentration and purity of DNA in the resulting eluate was determined spectrophotometrically, using the Synergy LX Multi-Mode Microplate Reader in conjunction with the Take3 Micro-Volume Plate (BioTek Instruments Inc; Winooski, VT, USA). Samples with a minimum volume of 20 µL and 10 ng/µL of DNA were stored at -80°C until the following day, per recommendation by the manufacturer (QIAGEN, Venlo, the Netherlands) as the final solution does not contain EDTA that prevents the degradation of DNA. Any samples that failed to meet these requirements were rejected and the DNA extraction cycle was repeated.

DNA extraction of rumen and fecal contents

Rumen and fecal DNA was extracted following the procedures previously described by Welch et al. (2020) with modifications. Briefly, this procedure uses 350 µL of rumen sample and 0.35 g of fecal sample placed in 2-mL Lysing Matrix E tubes (MP Biomedicals LLC, Irvine, CA, USA), which are homogenized using a QIAGEN vortex adapter (QIAGEN, Venlo, the Netherlands) to disrupt the cells. Enzymatic inhibition was achieved by using InhibitEX Buffer (QIAGEN, Venlo, the Netherlands), and DNA elution and purification were carried out using a spin column and a series of specialized buffers according to manufacturer's specifications (QIAamp Fast DNA Stool Mini Kit; QIAGEN, Venlo, the Netherlands). Calculation of DNA concentration and purity in the resulting eluate was performed spectrophotometrically using the Synergy LX Multi-Mode Microplate Reader in conjunction with the Take3 Micro-Volume Plate (BioTek

Instruments Inc; Winooski, VT, USA). Samples with a minimum volume of 20 µL and 10 ng/µL of DNA were stored at -80°C until the following day. Samples that failed to meet these requirements were rejected and subjected to a new DNA extraction cycle.

DNA data sequencing analysis

Following DNA extraction, samples were shipped overnight on dry ice to LC Sciences (Houston, TX, USA; https://lcsciences.com/services/dna-sequencing/microbial-sequencing/) for library preparation and 16S ribosomal ribonucleic acid (rRNA) gene sequencing. The library preparation step included polymerase chain reaction (PCR) replications using the forward: S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and reverse: S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) primer pairs (Klindworth et al., 2013), followed by a PCR clean-up using AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, IN, USA). A second PCR step was then carried out to attach Illumina's indices and sequencing adapters (Nextera XT Index Kit; Illumina Inc., San Diego, CA, USA), followed by another PCR clean-up step using AMPure XP beads. Following this final library clean up, the library was quantified using qPCR, and the nucleotides were sequenced using an Illumina NovaSeq instrument and a NovaSeq v2 reagent kit (Illumina Inc., San Diego, CA, USA). A well-characterized bacteriophage PhiX genome (PhiX Control v3 Library; Illumina Inc., San Diego, CA, USA) was used as a control for the sequencing runs.

Sequencing data were first demultiplexed before being converted into FASTQ files, and the paired-end sequences imported into QIIME 2 (Bolyen et al., 2019). The non-biological nucleotides were then removed, and sequences were denoised, dereplicated, and chimera-filtered using DADA2 (Callahan et al., 2016). Taxonomies were assigned to the

sequences by using a pre-trained Naive Bayes classifier trained on the SILVA 138 SSU database (Quast et al., 2013), and reads were classified by taxon using the fitted classifier (Pedregosa et al., 2011). For further analysis, the sequencing depth was set at 27,230 sequences per sample. The following alpha-diversity indices were computed for each sample: Shannon Diversity Index, Faith's Phylogenetic Diversity Index, and number of observed amplicon sequence variants (ASVs). For beta diversity, we present results only from the unweighted UniFrac distance matrix. Additionally, relative bacterial abundances were quantified at the phylum and genus levels.

Statistical analysis

Statistical analyses were performed using the software Minitab® v21 (Minitab LLC, State College, PA). Milk sample SCC, the alpha diversity indices, and relative bacterial abundances at the phylum levels in rumen, fecal, and milk samples were analyzed using ANOVA. Analysis of relative bacterial abundances at the genus level in milk samples was carried out by ANOVA using SCC, milk yield, and cultured bacteriology as factors. Resulting *P*-values were corrected by Tukey's method for multiple comparisons. The differences in beta-diversity were assessed using two-sample t-tests and *P*-values were corrected by Bonferroni's method for multiple comparisons. For all the statistical tests used, results were considered significant at $P \leq 0.05$ and were treated as trends when $0.05 < P < 0.10$.

Results and discussion

Microbial diversity

The number of sequences in all samples ranged from 33,192 to 104,655 and were rarefied to a sequencing depth of 27,230 sequences per sample. A total of 40 phyla, 320

families, 733 genera, and 377 species were detected. The rumen, fecal, and milk microbiotas of the dairy cattle were very different (1,236, 801, 421 observed ASVs, respectively). Beta diversity between all pairs of samples was calculated using QIIME's "beta_diversity_through_plots.py" script and results were visualized using unweighted UniFrac distances. Rumen, fecal, and milk samples were all significantly diverse from each other ($P < 0.001$; Figure 1). Milk samples were not as clustered in comparison to rumen and fecal samples, indicating that the diversity between milk samples is more spread out than in ruminal or fecal samples. Shannon index (Figure 2) was similar to the beta-diversity findings and further demonstrated that the ruminal, fecal, and milk microbiotas were significantly distinct. This index revealed that microbial diversity was greatest ($P < 0.001$) in ruminal microbiota compared with the fecal microbiota and milk microbiota of dairy cows, reiterating how complex the rumen environment is. A greater ruminal microbial diversity compared to the feces has been reported, and suggested to be due to nutrient availability in the rumen, where most nutrients in feedstuffs are absorbed before they reach the large intestine (Lourenco et al., 2020; Siciliano-Jones & Murphy, 1989). It is not surprising that milk microbiota samples were the least diverse, as milk contains far less bacterial DNA than rumen or fecal samples (Dill-McFarland et al., 2017).

Cows were classified based on milk SCC in groups for comparison with milk microbiota: low = SCC ≤ 200,000 cells/mL, medium = 201,000 cells/mL < SCC < 800,000 cells/mL, high = SCC ≥ 800,000 cells/mL. Table 1 summarizes the microbial richness and diversity metrics calculated for milk samples collected based on SCC values enumerated at the time of collection. The Shannon index decreased as SCC increased, while Faith's Phylogenetic Diversity increased as SCC increased, although neither were significant ($P >$

0.05). In contrast, milk samples from cows that had low SCC values were more evenly distributed in comparison to samples from cows with high SCC values ($P = 0.053$; Figure 3). The greater evenness observed in the milk microbiome of cows with low SCC values may be indicative that high SCC values contributes to a reduction in microbial evenness potentially due to the influx of white blood cells that kill and digest bacteria or presence of infecting pathogens, generating important differences between the microbiota of mastitic and non-mastitic cows. Differences could be due to pathogen abundance being increased in mastitic milk populations, how the infecting pathogen affects the abundance of non-pathogenic bacteria in normal milk populations, or how pathogens change the nutritional composition of the milk (e.g., competition for iron). It has been suggested that pathogens may suppress regrowth of commensal bacteria, resulting in detrimental effects on udder homeostasis (Derakhshani et al., 2018). However, the lack of significant differences in diversity in cows with differing SCC ranges may indicate that the presence of elevated SCC may not affect the overall milk microbial diversity. Although, in the current study 58% of the high SCC animals were identified as having IMI, and previous data demonstrated that mastitic quarters had a greater bacterial richness than healthy quarters (Ganda et al., 2016). It is likely that cows that had a high SCC but were culture-negative had an IMI that had already cleared, although SCC remained elevated. When a bacteriological cure is achieved, SCC gradually declines and the speed of return to a healthy level is influenced by the pathogen that caused the IMI (Ruegg, 2021). Cows identified to have a low SCC had less observed ASVs than cows with medium and high SCC values, although the difference was not significant. Rodrigues et al. (2017) found that bulk tank milk diversity was negatively correlated with bacterial richness, suggesting that

the milk microbiomes of high bacterial load are dominated by small groups of bacterial taxa. Furthermore, healthy quarters with a low milk SCC demonstrated a more diverse milk microbiome population than quarters with mastitis (Kuehn et al., 2013; Stephanie A Metzger, Hernandez, Skarlupka, et al., 2018; Oikonomou et al., 2014). Current results demonstrated a numerical decline in Shannon diversity as SCC levels increased, in agreement with previous studies.

Table 2 and 3 summarizes the microbial richness and diversity metrics calculated for rumen and fecal samples, respectively, based on SCC values enumerated at the time of collection for comparison with milk samples. In contrast to milk, rumen samples revealed no differences ($P > 0.05$) for any diversity metrics calculated. Thus, although there were differences in milk microbiota based on SCC range, this is not reflected in the ruminal samples of the dairy cattle. Past research found a significant shift in rumen microbiota associated with inflammation and immune responses during mastitis, and a decrease in diversity of rumen microbiota in cows with mastitis when compared to healthy cows (Wang et al., 2021). While an association of SCC and rumen microbiota may not be present in the current study, further investigation of differing SCC and IMI status with rumen diversity is necessary. Similar to ruminal microbiota, fecal samples revealed no differences ($P > 0.05$) for any diversity metrics calculated. Differences in milk microbiota based on SCC range was not reflected in the fecal samples of the dairy cattle and have not been previously investigated.

In the rumen, research has demonstrated that more efficient animals have lower bacterial diversity and richness, while the opposite is seen in the intestinal environment, with a higher bacterial richness and diversity linked to a higher feed efficiency (de Oliveira

et al., 2013; Shabat et al., 2016; Welch et al., 2020). With more investigation, researchers hope for use of these same diversity metrics to predict if cows are at a greater risk for developing IMI (Stephanie A Metzger, Hernandez, Suen, et al., 2018). It has been suggested that the incidence of mastitis is associated with altered composition and decreased diversity of intramammary microbiota, although whether this is a cause or effect is still to be determined (Derakhshani et al., 2018; Oikonomou et al., 2014). Currently, few studies has investigated milk microbiome richness and diversity in association with individual cow factors such as SCC (Stephanie A Metzger, Hernandez, Skarlupka, et al., 2018; Scarsella et al., 2021) that were not from bulk tank milk studies. However, none have investigated the associations of richness and diversity with both SCC and milk yield, and the addition of cultured mastitis pathogens, as in the current study. Scarsella et al (2021) investigated the association of the udder IMI status with feces and blood microbiomes, but like the current study lacking associations between SCC and rumen and fecal microbiomes, authors did not find diversity of these populations to be associated with SCC.

Bacterial abundance at the phylum level

Microbial composition at the phylum level for the rumen, fecal, and milk populations of Holstein dairy cattle is shown in Figure 4. Of the 8 main phyla comprising the microbiota of dairy cattle, 4 had significantly different ($P < 0.05$) abundances between the rumen, feces, and milk: Bacteroidota, Patescibacteria, Proteobacteria, and Spirochaetota. In the rumen, Fibrobacterota was detected at a higher abundance ($P < 0.001$) when compared to fecal and milk samples, whereas Firmicutes were found at greater abundance ($P < 0.001$) in feces when compared to rumen and milk samples. In previous studies investigating ruminal microbiota, Bacteriodota was the most prevalent phylum,

followed by Firmicutes (de Oliveira et al., 2013; Lourenco et al., 2020; McCann et al., 2014). In contrast, Firmicutes composed a greater amount of the microbial population in the feces, and was followed by Bacteriodota (de Oliveira et al., 2013). Members of the Bacteriodota phylum have many functions in the GIT, including degradation of carbohydrates such as complex plant cell walls and production of butyrate, a significant player in energy metabolism in the rumen (Miguel et al., 2019; Thomas et al., 2011). Members of the Firmicutes phylum also serve an important role in the degradation of fiber and starch, in addition to the production of butyrate (M. Kim et al., 2011). Firmicutes has been denoted as a critical component for the milk microbiota, and while its specific role still has not been determined, gram-positive Firmicutes were previously considered as contagious mastitis pathogens (Bhatt et al., 2012; Oikonomou et al., 2014; Rodrigues et al., 2017). While the ratio of phyla Firmicutes to Bacteroidota in the rumen has been correlated to milk fat yield (Jami et al., 2014), little is known about the role of Bacteriodota in the milk microbiome. Bacteriodota was in much smaller abundance in the milk microbiome and did not compose any of the top 3 most abundant phyla in milk, namely: Firmicutes, Actinobacteriota, and Proteobacteria. Proteobacteria consists of a wide variety of gram-negative species that are considered environmental mastitis pathogens (Bhatt et al., 2012; Hogan et al., 1999), although the diversity within this phylum is large and includes non-pathogenic bacteria as well. Actinobacteriota includes gram-positive bacteria that are regularly found in the rumen, although more investigated is needed on the ecology of this phylum in the rumen and how it may influence IMI in dairy cattle (Šuľák et al., 2012). Phyla Actinobacteriota and Chloroflexi were both present in milk microbiota at greater abundances ($P < 0.05$) than rumen and fecal microbiota. Chloroflexi contains

bacteria with a diversity of roles, including aerobic thermophiles who use oxygen for growth and anoxygenic phototrophs who use light for photosynthesis (Ward et al., 2018), but little is known about what roles these members play in cattle microbiota. Phylum Cyanobacteria was present in all three sample types, but no differences between populations were found ($P > 0.05$). Cyanobacteria consists of gram-negative bacteria that share similarities to eukaryotic algae and includes toxin-producing bacteria that may cause disease in livestock, such as blue-green algae toxicosis (McGorum et al., 2015; Puschner et al., 1998). More investigation is needed to determine the impacts of this phylum on animal welfare in terms of production. Both Cyanobacteria and Chloroflexi have been identified before in milk samples but in low levels (Ganda et al., 2016; Rodrigues et al., 2017; Verdier-Metz et al., 2012). Phylum Chloroflexi was only present in milk microbiota, while Fibrobacterota was the only phylum present in rumen that was not identified in milk or fecal microbiota. Fibrobacterota has been previously identified as a core rumen bacterial taxon in dairy cows, and suggested to be due to a higher forage-to-concentrate ratio (M. Xue et al., 2018), similarly used on the current study's farm. However, the milk and fecal populations do not reflect this, suggesting this phylum has a more essential role in the rumen community. Fecal microbiota did not have any phyla in abundance that were not found in rumen or milk populations. Rumen and milk populations both had presence of phyla Proteobacteria and Patescibacteria that were not identified in the fecal microbiota. Patescibacteria have been found to be prevalent in groundwater, sediment, and a variety of anoxic environments, but this phylum has not been well characterized in cattle microbiomes (Park et al., 2021; Tian et al., 2020). The role that some of these phyla play in cattle microbiomes is still undetermined, and more investigation is needed to discover

what influences they have on animal health and disease. Overall, it is unsurprising to find some similarities in phyla identified in rumen, fecal, and milk communities, as bacteria from the rumen end up in the feces, on the exterior of the animal, and in the surrounding environment the animal is housed in (Taponen et al., 2019).

In past research, Firmicutes, Proteobacteria, Bacteroidota, and Actinobacteriota have been identified as the most abundant phyla in milk (Bonsaglia et al., 2017; Derakhshani et al., 2018; E. et al., 2022; Ganda et al., 2016; Quigley et al., 2013; Rodrigues et al., 2017; Verdier-Metz et al., 2012). Although, Cyanobacteria has also been identified as one of the most prevalent phyla present in milk samples, over populations of Actinobacteriota and Bacteriodota (Bhatt et al., 2012; Rodrigues et al., 2017). Previous studies have indicated that the composition of bacterial communities in milk samples can differ between cows kept on different beddings and in different geographical locations, even with the milk is collected directly from the gland cistern, which may explain differences in microbiome populations of cows housed in different environments (Derakhshani et al., 2018; S A Metzger et al., 2018; Taponen et al., 2019). Past research found the most abundant phyla in milk samples were Proteobacteria, Bacteroidota, Actinobacteria, and Firmicutes (Scarsella et al., 2021), whereas Firmicutes and Actinobacteria were present in higher abundances compared to Proteobacteria and Bacteroidota in the current study. Differences may be due to geographical location (Italy vs U.S.) and/or bedding type (not described by Scarsella et al. (2021)). Additionally, Bhatt et al. (2012) found differences between the most abundant phyla in different breeds of cattle (Kankrej and Gir cattle; India), with Cyanobacteria only present in Kankrej cattle and Bacteroidetes not present in Gir cattle. The season of collection also has an impact on

the abundance microbiota, with proportions of Actinobacteria and Chloroflexi higher in the spring, whereas Bacteriodetes were more enriched in the fall season (E. et al., 2022). Furthermore, the number of observed OTUs was significantly higher in the spring than in the summer and fall (E. et al., 2022). While the current study collected samples in the fall, comparisons are difficult to make as the latter study focused on bulk tank milk samples. Most importantly, the technique of collection from the mammary gland produces different microbiota results. For example, researchers have investigated collection of microbiome samples directly from the milk, using teat canal swabs, and on the teat apex (skin), and found that each location has their own bacterial taxa exclusive to their environment, as well as differing diversities and abundances (Andrews et al., 2019; Derakhshani et al., 2020). The results presented herein specifically represent the milk microbiota, and comparison with other results must be done carefully as the milk microbiota may not reflect the entirety of the bacterial population in the mammary gland. Research has suggested the possibility of a pathway that transports GIT microbes to the mammary gland, assisting in establishment of the mammary gland/milk microbiome (Addis et al., 2016). In the current study, the presence of anaerobic bacteria (e.g., Bacteriodota) commonly found in the rumen and fecal microbiome was also in abundance in the milk microbiome. However, the presence of similar bacterial DNA between milk microbiota and ruminal and fecal microbiota does not confirm that bacteria is entering the mammary gland and milk from the GIT.

Bacterial abundance at the genus level

The lowest taxonomical level at which the samples in this study were classified was the genus level. The top 10 most abundant genera for rumen, fecal, and milk populations

of Holstein dairy cattle is shown in Figure 5. Rumen and fecal populations shared one genus in the top 10 most abundant, *Muribaculaceae*, that was present in similar levels (2.85% vs 2.86%). *Muribaculaceae,* member of the Bacteroidota phylum, is a prevalent group of bacteria found in the GIT microbiome of cattle (Lagkouvardos et al., 2019), but its role in the milk microbiota is still unknown. Fecal and milk populations shared the genera *UCG-005* and *UCG-010,* both which were present in higher abundance in the fecal microbiota. *UCG-005* and *UCG-010* are from the Ruminococcaceae family under phylum Firmicutes, and are obligate anaerobes that have been previously identified in the GIT of ruminant animals (Bach et al., 2019; Fomenky et al., 2018). Interestingly, there were no abundant genera that were shared between the rumen and milk microbiotas. The presence of genera in milk samples that are usually found in fecal microbial populations and the absence of similar genera between ruminal and milk microbiomes could suggest that the fecal microbiota may inform the milk population more than the ruminal microbiota.

The most prevalent genera previously identified in both milk samples and on the teat apex that were identified in milk samples in the current study include *Corynebacterium, Acinetobacter, Staphylococcus, and Streptococcus* (Bhatt et al., 2012; Bonsaglia et al., 2017; Gorik Braem et al., 2012; Derakhshani et al., 2018; Ganda et al., 2016; Kuehn et al., 2013; Oikonomou et al., 2014; Rodrigues et al., 2017; Vacheyrou et al., 2011; Verdier-Metz et al., 2012). *Corynebacterium*, which was identified as the most prevalent genera in milk, consists of a large variety of species that are frequently detected within cow's milk and assist in development of cheese flavor and aroma (Duthoit et al., 2003; Oikonomou et al., 2014; Quigley et al., 2013). Although, species of this genus can have contradictory functions with regard to protection against IMI (Derakhshani et al.,

2018; Hogan et al., 1999; Woodward et al., 1987). *Acinetobacter* is an important spoilage bacteria and can adapt to a variety of environmental conditions, but a new emergence of pathogens from this genus have been reported (Gurung et al., 2013; Rodrigues et al., 2017). While genera *Acinetobacter* and *Staphylococcus* have been frequently detected in raw milk from healthy cows, species from these genera can be involved with development of IMI in dairy cattle if favorable conditions for pathogen growth persist (Bonsaglia et al., 2017; G Braem et al., 2011; Gonçalves et al., 2016). The presence of *Streptococcus* as an abundant genus in milk has been previously identified in both healthy and mastitic milk samples, and while many of these species are considered pathogenic in terms of IMI, some are frequently isolated in milk and used as starter cultures in the manufacture of dairy products (Duthoit et al., 2003; Oikonomou et al., 2014; Quigley et al., 2013; Randazzo et al., 2002; Santarelli et al., 2008). *Halomonas* comprises gram-negative aerobic bacteria under phylum Proteobacteria and is an abundant genus found in both in milk and cheese (Coton et al., 2012; Pang et al., 2018), but the impact of these bacteria in terms of disease is still under investigation (K. K. Kim et al., 2013). The presence of *Trueperella, Ornithinimicrobium,* and *JG30-KF-CM45* as abundant genera were specific to our current study. *Trueperella pyogenes* is a common summer mastitis pathogen (Ishiyama et al., 2017), and was present in one of our IMI samples in high abundance, accounting for the abundance of this genera in the milk samples. *Ornithinimicrobium* has been detected previously in milk and bedding microbiome samples (Dean et al., 2021; Li et al., 2021), and was linked to be significantly higher in sand bedding in comparison to other beddings (Ray et al., 2022), which is the type of bedding used at the farm in the present study. Little is known about genus *JG30-*

KF-CM45, other than it falls under the phylum Chloroflexi (Speirs et al., 2019), which was an abundant phylum present in milk samples in the current study.

Milk microbiota and milk yield at the genus level

Cows were classified by milk yield into groups for comparison with milk microbiota: low = ≤ 65 lbs, medium = 65 lbs < x < 90 lbs, high = ≥ 90 lbs. The bacterial genera detected in the milk of dairy cattle that were associated with milk yield are listed in Table 4. Milk samples from cows that had high milk yield at the time of collection had higher amounts of bacteria genera *Escherichia-Shigella* and *Hymenobacter* present in their milk samples compared to cows that had medium milk yield ($P < 0.05$), and higher amounts of genus *Rheinheimera* in comparison to milk samples from cows that had medium and low milk yield ($P = 0.024$). *Hymenobacter*, under phylum Actinobacteria, has been previously identified in the teat canal microbiota (Falentin et al., 2016), although little is known about its role in cattle health. *Rheinheimera*, under phylum Proteobacteria, was previously demonstrated to be more abundant in ruminal samples from cows in late lactation compared to those in mid lactation (Lyons et al., 2018). Significant presence of *Rheinheimera* in cows in late lactation and those with high milk yield may suggest this genus plays a bigger role in cattle production than previously expected. Many members of genus *Escherichia-Shigella* are pathogenic, such as *Escherichia coli*, a common mastitis pathogen that can affect high producing cows in dairy herds and in severe cases result in death (Burvenich et al., 2003; Yu et al., 2021). It is unsurprising to find presence of this bacterial genus in milk samples as some cows in the present study were identified as having a gram-negative *E. coli* IMI. However, it is interesting that cows with high milk yield had significantly higher abundance of this genus, but only in comparison to cows with medium

milk yield. It is expected that lower production cows would be more influenced by pathogenic bacteria, but results demonstrate it may be more complicated than previously thought.

Milk samples from cows that had low milk yield at the time of collection had significantly higher amounts of bacteria genera *Bacteroides, Coprococcus,* and *UCG-005* present in their samples in comparison to milk samples from cows that had medium milk yield ($P < 0.05$). There was trend between cows with low milk yield having higher levels of bacterial genus *Coprococcus* in comparison to cows with high milk yield ($P = 0.079$). *Coprococcus* abundance in the rumen has been previously identified as having a negative correlation with milk production (Jami et al., 2014; Mu et al., 2019), and indicates the same relationship within the milk microbiota. *Bacteroides* species in the GIT maintain a generally beneficial relationship with the animal, however out of the GIT environment they can act as etiologic agents for bacteremia and foot rot (Berg & Loan, 1975; Laing & Egerton, 1978; Wexler, 2007). The abundance of *Coprococcus* and *Bacteroides* in low milk yield cows suggest that species of these genera may play a role in diminishing milk production and efficiency in cattle, and the pathology of these species in production diseases requires more investigation. Additionally, cows with low milk yield had significantly higher levels of bacteria genera *Chitinophaga, Parasutterella, Porphyrobacter, Sphingomonas*, and *UCG-010_1* in comparison to milk samples from cows with a high milk yield ($P < 0.05$). *Chitinophaga,* under phyla Bacteroidota, has been previously identified in the rumen microbiota but little is known about their role in animal health (Neves et al., 2020). *Porphyrobacter,* a genus of aerobic bacteria, has been previously identified in high relative prevalence in cows with metritis (Sicsic et al., 2018),

also known as inflammation of the uterus. *Sphingomonas* includes gram-negative aerobic bacteria that have been previously detected in dairy environments and milk microbiota samples without any associations with pathogenicity (Kuehn et al., 2013; Vacheyrou et al., 2011). *Parasutterella* is a genus of gram-negative bacteria from the Proteobacteria phylum that have been linked to modulation of the abundance and/or function of commensal bacteria in the GIT (Ju et al., 2019). Higher abundance of bacterial genera *Parasutterella* ($P = 0.002$) and UCG-010_1 ($P = 0.002$) was found in low milk yield cows in comparison to both medium and high milk yield cows. *Halomonas* was also a prevalent genus but was higher in medium milk yield cows in comparison to low milk yield cows ($P = 0.014$), with no significant difference with high milk yield cows. Genus *Ornithinicoccus* was present in higher abundance in cows with low milk yield in comparison to cows with medium milk yield, but little is known about the role of this genus in cattle.

Milk microbiota and SCC at the genus level

The bacterial genera detected in the milk of dairy cattle that were associated with SCC are listed in Table 5. Milk samples from cows identified as having low SCC values at the time of collection had higher levels of bacterial genus *Acholeplasma* in comparison to milk samples from cows identified as having high SCC values ($P < 0.05$). Species of this genera are common nonpathogenic contaminants found in milk, however have been previously isolated in mastitis milk as well (Boonyayatra et al., 2011). In addition, cows with low SCC values had numerically higher levels of bacteria genera *Sphingomonas* present in their samples in comparison to samples from cows with high SCC values, although cows with medium SCC values had the highest abundance of these genera present in comparison to cows with low SCC and high SCC ($P < 0.05$). Cows with medium SCC

values at the time of collection also had higher levels of bacterial genus *Brevundimonas* present in their milk samples in comparison to samples from cows with low SCC values ($P = 0.047$). Some *Brevundimonas* species have been linked with infection in humans, and shown potential for etiology of IMI in cattle (Han & Andrade, 2005; Stabler et al., 2018; R. Zhang et al., 2015). Previously, *Brevundimonas* was significantly more present in mastitic milk samples, as well as *Sphingomonas* (Kuehn et al., 2013). However, the variation of presence of these genera in the current study between different levels of SCC does not allow us to draw the conclusion that these genera are associated with mastitic milk.

Milk samples from cows identified as having high SCC values at the time of collection had higher levels of bacteria genus *Succinivibrionaceae_UCG-001* in comparison to milk samples from cows identified as having low and medium SCC values ($P < 0.05$). The Succinivibrionaceae family, a group of volatile fatty acid producing microbes, is associated with low methane production in the rumen, and additional investigation suggests the genus *Succininivibrionaceae_UCG-001* demonstrates the same pattern (Ramayo-Caldas et al., 2020). *Succinivibrio* species ferment glucose into acetic and succinate acids, which assist in the production of propionate, a major gluconeogenic substrate (Bryant and Small, 1956; Hailemariam et al., 2020; Lee et al., 2005). Little is known about this genus in the milk microbiota, however its presence in high abundance in cows with high SCC suggests it may play a different role in milk than in the rumen. Is it possible that commensal bacteria are increasing the production of glucose in order to supply energy to the immune cells (Kvidera et al., 2017) in high SCC cells to fight off infection? While genera *Alloiococcus, Marinobacter*, and *Thiopseudomonas* were

identified to be associated with SCC of cows at the time of collection, little is known about the role of these genera in cattle.

Milk microbiota and bacteriology at the genus level

Of the 51 cows enrolled in the study, 11 (22%) were identified through culture as having mastitis pathogens present in their milk samples. The most predominant pathogen identified was coagulase-negative staphylococci (CNS) (14%), then gram-negative (4%) bacteria, followed by *Staphylococcus aureus* (2%), and Streptococcus spp. (2%). The other 78% of cows were identified as culture negative. The Shannon diversity was highest in milk samples that were culture negative at the time of collection (Figure 6). Shannon diversity of the milk samples decreased in order of the following types of mastitis pathogens identified in samples: CNS, *Staph. aureus*, Streptococcus spp., gram-negative. Cows with gram-negative pathogen growth significantly decreased the Shannon index ($P < 0.001$). Similarly, milk samples that were culture negative at the time of collection were more evenly distributed than samples with cultured pathogen growth (Figure 7). Evenness decreased in the order of the following types of mastitis pathogens identified in samples: CNS, *Staph. aureus*, Streptococcus spp., gram-negative. Cows with gram-negative pathogen growth significantly decreased the evenness ($P < 0.001$). Results suggest that gram-negative pathogen presence has the largest influence on the normal host microbiota.

Tables 6 and 7 show the association of cultured mastitis pathogens with relative bacterial abundance at the genus level in milk samples, with only significant or trending genera. Genera presented in parts per million (ppm) in Table 7 were in small abundance. The presence of mastitis pathogens in the microbiome analysis was consistent with culture results, in line with previous studies using conventional culturing (Ganda et al., 2016;

Oikonomou et al., 2012; Taponen et al., 2019), with a few exceptions to be discussed. Milk samples from cows identified through culture as having *Staph. aureus* pathogen growth had significantly higher levels of bacteria genus *Acidibacter* present in their milk samples in comparison to cows that had no pathogen growth and cows with CNS, Streptococcus spp., or gram-negative pathogen growth ($P < 0.05$). Little is known about *Acidibacter* in cattle microbiota, although this group of ferric iron-reducing bacteria have been previously identified in soil and cow manure fertilizer (Falagán & Johnson, 2014; S. Zhang et al., 2020). Cows identified as having Staph. aureus pathogen growth also had significantly higher levels of bacteria genus *Staphylococcus* present in their milk samples in comparison to cows with no pathogen growth and gram-negative growth ($P = 0.001$). Milk samples from cows identified through culture as having CNS pathogen growth had significantly higher levels of bacteria genera *Chitinophaga* and *Porphyrobacter* present in their milk samples in comparison to cows that had no pathogen growth and cows with Streptococcus spp ($P < 0.05$). Identification of non-aureus staphylococci (e.g., CNS) in cases when *Staph. aureus* was abundant was previously demonstrated (Taponen et al., 2019). The present study demonstrated similar results, with a few exceptions of CNS and *Staph. aureus* cows not having similar levels of genera of lower abundances, such as *Acidibacter.* It is important for producers to note whether a cow has IMI due to Staph. aureus or CNS, as antibiotic treatment outcome for CNS is more successful (Taponen & Pyörälä, 2009). While identification down to the species level would be beneficial in these cases, the potential association of these IMI pathogens with specific bacterial genera may allow for some applicability of results. Taponen et al. (2019) also identified associations of *Staph. aureus* presence with *Cornyebacterium bovis,* and while genera *Corynebacterium* was

identified in abundance in milk samples, no significance was indicated with the abundance of this genus with levels of SCC, milk range, or cultured mastitis pathogens.

Milk samples from cows identified through culture as having Streptococcus spp. pathogen growth had significantly higher levels of bacteria genera *Flavonifractor, Lachnospiraceae_UCG-008, Muribaculaceae_1, Olsenella, Ruminobacter,* and *Streptococcus* present in their milk samples in comparison to cows that had no pathogen growth and cows with CNS, *Staph. aureus*, or gram-negative pathogen growth ($P < 0.05$). *Flavonifractor,* under phylum Firmicutes, has previously presented a greater abundance in cows under thermoneutral conditions being fed a high energy diet (Correia Sales et al., 2021). A main product of *Flavonifractor* metabolism is acetic acids, which cows use to make milk fat (Carlier et al., 2010; Daniel et al., 2013). While this genus plays a specific role in cattle metabolism, its role in pathogenicity and high abundance in cows with Streptococcus spp. needs to be investigated. *Olsenella* has been identified in abundance in the rumen of cattle, and the bacteria in this group are known as lactic acid bacteria (M. Kim et al., 2020; Kraatz et al., 2011). Lactic acid bacteria abundance in the dairy animal diet has been linked to improved feed efficiency and increased milk yield (Murad & Azzaz, 2019), however little is known about genus *Olsenella* specifically in terms of production and pathogenicity. Previously, loss of *Lachnospiraceae* was suggested to be associated with mastitis and critical to a healthy host state (Chen Ma et al., 2018), although the current study found that one genus from this family, *Lachnospiraceae_UCG-008,* was present in higher abundance in cows identified as having Streptococci spp. than those clear of mastitis infection. *Ruminobacter* are common colonizers of GIT microbiomes and play in important role in the digestive process of cattle, and the presence of this genus in milk microbiota

(Anderson, 1995; Scarsella et al., 2021) may provide evidence for the GIT translocation hypothesis. Cows identified as having Streptococcus spp. growth had significantly higher levels of bacteria genera *Massilia* in comparison to cows with CNS and *Staph.aureus*, and higher levels of *Monoglobus* in comparison to cows with CNS ($P < 0.05$). *Massilia* has been identified as a core genera in GIT microbiota, but have been isolated from environmental sources and could represent contamination of milk samples (Alipour et al., 2018). Genus *Monoglobus* has been previously identified in the nasopharynx, rumen, and vaginal microbial community of heifers, and was suggested to be primary degrader in the human GIT (Amat et al., 2021; C. C. Kim et al., 2019), suggesting that this bacteria may originate from the GIT in cattle as well.

Milk samples from cows identified through culture as having gram-negative pathogen growth had significantly higher levels of bacteria genera *Globicatella, Hymenobacter,* and *Trueperella* present in their milk samples in comparison to cows that had no pathogen growth and cows with CNS, Streptococcus spp., or *Staph. aureus* pathogen growth ($P < 0.05$). Previous research of *Globicatella* indicates that one species (of two) of this gram-positive genus is a new, rare pathogen in human infections that has been misdiagnosed with Streptococcus spp. (Miller et al., 2017; Seegmüller et al., 2007). *Globicatella* colonies appear very small on agar and could easily be misdiagnosed with standard culture techniques. The potential pathogenicity of this genus in dairy cattle is unknown but should be considered for investigation. Additionally, cows identified as having gram-negative pathogen growth had significantly higher levels of bacteria genus *Escherichia-Shigella* in comparison to cows with no pathogen growth, *Staph. aureus*, or Streptococcus spp. ($P = 0.043$). Genus *Lactococcus* was also present in higher abundance

in cows with gram-negative pathogen growth in comparison to cows that were culture-negative or had pathogen growth of any other type ($P = 0.018$). *Lactococcus* contains lactic acid bacteria that ferment lactose to lactate, and members are naturally present in raw milk (Quigley et al., 2013). The high abundance of this genus in cows with gram-negative pathogen growth could be due to pathogen influence on surrounding commensal microbiota.

Milk samples from cows identified through culture as culture negative had significantly higher levels of bacteria genus *Christensenellaceae_R-7_group* in comparison to cows that had CNS pathogen growth and *Staph. aureus* ($P = 0.03$). *Christensenellaceae* in the GIT has been linked to a healthier digestive system in humans and better feed efficiency in steers (Goodrich et al., 2014; Welch et al., 2021). Additionally, cows that were culture-negative had significantly higher levels of bacteria genus *UCG-010_1* in comparison to cows with Streptococcus spp. pathogen growth ($P = 0.009$). Previously, a general increase of *Enterococcus, Streptococcus*, and *Staphylococcus* and decrease in *Lactobacillus* was observed in both milk and gut microbiota when cows were suffering from mastitis (C Ma et al., 2016). In the current milk microbiome population, no association of *Enterococcus* and *Lactobacillus* with mastitis, SCC, or milk yield was identified. Genera *Branchiibius, Chloroplast_1, Desulfomonile, Haliangium, Luteolibacter, Pseudohongiella*, and *Vicinamibacteraceae_1* were all identified to be associated with IMI status, but little is known about these genera in terms of cattle and cattle health.

Mastitic milk has shown favor in growth of different bacterial genera as compared to genera in normal milk, where non-pathogenic bacteria are inhibited by mastitis milk and

growth of pathogenic bacteria is limited in normal milk (Fang et al., 1993; Kaartinen et al., 1989; Mattila-Sandholm et al., 1990). This change in composition of milk is suggested as an explanation for the differences between microbiomes in healthy and mastitic quarters (Taponen et al., 2019). Previously, genera *Staphylococcus, Enterobacter,* and *Streptococcus* were the most common pathogens isolated in mastitic milk samples (Taponen et al., 2019). While our study did identify *Staphylococcus* and *Streptococcus* to be the most common pathogens present in mastitic milk samples, we did not identify any abundance of *Enterobacter*, but instead genus *Trueperella*. In milk samples from IMI due to *Trueperella pyogenes* as diagnosed by culture, sequences of this pathogen were also among the most common in the milk microbiome (Oikonomou et al., 2012). Presently, *Trueperella* was not identified by conventional culture methods used, but was identified in greatest abundance within cows with gram-negative pathogen growth. However, *Trueperella* is a gram-positive bacterium, and likely some contamination occurred during conventional culturing that misdiagnosed this mastitis pathogen as another. There are limitations with culturing, especially when colonies from species like *Trueperella pyogenes* are very small (Sciences, 2021), and culturing only detects organisms that grow under laboratory conditions, thereby some bacterial groups go undetected with conventional culture techniques (Hugenholtz et al., 1998; Kuehn et al., 2013; Lima et al., 2017; Oikonomou et al., 2014; Rondon et al., 2000). In addition, contamination rates in culture-based mastitis studies can range from 5-15%, however without conventional culturing in microbiota research, interpretation of results can be difficult when contamination from outside the mammary gland may occur (Stephanie A Metzger, Hernandez, Suen, et al., 2018). The identification of the origin of bacterial DNA within

milk samples is critical, because if the bacterial DNA was not sourced from the milk within the mammary gland, it cannot be concluded that the bacteria are related to the outcomes of mammary health (Stephanie A Metzger, Hernandez, Suen, et al., 2018).

Conclusion

Collectively, our results demonstrated that the ruminal, fecal, and milk microbiotas of dairy cows were very distinct. Alpha and beta-diversities, and the individual microbial compositions at the phylum level agreed that the ruminal, fecal, and milk environments were dissimilar. However, some bacterial taxa were present in all of the environments, suggesting that there may be some relationship between these compartments and potentially how the milk microbiome is established. Furthermore, milk samples associated with higher SCC values decreased the evenness of the microbial population, likely due to domination of the environment by certain bacterial taxa. While there were specific bacterial genera associated with SCC range, milk yield range, and cultured pathogen presence, no phyla or genera were identified in significant abundance in both low SCC cows and high mild yield cows, a coveted pair of traits in the dairy industry. More precise investigation (e.g., PCR) of mastitis bacteriology in association with the milk microbiome should be done to determine if pathogens directly or indirectly alter the milk microbiome. Additionally, further research is needed to investigate the presence of a biological pathway between the rumen and fecal microbiomes and the milk microbiome.

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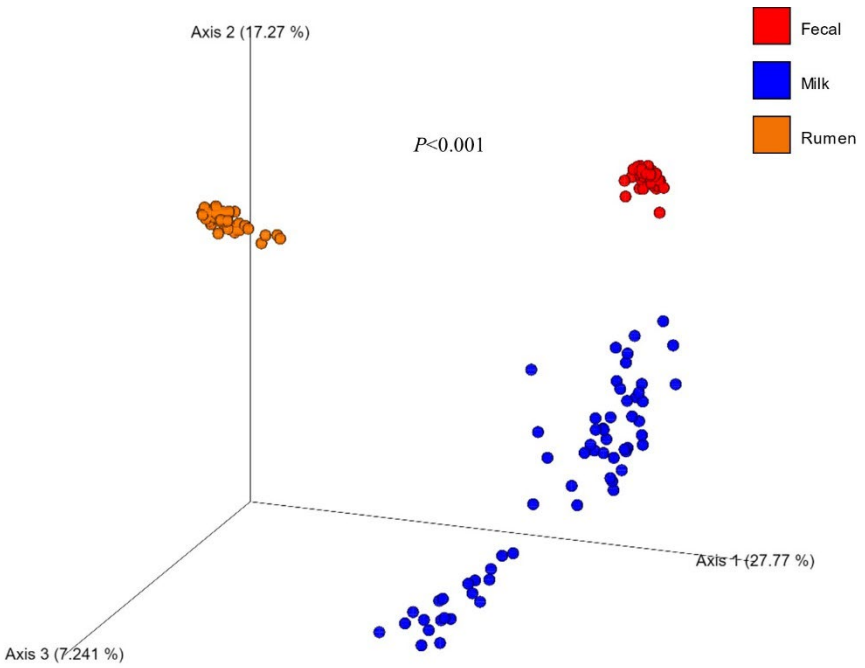

Figure 5.1. Principal coordinate analysis plot of beta diversity of rumen, fecal, and milk bacterial populations of Holstein dairy cattle (n=51) using the unweighted UniFrac distance matrix. P-values indicate a difference in beta diversity between sample types (milk, rumen, fecal).

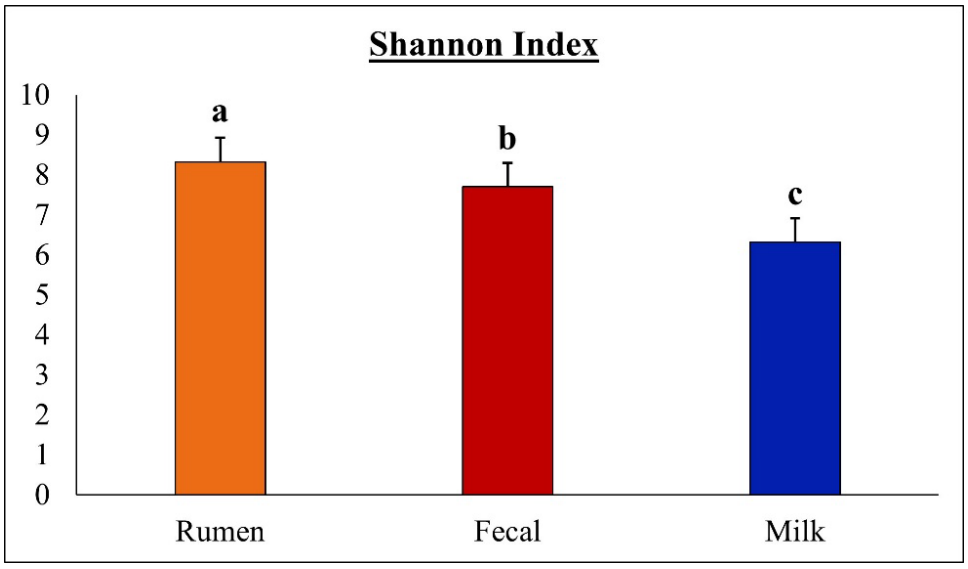

Figure 5.2. Comparison of Shannon diversity indices between rumen, fecal, and milk microbiota from Holstein dairy cattle. Error bars indicate the standard error. [a,b,c]Letters indicate statistical differences between samples ($P \leq 0.05$) according to Tukey's pairwise comparisons.

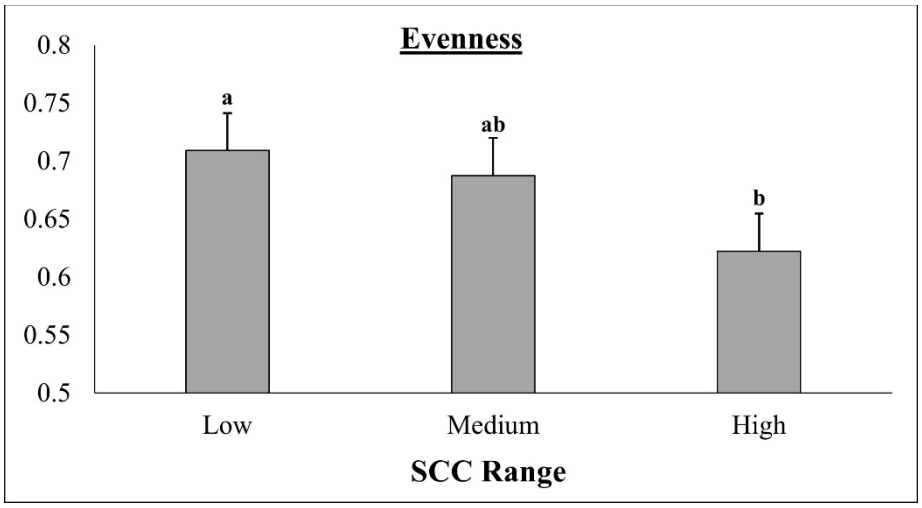

Figure 5.3. Comparison of evenness indices within milk microbiota of Holstein dairy cattle between somatic cell count range at sample collection: Low = SCC ≤ 200,000 cells/mL. Medium = 201,000 cells/mL < SCC < 800,000 cells/mL. High = SCC ≥ 800,000 cells/mL. Error bars indicate the standard error. [a,b]Letters indicate statistical differences between samples ($P \leq 0.05$) according to Tukey's pairwise comparisons.

Table 5.1. Alpha-diversity indices calculated for the milk samples of Holstein dairy cattle with different somatic cell count ranges at time of collection: low, medium, or high.

	SCC Range[1]				
Item	**Low**	**Medium**	**High**	**SE**	***P*-value**
Shannon Index	5.89	5.68	5.24	0.59	0.329
Faith's PD[2]	55.34	62.46	76.55	25.68	0.213
Evenness	0.709[a]	0.688[ab]	0.623[b]	0.032	0.053
Observed ASVs	332.90	367.83	373.27	177.79	0.831

[1]Low = SCC ≤ 200,000 cells/mL. Medium = 201,000 cells/mL < SCC < 800,000 cells/mL. High = SCC ≥ 800,000 cells/mL; [2]Faith's Phylogenetic Diversity. [a,b]Means within a row with different superscripts differ ($P \leq 0.05$) according to Tukey's pairwise comparisons.

Table 5.2. Alpha-diversity indices calculated for the rumen samples of Holstein dairy cattle with different somatic cell count ranges at time of collection: low, medium, or high.

	SCC Range[1]				
Item	**Low**	**Medium**	**High**	**SE**	***P*-value**
Shannon Index	9.46	9.80	9.49	0.24	0.232
Faith's PD[2]	60.18	65.63	62.25	3.09	0.154
Evenness	0.908	0.924	0.905	0.014	0.357
Observed ASVs	1380	1560	1440	106.5	0.179

[1]Low = SCC ≤ 200,000 cells/mL. Medium = 201,000 cells/mL < SCC < 800,000 cells/mL. High = SCC ≥ 800,000 cells/mL; [2]Faith's Phylogenetic Diversity. None of the means within each row were significantly different ($P \geq 0.05$) according to Tukey's pairwise comparisons.

Table 5.3. Alpha-diversity indices calculated for the fecal samples of Holstein dairy cattle with different somatic cell count ranges at time of collection: low, medium, or high.

	SCC Range[1]				
Item	**Low**	**Medium**	**High**	**SE**	***P*-value**
Shannon Index	8.86	8.88	9.06	0.12	0.227
Faith's PD[2]	40.18	40.3	41.58	1.51	0.625
Evenness	0.886	0.887	0.903	0.007	0.054
Observed ASVs	1031	1031.5	1065.3	54.67	0.795

[1]Low = SCC ≤ 200,000 cells/mL. Medium = 201,000 cells/mL < SCC < 800,000 cells/mL. High = SCC ≥ 800,000 cells/mL; [2]Faith's Phylogenetic Diversity. None of the means within each row were significantly different (P ≥ 0.05) according to Tukey's pairwise comparisons.

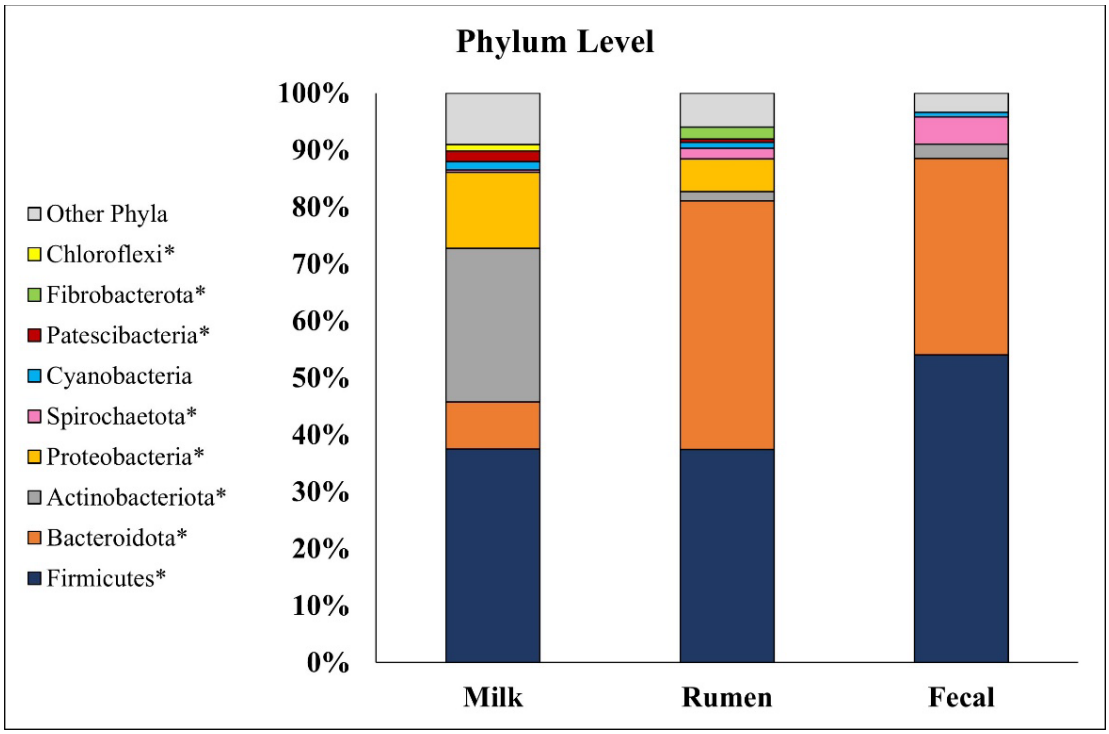

Figure 5.4. Relative bacterial abundance at the phylum level for Holstein dairy cattle in different samples (Milk, Rumen, Fecal; n=51): phyla with relative abundance ≥1% in one of the sample groups (group averages shown). * Samples within a phylum differ ($P \leq 0.05$) according to Tukey's pairwise comparisons.

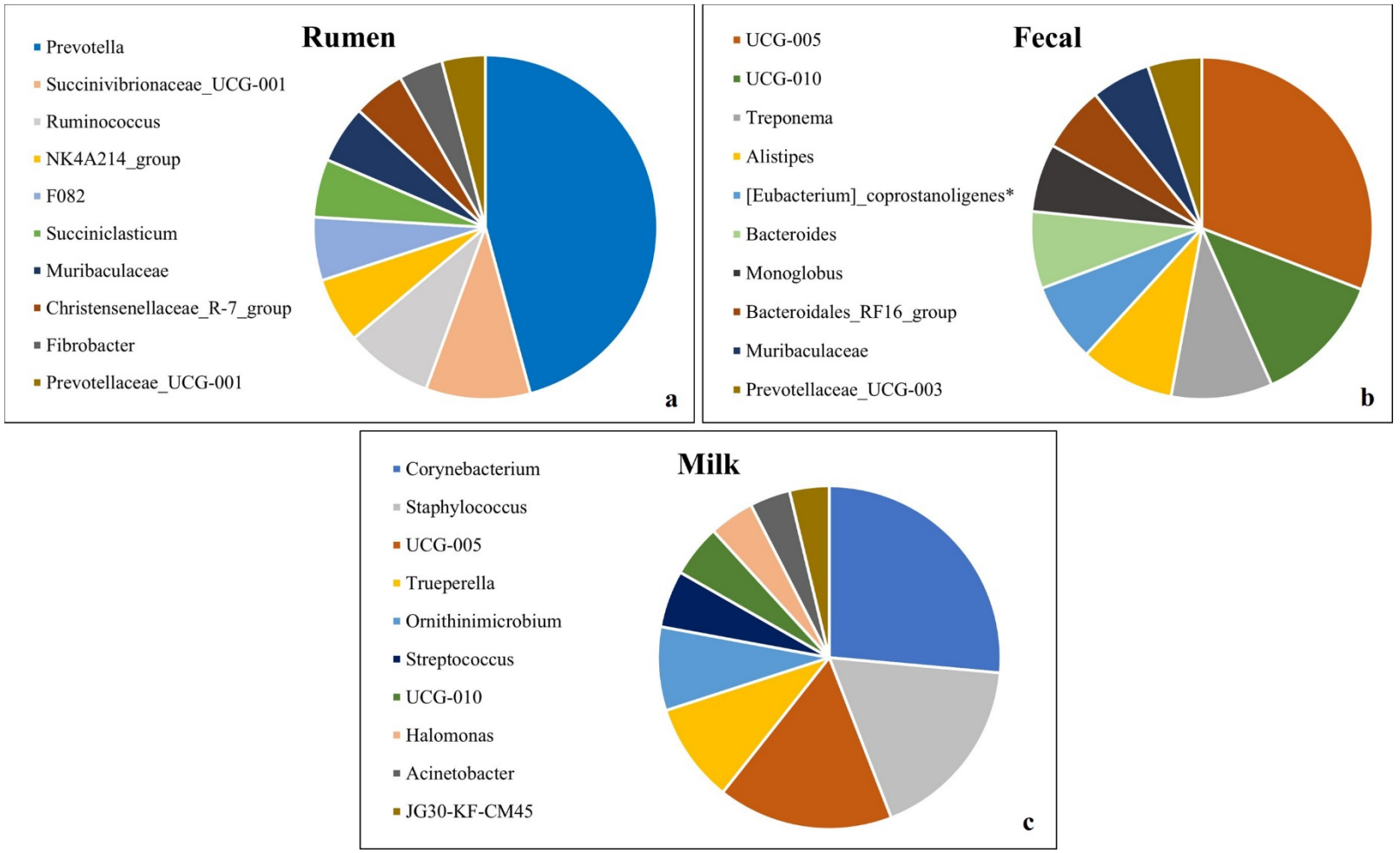

Figure 5.5. Top 10 most abundant bacterial genera detected in the rumen (**a**), fecal (**b**), and milk (**c**) samples of Holstein dairy cattle (*n*=51).

Table 5.4. Bacterial genera abundance (%) in the milk samples of Holstein dairy cattle associated with different milk yield ranges at time of collection: low, medium, and high.

	Milk Yield Range[1]				
Genera	**Low**	**Medium**	**High**	**SE**	***P*-Value**
Bacteroides	0.667[a]	0.088[b]	0.424 [ab]	0.237	0.047
Chitinophaga	0.586[a]	0.272[ab]	0.000[b]	0.190	0.02
Coprococcus	0.242[a]	0.000[b]	0.001 [ab]	0.097	0.012
Escherichia-Shigella	0.295[ab]	0.103[b]	0.574 [a]	0.225	0.033
Halomonas	0.235[b]	1.645[a]	1.117 [ab]	0.468	0.014
Hymenobacter	0.014[ab]	0.000[b]	0.025 [a]	0.010	0.033
Ornithinicoccus	1.081[a]	0.430 [b]	0.471 [ab]	0.264	0.028
Parasutterella	0.131[a]	0.000 [b]	0.000 [b]	0.050	0.002
Porphyrobacter	0.958[a]	0.405[ab]	0.000 [b]	0.313	0.016
Rheinheimera	0.000[b]	0.000 [b]	0.102[a]	0.069	0.024
Sphingomonas	1.133[a]	0.540 [ab]	0.183 [b]	0.351	0.046
UCG-005	5.851[a]	2.317 [b]	3.069 [ab]	1.205	0.01
UCG-010 1	1.616[a]	0.000 [b]	0.212 [b]	0.461	0.002

[1]Low = Yield ≤ 65 lbs. Medium = 65 lbs < Yield < 90 lbs. High = Yield ≥ 90 lbs. [a,b]Means within a row with different superscripts differ ($P \leq 0.05$) according to Tukey's pairwise comparisons.

Table 5.5. Bacterial genera abundance (%) in the milk samples of Holstein dairy cattle associated with different somatic cell count ranges at time of collection: low, medium, and high.

	SCC Range[1]				
Genera	**Low**	**Medium**	**High**	**SE**	***P*-Value**
Acholeplasma	0.327[a]	0.363[a]	0.032[b]	0.106	0.007
Alloiococcus	1.219[a]	0.876[ab]	0.356[b]	0.255	0.012
Brevundimonas	0.000[b]	0.120[a]	0.039[ab]	0.064	0.047
Marinobacter	0.856[ab]	1.449[a]	0.305[b]	0.486	0.012
Sphingomonas	0.712[ab]	1.034[a]	0.110[b]	0.361	0.045
Succinivibrionaceae_UCG-001	0.001[b]	0.007[b]	0.119[a]	0.042	0.027
Thiopseudomonas	0.260[b]	0.867[a]	0.062[b]	0.206	0.002

[1]Low = SCC ≤ 200,000 cells/mL. Medium = 201,000 cells/mL < SCC < 800,000 cells/mL. High = SCC ≥ 800,000 cells/mL. [a,b]Means within a row with different superscripts differ ($P \leq 0.05$) according to Tukey's pairwise comparisons.

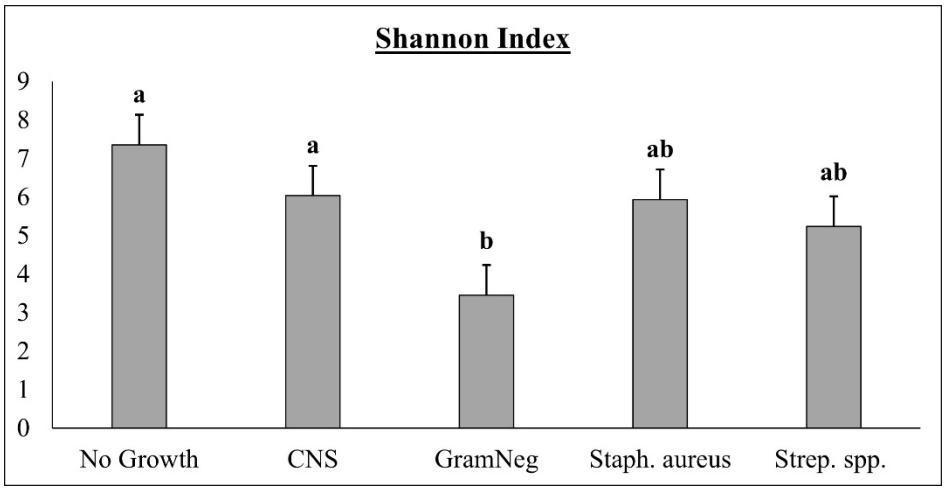

Figure 5.6. Comparison of Shannon diversity indices within milk microbiota of Holstein dairy cattle between cultured mastitis pathogens identified at sample collection: no growth, coagulase-negative staphylococci (CNS), gram-negative, *Staphylococcus aureus*, and streptococci spp. Error bars indicate the standard error. [a,b]Letters indicate statistical differences between samples ($P \leq 0.05$) according to Tukey's pairwise comparisons.

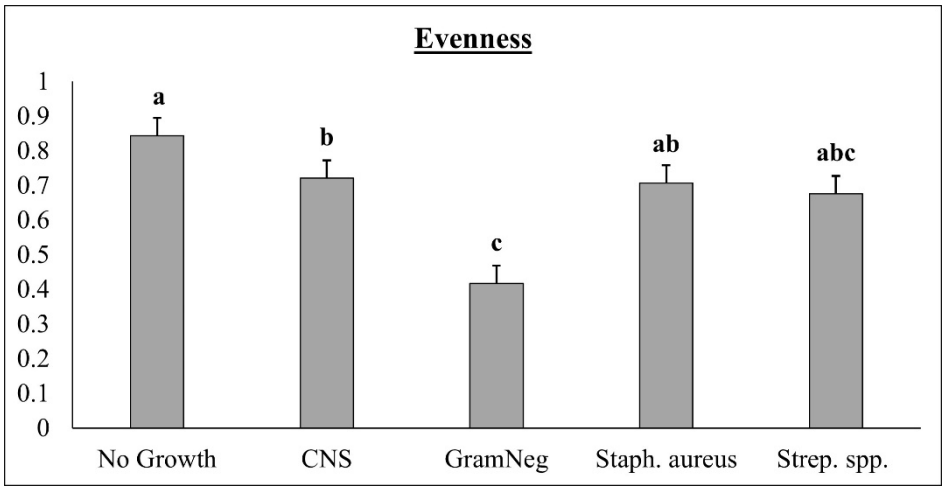

Figure 5.7. Comparison of evenness indices within milk microbiota of Holstein dairy cattle between cultured mastitis pathogens identified at sample collection: no growth, coagulase-negative staphylococci (CNS), gram-negative, *Staphylococcus aureus*, and streptococci spp. Error bars indicate the standard error. [a,b,c]Letters indicate statistical differences between samples ($P \leq 0.05$) according to Tukey's pairwise comparisons.

Table 5.6. Bacterial genera abundance (%) in the milk samples of Holstein dairy cattle associated with different cultured mastitis pathogens at the time of collection: no growth, CNS, gram-negative, *Staph.aureus*, and Streptococcus spp.

	Cultured Mastitis Pathogens						
Genera	**No Growth**	**CNS**	**GramNeg**	***Staph. aureus***	**Strep spp.**	**SE**	***P*-Value**
Acidibacter	0.019[b]	0.048[b]	0.001[b]	2.144[a]	0.069[b]	0.250	<0.001
Chitinophaga	0.000[b]	0.740[a]	0.256[ab]	0.528[ab]	0.000[b]	0.289	0.027
Chloroplast_1	0.249[b]	0.149[b]	4.250[a]	0.216[b]	0.000[b]	0.676	<0.001
Christensenellaceae_R-7_group	1.196[a]	0.138[b]	0.391[ab]	0.055[b]	0.887[ab]	0.409	0.03
Escherichia-Shigella	0.159[b]	0.290[ab]	1.142[a]	0.000[ab]	0.060[ab]	0.315	0.043
Haliangium	0.015[b]	0.011[b]	0.020[b]	0.788[a]	0.000[b]	0.089	<0.001
Lachnospiraceae_UCG-008	0.004[b]	0.003[b]	0.002[b]	0.000[b]	0.583[a]	0.063	<0.001
Lactococcus	0.268[b]	0.000[b]	1.168[a]	0.000[ab]	0.000[ab]	0.266	0.018
Luteolibacter	0.004[b]	0.009[b]	0.004[b]	1.163[a]	0.013[b]	0.127	<0.001
Massilia	0.282[ab]	0.000[b]	0.123[ab]	0.000[ab]	1.588[a]	0.369	0.042
Monoglobus	0.548[ab]	0.161[b]	0.075[ab]	0.088[ab]	1.799[a]	0.339	0.036
Muribaculaceae_1	0.358[b]	0.114[b]	0.086[b]	0.114[b]	1.562[a]	0.237	0.006
Porphyrobacter	0.000[b]	1.146[a]	0.419[ab]	0.818[ab]	0.000[b]	0.475	0.049
Pseudohongiella	0.016[b]	0.027[b]	0.010[b]	0.002[b]	0.208[a]	0.035	0.013
Ruminobacter	0.196[b]	0.160[b]	0.175[b]	0.278[b]	3.066[a]	0.362	<0.001
Staphylococcus	0.327[c]	18.985[a]	0.000[c]	24.278[ab]	10.442[abc]	6.258	0.001
Streptococcus	0.311[b]	1.482[b]	0.000[b]	0.000[b]	30.860[a]	2.368	<0.001
Trueperella	0.761[b]	0.208[b]	55.473[a]	0.000[b]	0.000[b]	4.792	<0.001
UCG-010_1	2.035[a]	0.591[ab]	0.737[ab]	1.034[ab]	0.000[b]	0.726	0.009
Vicinamibacteraceae_1	0.011[b]	0.029[b]	0.014[b]	3.636[a]	0.040[b]	0.396	<0.001

[a,b,c]Means within a row with different superscripts differ ($P \leq 0.05$) according to Tukey's pairwise comparisons.

Table 5.7. Bacterial genera abundance (ppm) in the milk samples of Holstein dairy cattle associated with different cultured mastitis pathogens at the time of collection: no growth, CNS, gram-negative, *Staph.aureus*, and Streptococcus spp.

	Cultured Mastitis Pathogens						
Genera	**No Growth**	**CNS**	**GramNeg**	***Staph. aureus***	**Strep spp.**	**SE**	***P*-Value**
Branchiibius	1.831b	4.737b	2.322b	602.574a	6.64b	65.677	<0.001
Desulfomonile	13.48b	25.94b	18.21b	23.41b	2293.21a	250.533	<0.001
Flavonifractor	40.69b	0.00b	0.00b	0.00b	2366.68a	267.883	<0.001
Globicatella	2.874b	0.00b	578.54a	0.00b	73.355b	91.986	<0.001
Hymenobacter	63.907b	0.00b	762.377a	0.00b	5.558b	149.825	0.001
Olsenella	1008.7b	0.00b	0.00b	0.00b	8541.19a	1312.194	0.002

[a,b]Means within a row with different superscripts differ ($P \leq 0.05$) according to Tukey's pairwise comparisons.

Supplemental Table 5.1. Composition of the diet used to feed the Holstein cattle.

UGA GW SS Mix 1						
Ingredient Name	**AF lb**	**DM %**	**DM lb**	**% of AF**	**Nutrient Analysis**	**(DM%)**
Corn, Ground Shelled	9.99	87	8.71	44.25	Dry matter (DM), %	88.43
Soybean Meal 48%	7.49	89	6.67	33.16	Net energy of lactation, Mcal/lb	0.9
Distillers Grains	2	87.5	1.75	8.85	Crude protein, % of DM	26.1
Molasses, Liquid	0.8	75	0.6	3.54	Neutral detergent fiber, % of DM	11.56
Calcium Carbonate	0.74	99	0.73	3.28	Acid detergent fiber, % of DM	5.44
Sodium Bicarb	0.5	99	0.5	2.21	Non-fiber carbohydrate, % of DM	46.71
Nurisol	0.29	98	0.28	1.28	Fat, % of DM	5.05
Palmit 80	0.2	99.5	0.2	0.88	Calcium, % of DM	1.73
Salt	0.17	99	0.17	0.75	Phosphorus, % of DM	0.44
Diamond V XPC	0.12	91	0.11	0.53	Lysine, % of DM	1.2
Urea	0.1	99	0.1	0.44	Methionine, % of DM	0.36
Magnesium Oxide 58%	0.08	95	0.08	0.35	Potassium, % of DM	1.01
Godfrey Warehouse Trace	0.04	98	0.04	0.19	Magnesium, %	0.43
Godfrey ADE	0.04	95	0.03	0.15	Sulfur, %	0.32
Availa-4	0.02	98	0.02	0.07	Added Salt, %	0.84
Selenium Yeast 600	0.02	97	0.01	0.07		
Total	22.6		19.99	100		

Supplemental Table 5.2. Composition of one ration (High) used to feed the Holstein cattle.

UGA Hi SS #1					
Ingredient Name	**AF lb**	**DM %**	**DM lb**	**Nutrient Analysis**	**(DM%)**
Sorghum Sudan Sil 12.21	54.07	34	18.38	Dry matter (DM), %	56.73
12.16.21 UGA GW SS Mix 1	22.6	88.43	19.99	Net energy of lactation, Mcal/lb	0.77
Hominy Feed	10	90.5	9.05	Crude protein, % of DM	16.5
Cottonseed, Whole	4	92	3.68	Neutral detergent fiber, % of DM	37.1
Bermuda hay	1	90	0.9	Acid detergent fiber, % of DM	22.37
Total	91.67		52	Non-fiber carbohydrate, % of DM	32.42

CHAPTER 6

ANTIMICROBIAL ACTIVITY OF D-LIMONENE AND ORANGE OIL ON RUMINAL AND FECAL VOLATILE FATTY ACID PRODUCTION IN VITRO

[1]Williamson, J. R., T. R. Callaway, J. M. Lourenco, V. E. Ryman. To be submitted to *Frontiers in Veterinary Science*.

Abstract

Antibiotic usage is a necessary strategy to protect animal health on many cattle operations, however, consumer demand has increased focus on investigating alternatives to antibiotics (ATA). Moreover, the microbiomes that exist on and within cattle may be negatively impacted by these antibiotics. In recent years, research demonstrated that essential oils possess the ability to modify microbial populations and functions. In particular, oils derived from citrus fruits provide an interesting area of investigation given the availability and usage of citrus-derived products in cattle feeds and rations. Initially, however, it is imperative to understand the potential impacts of citrus oils on ruminal and hindgut metabolism and performance, with a primary goal to avoid negatively impacting production and health. In addition, shedding light on the potential source of effects as a result of citrus oil inclusion, i.e. the contribution of the active ingredient, d-Limonene. The objective of this study was to determine the *in vitro* antimicrobial activity of the orange oil and its active ingredient, d-Limonene, on ruminal and fecal metabolism. We hypothesized that the inclusion of orange oil and d-Limonene to ruminal and fecal samples would not negatively impact animal metabolism. Approximately 1 liter of ruminal fluid and fecal fluid were collected from a cannulated Holstein steer. Samples were prepared for *in vitro* incubation with food grade d-Limonene and cold-pressed orange oil at varying concentrations, namely 0-, 32-, 64-, 125-, 250-, 500-, and 1000- mM in triplicates. Analysis of VFA content, ammonia, methane, and pH was performed on ruminal and fecal samples. In both ruminal and fecal samples, total VFA production, acetate:propionate ratio, ammonia, and pH did not significantly differ by no addition (0 mM) of both orange oil and d-Limonene and addition at 1000 mM. There was a numeric increase of methane

production with the inclusion of d-Limonene in ruminal samples, but the opposite was seen in fecal samples. *In vitro* fermentation of orange oil with fecal and ruminal samples did not significantly alter methane production. Most importantly, there was a significant increase in butyrate production in both ruminal and fecal samples when fermented *in vitro* with orange oil and d-Limonene. Butyrate positively impacts the development and function of the GIT, enhances animal growth and performance, and reduces inflammation, thereby reducing the risk of inflammatory diseases, such as mastitis, especially around the transition period of dairy cattle. More investigation on essential oil supplementation *in vivo* and resulting impacts on butyrate and methane production should be done to determine if orange oil and derivatives like d-Limonene can be used as an ATA.

Key words: rumen, fecal, milk, dairy cattle, antimicrobial activity, limonene, citrus, orange, oil, microbiome

Abbreviation key: ATA = alternative to antibiotics, **VFA** = volatile fatty acids

Introduction

Antibiotic usage is a necessary strategy to promote animal health and well-being on livestock operations, and is employed for improved feed efficiency, growth promotion, and most importantly disease prevention and treatment (Hao et al., 2014; Hong et al., 2013; Manyi-Loh et al., 2018; You & Silbergeld, 2014). However, increasing consumer demand for alternatives to antibiotics (ATA) has found producers investigating new options to confer protection from such diseases. Moreover, the microbiomes that exist on and within cattle may be negatively impacted by these antibiotics, such as reduced bacterial species diversity, promotion of antibiotic-resistant bacteria, and altered metabolic activity (Ramirez et al., 2020). Metabolic status is critical to the animal production industry, especially in dairy cattle, where a successful transition from late gestation to early lactation depends on proper metabolic function in order to support the cow's requirements when adapting to the new physiological state that is before and after parturition (Caixeta & Omontese, 2021). In dairy cattle where culling and mortality of cows during early-lactation is strongly linked to metabolic disorders (Chiumia et al., 2013; Pinedo et al., 2010), and disorders, culling, and death cause substantial losses to production (Caixeta & Omontese, 2021; Thomsen & Houe, 2006), it is pertinent that an ATA or feed additive that can enhance gut health and prevent metabolic diseases is established.

More recently, investigation of essential oils as an ATA has increased since many possess antioxidant, antimicrobial, and anti-inflammatory properties (Braun et al., 2019). Essential oils naturally occur in a variety of plants that are important for plant defense mechanisms, and show promise for usage in various applications including reducing

survival and growth of foodborne pathogens and spoilage bacteria (Barbosa et al., 2009; Dabbah et al., 1970; Viuda-Martos et al., 2008), as well as modification of fermentation patterns with effects on volatile fatty acid (VFA) production (Calsamiglia et al., 2007). Oils derived from citrus fruits provide an interesting area of investigation given the availability and usage of citrus-derived products in cattle feeds and rations as a low-cost nutritional supplement. For example, orange peel and orange pulp are a common by-product from juice that are fed to dairy cattle, and have a good nutritive value for ruminant animals (Arthington et al., 2002). In addition, d-Limonene, a terpene compound extracted from citrus (e.g., orange) fruits, has been shown to reduce populations of foodborne pathogenic bacteria *Escherichia coli O157:H7* and *Salmonella spp* in ruminants (Callaway et al., 2011a; Callaway et al., 2011b). However, there has been reports of associations between digestive disorders, such as rumen parakeratosis, with feeding citrus pulp in cattle at 60% or more of the concentrate mixture for cows fed fully in feedlot (Arthington et al., 2002). Although, if selected and used carefully, citrus by-products included within the diet can support growth and lactation with less negative effects on rumen fermentation than other feed products (Alnaimy, 2017; Bampidis & Robinson, 2006).

While citrus oil has demonstrated preservation of animal feed without impacting *in vitro* rumen fermentation (Nam et al., 2006), it is unknown how the extract d-Limonene impacts the rumen and fecal fermentation of dairy cattle, and at what concentrations it can safely be used without negatively impacting animal metabolism. Before investigating the use of a natural plant-derived additive for optimizing milk production and improving milk quality, and as a potential ATA in cases of disease, the impact of these additives on animal metabolism and performance must be evaluated. The objective of this study was to

determine the activity of food grade d-Limonene and orange oil on both rumen and fecal volatile fatty acid (VFA) output. We hypothesized that the inclusion of orange oil and d-Limonene to ruminal and fecal samples would not negatively impact animal metabolism, with potential benefits such as increasing total VFA. Following, results could be critical for understanding if we can modulate ruminal and fecal populations using an ATA derived from d-Limonene or orange oil to optimize milk composition, milk production, and mammary health, without affecting ruminal or fecal fermentation patterns.

Materials and methods

All procedures involving live animals were verified and approved by the University of Georgia's Office of Animal Care and Use (AUP # A2021 07-029-Y1-A0). The dairy cattle and steers used in this study were located at the University of Georgia Teaching Dairy in Winterville, GA (33°54'32.9"N 83°14'50.9"W).

Animal selection and collection

Ruminal and fecal contents were collected from a healthy, Holstein steer receiving the same diet as Holstein dairy cattle at the UGA Teaching Dairy. Nutrition information is available in Supplementary Tables 1-3. Approximately 1 liter of rumen contents were collected via the rumen cannula and squeezed through a cheesecloth into a thermos. Fecal matter was collected via digital palpation as described by Lourenco et al. (2020), and squeezed through a cheesecloth to collect approximately 1 liter of fecal fluid. Samples were immediately transported to the UGA lab for preparation of incubation.

In vitro fermentation and chemical analyses of rumen and fecal contents

Ruminal fluid and fecal fluid (1 part fecal sample solubilized in 3 parts sterile media) were prepared for incubation with food grade d-Limonene and cold pressed orange

oil (supplied by ADM Research Center; Decatur, IL, USA) according to previously described work by Castillejos et al. (2006). Orange oil is derived from citrus peels, which includes active components such as d-Limonene. Using purified d-Limonene, we can compare to non-purified essential oils from citrus peels. The ruminal fluid or fecal solution was mixed with anoxic media (33% v/v) and made oxygen-free through bubbling with CO_2 for 10 minutes then sealed. d-Limonene concentrations of 0, 32, 64, 125, 250, 500, and 1000 mg/L as requested by the supplier were added to the *in vitro* fermentation tubes and incubated for 0, 12, and 24 h in triplicate. The same concentrations and process were repeated for the addition of orange oil. After 12 h incubation, samples were centrifuged at 10,000 x g for 10 min before 4 mL of the supernatant was transferred to glass-vials and taken to a -20°C freezer for storage until VFA and NH_3 analysis were performed approximately a month later. After 24 h of incubation, methane (CH_4) and pH levels were determined before the freezing process.

Total gas production was measured by displacement using similar methods as previously described by Callaway and Martin (1996). A 0.5 mL gas sample was removed from each sample tube using a 60-mL glycerol-lubricated syringe and analyzed for hydrogen (H_2) and methane (CH_4) on a Gow Mac thermal conductivity series 580 gas chromatograph (Gow Mac Instrument, Bridgewater, NJ, USA) that is equipped with a Porapak Q column (60°C, 20 mL/min of N_2 carrier gas). The tubes were then uncapped, and sample pH was determined using a pH meter (pH 11 Series, Cole-Parmer Scientific; Vernon Hills, IL, USA). The rest of the sample was then centrifuged at 10,000 x g for 10 min before 4 mL of the supernatant was transferred to the glass-vials and frozen at -20°C for further analyses.

Rumen and fecal samples were then thawed and analyzed for VFA following procedures previously described by Lourenco et al. (2020). Briefly, 2 mL from each ruminal fluid or fecal sample was centrifuged at 10,000 x g for 10 min and 1 mL of the supernatant was mixed with 0.2 mL of 25% (wt/vol) meta-phosphoric acid, vortexed, and frozen overnight. The samples were then thawed and centrifuged at 10,000 x g for 10 min before 1 mL of the supernatant was transferred to screw-thread vials containing 2 mL of ethyl acetate. These samples were vortexed for 10 s and allowed to settle for at least 5 minutes to optimize separation. The upper layer was transferred (approximately 1 mL) into gas chromatography vials for VFA analysis using a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu Corporation Kyoto, Japan) equipped with a flame ionization detector and capillary column (Zebron ZB-FFAP; 30 m x 0.32 mm x 0.25 μm; Phenomenex Inc., Torrance, CA, USA). The sample injection volume was 1.0 μL, and helium was the carrier gas. The starting temperature of the column was set at 110°C and gradually increased to 200°C. The injector temperature was set at 250°C, and the detector temperature was set at 350°C. The output variables recorded were acetate, propionate, iso-butyrate, butyrate, iso-valerate, and valerate.

At each timepoint, ammonia (NH_3) was analyzed by colorimetry as previously described by Chaney and Marbach (1962). At timepoints 0 h and 12 h, 3 mL of a phenol reagent was added to microcentrifuge tubes containing 50 μL of sample and vortexed. Then, another 3 mL of hypochlorite reagent was added to the tubes, vortexed, and then incubated at 39°C for 20 min. The same procedures were followed for timepoint 24 h, but samples were diluted (1:10) with DI water prior to addition into microcentrifuge tubes.

After incubation, NH_3 was determined by spectrophotometry using the GENESYS 30 VIS Spectrophotometer (Thermo Scientific; Waltham, MA, USA).

Statistical analysis

Statistical analyses were performed using the software Minitab® v21 (Minitab LLC, State College, PA). Analysis of VFA concentrations, pH, CH_4, and NH_3 were carried out by ANOVA using the supplementation status, concentration, time, and the type of sample analyzed as factors, along with their interactions. The resulting P-values were corrected by Tukey's method for multiple comparisons. For all the statistical tests used, results were considered significant at $P \leq 0.05$ and were treated as trends when $0.05 < P < 0.10$.

Results

Ruminal Metabolism

Total VFA concentration after *in vitro* fermentation of orange oil and d-Limonene with ruminal fluid of dairy cattle is described in Figure 1. Inclusion of orange oil and d-Limonene at any concentration did not significantly change VFA production. The inclusion of d-Limonene at 24 hr increased the ruminal total VFA concentration *in vitro* when compared to d-Limonene at 12 hr and orange oil at 12 and 24 hr ($P = 0.036$). The ruminal acetate:propionate ratio after *in vitro* fermentation with orange oil or d-Limonene is described in Figure 2. *In vitro* fermentation at 24 hr increased the acetate:propionate ratio compared to fermentation at 12 hr ($P < 0.001$). Acetate:propionate ratios did not differ by 12 hr incubation with orange oil and d-Limonene concentrations ($P > 0.05$). Acetate:propionate ratios were highest at 0 and 1000 mM orange oil and d-Limonene concentrations and lowest at 125 mM ($P < 0.001$). Individual VFA concentrations (acetate,

propionate, isobutyrate, butyrate, isovalerate, valerate) after *in vitro* fermentation of orange oil and d-Limonene with rumen fluid at various concentrations can be found in Supplementary Table 4. Most notably, 24 hr fermentation of orange oil and d-Limonene with ruminal fluid increased the levels of butyrate in comparison to 12 hr fermentation ($P = 0.001$) but did not differ between orange oil or d-Limonene ($P > 0.05$). As concentrations of orange oil and d-Limonene increased, there was an overall increase in butyrate ($P < 0.001$; Figure 3). The inclusion of orange oil and d-Limonene at 1000 mM significantly increased butyrate fermentation in comparison to inclusion of oils at 0, 32, 64, and 125 mM.

Ruminal *in vitro* NH_3 production was measured at 12 hr and 24 hr (Table 1). Ruminal NH_3 concentrations were highest at 24 hr *in vitro* fermentation compared to 12 hr fermentation ($P < 0.001$). Inclusion of orange oil and d-Limonene at 1000 mM concentration increased NH_3 concentrations compared to 32 and 500 mM inclusion ($P = 0.025$). Total *in vitro* CH_4 gas production after 24 hr was measured for ruminal samples (Table 2). Ruminal CH_4 was not significantly affected by orange oil and d-Limonene inclusion at any concentration ($P > 0.05$), though d-Limonene inclusion at concentration 32, 64, 125, 250, 500, and 1000 mM did result in numerically higher CH_4 levels than d-Limonene at concentration 0 mM. Orange oil inclusion at concentration 64, 125, 250, 500, and 1000 mM resulted in lower levels of CH_4 output than no addition (0 mM). Sample pH was taken at 24 hr to evaluate changes in acidity after *in vitro* fermentation with d-Limonene and orange oil. Ruminal pH was not impacted by orange oil or d-Limonene concentration *in vitro* ($P > 0.05$; Table 3).

Fecal Metabolism

Total fecal VFA concentrations after *in vitro* fermentation of orange oil or d-Limonene with fecal fluid are described in Supplementary Table 5. Fecal fermentations were low in the hindgut, and inclusion of orange oil and d-Limonene yielded very few differences by treatment. Inclusion of d-Limonene increased total VFA concentration *in vitro* compared to orange oil ($P < 0.001$). *In vitro* fermentation at 24 hr increased total VFA concentration compared to fermentation at 12 hr ($P < 0.001$). Total VFA concentrations did not differ by any d-Limonene concentrations ($P > 0.05$) but decreased with the inclusion of orange oil (32, 64, 125, 250, 500, 1000 mM; $P = 0.014$). Orange oil increased the acetate:propionate ratio in comparison to d-Limonene ($P < 0.001$). *In vitro* fermentation at 12 hr increased acetate:propionate ratio compared to fermentation at 24 hr ($P < 0.001$). Acetate:propionate ratios did not differ by d-Limonene concentrations ($P > 0.05$). The most notable change in individual VFA concentrations was the increase in butyrate with the inclusion of orange oil and d-Limonene. Fermentation of orange oil and d-Limonene with fecal fluid for 24 hr increased the levels of butyrate in comparison to 12 hr fermentation ($P < 0.001$), and this increase was more robust in orange oil when compared to d-Limonene ($P < 0.001$). More importantly, butyrate concentrations increased as orange oil and d-Limonene concentrations increased ($P < 0.001$; Figure 3). The inclusion of orange oil and d-Limonene at 1000 and 500 mM significantly increased butyrate fermentation in comparison to inclusion of oils at 0, 32, 64, and 125 mM.

Fecal *in vitro* NH_3 production was measured at 12 hr and 24 hr (Table 1). Fecal NH_3 concentration was highest when fermented with d-Limonene at 24 hr, and lowest when fermented with either orange oil or d-Limonene at 12 hr ($P = 0.005$). Total *in vitro*

CH_4 gas production after 24 hr was measured for fecal samples (Table 2). The inclusion of d-Limonene resulted in higher fecal CH_4 across concentrations in comparison to the inclusion of orange oil ($P = 0.004$), although d-Limonene at 1000 mM had lower levels of CH4 than no addition (0 mM). Orange oil inclusion at concentration 32, 64, 125, 250, 500, and 1000 mM had lower CH_4 levels than orange oil at concentration 0 mM. Sample pH was taken at 24 hr to evaluate changes in acidity after *in vitro* fermentation with d-Limonene and orange oil. Fecal pH was not impacted by orange oil and d-Limonene concentration *in vitro* ($P > 0.05$; Table 3).

Discussion

One way to assess animal metabolism and performance is through the end products of normal cellular processes such as rumen and fecal microbial fermentation. The direct outputs of fermentation include short chain fatty acids, specifically VFA, that are absorbed and used as energy sources by the ruminant animal (Bergman, 1990). The rumen microbiota also metabolize nitrogen-containing compounds, such as NH_3, in order to supply microbial protein to the animal for milk synthesis (Bach et al., 2005). Assessing changes (increases or decreases) in VFA and other outputs including NH_3 will give a direct indication of the effect of d-Limonene or orange oil on its ability to inhibit and/or kill microbiota for use as an ATA. Little investigation has been done on fecal fermentation patterns with inclusion of orange oil and orange oil extracts, although the current study found no negative impacts to fecal fermentation when d-limonene or orange oil was included *in vitro*. While neither orange oil nor d-Limonene significantly impacted the concentrations of VFA, d-Limonene did result in higher total VFA *in vitro* in comparison to orange oil in both rumen and fecal samples. Previously, the addition of limonene at 1000

uL/L *in vitro* to a ruminal environment had no adverse effects on VFA production (Joch et al., 2016), similar to the current study, suggesting that at a high dosage, d-Limonene is not toxic to ruminal bacteria. However, Kamalak et al. (2011) found that ruminal VFA production was significantly decreased with increasing level of orange oil, although the study used ruminal fluid from sheep. As fermentation and absorption patterns are different in large and small ruminants (Bergman, 1990), it cannot be concluded that the results would be the same in an *in vivo* cattle study. When analyzing the acetate:propionate ratio, orange oil resulted in a higher ratio in comparison to d-Limonene in fecal samples, but no difference between orange oil and d-Limonene when looking at the acetate:propionate ratio in ruminal samples. An increased ruminal acetate:propionate ratio was previously seen with increasing level of orange oil (Kamalak et al., 2011), and while this was demonstrated in the fecal samples in the current study, differences in ruminal sample results may be due to species investigated (cattle vs sheep). Additionally, there was no difference seen in ruminal NH_3 production between oils, although fecal NH_3 production was increased when samples were fermented with d-Limonene in comparison to orange oil. This contrasts with Cattani et al. (2016), with *in vitro* inclusion of limonene (200 mg/L) reducing the NH_3 concentration of rumen fluid, however researchers used a substrate in fermentation that stimulated a dairy cow diet. Although, other researchers have demonstrated that supplementation of orange oil at 200 mg/L did not affect the NH_3 production after ruminal fluid *in vitro* fermentation (Benchaar et al., 2007), similar to the current study. In agreement with the present results, Cattani et al (2016) and Joch et al (2016) found no effects on the final pH of ruminal fermentation fluids when treated with limonene. Additionally, supplementation of an essential oil blend that included orange oil to sheep did not result in

any changes to ruminal pH (Passetti et al., 2021). It is important to note that the influence of essential oils like orange oil on rumen fermentation can be affected by ruminal pH, as it can impact the dissociated status of the orange oil (Passetti et al., 2021). Currently, pH did not influence the ruminal fermentation patterns *in vitro*.

The most notable difference between the inclusion of orange oil or d-Limonene was the production of CH_4. In the rumen, addition of orange oil at 64, 125, 250, 500, and 1000 mM resulted in lower CH_4 levels than no addition (0 mM). A negative correlation between orange oil and CH_4 production was previously reported, with CH_4 production in the rumen significantly decreasing as orange oil increased (Kamalak et al., 2011). The current study demonstrated that the addition of d-Limonene to ruminal samples across all concentrations numerically increased the levels of CH_4 in comparison to no addition (0 mM). This is in disagreement with past research evaluating the effects of limonene on ruminal CH_4 production *in vitro*, with the addition of 200 mg/L of limonene reducing the total gas and methane production (Cattani et al., 2016). Additionally, a study by Joch et al. (2016) found *in vitro* limonene addition at 1000 uL/L decreased methane production. Differences may be due to the conditions of the fermentation medium, as Joch et al. (2016) did not include a solvent, such as ethanol, with the addition of limonene as done in the current study. Additionally, the stimulation of the dairy cow diet *in vitro* by Cattani et al (2016) may account for the differences between the two studies. In the feces, addition of orange oil decreased CH_4 production across all concentrations in comparison to no addition (0 mM), while addition of d-Limonene only decreased CH_4 production when included at 32, 125, 250, 500, and 1000 mM. Although not significant, d-Limonene increasing CH4 production of the rumen *in vivo* would indicate metabolic inefficiencies, as CH4 is a by-product of

fermentation that is not used by the animal and represents an energy loss (Danielsson et al., 2017). In addition, methane emissions from livestock are a major contributing factor to global greenhouse gas emissions (Wolf et al., 2017). More investigation should be done in order to determine if inclusion of d-Limonene *in vivo* results in increased methanogenesis before its promotion as an ATA is considered.

In the present study, the inclusion of both d-Limonene and orange oil at varying concentrations into ruminal and fecal samples *in vitro* did not significantly impact VFA production, ammonia, or pH levels. This is agreement with previous studies finding *in vitro* supplementation of essential orange oil to not effect ammonia and total VFA production (Benchaar et al., 2007), and *in vivo* supplementation of limonene to not effect pH, ammonia, or total VFA production (Samii et al., 2016). In addition, Samii et al. (2016) found that *in vivo* supplementation of limonene linearly reduced ruminal proportions of propionate, and while not significant in the current study, there was a numeric reduction of propionate in both ruminal and fecal samples. In addition, the abundance of acetate was greatest with no addition (mM) of either orange oil or d-Limonene in both ruminal and fecal samples. A reduction of ruminal acetate and propionate *in vivo* was previously demonstrated by Wu et al. (2018) after addition of citrus essential oil, and these reductions coincided with a decline in the acetate:propionate ratio. When considering the acetate:propionate ratio in the current study, fermentation at 24 hr resulted in a greater ratio than fermentation at 12 hr, but there was a numeric decrease in the acetate:propionate ratio across concentrations for both orange oil and d-Limonene. While orange oil and d-Limonene were numerically decreasing both levels of acetate and propionate, they were conversely increasing the abundance of butyrate in samples. This is not unexpected, as

acetate, propionate, and butyrate usually present themselves in a molar ratio of 3:2:1 or 3:1:1 (den Besten et al., 2013; Hurst et al., 2014). An increase in ruminal butyrate concentration has been previously observed in dairy cows that were supplemented with dried citrus pulp (Gouvêa et al., 2016; Santos et al., 2014), and a trending increase in ruminal butyrate when limonene was supplemented *in vivo* in heifers (Samii et al., 2016). In the current study, levels of butyrate in both ruminal and fecal samples increased more significantly after 24 hr fermentation with orange oil and d-Limonene in comparison to 12 hr fermentation. There was not a significant difference between orange oil and d-Limonene in resulting butyrate concentrations in ruminal samples, however orange oil resulted in higher levels of butyrate in comparison to d-Limonene in fecal samples.

Butyrate is one of the major end products of bacterial carbohydrate fermentation in the gastrointestinal tract (GIT) and is produced mainly by bacteria from the phylum Firmicutes, although the list of butyrate-producing bacteria is growing longer (Bergman, 1990; Górka et al., 2018; Parada Venegas et al., 2019). Of the major VFA produced in the GIT, butyrate is the most dynamic and varies in abundance ranging from 5% to more than 20%, allowing its production in the GIT to be modulated to a great degree (Aschenbach et al., 2011; Górka et al., 2018; Plöger et al., 2012). Production of butyrate critical, as it is most closely related to the health status of GIT tissue and integrity (Górka et al., 2018; Kvidera et al., 2017; Plöger et al., 2012). Butyrate is the main stimulator of rumen epithelium development and function, the tissue that is responsible for absorbing VFA, including absorption of ruminal butyrate (Bergman, 1990; Górka et al., 2011). Ruminal butyrate is converted to the ketone β-hydroxybutyrate in epithelial tissues which provides energy to the epithelium (Guan et al., 2008). Thereby, butyrate is a primary energy source

for tissues of the GIT, and butyrate supplementation has demonstrated evidence for increasing the surface area in the rumen for nutrient absorption (Górka et al., 2018; Malhi et al., 2013; Shen et al., 2005).

In humans, the presence of butyrate has been linked to an increase in tight junction proteins and growth of epithelial cells in the lower GIT, making it a potential tool to treat inflammatory bowel diseases, such as acute gastroenteritis, cholera, congenital chloride diarrhea, Crohn's disease, and ulcerative colitis (Canani et al., 2011; Fleming & Arce, 1986; Plöger et al., 2012). Additionally, butyrate inhibits the growth of colonic cancer cells in humans and rats by decreasing cell proliferation and increasing apoptosis (Clarke et al., 2012; Hague & Paraskeva, 1995; Morita et al., 1982). Moreover, numerous studies have also shown that dietary butyrate supplementation in ruminant animals may accelerate GIT development and increase total nutrient digestibility, (Canani et al., 2011; Górka et al., 2018; Huhtanen et al., 1993), and has been linked with more feed efficient steers (Carberry et al., 2012; Guan et al., 2008). Not only can butyrate addition positively effect growth performance and nutrient digestion efficiency, but can alter immune responses through induction of intracellular or extracellular processes, translating into increased epithelial barrier function, elevated antimicrobial peptide production, and decreased inflammation (Górka et al., 2018; Hill et al., 2016; Hill et al., 2011a; 2011b; Parada Venegas et al., 2019). In broiler chickens, butyrate supplementation prevented and reduced colonization of the GIT with potentially harmful pathogens such as *Salmonella* (Cox et al., 1994; Fernández-Rubio et al., 2009), and supplementation in cows resulted in expansion of Firmicutes and other butyrate-producing bacteria (Li et al., 2012). However, when butyrate-producing

bacteria were depleted, there was increased aerobic expansion of *Salmonella* (Rivera-Chávez et al., 2016).

In the present study, addition of orange oil and d-Limonene significantly increased butyrate concentrations in both ruminal and fecal samples during an *in vitro* exposure. Increasing levels of butyrate reportedly contributes to positive effects on animal production, such as control of pathogens, improvement of GIT development, enhancement of performance, modification of gut microbiota, and reduction of inflammation (Bedford & Gong, 2018). For example, in dairy calves butyrate supplementation decreased the probability of digestive upset (Górka et al., 2011, 2018). Additionally, *in vitro* butyrate assessments demonstrated alleviation of mammary cell pro-inflammatory responses through mechanisms that diminished NF-kB signaling, a regulator of innate immunity that is essential for inflammatory responses (Jiang et al., 2020; Sun et al., 2020; Taniguchi & Karin, 2018). It was suggested that butyrate might represent a preventative tool to help the mammary gland confer protection against pathogens, such as those that cause mastitis (Jiang et al., 2020; Sun et al., 2020). Additionally, inclusion of butyrate into the diet has shown to increase milk fat production (Izumi et al., 2019), but more investigation is needed on the effects of butyrate on lactation performance. However, digestive disorders such as diarrhea, indigestion, and bloat are associated with decreased milk yield (Kirchman et al., 2017; Pérez-Báez et al., 2021), and diseases such as mastitis negatively impact milk composition and milk yield (Malek dos Reis et al., 2013; Rajala-Schultz et al., 1999). While little is known about how butyrate supplementation can impact milk production, protection against digestive disorders and diseases like mastitis will stimulate animal health.

Conclusion

In vitro fermentation of orange oil and d-Limonene with ruminal and fecal samples did not critically impact total VFA fermentation, NH_3 production, or pH of ruminal fluid or fecal fluid of Holstein cattle. The most important observation seen in both sample types was the increase in butyrate concentrations as orange oil and d-Limonene concentrations increased. Besides serving as an important energy source for the ruminant animal, butyrate is critical for the growth and function of the ruminal epithelium. Additionally, the potential for the use of butyrate as a tool to decrease inflammation in the GIT has been widely suggested. The data presented herein suggests that use of orange oil and d-Limonene as a feed additive may increase levels of butyrate in ruminal and fecal populations, and further studies should investigate if this increase is synonymous *in vivo* and can be used as an ATA to promote animal health and production efficiency such as increased milk yield.

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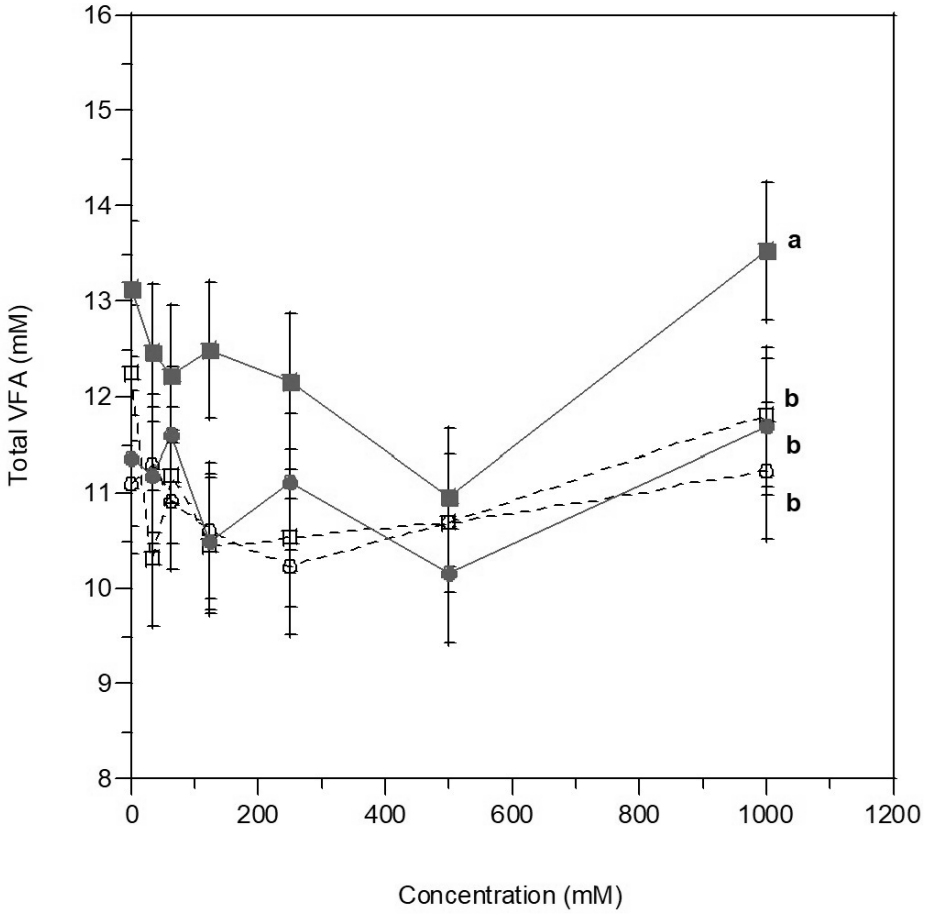

Figure 6.1. Total volatile fatty acid (VFA) concentration (mM) after *in vitro* fermentation of orange oil or d-Limonene with ruminal fluid of a Holstein steer (n = 42, P = 0.036). Open circle (○) indicates orange oil at 12 Hr, closed circle (●) indicates d-Limonene at 12 Hr, open square (□) indicates orange oil at 24 Hr, closed square (■) indicates d-Limonene at 24 hr. P-value indicates significant difference between oil by hour. Error bars indicate the standard error. [ab]Indicates a significant difference between oil and hour using Tukey's Pairwise comparison.

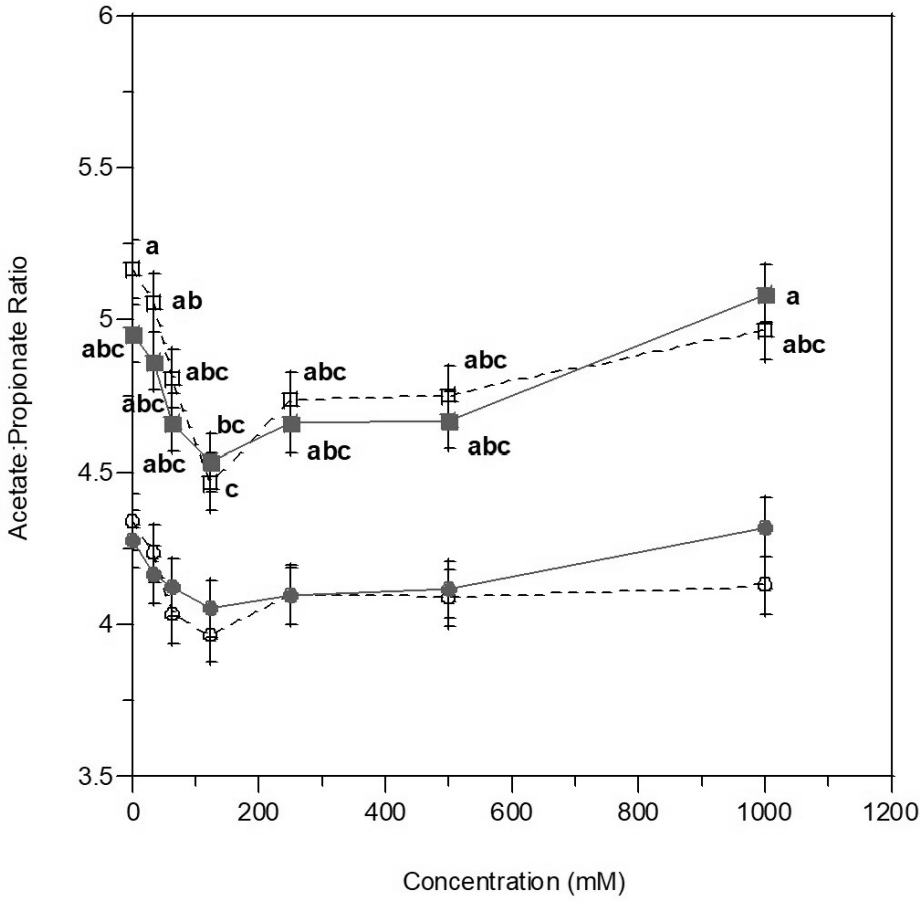

Figure 6.2. Acetate:propionate ratio after *in vitro* fermentation of orange oil or d-Limonene with ruminal fluid of a Holstein steer (n = 42). Open circle (○) indicates orange oil at 12 Hr, closed circle (●) indicates d-Limonene at 12 Hr, open square (□) indicates orange oil at 24 Hr, closed square (■) indicates d-Limonene at 24 hr. P-value indicates significant difference between concentration and hr ($P < 0.001$). Error bars indicate the standard error. [abc]Indicates a significant difference between orange oil and d-Limonene concentrations at 24 hr using Tukey's Pairwise comparison.

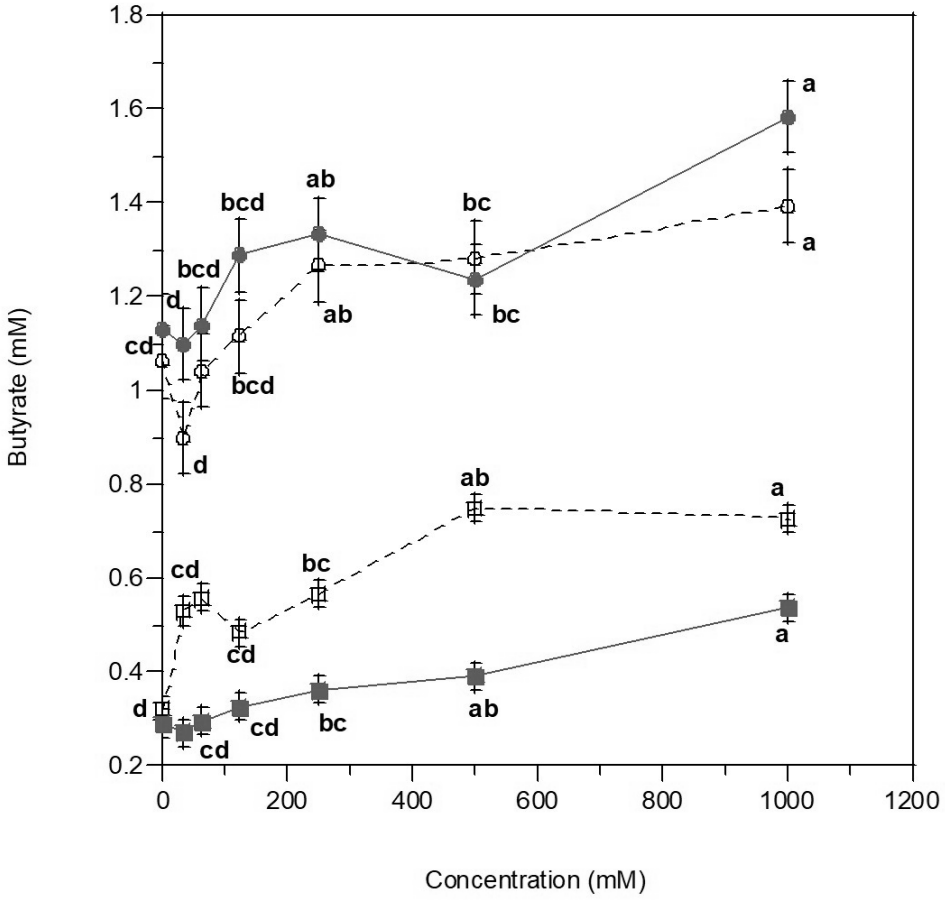

Figure 6.3. Butyrate concentrations (mM) after *in vitro* fermentation of orange oil or d-Limonene with ruminal fluid and fecal fluid of a Holstein steer (n = 42). Open circle (○) indicates orange oil at 24 Hr in rumen samples, closed circle (●) indicates d-Limonene at 24 Hr in rumen samples. Open square (□) indicates orange oil at 24 Hr in fecal samples, closed square (■) indicates d-Limonene at 24 Hr in fecal samples. P-value indicates significant difference between concentrations ($P < 0.001$). Error bars indicate the standard error. ^{abcd}Indicates a significant difference between oil concentrations at 24 hr using Tukey's Pairwise comparison.

Table 6.1. Ammonia (NH_3) concentrations (mg/L) at 12- and 24-hour *in vitro* fermentation of orange oil or d-Limonene with ruminal or fecal samples from Holstein steer.

Item	Time	Oil Concentration								*P*-Value*			
		0	32	64	125	250	500	1000					
		Orange							SEM	Oil	Conc	Hr	OilxHr
Rumen	12 Hr	154.9	107.4	145.6	172.9	111.9	145.3	162.5	43.8	0.184	0.025	<0.001	0.897
	24 Hr	359.7	379.8	376.3	374.3	364.7	362.2	434.3					
Fecal	12 Hr	177	165.6	169.6	173	159.8	190.2	175.4	43.2	<0.001	0.943	<0.001	0.005
	24 Hr	295.6	311.7	317.3	241.8	261.3	306.7	323.9					
		Limonene											
Rumen	12 Hr	131.3	150.9	142.1	178.3	151.8	159.1	257.8	43.8	0.184	0.025	<0.001	0.897
	24 Hr	380.3	357.6	366.2	433.3	393	330.4	531.2					
Fecal	12 Hr	201.5	180.2	227	196.3	249	196.1	245.6	43.2	<0.001	0.943	<0.001	0.005
	24 Hr	483.8	378.3	388.9	551.9	403.6	410.1	390.4					

*P-value for the repeated measures ANOVA using oil, concentration, and hour as factors. There were no significant interactions between OilxConc, ConcxHr, or OilxConcxHr.

Table 6.2. Methane (CH_4) concentrations (mM) at 24-hour *in vitro* fermentation of orange oil or d-Limonene with ruminal or fecal samples from Holstein steer.

Item	Oil Concentration								*P*-Value*		
	0	32	64	125	250	500	1000				
	Orange							SEM	Oil	Conc	OilxConc
Rumen	3.44	5.44	3.11	3.11	2	2.77	2.55	1.64	0.429	0.853	0.62
Fecal	0.888	0.444	0.555	0.555	0.555	0.666	0.666	0.27	0.335	0.004	0.578
	Limonene										
Rumen	2.55	4	3	3.44	5.11	2.89	6.44	1.64	0.429	0.853	0.62
Fecal	1.443	0.888	1.776	0.777	0.999	0.888	0.999	0.27	0.335	0.004	0.578

*P-value for the repeated measures ANOVA using oil and concentration as factors.

Table 6.3. Ruminal, fecal, and milk pH at 24-hour *in vitro* fermentation of orange oil or d-Limonene with samples from Holstein cattle.

Item	Oil Concentration								*P*-Value*		
	0	32	64	125	250	500	1000				
	Orange							SEM	Oil	Conc	OilxConc
Rumen	7.4433	7.5167	7.5067	7.4667	7.5167	7.4367	7.43	0.0522	0.002	0.887	0.734
Fecal	6.97	6.97	6.99	7.0033	7.0133	7.03	7.0733	0.014	<0.001	0.001	0.026
Milk	7.1733	7.17	7.16	7.18	7.1833	7.1533	7.1467	0.017	<0.001	0.001	<0.001
	Limonene										
Rumen	7.4433	7.3667	7.3867	7.36	7.36	7.3933	7.34	0.0522	0.002	0.887	0.734
Fecal	7.0867	7.0667	7.05	7.0567	7.06	7.07	7.0933	0.014	<0.001	0.001	0.026
Milk	7.2067	7.2	7.2133	7.3167	7.3167	7.3133	7.3	0.017	<0.001	0.001	<0.001

*P-value for the repeated measures ANOVA using oil and concentration as factors

Supplemental Table 6.1. Composition of the diet used to feed the Holstein steers.

UGA GW SS Mix 1						
Ingredient Name	**AF lb**	**DM %**	**DM lb**	**% of AF**	**Nutrient Analysis**	**(DM%)**
Corn, Ground Shelled	9.99	87	8.71	44.25	Dry matter (DM), %	88.43
Soybean Meal 48%	7.49	89	6.67	33.16	Net energy of lactation, Mcal/lb	0.9
Distillers Grains	2	87.5	1.75	8.85	Crude protein, % of DM	26.1
Molasses, Liquid	0.8	75	0.6	3.54	Neutral detergent fiber, % of DM	11.56
Calcium Carbonate	0.74	99	0.73	3.28	Acid detergent fiber, % of DM	5.44
Sodium Bicarb	0.5	99	0.5	2.21	Non-fiber carbohydrate, % of DM	46.71
Nurisol	0.29	98	0.28	1.28	Fat, % of DM	5.05
Palmit 80	0.2	99.5	0.2	0.88	Calcium, % of DM	1.73
Salt	0.17	99	0.17	0.75	Phosphorus, % of DM	0.44
Diamond V XPC	0.12	91	0.11	0.53	Lysine, % of DM	1.2
Urea	0.1	99	0.1	0.44	Methionine, % of DM	0.36
Magnesium Oxide 58%	0.08	95	0.08	0.35	Potassium, % of DM	1.01
Godfrey Warehouse Trace	0.04	98	0.04	0.19	Magnesium, %	0.43
Godfrey ADE	0.04	95	0.03	0.15	Sulfur, %	0.32
Availa-4	0.02	98	0.02	0.07	Added Salt, %	0.84
Selenium Yeast 600	0.02	97	0.01	0.07		
Total	22.6		19.99	100		

Supplemental Table 6.2. Composition of one ration (High) used to feed the Holstein steers.

UGA Hi SS #1					
Ingredient Name	**AF lb**	**DM %**	**DM lb**	**Nutrient Analysis**	**(DM%)**
Sorghum Sudan Sil 12.21	54.07	34	18.38	Dry matter (DM), %	56.73
12.16.21 UGA GW SS Mix 1	22.6	88.43	19.99	Net energy of lactation, Mcal/lb	0.77
Hominy Feed	10	90.5	9.05	Crude protein, % of DM	16.5
Cottonseed, Whole	4	92	3.68	Neutral detergent fiber, % of DM	37.1
Bermuda hay	1	90	0.9	Acid detergent fiber, % of DM	22.37
Total	91.67		52	Non-fiber carbohydrate, % of DM	32.42

Supplemental Table 6.3. Composition of one ration (Jersey) used to feed the Holstein steers.

UGA Jersey SS #1					
Ingredient Name	**AF lb**	**DM %**	**DM lb**	**Nutritent Analysis**	**(DM%)**
Sorghum Sudan Sil 12.21	44.12	34	15	Dry matter (DM), %	55.48
12.16.21 UGA GW SS Mix 1	17	88.43	15.03	Net energy of lactation, Mcal/lb	0.76
Hominy Feed	8	90.5	7.24	Crude protein, % of DM	16.06
Cottonseed, Whole	2	92	1.84	Neutral detergent fiber, % of DM	37.84
Bermuda hay	1	90	0.9	Acid detergent fiber, % of DM	22.57
Total	72.12		40.01	Non-fiber carbohydrate, % of DM	32.45

Supplemental Table 6.4. Volatile fatty acid (VFA) concentrations (mM) at 12- and 24-hour *in vitro* fermentation of orange oil or d-Limonene with ruminal samples from Holstein steer.

		Orange oil							d-Limonene								*P*-Value*				
		0	32	64	125	250	500	1000	0	32	64	125	250	500	1000	SEM	Oil	Conc	Hr	OilxConc	OilxHr	ConcxHr
Ace	**12 Hr**	7.887	7.980	7.598	7.283	7.003	7.319	7.730	8.049	7.876	8.122	7.259	7.668	7.006	8.062	0.511	0.004	0.008	0.001	0.866	0.039	0.828
	24 Hr	9.018	7.559	8.081	7.314	7.228	7.328	8.185	9.606	9.074	8.778	8.799	8.490	7.555	9.407							
Prop	**12 Hr**	1.824	1.888	1.883	1.835	1.707	1.792	1.869	1.878	1.891	1.972	1.791	1.872	1.703	1.859	0.105	0.002	0.168	0.007	0.802	0.012	0.835
	24 Hr	1.746	1.499	1.682	1.637	1.525	1.543	1.647	1.935	1.863	1.882	1.941	1.822	1.619	1.842							
Isobut	**12 Hr**	0.097	0.101	0.097	0.093	0.097	0.096	0.098	0.102	0.099	0.103	0.093	0.097	0.092	0.111	0.008	0.004	<0.001	<0.001	0.814	0.033	0.006
	24 Hr	0.108	0.085	0.094	0.094	0.114	0.113	0.132	0.123	0.107	0.108	0.112	0.122	0.122	0.158							
But	**12 Hr**	0.995	1.044	1.055	1.111	1.135	1.181	1.222	1.034	1.021	1.098	1.057	1.171	1.072	1.296	0.077	0.069	<0.001	0.001	0.642	0.075	0.271
	24 Hr	1.061	0.898	1.042	1.114	1.266	1.282	1.393	1.129	1.098	1.140	1.287	1.334	1.236	1.583							
Isoval	**12 Hr**	0.177	0.179	0.183	0.177	0.173	0.179	0.191	0.185	0.182	0.196	0.182	0.195	0.170	0.234	0.018	0.002	<0.001	<0.001	0.445	0.131	0.16
	24 Hr	0.201	0.176	0.176	0.176	0.205	0.208	0.244	0.222	0.201	0.203	0.218	0.240	0.219	0.320							
Val	**12 Hr**	0.082	0.081	0.082	0.078	0.086	0.086	0.085	0.080	0.079	0.087	0.080	0.084	0.078	0.110	0.008	0.472	<0.001	<0.001	0.012	0.979	<0.001
	24 Hr	0.089	0.071	0.084	0.085	0.166	0.170	0.168	0.094	0.090	0.095	0.108	0.117	0.165	0.180							

**P*-value for the repeated measures ANOVA using oil, concentration, and hour as factors. There were no significant three-way interactions.

Supplemental Table 6.5. Volatile fatty acid (VFA) concentrations (mM) at 12- and 24-hour *in vitro* fermentation of orange oil or d-Limonene with fecal samples from Holstein steer.

		Citrus							Limonene								*P*-Value*						
		0	32	64	125	250	500	1000	0	32	64	125	250	500	1000	SEM	Oil	Conc	Hr	OilxConc	OilxHr	ConcxHr	OilxConcxHr
Total	**12 Hr**	4.003	3.138	2.754	2.669	2.537	2.341	3.019	5.466	5.169	5.264	5.24	5.006	5.178	4.8	0.303	<0.001	<0.001	<0.001	0.014	0.778	0.892	0.989
	24 Hr	5.282	4.003	3.903	3.937	3.696	3.378	4.036	6.859	6.331	6.643	6.446	6.424	6.19	5.458								
A:P	**12 Hr**	3.021	3.161	3.252	3.762	4.473	4.48	3.585	3.047	2.73	2.708	2.756	2.578	2.534	2.802	0.172	<0.001	0.002	<0.001	<0.001	<0.001	0.004	0.086
	24 Hr	2.639	3.153	3.029	2.521	2.972	3.896	3.359	2.992	2.713	2.653	2.539	2.413	2.471	2.911								
Ace	**12 Hr**	2.804	2.221	1.950	1.922	1.906	1.761	2.155	3.853	3.552	3.601	3.583	3.349	3.434	3.238	0.218	<0.001	<0.001	<0.001	0.047	0.026	0.419	0.983
	24 Hr	3.455	2.439	2.312	2.280	2.074	1.771	2.208	4.734	4.275	4.441	4.213	4.100	3.924	3.374								
Prop	**12 Hr**	0.942	0.703	0.601	0.538	0.433	0.398	0.608	1.267	1.300	1.331	1.297	1.300	1.357	1.156	0.079	<0.001	<0.001	<0.001	<0.001	0.199	0.038	0.802
	24 Hr	1.310	0.774	0.764	0.898	0.733	0.461	0.670	1.582	1.575	1.673	1.653	1.698	1.590	1.167								
Isobut	**12 Hr**	0.038	0.031	0.030	0.033	0.035	0.033	0.038	0.054	0.048	0.049	0.052	0.048	0.048	0.050	0.005	<0.001	0.087	<0.001	0.389	<0.001	0.989	0.816
	24 Hr	0.056	0.043	0.038	0.043	0.039	0.042	0.056	0.089	0.078	0.087	0.091	0.086	0.084	0.083								
But	**12 Hr**	0.158	0.133	0.125	0.128	0.113	0.103	0.157	0.208	0.195	0.205	0.222	0.228	0.252	0.264	0.029	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	24 Hr	0.318	0.529	0.556	0.483	0.566	0.747	0.725	0.289	0.271	0.294	0.326	0.362	0.390	0.536								
Isoval	**12 Hr**	0.036	0.028	0.027	0.027	0.026	0.024	0.027	0.052	0.045	0.049	0.052	0.048	0.048	0.045	0.006	<0.001	0.112	<0.001	0.232	<0.001	0.747	0.866
	24 Hr	0.059	0.037	0.033	0.037	0.036	0.040	0.055	0.101	0.088	0.099	0.103	0.097	0.101	0.096								
Val	**12 Hr**	0.024	0.022	0.021	0.022	0.025	0.024	0.033	0.031	0.029	0.029	0.033	0.034	0.039	0.047	0.011	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	24 Hr	0.079	0.174	0.194	0.188	0.236	0.297	0.305	0.064	0.044	0.048	0.061	0.081	0.101	0.196								

*P-value for the repeated measures ANOVA using oil, concentration, and hour as factors.

CHAPTER 7

CONCLUSION

We sought to identify if antibiotic cure rates of mastitis could be improved by using a somatic cell count (SCC) threshold-based approach for treatment. While the present data supports that quarters with lower SCC prior to antibiotic treatment of mastitis are more likely to successfully cure than quarters with high SCC prior to treatment, limiting factors of utilizing a pre-treatment SCC threshold value are evident. Further examination of a SCC threshold with an increased sample size, differing antibiotic regimen, and various durations of antibiotic treatment is warranted to determine the practicality of using pre-treatment SCC values as a treatment basis fully. At the present time, current and previous literature suggest that decision-making strategies for mastitis include consideration of mammary health history (reflected by SCC and/or culture results) and causative pathogen. Additionally, the type of mastitis at the time of infection, lactation number, DIM, and infection chronicity should be considered when making future treatment decisions. With additional investigation, including SCC in this conversation may be viable, contributing to more judicious use of antibiotics in dairy cattle as it becomes increasingly demanded by consumers. In addition, investigating alternatives to antibiotics (ATA) may prove to be useful in combination with strategies that limit overall therapy for mammary diseases, either by preventing or supporting a natural, robust immune response.

Previous efforts for developing ATA have focused on manipulation of microbial populations, in particular the ruminal microbiota. To develop techniques that utilize ATA

in prevention and control of mammary diseases, like mastitis, a foundational investigation of mammary microbiota and its establishment is necessary. Collectively, our results demonstrated that the ruminal, fecal, and milk microbiotas of dairy cows were distinct. Alpha and beta-diversities, and the individual microbial compositions at the phylum level agreed that the ruminal, fecal, and milk environments were dissimilar. However, some bacterial groups were present in all of the environments, suggesting that there may be some relationship between these compartments and potentially how milk microbiota is established. More precise investigation (e.g., PCR) of mastitis bacteriology in association with the milk microbiome should be done to determine if pathogens directly or indirectly alter the milk microbiome. Additionally, further research is needed to investigate the possibility of rumen and fecal microbiota translocation to the mammary gland, establishing milk microbiomes. Determining how these populations are established, is critical to develop an ATA aimed at this compartment specifically.

Recently, essential oils have gained popularity as a major ATA. In cattle, we know some essential oils can impact ruminal function, both negatively and positively. In the present study, *in vitro* fermentation of orange oil and d-Limonene with ruminal and fecal samples did not critically impact total VFA fermentation, NH_3 production, or pH of ruminal fluid or fecal fluid of Holstein cattle. However, there was an increase in butyrate concentrations as orange oil and d-Limonene concentrations increased. Besides serving as an important energy source for the ruminant animal, butyrate is critical for the growth and function of the ruminal epithelium. Additionally, the potential for the use of butyrate as a tool to decrease inflammation in the GIT has been widely suggested. The data presented herein suggests that use of orange oil and d-Limonene as a feed additive may increase

levels of butyrate in ruminal and fecal populations. Further studies should investigate if this increase is synonymous *in vivo* and can be used as an ATA to promote animal health and production efficiency such as increased milk yield.

In conclusion, strategies utilizing current mammary health parameters are difficult to employ as treatment guidelines due to the complex nature of individual cow immune responses and limitations in early detection of mastitis. Thus, it is necessary to utilize novel means to maintain dairy cattle health and well-being. Manipulation of cattle microbiomes appears to be a promising tool for promotion of animal efficiency with limited to no negative effects on production metrics. Overall, our results indicate potential for plant-derived compounds as a preventative and/or treatment for various dairy production diseases, including mastitis, with the long-term goal of reducing antibiotic usage on dairy farms.