Abstract

TILLMAN, WARREN SCOTT. The development of a *Streptococcus uberis* mastitis challenge protocol. (Under the direction of Dr. Mitchell Hockett).

A series of experiments was conducted using a *S. uberis* bacterial isolate in order to produce a predictable and effective protocol for experimental induction of mastitis. A bacterial isolate was obtained from a clinical mastitis infection in a local, North Carolina dairy herd. *In vitro* growth of the bacterial isolate produced a predicted lag, log, stationary, and death phase. Comparison of optical absorbance values to bacterial concentrations resulted in a Pearson's correlation coefficient of 0.71. It was determined that time of growth would be a better indicator of bacterial concentration. *S. uberis* isolates were incubated for 3 hours of growth and placed on ice to observe temporal changes in bacterial concentration. Bacterial concentration did not differ over time (P = 0.96), indicating that ice storage maintained *S. uberis* concentrations for 3 hours. *S. uberis* bacterial concentrations deviated from predicted values in growth curves, so an alteration to the serial dilution protocol was performed in order to reach the desired concentration.

Four Holstein cows were challenged with 5, 400 cfu of *S. uberis* in 2 quarters that contained no mastitis pathogens and were compared to 4 Holstein control cows. The inoculum concentration produced a 100% incidence of clinical infection in all quarters challenged. The challenged group exhibited elevated quarter milk scores, quarter pain scores, attitude scores, and quarter size scores, with first clinical signs occurring at 36 hours. Rectal temperatures were highest at 36 hours (40.5 ± .3 ^{o} C vs. 38.5 ± .3 ^{o} C; P<0 .0001) (mean ± SEM) compared to control. Challenged quarter somatic cell counts (SCC) were elevated at 24 hours post-infusion in comparison to control quarter SCC at 0 (Challenge 24:

3.5 X 10^6 ± 5.6 X 10^6 vs. Control 0: 4.3 X 10^4 ± 3.1 X 10^4; P < 0.0001) and 24 hours (Challenge 24: 3.5 X 10^6 ± 5.6 X 10^6 vs. Control 24: 6.7 X 10^4 ± 5.0 X 10^4; P = 0.003). Challenge quarter SCC at 24 hours also differed from challenge SCC values at time of challenge (Challenge 24: 3.5 X 10^6 ± 5.6 X 10^6 vs. Challenge 0: 1.4 X 10^5 ± 2.1 X 10^5; P < 0.0001).

While a predictable mastitis model was acquired using 5,400 cfu, the response in challenged cows was more severe than preferred. In the attempt to obtain a clinical infection that was physiologically more benign (low-grade pyrexia, less severe quarter abnormalities, and faster response to treatment), it was determined that lower doses might still produce a high incidence of clinical infection while presenting less severe clinical signs. In this trial, 500 cfu (low dose; n=6 quarters in 3 cows), 1,950 cfu (medium dose; n=5 quarters in 3 cows), and 4,000 cfu (high dose; n=6 quarters in 3 cows) were inoculated into quarters that contained no intramammary pathogens. All cows (n=9) and 13/17 (76.5%) quarters developed a clinical infection. Regardless of dose, an overall elevation in SCC from baseline values was reported following challenge (P < 0.0001). SCC was found to be elevated in the high dose group on day 2, and in both the medium and high dose group on days 3 and 4 (P < .05). The low dose tended to be elevated 2 days post challenge (P < 0.1). Regardless of dose, peak rectal temperature after challenge (39.8 ± .2 ˚C) was higher (P = 0.03) than initial rectal temperature (39.1 ± .1 ˚C). Milk score, quarter pain score, quarter size score, attitude score, and appetite score were elevated following challenge for each dose. Udder temperature was elevated only in the low group (P < .05). Serum levels of IL-1β, TNF-α, and IL-8 were not elevated during the sampling period. Five hundred cfu of this *S. uberis*

strain produced a predictable clinical infection in challenged cows. Therefore, higher doses would not be needed to produce mastitis.

The Development of a *Streptococcus uberis* Mastitis Challenge Protocol

by

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Biography

Warren Scott Tillman was born, January 14, 1980, to the parents of Warren and Linda Tillman in Atlanta, GA. The good fortune of being an only child and raised in a household with two classically trained scientists, was soon realized as Scott quickly developed an intense fondness for the natural world. Scott attended high school at The Fayetteville Academy in Fayetteville, NC. In 1998, he graduated, Thomas Hurdle Science Award in hand, and tested fate at Elon College/University. As a Science Fellow and Presidential Scholar, Scott quenched not only his love of the sciences, but also the liberal arts. In 2002, he graduated from Elon with a Bachelor of Science degree in Biology and as is the case with many new graduates, found himself at a crossroads. Veterinary medicine was always Scott's goal, but more experience was needed in order for him to fulfill that dream. Once again, good fortune was found through an opportunity to work towards a Master of Science degree with Dr. Mitch Hockett in the Animal Science Department at North Carolina State University. Here, Scott worked in a number of areas including the impact of novel endophyte infected fescue on reproductive performance, slow-freeze vs. vitrification embryo viability, and most importantly, *S. uberis*. Upon completion of his Master's work, Scott will begin his next chapter at the College of Veterinary Medicine at North Carolina State University. Here, his dream will be fulfilled as he begins working on his Doctorate of Veterinary Medicine.

Acknowledgements

It is impossible to imagine my progression in life without considering the countless number of helping hands that followed me throughout my journey. While the list is virtually infinite, I must recognize certain individuals who provided motivation, support, experience, and knowledge throughout my graduate work. First and foremost, I would like to thank my major professor, Dr. Mitch Hockett, for his incomparable patience and never-ending focus on my success. He has been a mentor and a friend, and will always garner my utmost respect. I would also like to thank Dr. Scott Whisnant, Dr. Kevin Anderson, and Dr. Peter Farin for serving on my graduate committee. Their invested time and advice have helped me to appreciate the scientific method, the importance of the smaller details, and how to overcome Murphy's dreadful law.

I would also like to thank all of my fellow graduate students whose friendships I will keep forever. While each individual has had an important impact in my life, it was the memories that we shared as a group that I will always remember. Habitual lunches at Mitch's, the trip to Oak Island, occasional nights out on the town, and St. Louis will all define my graduate school experience. These times together provided the necessary stress-relief needed to maintain our wits in the often chaotic world of graduate research.

Finally, I would like to thank the most important people in my life. My fiancé and best friend, Lauren Radson, has had to live with the many ups and downs associated with the graduate school lifestyle. She somehow managed to take everything in stride with a smile on her face. Her unconditional love and support during this entire endeavor has been amazing, and I always wonder how I could have been so lucky to have ended up with her. And, to my parents, who always taught me the value of perseverance and integrity. They have always

and will forever be the biggest influence in my life, and I attribute everything I accomplish to their unending love and perpetual wisdom.

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Introduction

According to the latest USDA census, approximately $21 billion of milk was sold in the United States in 2002, and future outlooks predict even higher sales over the next decade. High milk production and reproductive efficiency are crucial to maintaining this upward trend in the United States' dairy sector. One of the biggest threats to this growth is mastitis.

Mastitis, or inflammation of the udder, is estimated to cost the dairy industry $2 billion per year (DeGraves and Fetrow, 1993). Lower milk yields due to decreased production and antibiotic contamination as well as reproductive inefficiencies such as increased days open are associated with this disease. To date, the specific pathway by which mastitis alters these reproductive parameters is undetermined. Since its development in 1969, the measures associated with the "five-point plan" have greatly reduced the incidence rate of certain contagious mastitis pathogens (Neave et al.). But, the overall incidence level of mastitis remains the same.

Environmental mastitis pathogens such as coliform species and environmental streptococci are now becoming increasingly prominent in the modern dairy industry. While a bulk of knowledge exists in regards to coliform mastitis, the pathogenesis of the environmental streptococci, namely *S. uberis,* remains incompletely understood.

S. uberis is a gram positive mastitis pathogen that is found throughout the dairy environment. Prevention of infection is extremely difficult because of its ability to replicate outside of the bovine mammary. *S. uberis* is responsible for both clinical and subclinical infections in lactating and non-lactating mammary glands, and induces differential physiological responses based on strain. Once infiltration and infection occurs in the mammary gland, the pathogenesis of *S. uberis* has not been well characterized.

In order to better study the effects of this important mastitis pathogen at North Carolina State University, a predictable, efficient, manageable challenge protocol must first be established. Therefore, the aim of this study was to isolate a challenge organism, characterize pathogen growth, and produce a predictable *S. uberis* challenge protocol for experimental infection.

Literature Review

Mastitis is the most costly disease in the dairy industry, primarily because of its frequency in modern, high producing herds. Initial expenses such as lowered milk yields, treatment, reproductive alterations and increased labor are only compounded by situations where culling becomes necessary. An understanding of the physiological effects of intramammary pathogens within the cow may alleviate the potential problems caused by mastitis.

Alterations in dairy production/management have greatly decreased the prevalence of contagious mastitis pathogens (Bramley and Dodd, 1984). However, mastitis due to environmental pathogens still remains difficult to prevent. In fact, it has been reported that the incidence of mastitis from environmental pathogens has increased while the overall incidence of mastitis remains the same (Hillerton et al., 1995).

Streptococcus uberis is a common, environmental mastitis pathogen that is found ubiquitously within the dairy environment (Kruze and Bramley, 1982; Zadoks et al., 2005). To date, its epidemiology and pathogenesis is incompletely understood. Because of its relative frequency in the dairy industry and the enormous costs associated with its control and treatment, *S. uberis* has become an increasing liability to the modern dairy farmer. The aim of this study was to characterize a *S. uberis* strain, gain an understanding of its pathogenesis within the dairy cow, and ultimately produce a predictable challenge protocol for future experimental use.

Economic Impact of Mastitis

While assessing the totality of the expenses that are involved with mastitis prevention, researchers have produced some values based on the direct effects of mastitis.

Miller et al. (1993) reported the cost of preventing mastitis was $14.50 per cow per year, but these measures are not effective against many of the environmental pathogens. The actual cost of treatment of clinical mastitis was $37.91 per case, and it has been determined that the average cost, per case, of mastitis in the United States is $107 (Miller et al., 1993). In England, the average cost per affected cow hovers at £218 (Kossaibati and Esselmont, 1997), or approximately $398 by 2006 currency conversion standards.

While the amount of milk loss equated with an intramammary infection is correlated to the severity of infection, time of infection, and pathogen, it was reported that an average of 341 kg. (≈750 lbs.) of milk was lost in the 60 days following clinical presentation (Miller et al., 1991). This loss was the result of both decreased milk production and milk disposal due to antibiotic contamination. Rajala-Schultz et al. (1999) showed a consistent, yet much larger milk loss range from 110 to 552 kg over the cow's entire lactation. Wilson et al. (1997) reported that total milk losses, assuming the milk is valued at $13.00/cwt, were as high as $350 per case per 305 day lactation. In total, it has been estimated that bovine mastitis costs the United States dairy industry $2 billion annually (DeGraves and Fetrow, 1993).

The indirect fiscal impact of mastitis is far reaching and perhaps more noteworthy. In many cases of infection, culling of high producing cows is necessary (Erb et al., 1985). Not only does this affect the milk yield of a given herd, it also affects future losses due to the inability of producing any offspring from those culled. There is also a growing body of evidence reporting that mastitis alters reproductive success, thus causing even greater economic loss for the farmer (Cullor, 1990; Moore et al., 1991; Barker et al., 1998; Oliver et al., 2000; Hockett et al., 2000, Hockett et al., 2005).

Mastitis Defined

Mastitis is defined as an inflammation of the mammary gland parenchyma (International Dairy Federation Guidelines, 1987). This inflammation is triggered through trauma, toxicity, or infection and often is the result of a cellular pathogen. These pathogens can be bacterial, viral, algal, or fungal and their characterization is often based on their pathology, etiology, and/or severity (Sordillo and Streicher, 2002).

The majority of mastitis cases in the bovine are the result of bacterial pathogens (Jain et al., 1979; Oliver and Mitchell, 1984; Wilson et al., 1997; Waage et al., 1999; Bradley, 2002). Oliver and Mitchell (1984) observed bacterial pathogens in approximately 92% of subclinical and clinical mastitis cases. Wilson et al. (1997) reported that 84% of all mastitis pathogens isolated from milk during a four year period in New York and Pennsylvania were of bacterial origin. But, because of current diagnostic techniques, rarely are pathogens other than bacterial species associated with mastitis (Jain, 1979). In fact, 25-28% of clinical cases have an unknown etiology (Elbers et al., 1998; Barkema, 1998). The incidence of viral mastitis is currently unknown, but viruses have been isolated from mastitic teats (as reviewed by Wellenberg et al., 2002). The incidence of fungal mastitis has been estimated at 2-7% of all mastitis infections (Kirk and Bartlett, 1986). Perhaps the most important of these fungal mastitis pathogens are yeast of the genus *Candida* (Krukowski et al., 2001; de Casia dos Santos and Marin, 2005). The genus, *Prototheca*, comprises the only known algal mastitis pathogens. While incidence levels are low in mild climates, an outbreak can occur in certain herds and is difficult to control using current antimicrobial therapies (Costa et al., 1996; Roesler and Hensel, 2003). In tropical environments such as Brazil, the incidence of fungal and algal infections can greatly increase. Here, mastitis of *Prototheca* etiology has been

shown to be as high as 41% while mastitis of fungal etiology hovered in the vicinity of 20-25% (Costa et al., 1998; de Casia dos Santos and Marin, 2005). Nevertheless, because of their abundance in today's industry and pertinence to this study, the bacterial pathogens will remain the primary focus of this review.

Characterization of Mastitis

The use of terms such as "clinical" and "sub-clinical" mastitis have become commonplace within the literature and the criteria that differentiates the two is relatively straightforward. Clinical mastitis is an infection or inflammation of the udder that is often accompanied with pyrexia, swollen, sensitive and even hard quarters, flakes, and clots. An elevation in inflammatory cells within the milk is also associated with mastitis and can be accounted for by observing the milk somatic cell count (SCC). These cases of mastitis are typically acute, but are generally cleared by the cow or through treatment. Conversely, subclinical mastitis exhibits no outward signs of infection, yet, if the milk is cultured, a pathogen would be present. Elevation in milk SCC is typical of these infections, although not to the extent with which you see in clinical mastitis (Reneau, 1986). Subclinical infections can progress as chronic infections, either because they are rarely detected by the farmer and/or because of their pathology in the udder. Therefore, subclinical cases can remain elusive to the dairy farmer and while their effects on the cow are more benign, many of the same physiological and agronomic setbacks can result (Philpot, 1967). After running the California Mastitis Test (CMT) on foremilk samples in a Louisiana herd, Philpot (1967) observed a decrease in milk production by as much as 45.5% for those quarters that exhibited the highest CMT reaction.

Mastitis can also be classified as either environmental or contagious based upon its etiology. Contagious mastitis pathogens such as *Staphylococcus aureus* and *Streptococcus agalactiae* are typically subclinical in nature, apt to survive the conditions associated with the bovine udder, and spread easily throughout a dairy herd (Sol et al., 1997; Estuningsih et al., 2002). Environmental pathogens are opportunistic; more often cause the clinical infections, and are often cleared by the cow very quickly (Smith et al., 1985). It must be noted that while contagious pathogens are usually subclinical, this dogma does not always hold (Erskine et al., 1988). The same can also be said for environmental pathogens and clinical infections.

Gram (+) and Gram (-) Pathogens

The prevalence of mastitis pathogens varies from herd to herd; however, bacterial pathogens appear to be the most prominent contributor to worldwide mastitis (Wilson et al., 1997; Bradley, 2002). Bacterial cells are often classified into two distinct subgroups based upon their cellular structure. The response of the cow is often times specific and predictable in association with any given bacterium and the cell wall structure of these pathogens plays a key role. Because of this differential cell wall morphology, gram staining, empirically developed by Christian Gram in the late 1800's, has become an important means of classification in regards to bacterial mastitis.

The gram-positive bacterial cell wall is composed almost wholly of peptidoglycan layers, a relatively complex polymer of sugars with amino acid linkages (as reviewed by Navarre and Schneewind, 1999). The thick peptidoglycan layer allows gram-positive bacteria the ability to withhold crystal violet stain (Beveridge and Davies, 1983). Uniquely, this group of bacteria often contains teichoic acid which is incorporated within the peptidoglycan. Some of the major gram-positive pathogens include *Staphylococcus aureus*,

Streptococcus agalactiae, *Streptococcus dysgalactiae*, and *Streptococcus uberis*. Fortunately, many of these infections are sensitive to antibiotics and can be treated rather effectively by the producer.

Gram-negative bacteria tend to have a more complex layering in their cell wall structure. While it does contain peptidoglycan, it also contains a complex and species unique, lipopolysaccharide layer (LPS) (as reviewed by Beveridge, 1999). LPS, or endotoxin, typically elicits an acute immune response in an infected animal. *E. coli* is perhaps the primary gram-negative contributor to mastitis in dairy cows. Because many antibiotics target peptidoglycan, treatment of gram-negative bacteria proves difficult in comparison to gram-positive pathogens (Pyorala et al., 1994).

Mastitis Inflammatory Response

Inflammation is noted by a number of predictable physiological characteristics, most of which are associated with the acute phase response. This response occurs at areas of pathogen infiltration or tissue damage, congruent with mastitis (Conner et al., 1986). The various signs of inflammation include edema, pyrexia, pain, and depression (Harmon, 1994). A number of chemical mediators appear to be involved in the activation of the acute phase response as well as in the maintenance of this heightened immunological state.

The inflammatory response is typically biphasic (Jain et al., 1972). The immediate inflammatory response induces an increased amount of blood flow and an increase in vessel permeability at the area of insult (Jain et al., 1972). Persson and Sondgren (1992) reported that total cell numbers as well as serum albumin numbers increase with the induction of endotoxin, indicative of increased blood flow and vessel permeability. Histamine, released by mast cells and basophils, is most likely one of the primary mediators of this vascular

alteration (Majno and Palade, 1961; Jain et al., 1972; Zia et al., 1987). Bradykinin, a potent vasodilator and pain promoter, is also involved in the inflammatory response, and increased levels have been reported during both clinical and subclinical mastitis (Eshraghi et al., 1999). There is a high probability that other kinins might also play a role in vasodilation during mastitis.

The secondary phase is associated with the chemotaxis of the various leukocytes into the area of insult. Endotoxin administration (Persson et al, 1992), coliform mastitis (Lehtolainen et al., 2003), and gram-positive pathogens (Atroshi et al., 1996) have all resulted in increased leukocyte concentrations in the infected quarter. Neutrophils remain the predominant leukocyte found in the infected udder (Nickerson and Pankey, 1984; Saad and Ostensson, 1990). Secondarily, eosinophils and monocytes/macrophages appear to also be involved in the acute phase response associated with mastitis. (Sordillo et al., 2002). The recruitment and diapedesis of these immune cells are most likely the result of chemokines released locally from mammary endothelium (Granger and Kubes., 1994).

Chemical mediation of the innate immune response becomes a crucial second messenger system, insuring that the systemic immune cells are targeted to the area of insult. Complement proteins have an important bactericidal role within the acute-phase response. Either through mannose-binding lectins (classical pathway) or through direct activation from invading bacteria (alternative pathway), these complement proteins opsonize bacterial cells, making them targets for phagocytosis (Cooper, 1985; Pangburn, 1988). Antigens on bacterial cell walls, namely LPS and peptidoglycan, also promote the expression of cytokines by local immune cells and epithelial cells (Gupta et al., 1999; Hoeben et al., 2000; Schmitz et al., 2004). These cytokines include interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-

α), interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interferon gamma (IFN-γ). Acting on vascular endothelium and on circulating neutrophils, these cytokines as well as the earlier mentioned chemokines (namely IL-8) help to direct phagocytotic immune cells to the area of insult.

Leukocyte trafficking is a vital aspect of the innate immune system and remains one of the primary defenses against invading bacterial pathogens. Once local tissues are activated, TNF-α, released by local macrophages or bacterial LPS, activate vascular endothelium. This activation promotes the endothelium to express selectin proteins (P-selectin and E-selectin) on their cellular surface (Gotsch et al., 1994; Ley and Tedder, 1995). Selectin expression can also be prompted through the release of histamine from localized mast cells (Jones et al., 1993) and the complement component, C5a (Foreman et al., 1994). TNF-α, IL-1β, and LPS also up-regulate the expression of another endothelial adhesion molecule, ICAM-1 (Myers et al., 1992). Because vessel dilation is a result of inflammation, leukocytes in systemic circulation are allowed to interact more readily with the vascular endothelium. Once activated, endothelial selectins weakly bind to a leukocyte carbohydrate ligand, the sialyl-Lewis moiety, causing what is referred to as "leukocyte rolling" (Misugi et al., 1995). As these weak bonds are formed, stronger bonds between the endothelial ICAM-1 and leukocyte integrin proteins, notably LFA-1 (CD11a/CD18), develop (Simmons et al., 1988). Chemoattractant molecules such as IL-8, leukotriene-B4, and Gro-α greatly upregulate the adhesion of LFA-1 to ICAM-1 through leukocyte receptors (Seo et al., 2001). Once these leukocytes are bound to the vascular endothelium, diapedesis into the extracellular matrix occurs. From here, the leukocyte follows a chemokine concentration gradient (IL-8) to the area of insult (Middleton et al., 1997).

Inflammatory cytokines also have a number of systemic effects. By acting on the hypothalamus, TNF-α, IL-6, and IL-1β induce a pyretic response (Long et al., 1990; as reviewed by Rothwell, 1997). Inflammatory cytokines mobilize energy stores in the body (Zoccali et al., 2003). Through their action in the liver, these cytokines also induce the acute-phase response. This response elicits the production and release of various hepatic proteins such as mannan-binding lectin (Aittoniemi et al., 1997), C-reactive protein (Horadagoda et al., 1999, and lipopolysaccharide binding protein (LBP) (Wright et al., 1989; Bannerman et al., 2004a). These help to opsonize invading pathogens while also activating immune cells.

Gram Positive Mastitis

Current techniques have significantly reduced the number of mastitis occurrences involving contagious pathogens. However, these techniques have not greatly impacted the frequency of environmental streptococcal and coliform mastitis. (Smith, 1983; Oliver, 1988). As mentioned previously, distinctions exist between the epidemiology and pathogenesis of gram positive and gram-negative bacterial pathogens. While a large amount of work has been done using coliform pathogens and endotoxin, the literature is still lacking in regards to environmental streptococcal pathogens. Therefore, in this study, *Streptococcus uberis*, a gram-positive pathogen, will be our mastitis causing agent. Thus, an understanding of the gram (+) specific inflammatory response as well as its divarication from gram (-) pathogenesis is necessary. Second, while the literature still remains incomplete, the physiological effects of *S. uberis* will be examined, including correlates with reproductive function.

In comparison to the well-studied coliform pathogens, mastitis caused by gram positive bacteria, in general terms, are typically found to be much more benign (Bannerman

et al., 2004b; Riollet et al., 2000; Hoeben et al., 2000). Nevertheless, these generalizations often rely on a number of factors including dairy environment, strain heterogeneity, and antibiotic resistance.

Gram negative pathogens, specifically *E. coli*, are often associated with acute, toxic mastitis in dairy cattle. A study in Ireland showed that of all toxic mastitis cases, greater than 50% of them were of *E. coli* origin (Menzies et al., 2003). In contrast, only 13.4% of the cases were associated solely with gram-positive pathogens. When studying SCC patterns concurrent with various pathogens, it has been reported and often generalized, that gram negative pathogens induce a much faster immune response than do the gram (+) counterparts (de Haas et al., 2004; de Haas et al., 2002; Vaarst and Enevoldsen, 1997). de Haas et al. (2004) reported that *E. coli* mastitis tends to follow a pattern that exhibits a high spike in SCC coupled with a quick recovery. *S. uberis* and *S. aureus* mastitis were reported to have a more prolonged increase in SCC levels. Bannerman et al. (2004a) confirmed this when comparing *S. uberis* mastitis to *Serratia marcescens*, a gram-negative pathogen.

Streptococcal Bacteria

Streptococcal bacteria are highly diverse. However, beyond their gram-positive, cocci morphology, some other similarities can be found. These bacteria tend to proliferate in a "beaded" manner, remaining attached and within a single plane (Groisman, 2001). As replication continues, long continuous chains of a given species form. Streptococci can reproduce and survive in oxygen rich environments, but are more successful in anaerobic conditions, thus making them facultative-anaerobes (Facklam, 2002). All streptococci are also called lactic acid bacteria because lactic acid is their primary fermentative end product (as reviewed by Hardie and Whiley, 1997).

In the past 20 years, taxonomic changes have been made within the streptococcal grouping. Currently, there are 40 different identified streptococcal species, and it is this diversity that makes grouping difficult (as reviewed by Hardie and Whiley, 1997). Initial classification was based on the hemolytic activity of various streptococci strains. Hemolytic analysis is performed by culturing bacterial species on blood agar plates and then observing agarose morphology. Alpha-hemolytic streptococci partially digest red blood cells, leaving a green residue whereas beta-hemolytic streptococci completely digest red blood cells, leaving a clearing in the agarose plate. Gamma-hemolytic streptococci do not digest red blood cells and therefore, no change is noted on the agarose plate (Shottmuller, 1903). Soon after, different carbohydrate antigens on the streptococcal cell wall were identified and used as distinguishing factors (Lancefield, 1933). Combining these two classification systems along with other phenotypic tests provided the only real means of identifying streptococcal species. However, within the past couple of decades, thanks to the advent of genetic typing, definitive differentiation between differing species is possible (Hardie and Whiley, 1997). Secondarily, this genetic analysis has also helped to reclassify those bacteria that were once incorrectly grouped with streptococci.

Streptococcus uberis

Streptococcus uberis is a unique streptococcal species that does not consistently react to any one Lancefield-group antisera, making definitive microbiological identification difficult. As is the case with all Streptococcal species, *S. uberis* can be identified by its gram-positive cocci appearance and the fact that it is catalase negative. Species differentiation is often performed by observing esculin hydrolysis determining if the pathogen undergoes the CAMP reaction (Darling, 1975; Harmon et al., 1990). The species

itself is highly variable, as a number of different strains have been identified (Kahn et al., 2003).

S. uberis is one of the most common environmental mastitis pathogens found in dairy herds throughout the world. In a study of 274 herds in the Netherlands, Barkema et al. (1998) reported a clinical *S. uberis* mastitis incidence rate of 18.2%. A retrospective study from Sweden found similar results, whereby *Streptococcus uberis* accounted for 19.6% of its clinical cases (Jonsson et al., 1991). In a 16 herd study within the UK, *S. uberis* incidence levels were found to comprise anywhere between 15% and 45% of all new clinical and subclinical mastitis infections (Neave et al., 1969). *S. uberis* has also been found at varying incidence levels within the United States. Over a seven year period, Todhunter et al. (1995) saw that *S. uberis* comprised 18.2% of all environmental streptococcal intramammary infections isolated from an Ohio herd. Over a five year period in Knoxville, TN, a *S. uberis* incidence rate of 14.2% per lactation was reported (Jayarao et al., 1999) and was similarly confirmed by Barker et al. (1998) with an incidence rate of 14.7%.

The epidemiology of *Streptococcus uberis* is still incompletely understood. *Streptococcus uberis* mastitis can occur not only in lactating cows, but also in dry and periparturient cows (Todhunter et al., 1995). Therefore, it is conceivable that disease transmission is not solely associated with dairy parlor interaction, but also other components of the cow's environment. *S. uberis* can be found almost ubiquitously throughout the dairy environment, and has been isolated from the vulva and the rectum (Kruze, 1982) as well as the tonsils, rumen, and coat of dairy cows (Cullen, 1969; Cullen and Little, 1969; Cruz-Colque et al., 1993). Concurrently, considerable levels of the pathogen have been isolated from straw bedding (Bramley, 1982) and have been shown to readily grow in recycled

manure bedding (Zehner et al., 1986). Even on pasture-based dairies, the bacteria have been isolated fairly readily (Leigh, 2003). Therefore, as is the case with most environmental pathogens, removal of *Streptococcus uberis* from the dairy environment is extremely difficult, if not impossible.

Streptococcus uberis is responsible for both clinical and subclinical infections, the former typically being much more acute while the later being more persistent (Zadoks et al., 2003; Todhunter et al., 1995; Kahn et al., 2003). Distinguishing which infection type predominates (clinical vs. subclinical) is difficult and is perhaps strain dependent (Todhunter et al., 1995, Phuektes et al., 2001). However, the current literature suggests that from a holistic viewpoint, *S. uberis* probably is more attributed to infections that are subclinical in nature (Kahn et al., 2003, Zadoks et al., 2003).

The pathogenesis of *S. uberis* is not completely understood. However, many of the patterns associated with the inflammatory response apply. *S. uberis* is shown to initiate increased SCC, abnormal milk consistency (flakes, clots, and clumps), edema, pyrexia, and pain in and around the infected quarter (Thomas, et al., 1994, Pedersen et al., 2003, Rambeaud et al., 2003 Bannerman et al., 2004a). Pathological areas of necrosis in ductal and alveolar tissue, fibrin accumulation, and localized lymph node swelling are also noted (Thomas et al., 1994, Pedersen et al., 2003). The histological chemotaxis of monocyte/macrophages and leukocytes from the blood to the udder, like with most inflammation, is also characteristic of *S. uberis*. However, unlike in other cases of mastitis, the role of neutrophils in the control and clearance of *S. uberis* mastitis is questionable. While Pedersen et al. (2003) did show the phagocytosis of *S. uberis* by both macrophages and leukocytes in infected mammary tissue, Thomas et al. (1994) observed only a small

percentage of phagocytosed bacterial cells in neutrophils. Contrary to other mastitis pathogens, Rambeaud et al. (2003) as well as Bannerman et al. (2004a) observed an increase in bacterial cell numbers concurrent with an increase in SCC, further strengthening the premise that neutrophils perhaps play a minimal role in *S. uberis* control. The insertion of intramammary devices to increase SCC and ultimately prevent mastitis infection was attempted using *S. uberis (*Nickerson et al., 1990*)*. While this intramammary device has been shown to effectively prevent infection with other mastitis pathogens, and while SCC was increased in this study, the device was not shown to prevent *S. uberis*. *S. uberis* strain differences may in fact play a crucial role in neutrophil clearance (Leigh et al., 1990; Leigh and Field, 1991; Leigh, 2003). The importance of these neutrophils to the innate immune system's control of *S. uberis in vivo* has not been determined.

S. uberis mastitis elicits the release of a number of inflammatory mediators, similar to that of other mastitis pathogens. TNF-α, IL-2, and IL-8 were all found to be involved in the immune response to clinical *S. uberis* mastitis (Rambeaud et al., 2003; Bannerman et al., 2004a). Rambeaud et al. (2003) did not observe detectable levels of these cytokines until approximately 66 hours post challenge, perhaps a result of increased SCC. Bannerman et al. (2004a) observed an increase in SCC at 30 hours post-infusion, concomitant with increases in milk IL-8, IL-12, and IFN-γ. But, IL-1β and TNF-α levels were not elevated in milk until 60 and 72 hours respectively. Other mediators, such as acute phase proteins haptoglobin, LBP, and serum amyloid A, have been observed in milk, further indicative of a strong immune response (Pedersen et al., 2003; Bannerman et al., 2004a). Even upregulation of the macrophage LBP receptor, CD14, is reported (Bannerman et al., 2004a). Cortisol, an important stress hormone, has shown significantly increased levels with a *S. uberis* challenge

(Hockett et al., 2000). In this same study, it was also shown that under oxytocin stimulation, $PGF_{2\alpha}$ levels were elevated significantly higher than the unchallenged controls. Unfortunately, because of the lack of *S. uberis* pathogenesis literature, it is difficult to produce dogma based on this evidence. Nevertheless, it provides an important step in the understanding of *S. uberis* mastitis.

Pathogenecity of S. uberis

Because of the successful implementation of the "five point mastitis control plan" (Neave et al, 1969), the outbreak of mastitis due to contagious pathogens has been greatly reduced (Hillerton et al., 1995). This has prompted current research to focus primarily on the epidemiology and pathogenesis of environmental pathogens such as *E. coli* and *S. uberis* (Burvenich et al., 2003). A large majority of the research dealing with environmental pathogens has been performed with regard to *E. coli* and/or endotoxin induced mastitis (Giri et al., 1984; Shuster and Harmon, 1992; Shuster et al., 1993; Hoeben et al., 2000). However, the physiological response to *E. coli* mastitis differs from that of the gram-positive pathogens responsible for mastitis such as *S. uberis*. Therefore, making generalizations about environmental mastitis pathogenesis is very difficult, and an understanding of the similarities and differences is important to the context of this study. It must also be understood that this discussion will make further generalizations about *E. coli* and *S. uberis* mastitis, assuming that each species has a standardized infectivity and pathogenesis. While the literature does show clear intraspecies distinctions, generalized differences may exist amongst different species strains. Regardless, distinctions can be made between these two mastitis pathogens.

Pathogenesis of *S. uberis* and its divarication from *E. coli*

Because both *E. coli* and *S. uberis* are environmental pathogens, their epidemiology is fairly similar. As has been mentioned previously, *S. uberis* can be found ubiquitously throughout the dairy environment. The same can also be said of *E. coli*, which originates from the gastrointestinal tract of the dairy animal and is shed in manure (Hancock et al., 1994; Hogan and Smith, 2003). Increased intramammary infections with these pathogens have been attributed strongly to heavy fecal contamination of both the animals and their environment (Faull et al., 1983; Barkema et al., 1998; Ward et al., 2002). Bedding material also provides the appropriate environment for bacterial replication, allowing for prolonged exposure, especially in confinement based dairies (Zehner et al., 1986; Ward et al., 2002). Therefore, considering the current trends in dairy management systems, exposure is almost inevitable.

Once entrance via the teat orifice has occurred, the physiological responses of the animals to *S. uberis* and *E. coli* mastitis begin to diverge. This is perhaps the result of mammary epithelial binding (or lack there of). It appears that epithelial adherence and internalization of *S. uberis* in the mammary gland is important to its infectivity (Matthews et al., 1994; Almeida et al., 1996). This adhesion is reported to be the result of pathogen/host cell interactions through heparin sulfate proteoglycan receptors (Almeida et al., 1999a; Almeida et al., 2003) and is aided by milk lactoferrin (Fang et al., 2000) and/or extracellular matrix proteins (Almeida et al., 1999b). This adhesion does not appear to be necessary for the development of an *E. coli* intramammary infection (Frost et al., 1975), although an *in vitro* study by Dopfer et al. (2001) demonstrated that it can occur, and with greater success

than certain *S. uberis* strains. Ultimately, *E. coli* adhesion is highly variable and strain dependent (Lammers et al., 2001).

Macrophages and neutrophils act as the initial mediators of an innate immune response in the mammary gland (Sordillo et al., 1997). Both clinical and subclinical *E. coli* and *S. uberis* mastitis have been reported to increase milk somatic cell count (SCC) (Shuster et al., 1991; Kehrli and Shuster, 1994; Schrick et al., 2001; Rambeaud et al., 2003; Bannerman et al., 2004a; Hockett et al., 2005). As has been alluded to earlier, the direct role of neutrophils is questionable in *S. uberis* mastitis (Thomas et al., 1994; Rambeaud et al., 2003). This is in spite of neutrophil diapedesis and upregulation of leukocyte integrin protein CD11a (Smits et al., 1998; Rambeaud et al., 2002) Interestingly, the literature appears to be conflicting regarding CD18, a second integrin which forms the LFA-1 complex. While Smits et al. (1998) showed a significant increase in CD18 at 24 hours and 60 hours post infusion, Rambeaud et al. (2002) observed a sharp decrease at 96 hours post infusion, and these low levels remained throughout the remainder of the study. This decrease could be the result of regulatory cytokines attenuating the immune response, or it could be a mechanism by which *S. uberis* evades the immune system. As is the case with a number of other mastitis pathogens, neutrophils are a key component towards the control of *E. coli* mastitis (Hill et al., 1978; Hill et al., 1983) and weak neutrophil recruitment appears to increase infection severity (Shuster et al., 1996). Endotoxin has been shown to upregulate the expression of CD11/CD18 adhesion molecules, further substantiating the claim that *E. coli* induces a strong neutrophil response (Roets et al., 1999). Therefore, perhaps SCC prior to intramammary infection is important to that cow's susceptibility to *E. coli*, whereas it could have less of an impact on *S. uberis* susceptibility. Nevertheless, the fact that certain strains

of *S. uberis* are resistant to neutrophils phagocytosis is a crucial distinction between coliform mastitis. Also, the secondary effects that arise from this can further differentiate the two forms of mastitis.

The onset of systemic clinical signs with *S. uberis* has been found to exhibit a lag phase, at least in certain strains. In quarters infected with 6,650-10,500 colony forming units (cfu), Rambeaud et al. (2003) did not observe changes in mammary or milk scores until 84 hours post infusion. Similarly, using the same strain (UT888), Hockett et al. (2000) did not notice a significant change in clinical signs until day 3 post bacterial infusion (approx. 5,000 cfu). In a later study, similar results were found where temperature and mammary scores did not differ from controls until day 3 (Hockett et al., 2005). When challenging with large doses of *S. uberis*, (approximately 1 X 10^7 cfu), acute clinical symptoms can arise within a day (Thomas et al., 1994) and as early as 4 hours post infusion (Pedersen et al., 2003). Using strain O140J isolated in Great Britain, doses as low as 500 cfu produced localized effects seen as early as 24 hours (Smits et al., 1998). After challenging just one quarter with 220 cfu of strain 0140, Bannerman et al. (2004a) observed increases in SCC within 24 hours, with outward clinical signs appearing within 30 hours. It appears that strain variation is directly correlated to physiological virulence.

Contrary to this, the effects of *E. coli*, both localized and systemic, can be observed as early as 8-12 hours post infusion (Riollet et al., 2000; Bannerman et al., 2004b). This same trend holds, even when challenged with as few as 50 cfu (Tomita et al., 1998). With higher doses of *E.coli* (10,000 cfu), a further shortening of onset of infection can be observed (Lohuis et al., 1990). When inducing experimental mastitis with LPS, the key molecule involved in *E. coli* pathogenicity, an even shorter response time is seen. Moussaoui et al.

(2002) infused 10 μg of *E. coli* LPS and observed elevations in rectal temperature as early as 2 hours, and even observed localized edema within an hour. Using the same concentration of LPS, Shuster et al. (1993) observed swelling and increased serum albumin within the same time frame, although pyrexia was only observed in one cow. Based on general trends, it does appear that *E. coli* tends to have an earlier onset of pathogenicity once introduced into the mammary gland.

Duration of infection also appears to differ between the two pathogens. In a study observing the dynamics of environmental streptococcal infections in a single herd over seven years, it was shown that 41.1% exhibited an infection (whether clinical or subclinical) less than 8 days, whereas only 61.8% exhibited infections lasting less than 31 days, and 15% persisted for more than 90 days (Todhunter et al., 1995). The mean in this study was 12.3 days and bacterial species did not seem to have an effect. In a study observing selenium's effects on *E. coli* mastitis, challenged cows in the Se deficient group cleared bacterial milk concentrations, on average, within 162 hours while the Se supplemented group cleared in less time (Erskine et al., 1989). Even in compromised nutrient deficiency, cows were able to clear infection within a week. Hill et al. (1984) observed the length of E. *coli* infection lasting no longer than 13 days and Shuster et al. (1997) saw complete eradication of the pathogen within a week after experimental challenge.

E. coli is considered to be an acute, yet mild mastitis pathogen that is only severe in immunosuppressed animals (Vaarst and Enevoldsen, 1997). Much of this is probably due to the ability of polymorphonuclear leukocytes, namely the neutrophil's, ability to decrease cellular numbers. Concurrently, the inability of certain *E. coli strains* to adhere to mammary epithelium may also make it more vulnerable to the mammary environment and its immune

mediators, further shortening duration of infection. Following the same principles, the increased duration of *S. uberis* infections is perhaps a direct result of the pathogens ability to adhere to mammary epithelium and its avoidance of neutrophil phagocytosis.

While very little work has been done regarding the chemical mediators involved in *S. uberis* mastitis, further distinctions can be found in comparison to *E. coli*. As previously mentioned Rambeaud et al. (2003) observed increases in whey TNF-α, IL-1β, and IL-8 starting at 66 hours post experimental infusion. TNF-α levels remained elevated throughout the remainder of the study (168 hours post infusion). IL-1 levels were elevated to 144 hours post infusion, while IL-8 levels fluctuated after 66 hours, although peaking at 122 hours. Bannerman et al. (2004a) did not notice elevations in milk TNF-α and IL-1β until 72 and 60 hours respectively. However, elevation in milk IL-8, C5a, IFN-γ, and IL-12 were observed at 30 hours post infusion.

Hoeben et al. (1997) observed an increase in both milk and plasma TNF-α samples, starting at 10 hours post *E. coli* infusion. In the same study, LPS elicited an increase in TNF-α as early as 4 hours post infusion in both milk and plasma. Shuster et al. (1997) observed a very similar trend with whey TNF- α post *E. coli* infusion, however a large amount of variation was shown to exist between the cows and no increase in TNF- α levels was observed in the blood plasma. In this same study, Shuster et al. observed a sharp increase and peak of whey IL-1β around 12 hours and a gradual increase in whey IL-8 starting at 14-16 hours and peaking at 36 hours post infusion. While the pathogen used by Rambeaud et al. (2003) was found to be less virulent, even compared to cytokine levels found with the strain used by Bannerman et al. (2004a), distinctions regarding cytokine expression between *E. coli*

and *S. uberis* are obvious. This data further substantiates the idea that *E. coli* is a much more acute mastitis pathogen than *S. uberis*.

Because there appears to be a great deal of strain variation, it is difficult to make gross generalizations regarding a "typical" *S. uberis* mastitis infection. However, given the research available, it appears that a distinction is apparent between *S. uberis* and other environmental mastitis pathogens, namely *E. coli*. Similarly, this leads to the assumption that perhaps *S. uberis* mastitis might exhibit a differential effect on reproductive efficacy in dairy species, or at least may act through different mechanisms. Therefore, a guarded approach is necessary when considering the bulk of literature regarding the effects of mastitis on reproductive success.

Effects of Mastitis on Reproduction

The effects of intramammary infections on various reproductive parameters have recently been established. These effects were first implicated with gram-negative pathogens. Cullor (1990) first reported shortened interestrus intervals in a herd that had a high rate of gram-negative mastitis. No significant difference in interestrus interval was reported in a herd whose primary pathogen was *S. aureus*. Studying the same herds, Moore et al. (1991) reported that herd 1 (gram-positive herd) exhibited no difference in interestrus intervals between those infected and those uninfected. However, cows with mastitis in herd 2 (gram-negative herd) were reported to be 1.6 times more likely to have an altered (shortened) interestrus interval than those that were uninfected. Barker et al. (1998) observed various reproductive performance criteria in a retrospective study. Here, it was reported that clinical mastitis obtained prior to first artificial insemination (AI) showed an increase in number of days to first AI and an increase in days to conception. It was also reported that cows that

developed mastitis between first AI and establishment of pregnancy showed an increase in number of inseminations needed for pregnancy, an increase in the breeding period, and ultimately an increase in days to conception. Similarly, Risco et al. (1999) reported that cows with mastitis during the first 45 days of lactation had a 2.7 times higher likelihood of abortion. Schrick et al. (2001) observed increased days to first AI (service), days open, and services per conception, not only for those with clinical mastitis infection, but also for those with subclinical mastitis during early lactation. Reproductive abnormalities appear to arise more frequently during cases of mastitis that occur prior to first artificial insemination, and it is during this time frame that mastitis incidence level is highest (Kelton et al., 2001; Schrick et al., 2001; Santos et al., 2004).

As mentioned earlier, elevated cortisol has been associated with *S. uberis* mastitis (Hockett et al., 2000), and was also observed during endotoxin induced mastitis (Shuster and Harmon, 1992), and *E. coli* mastitis (Hirvonen et al., 1999; Roets et al., 1999). Cortisol has been shown to decrease LH pulsatility in the ovine model and is hypothesized to reduce estradiol's negative feedback threshold on the pituitary (Daley et al., 1999). Exogenous administration of dexamethasone has been reported to completely block, or shorten estrus behavior (Ehnert and Moberg, 1991). This decreased LH pulsatility and maximum decreased LH concentrations have also been observed in *S. uberis* challenged cows (Hockett et al., 2005). In these studies, estradiol levels were reported to remain unchanged leading up to the LH surge necessary for ovulation.

Because $PGF_{2\alpha}$, a luteolytic inflammatory cytokine, is shown to increase in the udder as a result of endotoxin challenge (Giri et al., 1984), it was hypothesized that this pathway may be the primary contribution to altered reproductive efficiency (Moore and O'Conner,

1993). Hockett et al. (2000) experimentally induced *S. uberis* mastitis in cows, and then subsequently challenged with oxytocin. The oxytocin challenge elevated serum levels of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM), a $PGF_{2\alpha}$ metabolite, in the mastitic cows to a greater degree than the controls. However, baseline values were not different between the two treatment groups. Congruent with Hockett et al. (2000), Jackson et al. (1990) reported no significant elevation in systemic PGFM levels following intramammary endotoxin challenge in the absence of oxytocin challenge. Intravenous injection of peptidoglycan in ewes did show elevated systemic PGFM levels and pregnancy rates were negatively correlated to both peptidoglycan dose and PGFM levels (Stewart et al., 2003).

As mentioned previously, gram-negative, gram-positive, and endotoxin induced mastitis have been shown to increase the concentration, at least locally, of TNF-α and IL-1β (Hoeben et al., 1997; Shuster et al., 1997 Rambeaud et al., 2003). Acosta et al. (1998) reported that TNF-α did not have a direct effect on *in vitro* bovine follicular estradiol, progesterone, or androstenedione release. But, TNF-α did attenuate the LH induced release of estradiol and androstenedione. In the same study, IL-1β stimulated estradiol release while inhibiting the release of androstenedione. IL-1β has been implicated as being a primary inflammatory mediator of ovulation, at least in the rat model (Bränström and Normanl., 1993). A TNF-α specific, dose-dependent and time-dependent cytotoxic effect was reported with bovine luteal endothelial cells (Friedman et al., 2000). Friedman et al. (2000) also reported that TNF-α also had no effect on luteinized steroidogenic cells and addition of progesterone prevented the cytotoxic effect on luteal endothelium. TNF-α has been shown to greatly upregulate $PGF_{2\alpha}$ release by the endometrium *in vitro* at all estrous cycle stages (Miyamoto et al., 2000). Cultured bovine luteal cells exposed to IL-1β exhibited an

upregulation of $PGF_{2\alpha}$ production and were reported to have a synergistic effect with arachidonic acid (a $PGF_{2\alpha}$ precursor) on $PGF_{2\alpha}$ production (Townson and Pate, 1994).

The actual mechanism by which mastitis affects reproductive function is still undetermined. Normal reproductive function involves various immune mediators ($PGF_{2\alpha}$, TNF-α, and IL-1β) and in case of inflammation, increasing concentrations of these immune mediators could ultimately skew normal ovarian/uterine function. Furthermore, these mediators along with stress hormones could act directly on reproductive organs, or indirectly through alteration of hypothalamic/pituitary hormones. With an estimated cost of $.25 to $4.68 per day, >84 days open beyond parturition (Stevenson and Pursley, 1994), the cost of altered reproductive efficiency associated with mastitis is even further emphasized.

Need for a *S. uberis* Challenge Protocol

Control of environmental pathogens such as *S. uberis* is of crucial importance to the dairy industry. This pathogen is found throughout the dairy environment, can induce both clinical and subclinical infections, and ultimately can diminish dairy profits from both a production and a reproduction standpoint. There is a need to fully understand the pathogenesis of *S. uberis* in order to reduce its financial impact on the producer. To do so at a research institution, a protocol must first be developed in order to produce a predictable infection. Only after this is established will further investigation into *S. uberis* pathogenecity be viable.

Materials and Methods

Bacterial Strain Isolation & Identification

A *Streptococcus uberis* isolate was obtained from the left front quarter of a clinically infected Holstein dairy cow in a North Carolina dairy herd. Milk was plated on tryptic soy agar (TSA; Becton, Dickinson and Company, Sparks, MD, USA) and incubated for 24 hours at 37^{o} C. Gram staining and catalase testing were performed. An API Strep test (bioMérieux, Nuertingen, Germany) was used to identify the streptococcal species in the Milk Microbiology and Mastitis Laboratory at North Carolina State University College of Veterinary Medicine. The Clinical Microbiology lab at North Carolina State University College of Veterinary Medicine Teaching Hospital verified this identification.

Once bacteria were isolated, a colony of the identified *Streptococcus uberis* was placed in Brain Heart Infusion broth and grown in a 36^{o} C water bath and shaken at 100 rpm. After 12 hours, 1.275 mLs of cultured broth was aliquoted to each of 50 vials containing .225 mLs of sterile glycerol. Vials were stored at -80 ^{o} C.

Bacterial *In Vitro* Growth Characterization

Bacterial growth was determined and replicated five times as follows. Frozen bacterial aliquots were thawed by immersion in warm tap water. One mL of aliquot was placed in 200 mLs of Brain Heart Infusion broth (Becton, Dickinson and Company) that was equilibrated to 36 ^{o} C in a water bath. This inoculum was grown for 24 hours with continual shaking at 100 rpms. Samples were removed from broth at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, and 24 hours for growth and optical density analysis.

At each time point, 100 µL of inoculum was diluted in 900 µL of sterile, .85% saline solution. Serial dilutions were performed in order to quantify bacterial concentrations using

a modification of a technique established by Miles and Misra (1938) (Anderson, 1984). In brief, 1:10 serial dilutions were performed by transferring 100 µL of each vial to the subsequent vial containing 900 µL of saline giving a final volume of 1000 µL. This was performed 8 times, making the final dilution 1:100,000,000. After vortexing, 10 µL from each of the 8 vials was plated on a TSA plate and then incubated for 24 hours at 36 ^{o} C (Figure 1). At 24 hours, colonies were counted on regions showing growth between 20-200 colony forming units (cfu). Total bacterial cell concentration of inoculum was established based on serial dilutions.

At each time point, 1 mL of inoculum was placed in a cuvette. Optical transmittance at 525 nm was read by spectrophotometer and then standardized to a sterile sample of brain heart infusion broth. Optical transmittance was recorded for all five replicates of bacterial growth.

A growth curve was established in order to characterize *in vitro* growth. An optical transmittance graph was also created in order to observe trends that existed between growth and transmittance.

S. uberis Storage on Ice and Serial Dilution Confirmation

After analyzing the data collected for growth characterization, a model was developed to establish a predictable target challenge concentration of *S. uberis* as well as ensure growth was arrested when placed on ice. 5,000 cfu/5 mL was the chosen target concentration due to efficiency of this challenge with a different *S. uberis* strain (Hockett et al., 2000; Hockett et al., 2005).

S. uberis was grown in bovine heart infusion broth as described previously. A consistent and manageable bacterial concentration that was in its log phase of growth was

observed at 3 hours. Visual differentiation could be made between growth curves 1, 4, and 5 and growth curves 2 and 3 (Figure 2). Because growth curves 2 and 3 were performed on the same weekend, and water bath temperatures were found to be elevated during this time, serial dilutions for this study were based upon curves 1, 4, and 5. The mean bacterial concentration of the inoculated broth was 5×10^7 cfu/mL ± 1.7×10^7 cfu/mL (mean ± SEM) at 3 hrs. Therefore, at 3 hrs of growth a 1:500 dilution of the inoculum was made in .85% sterile saline solution for a total theoretical concentration of 1×10^5 cfu/mL in 5 mL. This dilution was vortexed for 10 seconds, and a subsequent 1:10 dilution was performed, in duplicate, using .85% sterile saline solution that had been on ice for at least 30 minutes. The total theoretical concentration for the second dilution was 1×10^4 cfu/mL in 6 mL for each duplicate. These two final dilutions were left on ice for the remainder of the study.

At 0, .5, 1, 2, and 3 hrs, the 1×10^4 cfu/mL aliquot was vortexed and 1:10 and 1:1000 dilutions were performed using .85% saline that had been on ice for at least 30 minutes. Using the 1:10 dilution, 100 μL and 10 μL were plated directly onto a TSA agarose plate to produce the expected 100 cfu and 10 cfu, respectively. 10 μL from the 1:1000 dilution was directly plated onto a TSA agarose plate to produce the expected 1 cfu. Plates were incubated at 36 ^{o} C for 24 hours and colony counts were performed and annotated. 3 replicates were performed during this trial, and duplicates were performed for each.

Target bacterial concentrations were not obtained after performing these procedures and adjustments were made to bacterial dilution. Bacteria were grown to 3 hours and the initial serial dilution factor was reduced to 1: 300 in .85% saline. All other methods remained the same and 2 replicates were performed using new dilution factor.

Physiological Response to *S. uberis*

One month prior to study, aseptic milk samples for microbiology and milk samples for somatic cell counts (SCC) were obtained from each lactating quarter on eighteen multiparous, Holstein cows at the Umstead Research Farm Dairy Unit in Butner, NC. In brief, cows were prepped with iodine-based pre-milking dip and each quarter was then dried with a clean paper towel. Each teat was stripped and cleaned thoroughly with 70% isopropyl alcohol swabs (Fisher Scientific, Hampton, NH, USA). Milk samples were placed in sterile snap-top sample vials (Fisher Scientific) for microbiological analysis and large sample tubes containing potassium dichromate pills (obtained from Virginia Tech University's Dairy Improvement Association Lab) for SCC. Milk samples for microbiology were placed on ice and frozen at –20 ^{o} C.

Microbiology analysis was performed at the North Carolina State University College of Veterinary Medicine Milk Microbiology and Mastitis Laboratory. Frozen milk samples were thawed in tap water at room temperature. Ten μL of each milk sample was plated on TSA plates using sterile loops and incubated at 36 ^{o} C for 24 hours. If growth was not observed after 24 hours, incubation time was extended for another 24 hours. During this time, bacterial growth, if any, was observed for each lactating quarter. If bacterial growth was apparent, a catalase test and gram staining were performed. SCC analysis was performed at Virginia Tech University's Dairy Herd Improvement Association Lab. Based upon microbiological analysis and SCC, 10 cows were culled from study as a result of subclinical intramammary infections. The remaining cows exhibiting no clinical or subclinical intramammary infection were randomly separated into a control group (n=4) and a challenge group (n=4).

On the day of challenge, *S. uberis* was grown for 3 hours and a 1:30000 dilution was performed in duplicate, using .85% sterile saline solution. This dilution was based on data obtained in previous study assuming a target inoculum concentration of 5,000 cfu/5 mL. Each duplicate inoculum had a total volume of 50 mL that had been on ice for at least 30 minutes. To determine the actual bacterial concentration, 1:10, 1:100, and 1:1000 dilutions of each inoculum was plated on TSA agarose plates in the laboratory. These same dilutions were plated at time of challenge to ensure *S. uberis* concentration remained constant.

Prior to challenge, milk samples were obtained from all quarters of each cow for microbiological analysis and SCC as described previously. Rectal temperatures were recorded and scoring (Table 1) was performed for Appetite, Attitude, Udder Temperature, Udder Pain and Quarter Size by the same farm technician over the trial period. A 5-point scale was implemented for each criterion, 1 being normal and 5 being the most severe. Although slightly modified, this scale was based upon a previous scoring schematic developed by Anderson et al. (1986). Cows were milked and prepared for *S. uberis* challenge.

Cows to be challenged (n=4) were milked and two lactating quarters absent of an intramammary infection were cleaned with sterile, alcohol swabs. Five mLs of thoroughly mixed bacterial inoculum was obtained from the centrifuge tubes using a syringe. A sterile infusion cannula was placed on the tip of the syringe and the challenge inoculum was infused into two desired quarters on each challenged cow. The teat end was then clamped off using the thumb and index finger, and the solution was massaged up the teat cistern and into udder. Control cows (n=4) were not challenged with bacteria and had samples collected at the same times as challenge cows.

Every 12 hours for 84 hours, rectal temperature and scores were recorded. Blood samples were collected and milk samples for microbiological analysis and SCC determination were obtained daily as described previously. If clinical mastitis was observed at 2 consecutive milkings, quarter antibiotic treatment was performed. Cows with rectal temperatures > 41°C (n = 3) received 500 mg flunixin meglumine intramuscularly at each milking (Banamine®, Schering-Plough, Kenilworth, NJ). Administration of .36 mg of oxytocin and a single dose of 20000 mg oxytetracycline (Liquamycin LA-200®, Pfizer Animal Health, New York, NY) was performed on each cow. Four hundred mgs of cephapirin sodium (Cefa-Lak®, Fort Dodge Animal Health, Fort Dodge, IA) was infused into clinical quarters.

Microbiological analysis was done as previously described. However, if *S. uberis* pathogens were suspected, esculin plating was used to validate at first observation. During the esculin plating, in brief, bacteria were plated perpendicular to a known CAMP-positive *S. aureus* smear on esculin agarose plates (Becton, Dickinson and Company, Mountain View, CA). Typical "arrowhead" hemolysis was indicative of a CAMP-positive pathogen (Darling, 1975) while blackening of the esculin plate was indicative of esculin hydrolysis (Livingston et al., 1978). Both characteristics confer with a preliminary *S. uberis* identification. Secondly, API Strep tests were used to make a more definitive identification. All subsequent observations with similar gram staining morphology and catalase results were assumed to be *S. uberis*.

Dose Trial

Because it was determined that we could experimentally induce mastitis with this particular *S. uberis* strain, the goal was then to determine if a smaller dose would produce a

reliable challenge with less systemic, acute infections (low-grade pyrexia, less severe quarter abnormalities, and faster response to treatment). Nine multiparous, Holstein cows were selected at the Piedmont Research Station in Salisbury, NC. These cows were randomly allocated into three treatment groups. Milk samples were obtained aseptically for microbiological analysis on two occasions, 21 and 7 days prior to challenge to determine the presence or absence of intramammary pathogens. Quarters without an intramammary infection were selected for the study. Milk samples for SCC were also obtained one week prior to challenge and analyzed by Virginia Tech University's Dairy Herd Improvement Association Lab for results.

For the challenge, the target bacterial concentrations desired were 500 cfu/5mL (low dose), 2,500 cfu/5mL (medium dose), and 5,000 cfu/5mL (high dose). In order to obtain similar concentrations, bacteria were grown in brain heart infusion broth as described previously. At 3 hours, 1: 30000 (high), 1: 60000 (medium), and 1: 300000 (low) serial dilutions were performed in .85% saline solution that had been on ice for at least 30 minutes. In order to obtain actual inoculums concentrations, prepared inoculums for each dose were plated on TSA plates using serial dilution methods described previously. Challenge inoculum was plated in the laboratory prior to challenge and again following challenge at the farm to ensure inoculum consistency. The inoculum solutions were maintained on ice for the entirety of this time frame.

Once on the farm, scoring was performed and milk samples were obtained for microbiological analysis and SCC as described previously. Cows were milked, and led to a confinement area for treatment. Rectal temperatures were recorded and blood samples were obtained as previously described. At the same time, teats were prepared for challenge and 1-

2 quarters were infused with a predetermined dose. The challenged teats were selected based upon SCC and the absence of any major intramammary pathogens. Of the 9 cows, 3 received the low dose (6 quarters), 3 received the medium dose (5 quarters), and 3 received the high dose (6 quarters).

Every 12 hours for 96 hours, farm technicians obtained rectal temperature and infection severity scores (Table 1). One technician was given the responsibility of reviewing the severity scores to ensure consistency in scoring criteria. Every 24 hours, blood samples were collected in serum separator tubes and milk samples for microbiological analysis and SCC determination were obtained. Treatment was allowed after clinical mastitis was observed at 2 consecutive milkings, and was based upon the perceived severity of infection. Beyond these criteria, treatment options were allowed at the discretion of the farm, and were based upon the farm's standard operation procedure. Four hundred mgs of cephapirin sodium (ToDay®, Fort Dodge Animal Health, Fort Dodge, IA; Cefa-Lak®, Fort Dodge Animal Health, Fort Dodge, IA) and 125 mgs of amoxicillin (Amoxi-Mast®, Schering-Plough, Kenilworth, NJ) were used daily to treat infected quarters. Systemic treatment with 60 mg of dexamethasone (compounded by Rowan Animal Clinic, Salisbury, NC) was used intramuscularly on hours 84 and 96 in cows still presenting with clinical infection. Following challenge, 1000 mgs of ceftiofur sodium (Excenel®, Pfizer Animal Health, New York, NY), 1,250 mgs of flunixin meglumine (Banamine®, Schering-Plough, Kennilworth, NJ) intramuscularly, and bolus of 960 mgs of aspirin were used in cows exhibiting a febrile response > 41°C.

Microbiological analysis was performed as previously described. If *S. uberis* pathogens were suspected, esculin plating and API strep tests were used to validate at first

observation. All subsequent observations with similar gram staining morphology and catalase results were assumed to be *S. uberis*.

Serum samples were analyzed for concentrations of IL-8, IL-1β, and TNF-α. Analysis of these cytokines could potentially elucidate the mechanism behind a systemic response with this strain. IL-8 concentrations were determined using the Quantikine human IL-8 ELISA (R & D Systems, Minneapolis, MN, USA). This assay was performed in order to observe temporal, systemic IL-8 levels, and because of conserved homology between the bovine and human IL-8 isoforms and proven success using this kit with bovine serum, accurate measures have been observed previously (Hassfurther et al., 1994; Rambeaud et al., 2003). In brief, assay diluent was added to microplates coated with mouse anti-human antibodies against IL-8. Standards were added to wells in triplicate while serum samples were added to wells in duplicate and allowed to incubate for 2 hours at room temperature. Wells were aspirated and washed 4 times with wash buffer. An IL-8 conjugate was added (IL-8 antibody coupled to horseradish peroxidase) and allowed to incubate for 1 hour at room temperature. Wells were once again aspirated and washed 4 times with buffer. A 1:1 H_2O_2:chromogen color reagent was added to each well and allowed to incubate for 30 minutes at room temperature, and wells were protected from direct light. Finally, 2 N sulfuric acid was added to each well in order to stop the color reaction. Plates were read in a Spectramax 250 microplate reader (Molecular Devices Corp., Palo Alto, CA, USA) at 450 nm. Wavelength correction was performed at 540 nm in order to obtain more accurate readings. Softmax Pro software v 1.2 (Molecular Devices Corp., Palo Alto, CA, USA) was used to compare serum absorbance values to the standard curve. The interassay coefficient of variation (CV) for this assay was 0.051 (n = 2 assays).

Bovine IL-1β concentrations were measured in sera using a sandwich ELISA protocol described by Rambeaud et al. (2003) with minor deviations. In brief, 96-well flat bottom, medium binding plates (Corning Inc., Corning, NY, USA) were coated with 100 µL mouse anti-ovine IL-1β (Serotec Inc, Raleigh, NC, USA) diluted in phosphate buffered saline (PBS) as indicated by manufacturer (5µg/mL). Coated plates were incubated overnight at 4°C, then washed three times with .01% tween 20 - phosphate buffered saline (PBST). Plates were blocked with 200 µL of PBST- 5% bovine serum albumin (BSA) and incubated for 1 hr at 37°C. Plates were washed 3 times with PBST, and 100 µL of collected serum samples were added to the corresponding wells. Recombinant bovine IL-1β (Serotec Inc., Raleigh, NC USA) was used as the standards, and 1:2 dilutions were established with a range of 10 - 0.31 ng/mL. 100 µL of the standards and unknowns were pippetted, in triplicate, and the plates were incubated for 1 hr at 37°C. Plates were washed with PBST and 100 µL of rabbit anti-ovine IL-1β (Serotec Inc., Raleigh, NC, USA), diluted per manufacturer's recommendations (1:500) in PBST - 1% BSA, were added to each well. The plates were then incubated 1 hr at 37°C and washed three times with PBST. Goat anti-rabbit IgG (H+L) bound to horseradish peroxidase (Jackson Immunoresearch, West Grove, PA, USA) was diluted in PBST – 1% BSA to a 1:10000 dilution and 100 µL were added to each well. The plates were incubated 1 hr at 37°C and washed three times with PBST. 100 µL of pre-made, 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) liquid substrate system (Sigma, St. Louis, MO) was added to each well. Plates were protected from light, and absorbance readings were taken at 405 nm in a Spectramax 250 microplate reader (Molecular Devices Corp., Palo Alto, CA, USA) within 30 minutes of ABTS addition. Softmax Pro software v 1.2 (Molecular

Devices Corp., Palo Alto, CA, USA) was used to compare serum absorbance values to the standard curve. The interassay CV for this assay was 0.071.

TNF-α concentrations were determined in serum by Dr. T.H. Elsasser's laboratory (USDA's Beltsville, MD). Sera TNF-α levels were enumerated using a radioimmunoassay (RIA) protocol established by Kenison et al. (1990). In brief, this RIA was run as a nonequilibrium assay with the addition of the radiolabelled tracer 24 h after the initial setup. Following the addition of the tracer, the assay was incubated for another 24 hours at ambient room temperature. The assay volume was 400 µL: 100 µl antibody, 100 µl sample, 100 µl buffer and 100 µl tracer. The minimum detectable concentration was ≈0.02 ng/ml. The antibody was rabbit anti-bovine TNF-α (R-1-7-93) run at a final dilution of 1:200,000. The interassay CV for this assay was 0.072.

Statistical Analysis

Analysis of variance using the mixed model procedure of SAS (SAS 8.2, SAS Institute, Cary, NC) was performed to compare temporal changes in temperature, SCC, and scoring criteria, for both the *S. uberis* pathogenesis and dose trial. Because the scoring data had a large number of values, treating it as continuous data using parametric analysis was deemed the more conservative approach (Hogan et al., 1995; Schreiner and Ruegg, 2003; Hockett et al., 2005). A $\log_{10}$ transformation was performed on SCC values in order to normalize data. The mixed procedure of SAS was also used to analyze IL-1β, IL-8, and TNF-α between dosage groups and from baseline values. SCC least squares means between treatment/dosage groups were compared using the Bonferonni method due to high variation. Differences of least square means were used to compare all scoring criteria. Treatment was a nested variable for a given cow, and quarter was nested within cow for the analysis of milk

scores, quarter pain scores, and quarter size scores. In order to determine the differences regarding DIM, parity, and milk weight prior to the trial, treatment group/dose groups were compared using the GLM procedure of SAS. If differences between groups were observed, these criteria were then added to the model.

Analysis between peak temperature means and baseline temperature means in the dose trial was performed using a pooled T-test with the T-test procedure of SAS. The relationship between bacterial concentration and optical transmittance was determined using the Pearson Correlation Coefficient with the correlation procedure of SAS. The frequency procedure in SAS was used to perform a chi-square analysis of quarter incidence of clinical infection by dose and the effect of SCC on incidence of clinical infection.

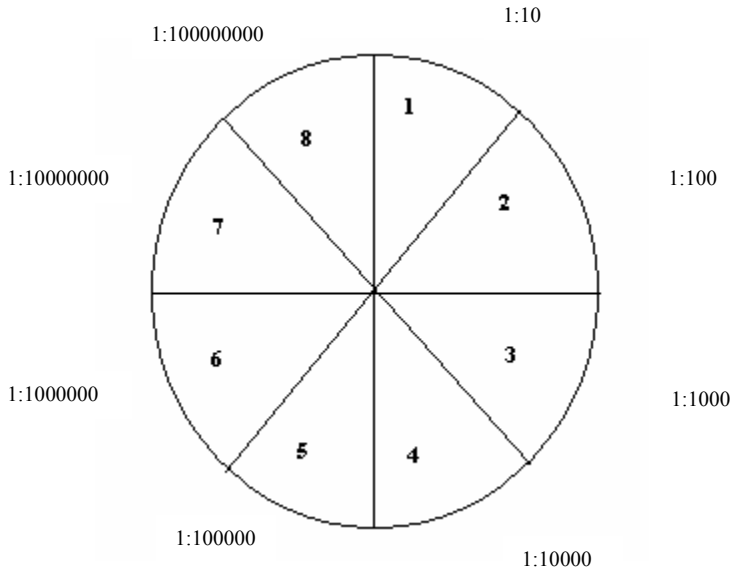

Figure 1: Schematic representation of TSA agarose plate for serial dilutions using a modified Miles and Misra (1938) protocol (Anderson, 1984).

Table 1: Scoring criteria for the determination of the severity of intramammary infection using criteria based on a modified scoring system by Anderson et al. (1986).

Scoring Scale	Attitude	Appetite	Milk
1	Normal	Normal	Normal
2	Slight Depression	Slight Decrease	Small Flakes
3	Moderate Depression	Moderate Decrease	Clots
4	Marked Depression	Marked Decrease	Serous (w/ or w/out clots)
5	Severe Depression	Severe Anorexia	Hemorrhagic

Scoring Scale	Udder Pain	Udder Size	Udder Temperature
1	None	None	Normal
2	Slight	Slight Increase (10% - 33%)	Slight Warmth
3	Moderate	Moderate Increase (34% - 66%)	Moderate Warmth
4	Marked	Marked Increase (67% - 100%)	Marked Warmth
5	Severe	Decreased	Cold

Results and Discussion

Bacteria Characterization

The *S. uberis* inoculum exhibited a standard, *in vitro*, temporal bacterial growth curve with the characteristic lag, log, stationary, and death phase (Figure 2). Visually, deviation is observed between growth curves 1, 4, and 5 and growth curves 2 and 3. Growth curves 2 and 3 were performed during the same weekend, and water bath temperature regulation was a problem during this time. It was determined that these two growth curves might not be indicative of actual *in vitro* growth. Therefore, serial dilution techniques were based solely on growth curves 1, 4, and 5. At 3 hours, mean bacterial concentration for these growth curves was 5×10^7 cfu/mL ± 1.7×10^7 cfu/mL (mean ± SEM). At this time point, bacteria were in log growth, a characteristic desired for challenge. Also, variation beyond 3 hours increases, making predictions regarding bacterial concentrations difficult.

Bacterial Turbidity

The analysis of solution turbidity via optical transmittance to predict bacterial concentrations was performed during the process of characterizing bacterial growth. Because culture turbidity increases with bacterial growth, this technique is an indirect method of making inferences regarding bacterial concentrations (Gerhardt et al., 1981). The benefit of using turbidity as a measure of bacterial concentration is that it minimizes time constraints. Therefore, growth could potentially be arrested at any point prior to the "death phase," and serial dilutions could be performed to obtain a predictable bacterial concentration. The "death phase," or phase at which bacterial numbers diminish, appears to begin at approximately 8 hours of growth in the current laboratory conditions. Bacterial pigmentation and cellular debris interfere with turbidity. Therefore, values beyond 8 hours were not used in

order to analyze correlation. The Pearson correlation coefficient of -0.71 is obtained comparing cfu to optical transmittance (Figure 3). However, residual plots show a definite increase in variation once transmittance values decrease. This high variation, especially with increasing bacterial numbers, is probably the result of cellular death. Therefore, it was determined that transmittance, while correlated to bacterial concentrations, would be a less accurate indicator of *S. uberis* concentrations. It was determined that future predictions of bacterial concentrations should be based upon incubation time (i.e. growth curve) as opposed to optical transmittance.

S. uberis Storage on Ice

Ice storage prevented bacterial concentration from changing over time (P=0.96). Bacterial concentrations after serial dilutions (theoretical expectation of 100 cfu) can be seen for each replicate (Figure 4). Using the Tukey-Kramer method to compare the least square means of replicate bacterial concentrations, replicates 3 and 4 differed from replicates 1,2,5 and 6 ($P < 0.0001$). Power outages occurred frequently during replicates 3 and 4. Power loss to the hot water bath could potentially alter bacterial growth. Incubation temperature would possibly decrease while shaking would be halted. The oxygen concentration within the media could decrease quickly without constant agitation and mixing of the inoculum, all while ambient temperature is dropping. Ultimately, an optimal environment for bacterial growth would be lost, explaining the lower concentrations that were obtained.

Due to minimized laboratory space and equipment, the development of a *S. uberis* inoculum at the research facilities was not a viable option Secondarily, portable incubation was not available for travel. Because travel to research facilities and preparation time could take as long as 3 hours, maintenance of bacterial concentration is crucial to the predictability

of this strain. Any increase in the bacterial population may prompt a more acute infection while a decreasing bacterial population could lower incidences of clinical infection (as will be examined in a later study). This data indicates that ice storage will maintain the same bacterial concentrations produced in the laboratory during this time frame.

The mean bacterial concentration of inoculum for replicates 1, 2, 5, and 6 was 2.5 X 10^7 cfu/mL ± 1.7 X 10^6 cfu/mL. Due to the fact that mean varied from the expected 5.0 X 10^7 cfu/mL, 4 replicates were performed using an altered dilution protocol with an initial dilution of 1:300. This change resulted in colony forming unit concentrations similar to the actual target concentration (Table 2). After a series of serial dilutions, a mean of 106 colony forming units were obtained for dilutions with an expected 100 cfu and a mean of 12 cfu were obtained for an expected 10 cfu.

Physiological Response to *S. uberis*

In this study, the control group had a mean parity of 2.75 ± 0.75, mean days in milk (DIM) of 230.8 d ± 72.8 d, and a mean daily milk production during the current lactation of 63.25 lbs. ± 6.78 lbs. Milk production prior to challenge did differ between the control (51.3 ± 8.6 lbs./day) and treatment group (107.3 ± 8.6 lbs./day) (P = 0.004).

Plating of inoculums revealed a mean of 5, 400 cfu as the actual challenge dose for each quarter in the challenge group. Microbiological analysis of milk from challenged quarters showed concentrations of *S. uberis* 24 and 48 hours post-challenge and all challenged quarters showed clinical signs of infection. Five of the 8 quarters and 3 of 4 cows challenged had *S. uberis* concentrations in milk samples at the end of sampling, regardless of antibiotic treatment (72 hr). Milk scores ≥ 2 for two consecutive milkings and isolation of *S.*

uberis were indicative of a quarter with a clinical infection. Using this criterion, all 8 challenged quarters became clinical, producing an experimental infection rate of 100%.

All control cows maintained scores of 1 for severity criteria throughout trial. Mean milk scores in challenged quarters were higher ($P < 0.01$) than the controls from 36 hours post-challenge to the end of the study (Table 3). Average quarter size in challenged quarters were elevated at 36 ($P < 0.001$) and 60 ($P < 0.001$) hours in comparison to controls (Table 3). Average quarter pain scores in challenged quarters were elevated at 36 ($P < 0.001$) and 60 ($P < 0.001$) hours in comparison to controls (Table 3). Mean attitude scores of challenged cows were elevated ($P < .05$) at 36 and 48 hours post infusion in comparison to controls (Table 3). Appetite scores remained at 1 for the entirety of the study.

Rectal temperatures were higher in challenged cows than controls at 24 ($40.1 \pm .3^{o}$ C vs. $38.6 \pm .3^{o}$ C respectively; $P = 0.0007$) and 36 hours ($40.5 \pm .3^{o}$ C vs. $38.5 \pm .3$ respectively ^{o} C; $P < 0.0001$) following *S. uberis* infusion. Mean SCC between the challenged quarters in the challenge group ($1.4 \times 10^5 \pm 2.1 \times 10^5$) did not differ ($P=0.92$) from the control group ($4.3 \times 10^4 \pm 3.1 \times 10^4$) at time 0. At 24 hours, challenged quarter SCC scores ($3.5 \times 10^6 \pm 5.6 \times 10^6$) were elevated ($P < .0001$) from baseline ($1.4 \times 10^5 \pm 2.1 \times 10^5$). At 24 hours, challenged quarter SCC scores where higher than control SCC scores ($3.5 \times 10^6 \pm 5.6 \times 10^6$ vs. $6.7 \times 10^4 \pm 5.0 \times 10^4$ respectively; $P=0.003$).

While all attempts were made to randomly allocate individuals into treatment groups, milk yield prior to the study differed between the control and treatment groups. However, the differences in milk weight had no effect on scoring criteria ($P \geq 0.22$), SCC ($P = 0.45$), and temperature ($P = 0.89$) between the control and treatment group. Higher milk yield has been associated with increased mastitis susceptibility (Gröhn et al., 1995). But, to the

author's knowledge, there is no data implicating that higher milk yields increase the susceptibility of a cow to experimentally induced mastitis.

Cows in the treatment group developed clinical mastitis based on severity of milk scores (score ≥ 2 at 2 consecutive milkings) and isolation of *S. uberis* from a collected milk sample. Because a febrile response was observed, the clinical infection had a systemic effect. While some researchers have reported that experimental challenge with *S. uberis* did not elicit clinical symptoms until 3 days post-challenge (Hockett et al., 2000; Rambeaud et al., 2002; Hockett et al., 2005), clinical symptoms were observed within 2 days during this study. High doses of *S. uberis* (1 X 10^7 cfu) have been shown to elicit an immediate systemic response (Thomas et al., 1994; Pedersen et al., 2003) and localized effects have been noted within 24 hours with very small doses (500 cfu) (Smits et al., 1998). In our study, even following antibiotic treatment, bacterial numbers were isolated in 5 of 8 quarters at the end of the trial. While antibiotic treatment was not performed during data analysis, Bannerman et al. (2004a) observed "severe clinical mastitis and distress" in cows challenged with 220 cfu of the 0140 *S. uberis* strain. This caused a premature end to study, at 7 days, so cows could be treated. First clinical signs in this study were reported at 24-30 hours. Leigh et al. (1990) have showed differing infectivity patterns in *S. uberis* strains, and have attributed the more virulent pathogens with their ability to avoid neutrophil phagocytosis. Ultimately, characterizing the pathogenesis of *S. uberis* species as a whole is difficult because of the variation found between different strains. Therefore, understanding the virulence of any strain is necessary to producing a predictable challenge model that can be used for future research.

Of the 4 challenged cows, three exhibited a temperature spike > 41.1 ^{o} C and each of these had pretreatment SCC in at least one challenged quarter that were ≤ 5,000. Milk SCC is correlated to the number of polymorphonuclear neutrophils and macrophages that migrate into quarter milk (as reviewed by Harmon, 1994). Because SCC levels were so low in 3 of the 4 challenge cows, and these cows with low SCC exhibited a high temperature spike, perhaps SCC is correlated to severity of infection. Going by this assumption, one may assume that neutrophils do contribute to the control of this particular strain. This is contradictory to previous reports indicating a minimal role to *in vivo S. uberis* control (Leigh et al., 1990; Thomas et al., 1994; Rambeaud et al., 2003). Given the small sample size in this study, this may well be purely coincidental and is especially surprising given the lingering bacterial numbers in milk samples and prolonged infection rate. Ultimately, further research would be needed before any conclusions could be drawn.

The desired 100% incidence of clinical infection was achieved with this dose and strain. However, the response in challenged cows was more acute than preferred. As mentioned previously, strain differences are a contributor to the physiological response of this mastitis pathogen (Leigh et al., 1990; Smits et al., 1998; Hockett et al., 2000; Rambeaud et al., 2003; Bannerman et al., 2004a). Regardless of treatment, bacterial numbers and clinical signs were observed in all quarters by the completion of the trial. While this provided a predictable model to use for future challenge protocols, the severity of infection was a concern. Therefore, in the attempt to obtain a clinical infection that was physiologically more benign, it was hypothesized that lower doses may produce a high rate of clinical mastitis with less severe clinical symptoms.

Dose Trial

The cows in the high dose group (n=3) had a mean parity of 3.0 ± 0.8, mean DIM of 448.7 d ± 69.7 d, and average milk weights prior to challenge of 49.3 ± 2.7 lbs./day. The cows in the medium dose group (n=3) had a mean parity of 2.7 ± 0.8, mean DIM of 442.3 d ± 69.7 d, and average milk weights prior to challenge of 38.3 lbs. ± 2.7 lbs./day. The cows in the low dose group (n=3) had a mean parity of 2.0 ± 0.8,mean DIM of 337.3 d ± 69.7 d, and average milk weights prior to challenge 32.3 lbs. ± 2.7 lbs./day. No differences in DIM (P = 0.67) and parity (P = 0.49) were reported. However, mean daily milk production during the current lactation (P = 0.01) once again differed between treatment groups.

Microbiological analysis indicated that the actual *S. uberis* challenge inoculum concentrations were 500 cfu for the low dose, 1,950 cfu for the medium dose, and 4,000 cfu for the high dose. Microbiological analysis of milk from challenged quarters showed *S. uberis* in 16 of 17 challenged quarters 24 hours after inoculation. The one quarter that did not have *S. uberis* colonies in milk analysis was from the low dose treatment group. All cows in the study developed a clinical infection. Of the 17 quarters challenged, 13 became clinical producing a 76.5% incidence of clinical infection. Challenge dose (P = 0.17) and pre-challenge SCC (P = 0.48) did not have an effect on rate of infection. Of those quarters that became infected, 4 out of 6 quarters administered were in the low dose group, 4 of 5 quarters were in the medium dose group, and 5 out of 6 quarters were in the high dose group. Furthermore, the quarter administered the high dose inoculum that did not become clinical presented with the lowest pre-challenge SCC (9000/mL). While only one observation, this contradicts our earlier premise that pre-challenge SCC might play a role in acute infection with this strain.

An overall elevation in SCC in comparison to baseline values was observed for the infused quarters ($p < 0.0001$). In the low group on day 2 post-challenge, there was a tendency for SCC to be elevated in comparison to baseline values (Figure 5, Table 4). In the high dose treatment group, SCC differed from baseline values on days 1, 2, 3, and 4. In the medium dose group, SCC differed from baseline on days 2, 3, and 4. No differences were observed between treatment groups at each time point post-infusion.

Based on SCC, the lower dose does not promote leukocyte chemotaxis in the udder to the extent that the medium and high dose treatment groups did. However, the severity of infection, at least locally, does not appear to differ by group. High variation and low animal numbers in this trial make it difficult to observe differences between treatment groups, although SCC was elevated from baseline levels for each treatment group.

Measuring SCC in milk samples was impacted by the high concentration of clots, clumps, and flakes found in clinically infected quarters. The determination of somatic cell counts in large dairy herd improvement labs typically rely on electronic cell counters. Milk with a high content of flakes, clots, and clumps are difficult to analyze using these cell counters (Zadoks et al., 2000). Because all infused quarters that became clinical contained these milk abnormalities, determining their SCC was not possible for many samples. It can be speculated that those unreadable milk samples were perhaps the most severely infected and therefore, had the highest SCC. Future studies would require larger sample sizes in order to fully elucidate the effects of dose on SCC. Secondarily, other indirect means of measuring SCC such as the California Mastitis Test might prove more effective.

No difference in temperature was found within each group when comparing to baseline values. Similarly, no temperature difference was observed between treatment

groups at any time points post-challenge. The difference between mean peak temperature and mean initial temperature were not different (P = 0.88) when comparing treatment groups. Overall, temperature did increase (P = 0.032) from the mean baseline (39.1 ± .1°C) compared to mean peak temperature (39.8± .3 °C) amongst all cows infected. These data show that temperature elevations are observed when comparing all cows, disregarding number of bacteria inoculated into quarter. Treatment of cows occurred at different times depending upon onset of clinical signs. This may have impacted the ability to observe temperature differences between groups receiving different inoculum dosages.

Milk scores for the high dose treatment group were higher at 48 hours in comparison to baseline values and remained elevated throughout the remainder of the study (Table 5). Elevations from baseline quarter milk scores were observed in the medium group at 48 60, 84, and 96 hours. The quarter scores for the low dose treatment groups were greater than the baseline score at 36 hours and then at 60 and 72 hours. Flakes, clots, and serous in milk are all consistent with an inflammatory response in the mammary gland. Because elevation in milk score is observed in all treatment groups, it is evident that a challenge dose of 500 cfu can produce a clinical mastitis infection.

Udder temperature score elevation is observed based on palpation, in the low dose treatment group at 60, 72, and 96 hours post challenge and in the high dose group at 72 hours (Table 6). Udder temperature is an indicator of overall body temperature (Bitman et al., 1984), and is indicative of mastitis (Younis et al., 2003). Quarter pain was elevated in all treatment groups at 36 hours post-infusion. At 48, 72, 84, and 96 hours, quarter pain was also elevated from baseline levels in the low and medium dose groups (Table 5). Algesia is another primary indicator of inflammation (Portanova et al., 1996) and indicative of mastitis.

Quarter size score was elevated in the low dose group at 60 hours and the high dose group at 48 and 96 hours. Elevation in the medium dose group was observed at 84 hours. An inflammatory response increases vascular permeability in localized tissues (Jain et al., 1972). This allows for the various inflammatory mediators and immune cells to migrate into the extracellular matrix to combat potential pathogens, prompting edema or swelling. Because elevations in all of these scores were observed in the low dose group, it is indicated that 500 cfu can further produce localized inflammation in the mammary gland.

Cow attitude score and appetite score was elevated for all treatment groups from 72 hours post-challenge to the end of the trial in comparison to baseline values (Table 6). Acute phase proteins, through cytokine activation, have been reported to decrease appetite (Moldawer et al., 1988). Similarly, systemic levels of the inflammatory cytokines act on the brain, and promote a general state of malaise (as reviewed by Larson and Dunn, 2001). Elevations in the appetite and attitude scores in all treatment groups indicate that a 500 cfu dose can produce not only a localized clinical infection, but can also prompt a systemic inflammatory response.

Scoring criteria show that cows from all treatment groups successfully acquired a clinical, *S. uberis*, intramammary infection. Dissimilarity in scoring criteria can be observed between the different treatment groups (Table 5, Table 6). But, overall consistencies are not observed between the doses. It is difficult to discern which criteria should be weighted more heavily in regards to severity of infection. Therefore, no conclusions can be made regarding physiological response to the different challenge concentrations.

Once again, even when cows were placed into groups at random, milk yield prior to challenge differed between groups. However, these differences had no effect on the various

scoring criteria ($P \geq 0.38$), SCC ($P = 0.14$), and rectal temperature ($P = 0.44$). Therefore, it is doubtful that these differences in milk weights at time of infection had any impact on infection severity.

The number of clinical infections during this trial was lower than in the previous study (76.5% vs. 100%). Again, because of lower numbers, it is difficult to assess whether challenge dose played a role in the lower incidences of clinical infection. Regardless, clinical mastitis was observed for all cows in each treatment groups, which gives evidence to the fact that a challenge dose of approximately 500 cfu will produce the desired intramammary infection.

Serum IL-1β and TNF-α concentrations remained unchanged throughout the entire study and no dose effect was observed. This is consistent with other data that shows no systemic elevation of these cytokines with *S. uberis* challenge (Rambeaud et al, 2003; Bannerman et al., 2004a). However, in these studies, elevation in milk IL-1β and TNF-α is reported. Because a febrile response is observed in at least 3 animals ($> 39.7^{o}$ C), it is assumed that inflammatory mediators were elevated systemically. Both of these cytokines are directly associated to pyrexia, and inhibition of them decreases body temperature (Luheshi et al., 1997). Elevations of these cytokines in milk have been directly attributed to a febrile response, even with absent systemic levels (Bannerman et al., 2004a). The serum half-life of TNF- α and IL-1β is extremely short (6-20 minutes) and is quickly bound by high affinity binding proteins (as reviewed by Tracey and Cerami, 1993). The sampling period of the study was designed in order to accommodate the primary goal of dose severity determination. Because sampling time in challenge trials was every 24 hours, systemic elevation in these cytokines could have been missed. Although it has been proposed that

cytokines are a viable indicator of infection severity during mastitis (Burvenich et al., 2003), other physiological parameters (pyrexia, scoring, SCC) appear to be more useful indicators of infection severity. In order to obtain systemic cytokine dynamics with this strain in future studies, more frequent sampling would be needed.

Conclusions

In vitro growth of *S. uberis* provided a predictable means to obtain a desired concentration. This pathogen could also be maintained on ice for at least 3 hours without altering inoculum concentration. This *S. uberis* strain was successful at providing a consistent and predictable mastitis challenge, even at doses as low as 500 cfu. Clinical signs of mastitis including milk alterations and quarter inflammation were detected as early as 24 hours post-infusion, and were detected in all successfully infected cows by 36 hours post infusion. Both a localized and systemic inflammatory response was produced, allowing for future study of induced *S. uberis* effects on mammary function.

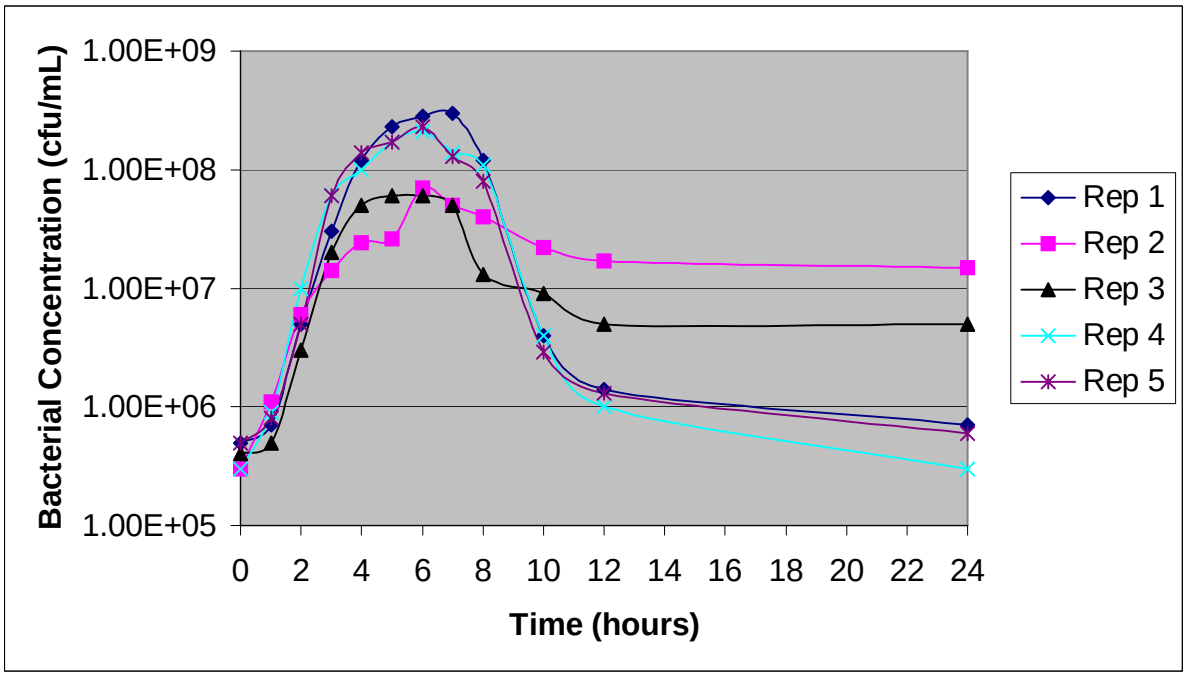

Figure 2: *S. uberis* growth, *in vitro*, over 24 hours.

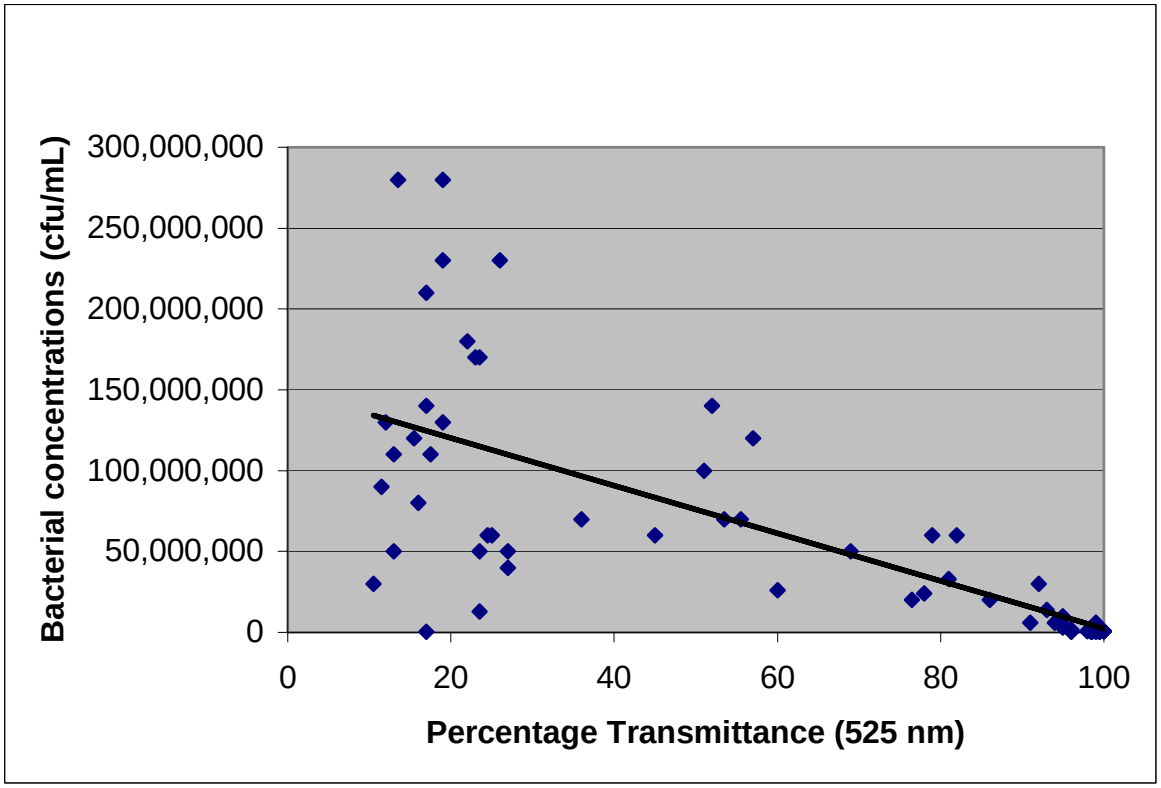

Figure 3: Optical transmittance of bacterial suspension from 0 – 8 hours for all replicates.

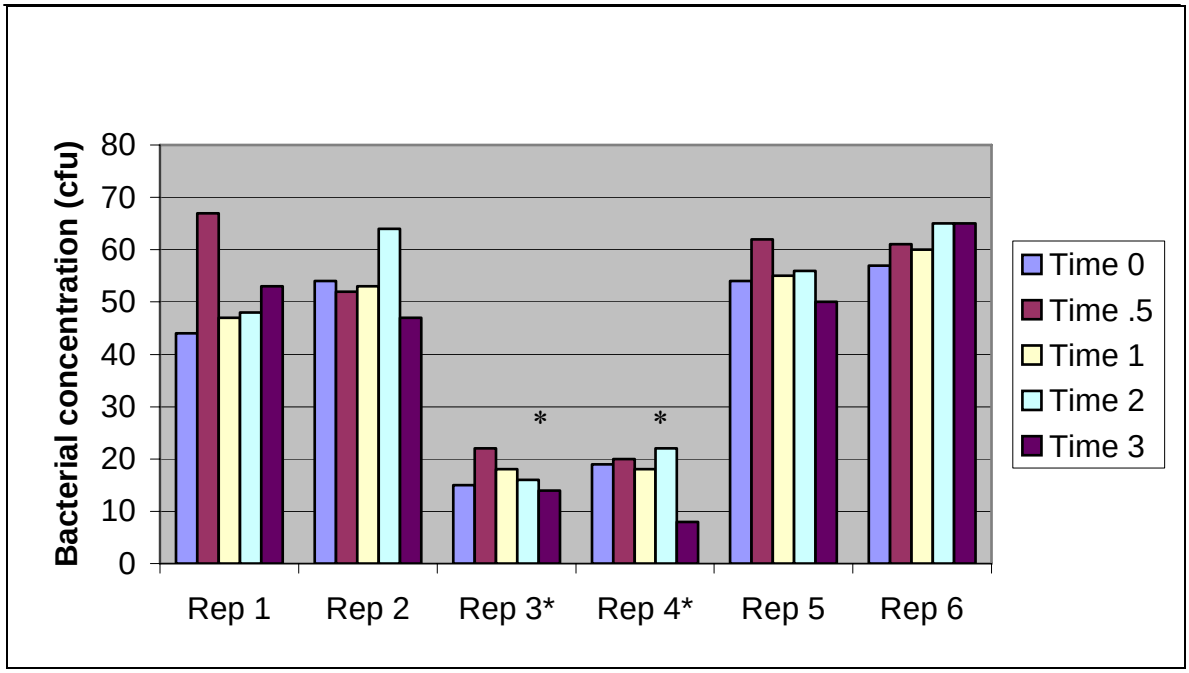

Figure 4: *S. uberis* bacterial maintenance, on ice, after 3 hour incubation period in 36°C BHI broth. Time .5 is 30 minutes on ice, whereas times 1, 2, and 3 are 1, 2, and 3 hours on ice respectively. Bacterial numbers were maintained on ice during the 3 hour period (P=0.96) The asterisked replicates (Rep 3 and Rep 4) represent a power loss during the trial, and perhaps explain the much lower bacterial numbers. Rep 3 and Rep 4 differed significantly from Rep 1,2,5, and 6 ($p < .0001$).

Table 2: Colony forming units after 3 hours growth with a theoretical inoculum concentration of 3 X 10^7 cfu/mL.

	Dilution	
Replicate	**1:300000**	**1:3000000**
1	99	14
2	111	10
3	108	15
4	105	10
Mean	106	12
Expected	100	10

Table 3: Quarter scores[1] of challenged cows during physiology trial using criteria listed in Table 1.

Criterion[2]	Time Post Challenge (hr)						
	Challange	12	24	36	48	60	72
Milk[3]	1.0[a]	1.0[a]	1.25[a]	2.75[c]	2[b]	3.5[d]	2[b]
Size[4]	1.0[e]	1.0[e]	1.0[e]	2[f]	1.25[e]	1.75[f]	1.13[e]
Pain[5]	1.0[g]	1.0[g]	1.25[g]	2.25[h]	1.5[g]	1.75[h]	1.5[g]
Attitude[6]	1.0[h]	1.0[h]	1.0[h]	1.75[i]	1.75[i]	1.0[h]	1.0[h]

[1]All control quarters had a score of 0 throughout the study for each criterion
[2]Severity of intramammary infection scores based on criteria in Table 1
[3]Average milk scores of challenged quarters, different superscripts indicate significant difference ($P < .01$) SEM = .18
[4]Average size scores of challenged quarters, different superscripts indicate significant difference ($P < .001$) SEM = .13
[5]Average pain scores of challenged quarters, different superscripts indicate significant difference from control ($P < .001$) SEM = .12
[6]Average attitude score of challenged cows, different superscripts indicate significant difference from control ($P < .05$) SEM = .34

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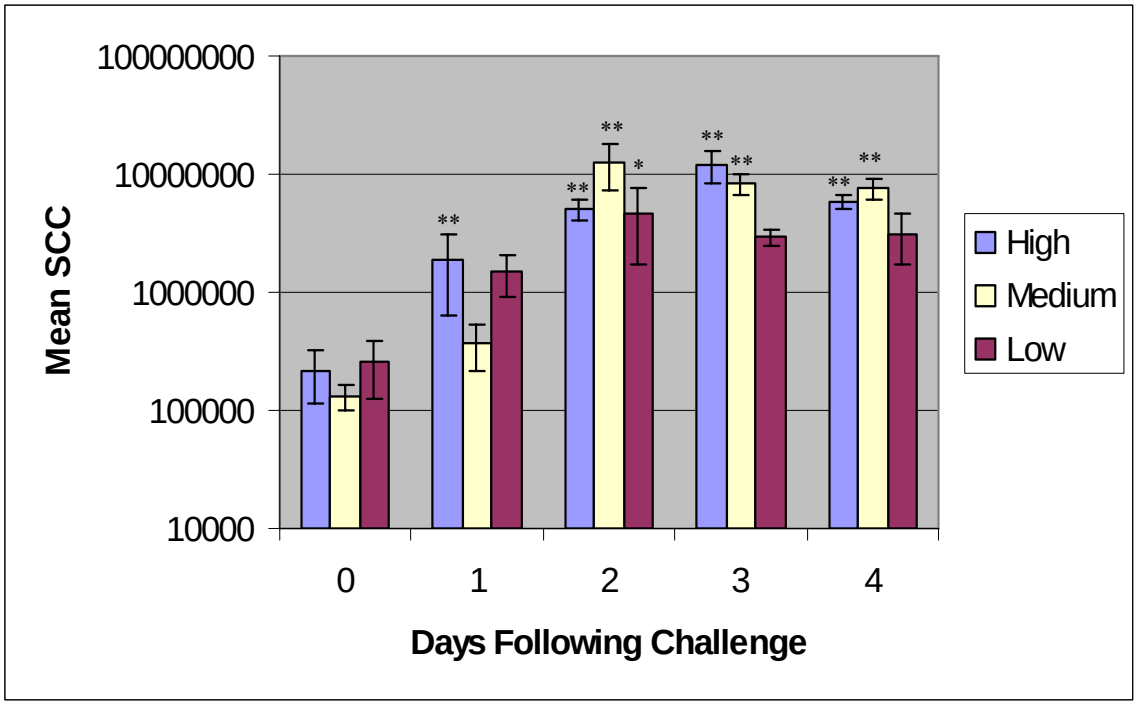

Figure 5: Mean somatic cell counts (SCC) of challenged quarters for each treatment group for the dose study. Day 0 represents the day of challenge. One asterisk indicates a change from baseline within each treatment group at P = 0.10. Two asterisks indicate a change from baseline within each treatment group at P= 0.05.

Table 4: A $\log_{10}$ transformation of SCC means ± SEM[1] of the challenged quarters for each treatment group.

		Dose[3]	
Time[2]	**High**	**Medium**	**Low**
0	4.91 ± .23	5.12 ± .25	5.13± .23
1	5.87 ± .23**	5.47 ± .25	5.99 ± .23
2	6.78 ± .36**	6.98 ± .30**	6.28 ± .23*
3	7.06 ± .31**	6.90 ± .36**	6.12 ± .30
4	6.83 ± .31**	6.87 ± .37**	6.25 ± .27

[1]One asterisk indicates a change from baseline within each treatment group at P = 0.10. Two asterisks indicate a change from baseline within each treatment group at P= 0.05.
[2]Days following challenge (Time 0 represents day of challenge)
[3]Dose challenged in each quarter. Low = 500 cfu, Medium = 1,950 cfu, High = 4,000 cfu

Table 5: Scores[1] of challenged quarters during dose trial for criteria listed in Table 1.

		Time[4]									
Criterion[2]	**Dose[3]**	**Ch**	**Ch+12**	**Ch+24**	**Ch+36**	**Ch+48**	**Ch+60**	**Ch+72**	**Ch+84**	**Ch+96**	**SEM +/-**
Milk[5]	Low	1	1	1	**3[b]	1.83[c]	*2.5	*2	2	1.83	.4319
	Medium	1	1	1	2[ab]	**3.2[d]	*2.4	2.2	**3.2	**3	.4731
	High	1	1	1	1.67[a]	**3[cd]	*2.5	**2.67	*2.33	**2.67	.4319
Quarter Pain[6]	Low	1	1	1.33	**3.17	**2[ef]	1.33	**2.33	**2	**2	.3241
	Medium	1	1	1.2	**3.5	**2.6[f]	1	*1.8	*1.8	**2.4	.3441
	High	1	1	1.17	**3.08	1.42[e]	1	1.67	1.67	1.67	.3241
Quarter Size[7]	Low	1	1	1.33	1.17	1.33	**1.83	1.33	1.17[gh]	1.33[ij]	.2291
	Medium	1	1	1.2	1.6	1.6	1.2	1.4	*1.8[h]	1[i]	.2492
	High	1	1	1.17	1.17	**1.83	1.5	1	1[g]	**1.83[j]	.2291

[1]One Asterisk * indicates significant elevation from baseline values (Ch) P<0.05. Two asterisks ** indicate elevation from baseline values (Ch) P < 0.01. Differences within group indicated by differing letters.
[2]Severity of intramammary infection scores based on criteria in Table 1
[3]Dose challenged in each quarter. Low = 500 cfu, Medium = 1,950 cfu, High = 4,000 cfu
[4]Time of sample collection, Ch is time of challenge + hours post challenge
[5]Mean milk scores of challenged quarters. Differing letters indicate difference between dose treatments.
Ch+36 (a,b) – Low vs. High (P=.031; SE=..61)
Ch+48 (c,d) – Low vs. Medium (P=.035; SE=.64)
[6]Mean quarter pain scores of challenged quarters. Differing letters indicate difference between dose treatments.
Ch+48 (e, f) – Medium vs. High (P=.019; SE=.47)
[7]Mean quarter size scores of challenged quarters. Differing letters indicate difference between dose treatments.
Ch+84 (g, h) – Medium vs. High (P=.021; SE=.34)
Ch+96 (i, j) – Medium vs. High (P=.014; SE=.34)

Table 6: Scores[1] of Challenged cows during dose trial for criteria listed in Table 1.

		Time[4]								
Criterion[2]	**Dose[3]**	**Ch**	**Ch+12**	**Ch+24**	**Ch+36**	**Ch+48**	**Ch+60**	**Ch+72**	**Ch+84**	**Ch+96**
Attitude[5]	Low	1	1	1	1	1.33	1.33	**2	**2	**3
	Medium	1	1	1	1.67	1.67	1.67	**2	**2	**3
	High	1	1	1.67	1.33	1.67	1.33	**2	**2	**3
Appetite[6]	Low	1	1	1[a]	1	1[c]	1.33	**2	**2	**2
	Medium	1	1	1[a]	1	1[c]	1.33	**2	**2	**2
	High	1	1	*1.67[b]	1.33	*1.67[d]	1.33	**2	**2	**2
Udder Temp[7]	Low	1	1	1	1.67	1.67	**2.67[e]	*2.33	1.67	**2.67[h]
	Medium	1	1	1	1	1	1.33[f]	1.33	1.67	1[g]
	High	1	1	1	1.67	1.67	1.67[ef]	*2.33	1.33	1.33[g]

[1]One Asterisk indicates significant elevation from baseline values (Ch) P<0.05. Two asterisks indicate elevation from baseline values (Ch) P<0.01. Elevations within group indicated by differing

[2]Severity of intramammary infection scores based on criteria in Table 1

[3]Dose challenged in each quarter. Low = 500 cfu, Medium = 1,950 cfu, High = 4,000 cfu

[4]Time of sample collection, Ch is time of challenge + hours post challenge

[5]Average attitude score of challenged cows. SEM=.35

[6]Average appetite score of challenged cows. Differing letters indicate significant difference between dose treatments.

Ch+24 (a,b) High vs. Low/Medium (P=.039; SEM=.31)

Ch+48 (c, d) High vs. Low/Medium (P=.039; SEM=.31)

[7]Average udder temperature of challenged cows. Differing letters indicate significant difference between dose treatments.

Ch+60 (e, f) – Low vs. Medium (P=.018; SEM=.54)

Ch+96 (g, h) – Low vs. Medium (P=.0034; SEM=.54),

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