ABSTRACT

Title of Thesis: IMMUNE RESPONSE AFTER INTRAMAMMARY CHALLENGE WITH *STREPTOCOCCUS UBERIS* MASTITIS FOR COWS FED OMNIGEN-AF® DURING MID-LACTATION

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Mastitis is one of the costliest diseases in the dairy cattle industry. Environmental pathogens, such as *Streptococcus uberis* (*Strep. uberis*), are the most prevalent causes of mastitis infections, while contagious pathogen mastitis has declined in incidences due to improvement in management protocols. Antimicrobials and antibiotics are the primary therapies currently utilized in the dairy industry to treat mastitis. However, the concern for antibiotic overuse and potential bacterial resistance due to improper use of these therapies has steered research in exploration of alternatives to antibiotics or other strategies. One potential alternative is supplementation of an immunomodulatory feed additive to daily cattle total mixed rations (TMR). A current immunomodulator is OmniGen-AF ® (OMN) produced by Phibro Animal Health Corporation (Teaneck, NJ) and has been explored being fed to lactating dairy cattle by previous research groups. OmniGen- AF ® has been reported to improve initial innate immune response during infection. However, it is unknown how OMN influences the innate immune system *in*

vivo to a *S. uberis* mastitis infection. The ability of OMN to modulate immune function during an environmental mastitis infection was tested compared to control groups. Cows fed OmniGen-AF® and challenged with *Strep. uberis* had numerically higher least squared mean Log somatic cell count compared to the control group that was not fed OMN and challenged with *Strep. uberis*. OMN fed cows displayed numerically higher average daily feed intake and fluid milk yield values compared to the control group. Further analysis of milk and blood samples using immunoassays to monitor the effects OMN has on cytokine and cortisol levels throughout mastitis infection is needed to determine innate immune response. In conclusion, OmniGen-AF® has the potential as an immunomodulator that improves innate immune system activity with continuous supplementation in the diet to prevention of dairy cattle environmental mastitis.

Immune response after intramammary challenge with *Streptococcus uberis* mastitis for cows fed OmniGen-AF® during mid-lactation

by

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of requirement for the degree of
Master of Science
2021

Advisory Committee:
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Dedication

This thesis is dedicated to my mother, Sandra Fischer, who has taught me true strength and resilience and my grandfather, Dan Holsapple, whose wisdom, and clarity I've always strived to emulate. I will forever be grateful to them, and I owe my successes to their constant love, support, and guidance.

Acknowledgements

First and foremost, I would like to thank my major professor, Dr. Kasey Moyes, for allowing me to be a part of her research and granting me this opportunity. I would like to thank the members of my committee, Dr. Bob Peters, and Dr. Daniel Nelson, for devoting their patience, time, and support towards my thesis research. I would like to thank Dr. Rick Kohn for his guidance especially in the last year of my degree and advising me throughout my project. I am truly grateful for your support and understanding. In addition, I would like to thank Dr. Carol Keefer for her support throughout my master's degree and dedication to assisting me in some harrowing moments in my academic career. I would also like to thank Ms. Sheryl Grey for being a caring and loving presence during my time in the Animal Science Department. Your constant support towards the entire 4111 office will always be a memory I hold close to my heart. You were a positive light in my University of Maryland experience. I would like to thank the Department of Animal and Avian Sciences for providing me the opportunity and financial assistance to complete my master's degree.

I will forever be grateful to my fellow "Moyes Lab" members, Cynthia Scholte, Maura McGraw, and Paola Martinez and would like to thank them for their assistance, support, and friendship throughout my degree. I honestly could not have finished my research without you all. Additionally, I would like to extend my gratitude to everyone at the Central Maryland Research and Education Center (CMREC) for their encouragement and assistance during my many cow trials. The CMREC crew truly added unforgettable memories to my experience and the farm felt like a home away from home.

To my best friends I consider the absolute rocks in my life, Bryce Cullen, and Marie Iwaniuk, thank you for infinite support and unconditional love. You both are the true backbones to my sanity, and I am incredibly lucky to have not one, but two people who truly understand me and I know I can rely on without fail. I cannot put into words how much I love you and how much you mean to me. Thank you from the bottom of my heart for absolutely everything you have done for me.

I would also like to thank the friends who helped me through some of the hardest days and made sure I never gave up on my goals. I am sincerely grateful to Jarvis Scott, Chaitra Surugihalli, Rachel Rha, Ronique Beckford, Sarah Ireland, and Jacob Gill. I am very lucky to have a deeply supportive, caring, and wonderful group of friends. You truly enhanced my graduate school experience, and I would not have been able to complete my degree without you all.

I would also like to thank the Kinlein family for "adopting" me when I moved to Maryland. Your support and love were an absolute gift throughout my graduate school experience. I cannot thank you enough for everything you have done for me.

Of course, all my success would not have been possible without the love and support of my family. I would like to thank my father, Michael Fischer, for teaching me the importance of hard work and always supporting my career decisions. I would also like to thank my mother, Sandy Fischer, and stepdad, Robert Hartman for always encouraging me to pursue my dreams and guiding me throughout my degree. I want to thank my sister, Danielle Fischer, for her endless love and support as well. I am truly blessed to have such a strong and amazing family. Thank you for always being in my corner and loving my unconditionally.

I can say that I am incredibly lucky to have absolute dimes of human beings as friends and family. I am forever grateful to have such kind, wonderful, and inspiring individuals in my life. I am blessed to know everyone who has helped me along the way.

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List of Abbreviations

CD14	cluster of differentiation 14, macrophage protein
CFU	colony forming unit
CON	control treatment, sterile PBS
d	day
DEX	dexamethasone
DIM	days in milk
h	hour
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MEC	mammary epithelial cell
OMN	OmniGen-AF®
OxPAPC	1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphorylcholine, phospholipid
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffer solution
PMN	polymorphonuclear leukocyte
PRR	pattern recognition receptors
ROS	reactive oxygen species
SCC	somatic cell count
SCFA	short-chain fatty acids
TLR	toll-like receptor
TMR	total mixed ration

Microorganisms

E. coli	*Escherichia coli*
S. aureus	*Staphylococcus aureus*
S. uberis	*Streptococcus uberis*
S. cerevisiae	*Saccharomyces cerevisiae*

Introduction

Bovine mastitis, inflammation of the mammary gland, is the most prevalent and economically significant disease of dairy cattle in the United States and worldwide (USDA, 2009). Pathogens that are responsible for the cause of mastitis are streptococci, staphylococci, and coliforms. The source of these pathogens are considered to be either contagious or environmental depending on when and where the infection has occurred. Environmental bacteria have become the leading cause of mastitis while post-milking teat dipping, and blanket dry cow therapy have decreased mastitis caused by contagious bacterial pathogens. To combat environmental pathogens, antimicrobial agents have been investigated by several groups as a treatment and control method. There is a lack of information on controlling environmental mastitis through alternatives to antimicrobial drugs allows for exploration of research involving possible new therapies.

Attention to animal health has major animal welfare implications. Important on-farm practices include development of standard protocols of humane management of diseased animals, and judicious use of antibiotic therapy. While current technology is helpful, innovations in alternative methods to prevent environmental mastitis infections are still needed. Currently, the primary strategy for mastitis treatment is antibiotic therapy. Producers treat subclinical and clinical mastitis depending on severity of symptoms. Subclinical mastitis measuring somatic cell count (SCC) using a California Mastitis Test or DHI testing, and loss in milk production. Clinical mastitis can be observed in cattle as inflammation of the udder (redness, pain, swelling), physical changes in milk secretions, decrease in production, increased SCC, or systemic changes such as decreased appetite, fever, and/or lethargy. Many producers will monitor a

subclinical infection with a California Mastitis Test, but antibiotics are administered when clinical symptoms arise. Although antibiotic treatment can be costly, it is the most successful method to cure infection in the bovine industry. However, potential of drug residue in milk and meat produced for human consumption is a current concern among some consumers. The pressure from government legislative changes, emergence of antibiotic resistance, and the cost of use have contributed to the industry actively striving to reduce overall use of antibiotics. This has allowed for the development and implementation of alternative therapies to manage herd mastitis cases and minimize antibiotic use in food-producing animals with government and consumer concerns in mind.

Antimicrobial alternatives that aid in mastitis prevention have been discussed at length and promising products have been explored, some of which are nutritional feed additives that have recently been investigated to determine their validity when cattle are exposed to different stressors. OmniGen-AF® is an immune modulatory feed additive in the global dairy industry that is known to support healthy immune function in cattle. This modulator is produced by Phibro Animal Health Corporation (Teaneck, NJ) and is recommended to be fed to dry, pre-fresh or lactating dairy cattle. The addition of OmniGen-AF® to a total mixed ration (TMR) has the possible ability to boost immune function during an environmental mastitis outbreak, including influence on milk productivity, somatic cell counts, and increased neutrophil isolation to site of infection. An immunomodulatory additive may be useful as an alternative antibiotic therapy for bovine mastitis or can attribute to increased reduction in prolonged antimicrobial treatment.

Mastitis

Bovine mastitis begins with the presence and invasion of a foreign infectious pathogen, when the mammary gland encounters a microbial agent that then enters through the streak canal at the distal end of the teat. The physical line of defense in place to protect against invading pathogens is the keratin plug created by the turnover of the stratified squamous epithelial lining of the teat end. Over-milking or trauma can cause damage to the teat's ability to close after milking via this keratin plug, thus allowing pathogens to invade past the canal into the teat cistern causing mammary inflammation. When a foreign bacterium penetrates past the body's defenses and enters the mammary gland the result is either a subclinical or clinical infection. A clinical infection can present as mild, moderate, or severe based on symptom severity and duration. Some cases may become chronic in some cattle, in which mild subclinical symptoms may persist with occasional moderate to severe clinical appearances over time. Clinical mammary infections and symptoms are easily diagnosed and treated by producers in the dairy industry, although subclinical cases can be a greater concern for dairy herds as subclinical case prevalence can range from 5-75% (Erskine, 2016) and harder to define. The estimated losses from mastitis treatment, including discarded milk, antibiotic therapy, and increased labor, in the United States exceed $2 billion per year with each clinical case costing approximately $179 (Cha et al., 2011) with the majority of financial losses attributed to reduced fluid milk yield caused by asymptomatic, subclinical mastitis (Gill et al., 1990). Subclinical mastitis cases tend to be diagnosed through observed altered milk composition, decreased milk yield, and increased milk somatic cell count (SCC) and bacterial count (Blowey and Edmonson, 2010). Since these cows appear to be otherwise

healthy and have normal milk these cases can be overlooked by farm management resulting in non-diagnosis or treatment. Additional costs of mastitis have been reported to be attributed to loss of milk quality premiums, administration of treatment, reduced milk quality, veterinary care, increased cull rate, increased labor costs, and milk discarded from antibiotic-treated cattle during the Food and Drug Administration (FDA) mandated withdrawal period that follows the end of antibiotic use.

Diagnosis of a mammary infection is typically determined through observation of cattle displaying clinical symptoms and further testing is done. Changes that are observed through diagnostic testing are milk composition, bacterial count, and somatic cell count in the milk. Detection of clinical mastitis can be based on visual indicators as well, such as, mammary gland may turn red in color, may feel hot and hard to the touch due to blood flow change to the inflamed area (Erskine, 2016). Some cases may cause udder palpations to be painful for the animal. Other symptoms include abnormal milk from the infected mammary gland quarter during milking, which may be abnormal in color, contain flakes, clots, or blood. These abnormalities are observable when the milking technician strips milk from each quarter to induce milk let down at the beginning of milking protocol. Technicians may notice these animals have a decreased total milk yield due to the inflammation (Gill et al., 1990). For instances where clinical symptoms are not present, off-farm testing of milk can be administered for mastitis diagnosis. State or privately-owned laboratories can determine possible pathogens, abnormal composition, and high SCC concentrations in the milk samples sent from the bulk tank or suspected infected animals. Somatic cell counts are considered elevated when greater than 200,000 cells/mL is reported for composite foremilk and 100,000 cells/mL is reported for a

quarter foremilk sample. Somatic cell score can also indicate elevation if the score is greater than 4 for either a composite or individual quarter milk sample (Pighetti et al., 2007). Both methods are proven methods to confirm the presence of mastitis in a lactating dairy cow.

To treat and manage mastitis appropriately there are several factors that need to be assessed to determine the nature of the infection. Mastitis causing pathogens are categorized as contagious or environmental, with environmental intruders are opportunistic, typically, invading and multiplying within the host to elicit an immune response resulting in elimination (Bradley, 2002). These pathogens can include bacteria, viruses, yeast, and algae that all have the ability to cause subclinical or clinical presentations of mastitis in an animal with differing severity (Wagner and Erskine, 2009). There are five bacteria known to cause clinical cases of mammary infections: the contagious pathogens *Streptococcus agalactiae* and *Streptococcus aureus*; along with the environmental pathogens *Escherichia coli*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* (Bradley, 2002). There has been documented decline in the prevalence of clinical mastitis caused by contagious pathogens due to the industry's adaption to improved management practices, such as post-milking teat disinfection, culling chronic mastitis infected cattle, dry cow therapy, and increased hygienic protocols for areas cows frequent the most (i.e., stalls, allies, parlor, and holding pens) (Blowey and Edmondson, 2010). This same decline, however, has not been observed in clinical cases caused by environmental pathogens even with the addition of these improvements to overall dairy cow management (Bradley et al., 2007).

Environmental Mastitis

Clinical mastitis is most often caused by environmental bacteria and is a serious concern for even well-managed dairy operations. These bacteria are typically gram-negative meaning they have a single layer cell wall and are considered environmental mastitis pathogens, such as *E. coli*, *Klebsiella,* and *Enterobacter* (National Mastitis Council, 2004). Gram-negative bacteria often can be found in feces, soil, contaminated water, or organic matter (bedding). Once the bacteria have gained entry through the teat canal it will rapidly adjust to the environment of the mammary gland where it will multiply under the anaerobic conditions. Intramammary infections caused by gram-negative environmental bacteria can be difficult to diagnose due to low shedding rates and sample contamination (Hogan and Smith, 2003).

Environmental mastitis can also originate from gram-positive bacteria known as Streptococcal species. *Streptococcus uberis* is a gram-positive organism known to be one of the most prevalent mastitis causing environmental pathogens and accounts for 10-50% of bovine mastitis infections (Jayarao et al., 1999; Phuektes et al., 2001). Practices that include sanitizing housing environments (i.e., free stall barn, allies, and parlor) and disinfecting agents pre and post milking are used to decrease mastitis prevalence but have a reduced efficiency when trying to control *S. uberis*-associated infections compared to other mastitis causing pathogens. *S. uberis* causes intramammary infections that are predominately subclinical but is also responsible for up to 16% of clinical cases per year in the U.S. (Hilerton et al., 1993; Jayarao et al., 1999) and can turn to chronic infections (Sordillo et al., 1997). Once the bacteria have entered the mammary gland through the teat canal species like *S. uberis* can have a longer adjustment in order to establish an

infection compared to gram-negative bacteria. *S. uberis* infections are reported to initiate an immune response within a day of post infection and colony forming units (CFUs) in milk peak at 10^4 CFU/mL within 5-6 days post infection (Pedersen et al., 2003; Rambeaud et al., 2003). The strain 0140J of *S. uberis* is known to be the most virulent due to it being resistant to phagocytosis by macrophages and neutrophils due to encapsulation which results in more severe infection in comparison to other strains (Hill, 1988; Leigh and Field, 1994). This virulency and widespread presence on dairy farming systems makes *S. uberis* a formidable pathogen to determine relevancy for the potential benefits of immunoregulatory feed additives aiding in immune function during mastitis infections.

Innate Immune Response to Mastitis

The innate immune system is the first line of defense for any possible foreign bacteria, virus, fungus, or microorganism invasion. At the moment of infection, the immune system is triggered to respond to eliminate the invading pathogen from the host. Polymorphonuclear cell (i.e., neutrophils, eosinophils, and macrophages) migration to the udder is the result of rapid bacterial multiplication in bovine milk following a contaminate entering the teat orifice (Bradley, 2002). The term milk somatic cells was coined to describe the presence of epithelial cells in milk, with normal milk total cell population predominately made up of 30-74% macrophages for noninfected quarters (Paape et al., 1963, Östensson et al., 1988). Implementation of improved management practices including increased hygiene on farms and use of inorganic bedding material have aided in a decreased prevalence of contagious pathogen caused mastitis and low

bulk tank somatic cell counts (SCC). However, studies have suggested low bulk tank SCC scores are associated with increased risk of clinical mastitis, specifically environmental bacteria were linked to causing higher incidences of clinical mastitis cases in herds (Suriyasathapron et al., 2000). Severity of the mastitis infection and the possible repercussions to cow health post-infection is heavily influenced by the animal's innate immune system's ability to promptly recruit necessary cells to the mammary gland (De Cueninck, 1979; Hill, 1981). Animals that recover rapidly from mastitis and tend not to develop chronic infections are genuinely more productive and have longevity in the herd.

The most effective method to defend against a bacterial infection is phagocytosis by polymorphonuclear neutrophils. Bacteria will attach to the surface of a polymorphonuclear leukocyte (PMN) causing oxidants to be released that coat and kill the bacteria while being ingested by the cell. Due to the potent nature of the oxidants tissue in the immediate area is also destroyed in the process of exterminating the bacteria and the PMN die after the invader is destroyed (Paape et al., 2002). Bovine PMN leukocytes have numerous plasma membrane receptors important for migration to sites of infection, such as adhesion molecules L-selectin and β_2-integrin that are associated with PMN binding to endothelial cells (Zimmerman, 1992, Kishimoto et al., 1989). When a pathogen is recognized by a PMN neutrophil membrane receptors immunoglobulin G_2 (IgG_2) and immunoglobulin M (IgM) trigger the complement pathway when they bind with C1 complex initiating the cleavage of protein C3 releasing fragment peptide C3b, which is an important protein component (opsonin) that will bind to an invading pathogen to begin the promotion of phagocytosis by recognizing antigens (Paape et al., 1991). This process causes what is known in the dairy industry as an increase in somatic

cell count. The phagocytic cells, such as neutrophils and macrophages, have a C3b receptor that when it interacts with the fragment peptide C3b which allows the cells to begin engulfing the foreign pathogen. The mammary gland is equipped with pattern recognition receptors (PRRs) on mammary epithelial cells (MEC) that recognize and identify pathogens by pathogen associated molecular patterns (PAMPs), that are powerful stimulators of innate immunity initiating the host response to a foreign bacterium (Lahouassa et al., 2007, Bannerman et al., 2004b). Receptors for PAMPs known as ficolins that are found on bacteria and are expressed by phagocytic cells during infection (Runza et al., 2008; de Greeff et al., 2013). These phagocytic cells are most often neutrophils which influx to the area they are most needed during a pathogen invasion (de Greeff et al., 2013). Another characteristic of ficolins which allow them to assist with gram-positive pathogen recognition is the ligands are monosaccharides which have been reported to abundantly present in capsules of gram-positive bacteria (Weis et al., 1992; de Greeff et al., 2013). The biding of these receptors can cause a complement cascade triggering an upregulation of genes in the innate immune system (Runza et al., 2008; de Greeff et al., 2013).

Specific bacterial components such as lipopolysaccharides (LPS), bacterial lipoproteins that are released from gram-negative bacteria (Berczi, 1998), are recognized by a family of PRRs known as toll-like receptors (TLRs) resulting in signaling host cells to produce cytokines (Lahouassa et al., 2007, Bannerman et al., 2004b). Typically, gram-positive organisms do not contain LPS on their cell walls, it is found on environmental pathogens such as *Escherichia coli* (Berczi, 1998). Due to this characteristic, *Streptococcus uberis* creates a different set of PRRs to be upregulated during infection

compared to other environmental pathogens, more specifically toll-like receptor 2, LPS-binding protein (LBP), and ficolin (Czabanska et al., 2012). Toll-like receptor 2 (TLR2) is upregulated during *S. uberis* infection which is indicated by the presence of T-helper 2 cells, secreting the inflammatory mediator interleukin-10 (IL-10) (Moyes et al., 2009). TLR2 recognizes the bacterial ligands known as lipoteichoic acid (LTA) found on the cell wall of *S. uberis* (Czabanska et al., 2012). It is suggested by de Greeff et al. (2013) that during a *S. uberis* infection in vivo the recognition of the bacteria upregulates TLR2 signaling. An alternative is also proposed, the upregulation of TLR2 could possibly be involved in LBP signaling meaning that regulation is controlled indirectly by recognition of LTA by pattern recognition receptors LPB, CD14, and TLR2 (Schröder et al., 2003; de Greeff et al., 2013). This is further enforced as possible method of pathogen recognition, especially during a *S. uberis* infection, when it was discovered that LBP can bind similarly as it does with LPS with the abundant LTA on the cell wall of streptococci (Muller et al., 2006; de Greeff et al., 2013). The recognition might be affected by upregulation of CD14 and TLR2 De Greeff et al. suggests (2013) and a previous study by Bannerman et al. (2004) reported that 48 h after intramammary challenge with S. uberis (specifically strain O140J) an increase in soluble CD14 concentrations was observed. These findings all point to the importance of understanding gram-positive pathogen recognition since other common mastitis causing bacteria have a confirmed complement pathway that trigger the innate immune response, but although *S. uberis* is one of the most common environmental mastitis causing pathogens it still is not completely understood how an infection effects all immune cells and genes. The process of recovery

from an infection is only quickened when the pathogen recognition method is efficient and timely.

Mammary tissue is also threatened when there is an infection. The combination of the high volume of somatic cells and the present *S. uberis* pathogen in the udder causes tissue damage and can potentially become dangerous if not treated. Three genes, superoxide dismutase (SOD), glutathione peroxidase, and indoleamine 2,3-dioxygenase (IDO), are expressed and upregulated during an udder infection to trigger invading pathogens to be killed. When these genes are expressed the formation of reactive oxygen species (ROS) occurs which kill foreign pathogens or bacteria (de Greeff et al., 2013). A key component of effectiveness of the inflammatory response when using antimicrobials is the production of ROS by phagocytes, although there is a risk of host cells being affected by ROS when trying to eliminate the bacteria (Erridge et al., 2008; de Greeff et al., 2013). Furthermore, damage to the host cells caused by ROS causes oxidized phospholipids to form, specifically one called 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphorylcholine (OxPAPC). OxPAPC has been reported to play a role in negatively affecting inflammatory response by inhibiting TLR2 and TLR4 signaling. The downregulation by OxPAPC is inhibited by CD14 and LBP, which have been reported to be upregulated during a *S. uberis* infection (Erridge et al., 2008; de Greeff et al., 2013). According to these results, the host's ability to recognize the S. uberis pathogen in order to initiate an immune response may also be a limiting factor in the host's ability to control damage caused by the immune response, such as ROS (de Greeff et al., 2013).

Mastitis Treatment with Antibiotics

Once mastitis symptoms are detected and infection is confirmed, the conventional method of treatment in the dairy sector is antibiotics (Oliver et. al., 2011). Intramammary infusion of a β-lactam, an U.S. Food and Drug Administration (FDA) approved cell wall disruptor typically a ceftiofur, cephalosporin, or cephapirin suspension (US Department of Agriculture, 2007) is the commonly used treatment for mastitis, but severe cases may need additional antibiotic treatment with intravenous injections of ampicillin, cloxacillin, penicillin, streptomycin, or tetracycline (Bhosale et al., 2014). Antibiotic use as a primary solution for combating infections in food producing animals has come under scrutiny and pressure to reduce overall use has become a challenge for modern dairy industry producers (Bradley 2002). With any use of antibiotic treatment, the possibility of inappropriate or overuse of drugs is a concern due to the possible development of resistant bacterial strains and decreased effectiveness. Resistant bacteria can evade death, multiply, and cause increased harm to the animal. Although growing concern of the detrimental effects of increased antimicrobial resistant pathogen prevalence on dairy farms is a result of antibiotic use for mastitis infections has no compelling evidence the push for alternatives is rising (Erskine et al., 2002; Rajala-Schultz et al., 2004; Pol and Ruegg, 2007). In 2015, the *National Action Plan for Combating Antibiotic Resistant Bacteria* was released containing implementations set to combat emergences of antibiotic-resistant bacteria by improved on-farm antibiotic stewardship, increased bacterial resistant species monitoring, and increased research on potential antibiotics alternatives and development for food animal production. Concern among consumers, health professionals, and government officials regarding antibiotic use posing a risk of

residue presences in meat or milk products is pushing the exploration of alternative strategies for mastitis maintenance as well.

Alternatives to Antimicrobials

Strategies to prevent and control mastitis on dairy operations have been explored for decades with most success seen with improved management practices such as disinfectant teat dipping and increased employee hygiene practices. Other preventative strategies include vaccinations and antimicrobial intramammary treatment. Vaccines have been shown to reduce the prominence of some coliform causing mastitis by decreasing the rate of incidence and severity. One of the most successful ones being *E. coli* J5 vaccine and another common mastitis causing pathogen *Staphylococcus aureus* has a few vaccines with differing effectiveness due to factors including cow age, health, environmental conditions, and pathogen species (Hogan et al., 1992; Hoedemaker et al., 2001; Kazemi et al., 2014). Although these therapies are beneficial, they lack the ability to provide sufficient protection to be deemed a solution for alternatives for antimicrobial treatment and can be costly to implement, especially for large operations (Sharun et al., 2021). Nutritional based alternative therapies have gained interest due to their ease of use and cost-efficiency with promising potential seen in immunomodulation in decreasing disease prevalence in dairy herds and new research targeting mastitis control.

Probiotics and Immunomodulatory Additives

Another solution that has gained increasing interest in the dairy industry includes the use of probiotics. Havenaar et al. (1992) defines probiotics as “mono or mixed

cultures of live microorganisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora". The most prominent bacterial species used are *Lactobacillus spp.* and *Bifidobacterium* which are lactic acid producing bacteria (Chiquette, 2009). Research has shown that lactic acid inhibits coliform growth in gastrointestinal tracts of piglets, reducing pH making an acidic environment that is inhabitable for many pathogens (Fuller, 1977; Chiquette, 2009). These findings along with a study by Schuijt et al. (2016), that demonstrated the protective ability of gut microbiota against *Streptococcus pneumoniae* induced pneumonia, have suggested the gut microbiome can have a significant effect on infectious diseases and possibly mastitis (Hu et al., 2019).

Gut metabolites such as lipopolysaccharides (LPS) and short-chain fatty acids (SCFAs) have since been proven to are involved in mastitis (Jin et al., 2016a; Zhang et al., 2016a; Wang et al., 2017a; Wang et al., 2017b). When rumen derived LPS crosses the epithelium, it then enters the bloodstream where it soon passes to organs and tissues throughout the body (Hu et al., 2019). When a dairy cow overproduces LPS damage to rumen epithelium occurs and excess LPS enters the blood traveling to the mammary gland which leads to inflammation (Zhang et al., 2016a; Hu et al., 2019). However, SCFAs have been shown to decrease the damage caused by LPS when dietary fiber in the gut is fermented by the microbiome producing SCFAs that have anti-inflammatory properties (Wang et al., 2017b; Hu et al., 2019). This process disrupts the blood-milk barrier, which consists of mammary epithelial cells (MECs) and tight junctions, that is typically is maintained by commensal microflora that act as a barrier to the host animal (Stecher et al., 2010; Hu et al., 2019). Bacteria with the potential to produce increased

levels of LPS in the gastrointestinal tract can be proliferated due to changes in the gut microbiome flora and potentially can enter the mammary gland (Hu et al., 2019; Zebeli et al., 2009). The higher levels of LPS decrease PMN ability to cross the blood-milk barrier triggering accumulation of PMN in the mammary gland which is manifests as elevated somatic cell counts and in turn increases the cow's susceptibility to mastitis (Kobayashi et al., 2013a; Kelly et al., 2015; Hu et al., 2019). Short chain fatty acids inhibit LPS caused change in the blood-milk barrier due to its importance as an energy source and was shown to regulate tight junction protein changes as well (Wang et al., 2017a; Wang et al., 2017b; Hu et al., 2019). These findings led Hu et al. (2019) to suggest further research in the role of gut microbiota on the development of mastitis with the possibility of the development of a new approach in mastitis prevention and treatment through modulation of the gut microbiome.

Yeasts

Although bacterial probiotics are studied more often, the most commonly used microorganism in ruminant probiotics are yeast preparations such as *Saccharomyces cerevisiae* and *Aspergillus oryzae*. Different aspects of the benefits of yeast probiotics have been studied including pathogen load and total bacterial growth in the rumen (Chiquette, 2009). *E. coli* (strain 0157:H7) and *Listeria monocytogenes* have shown to have decreased growth and viability in vitro when cultured in the presence of yeasts. A strain of *Saccharomyces* (*S. boulardii*) has also been reported to degrade toxins produced by *Clostridium difficile* and has effective properties against *Salmonella* and *E. coli*. The data also suggests *Saccharomyces* is involved with competitive exclusion and cell

binding that decrease pathogen load (Chaucheyras-Durand et al., 2008: Chiquette, 2009). Rumen bacterial growth is stimulated by the addition of yeast consistently over multiple studies due to the supply of growth factors such as B-vitamins, organic acids, and amino acid. Since yeast is a known aerobic microorganism, it establishes an environment within the rumen that is more favorable for the commensal bacteria (Jouany et al., 2006; Chiquette, 2009). The possibility of yeast as a microbe modulator in ruminant animals, specifically dairy cattle, to maintain health and prevent disease is promising and the increasing amount of knowledge regarding the composition of the ruminal microbe will help facilitate future studies (Chiquette, 2009).

Benefits of Yeast Supplementation

The rumen has a delicate ecosystem of microorganisms consisting mostly of protozoa and bacteria that work together in order to digest dietary components to promote performance and obtain useable energy (Desnoyers et. al., 2009). Yeast has been reviewed for its influence on rumen properties, such as digestion, pH, and fermentation. It has been reported that yeast has the ability to create a more anaerobic environment which stimulates cellulolytic bacteria growth in the rumen by attaching to fiber particles resulting in increased rates of cellulose digestion (Jouany et al., 1999a; Roger et al., 1990). The stimulation of bacterial growth in a progressively anaerobic rumen environment is a possible explanation for increased feed intakes seen with yeast supplementation (Jouany et al., 1999a; Roger et al., 1990; Chiquette, 2009). Yeasts influence the stabilization of rumen pH by decreasing the partial oxygen pressure caused by propionate to lactate conversion in the rumen to favor fermentation and acid sensitive

cellulolytic microorganisms' benefit (Chiquette, 2009). Reports from Desnoyers et. al. (2009) confirmed findings from previous experiments by Robinson (2002) to confirm that supplementation of yeast in diets of ruminants increases rumen pH, organic matter digestibility, VFA concentration and tended to decrease lactate concentration. When rumen pH is below 6.0 the lactate utilizing bacteria in the rumen are reported to disappear, resulting in cows developing acidosis and can further effect fluid milk yield or cause death if not treated (Chiquette, 2009).

Yeast supplementation has been reported to positively effect fiber degradation in the rumen as well. Dairy cow dietary dry matter typically consists of approximately 30% cellulose which the rumen microbiota breakdown because the cow lacks the enzymes to do so itself (Chiquette, 2009). In vitro studies by Mosoni et al. (2007) showed when sheep are fed probiotic yeasts there was a two to four-fold increase in the copies of 16 S-RNA of two important cellulolytic bacteria, *Ruminococcus albus* and *R. flavefaciens* (Chiquette, 2009). These cellulolytic bacteria require an anerobic environment to survive and the ability of yeast to use ruminal O_2 helps the bacterial population prosper (Mosoni et. al., 2007; Chiquette, 2009). The promotion of growth for cellulolytic bacteria by dietary supplementation of yeast increases the ruminant's ability to digest fiber which has been connected to the animal's ability to increase dry matter intake (Chiquette, 2009). This is important in lactating dairy cow industry, especially, since the consumption of dry matter is used for energy that is utilized by the body to produce milk.

Assessment of OmniGen-AF

A potential strategy to aid combating mastitis by supporting the innate immune system is inclusion of OmniGen-AF® in the feeding regimen on commercial dairy farms. OmniGen-AF® (OMN) is an immune modulatory feed additive containing active dried *Saccharomyces cerevisiae* in combination with other minerals and vitamins that have the potential to improve overall immune function of dairy cattle, specifically while fighting mastitis caused by a bacterial infection (Buntyn et. al., 2016). *Saccharomyces cerevisiae* is a well-known yeast that will scavenge for oxygen when introduced to the rumen, creating an increased anaerobic environment for gut microbiota (Desnoyers et. al., 2009: Moya et. al., 2009; Uyeno et al., 2015). According to Uyeno et al. (2015), *S. cerevisiae* also plays a role in providing growth factors to rumen microbes including oligosaccharides, B vitamins, and amino acids that stimulate rumen microbiome growth by acting as a probiotic, preparing the immune system to defend against a potential threat. OMN use in dairy cattle has been reported to improve leukocyte function, effect surface L-selectin concentration, and phagocytosis of extracellular pathogens (Brandão et al., 2019).

According to Wang et al. (2007), when OMN was supplemented into the diet of sheep immunosuppressed with dexamethasone, a corticosteroid used to cause immunosuppression, the levels of neutrophil L-selectin (CD62L) surface protein and cellular interleukin-1 beta (IL-1β) of animals not supplemented with OMN were significantly reduced by 62% and 99% respectfully, compared to the control. When sheep immunosuppressed using dexamethasone (DEX) were fed OmniGen-AF® the expression of CD62L and production of IL-1β returned to normalcy. Furthermore, it was reported

that OMN increased the number of neutrophils and lymphocytes in circulation compared to control animals (Wang et al., 2007). CD62L is an important L-selectin cell adhesion molecule typically found on leukocytes, or white blood cells, which protect the body from foreign infections. IL-1β is essential for suppressing the immune response and modulation of autoimmune inflammation. Further investigation using 15 hours post-partum Jersey cows were supplemented with OMN or a control diet 30 days prior to parturition and gene expression was assessed in harvested neutrophils (Wang et al., 2009). Data indicated that OmniGen-AF® facilitated increased expression of several genes that identified stronger inflammatory activity and inflammatory regulation in dairy cows (Wang et al., 2009). Another study utilizing dexamethasone (DEX) to initiate immunosuppression conducted by Ortiz-Marty et al. (2016), supplemented wild-type knockout mice with OmniGen-AF® and challenged with lipopolysaccharides, which has been reported to cause apoptosis and elimination of polymorphonuclear leukocytes (PMNs), after DEX treatment. When the immunosuppressed mice were challenged a reduction of L-selectin and CCL5, a chemokine gene known to play a role in leukocyte recruitment, was observed in control mice but not in mice supplemented with OMN. It was determined that OMN restored ability of PMNs to respond to the lipopolysaccharide challenge, agreeing with Wang et al. (2009) that OMN restores L-selectin expression (Ortiz-Marty et al., 2017). Since L-selectin is vital in regulation of leukocyte migration to sites of inflammation and recirculation of lymphocytes between blood and lymphoid tissue the data suggests that OMN restoration of L-selectin expression in immunosuppressed animals may be beneficial for potential mastitis control.

Blood leukocyte activity has been recorded to be modulated by OMN as well in pre-pubertal and pregnant Holstein dairy heifers by Ryman et al. (2013). This study showed that along with an increase to CD62L levels, interleukin 8 receptor (IL-8R) showed a significant increase as well. The ability of neutrophils to bind to bacteria increased along with reactive oxidative species (ROS) production and phagocytic activity compared to control animals (Ryman et al., 2013; Hurley et al., 2019). Ryman et al. (2013) determined that heifers supplemented with OMN had an enhanced innate immune capacity and lower instances of post-partum mastitis were observed, along with higher fluid milk yield levels during the initial start of the animal's lactation compared to controls (Hurley et al., 2019). The conclusion of the study indicated that enhancing innate immune response may aid in mastitis control (Ryman et al., 2013). Nace et al. (2014) found similar conclusions when OMN was fed to first calf heifers and a tendency was observed that the animals had a lower prevalence of post-partum mastitis compared to control animals. During the study immune cell trafficking markers, such as CD11c and CD62L, were evaluated along with ROS production and phagocytic activity, similarly to the Ryman study. These two studies point to a pattern of amplified innate immune function which was observed in phagocytic activity, gene expression, surface proteins, and radical production (Hurley et al., 2019; Ryman et al., 2013; Nace et al., 2014). These findings compounded with the decreased prevalence of mastitis in post-partum heifers is an indicator of innate immune system activity to potentially prevent intramammary infections and inflammation after calving can be modulated by feeding OMN, while potentially impacting milk production and quality positively (Hurley et al., 2019).

Another study looked at how immune marker production of CD62L and IL-8R while Holstein cows was affected when supplemented with OMN. Neutrophil function was also observed to determine if OMN could enhance the *in vitro* phagocytosis of *S. uberis* since previous studies have observed increased neutrophil function and innate immunity markers. Results found blood levels of IL-8R and CD62L were greater in cows fed OMN and there was a gradual increase throughout the entire 61 day feeding period. Once OMN was withdrawn from the diet there was a rapid decline of CD62L levels, proving continuous supplementation is needed to maintain results. Neutrophils from cows supplemented with OMN were harvested and demonstrated a greater ability to phagocytize *S. uberis* compared to control cow neutrophils. This study concluded that specific immune markers could be stimulated in combination with improving neutrophil function by supplementing OMN into a lactating cow diet, along with demonstrating oral administration of immunomodulators can be effective in ruminants (Corbett et al., 2008).

The previous reports strongly suggest that OMN has the potential to aid in the industry's goal to control mastitis in dairy cattle. However, no research has fully established the overall immunomodulatory properties of OMN in lactating dairy cattle while challenged with a live pathogen to illicit a mastitis infection *in vivo*. In this study, the ability of this immunomodulator (OMN) to boost the innate immune response in lactating Holstein cows during pre-mastitis infection, post-infection, and antibiotic treatment was evaluated.

Materials and Methods

Animals and Treatment Assignment

The original proposal for this study consisted of three trial rounds of 16 multiparous mid-lactation Holstein cows with n=48 (n=12 per treatment; 4 treatments total), unfortunately a contamination during the second round rendered the data collected as incomplete and the third round of the study was halted due to COVID-19 restrictions.

All procedures involving the use of live animals were approved in accordance with the regulations and guidelines set forth by the University of Maryland Animal Care and Use Committee. Sixteen, healthy multiparous Holstein cows in mid-lactation (>100 DIM) were used from the University of Maryland Central Maryland Research Extension Center (CMREC) dairy research facility in Clarksville, MD. Prior to enrollment, aseptic quarter foremilk samples were collected for bacteriological examination following the National Mastitis Council guidelines and somatic cell count (SCC) using the Lancaster Dairy Herd Improvement Association (DHIA) analysis. All eligible cows displayed no clinical signs of mammary or systemic disease, and all mammary quarters were bacteriologically negative with quarter somatic cell counts <100,000 cells/mL.

Cows were fed a standard lactation total mixed ration (TMR) with the addition of OmniGen-AF® (OMN) or without it (CON) for forty-two days prior to intramammary infection challenge with *Streptococcus uberis.* Cows were fed daily a fresh TMR into an assigned calan box. Cows were randomly assigned within dietary treatment to be inoculated with 4,000 colony forming units of *S. uberis* (strain 0410J; CON-YES or OMN-YES) or received saline (0.9% NaCl; CON-NO OR OMN-NO) solution into one rear mammary quarter following methods previously described (Moyes et. al., 2009).

There were eight cows per treatment with four total treatments and cows were not treated for clinical mastitis symptoms or any other disease prior to inoculation. The cattle were housed and fed in a Calan Broadbent Feeding System barn at the CMREC dairy unit in Clarksville, MD where the infected animals were separated from the control animals by a gate. The Calan Broadbent Feeding System is an electronic research equipment system that allows for individual diets to be administered to cattle. The electrical feeding door system has a circuit board in the door that reads when the key hanging from a neck cord on each cow is in close proximity and unlocks the door to allow the cow to access its feed. Each cow's daily feed intake is easily monitored using this system due to the inability for other cows to steal rations (American Calan, 1977). The animals had free access to water that is located at both ends of the Calan Broadbent Feeding System barn that had free stalls with water mattresses for cow comfort. Daily total mixed ration formulated to meet requirements and 10% refusals were fed into individual Calan boxes every morning in order to monitor and record feed intake. The cows that were fed OmniGen-AF® received a premeasured ~0.5 lb. of the additive top dressed to the TMR daily. Around 0.5 lb. of the control additive was administered in the same manner.

Figure 1.1: Treatment ingredients of dietary feed additives OmniGen-AF® (OMN) and Control (CON)

Treatment Ingredients	
OmniGen-AF®	**Control**
Processed grain by-products	Processed grain by-products
Silicon dioxide	Iron oxide
Calcium aluminosilicate	
Sodium aluminosilicate	
Brewers dehydrated yeast	
Yeast extract	
Niacin supplement	
D-calcium pantothenate	
Choline chloride	
Vitamin B-12 supplement	
Iron oxide	

At time of milking, study animals were separated from the regular herd in the holding pen by the crowd gate. Inoculated cattle were placed on one side of the double eight parallel parlor and the control on the opposite side to ensure no cross contamination. Cows were milked twice daily at the Central Maryland Research and Education Center, Clarksville dairy facility at 0630 and 1600 h.

Bacterial Inoculation

To reproduce clinical signs of mastitis seen in pilot studies, *Streptococcus uberis* strain 0140J (ATCC # BAA-854) was used as the mastitis causing pathogen which was obtained from Dr. Daniel Nelson's Laboratory (Institute of Bioscience and Biotechnology Research. Rockville, MD). The strain was maintained in a cryovial at -20°C. A stock of bacteria was sub-cultured in a shaking water bath of 37°C in Todd Hewitt broth for 5-6 h then periodically transferred to cuvettes to monitor optical density

(OD) peaks at approximately 0.5 in the Spectrophotometer (Shimadzu UV-1280 Serial # A120653). Once incubation peak, Todd Hewitt broth inoculant was diluted starting with a 1:10 dilution in sterile Ringer's Solution until coliform forming units equal approximately 1000 CFU per milliliter. Sub-cultures were made and plated on Tryptic Soy Agar with 5% sheep's blood in triplicate to detect possible contamination and calculate total volume of inoculant.

Dilutions for total volume for eight cows of inoculum was calculated prior to the inoculation date (day 42) and then reproduced the night before inoculation.

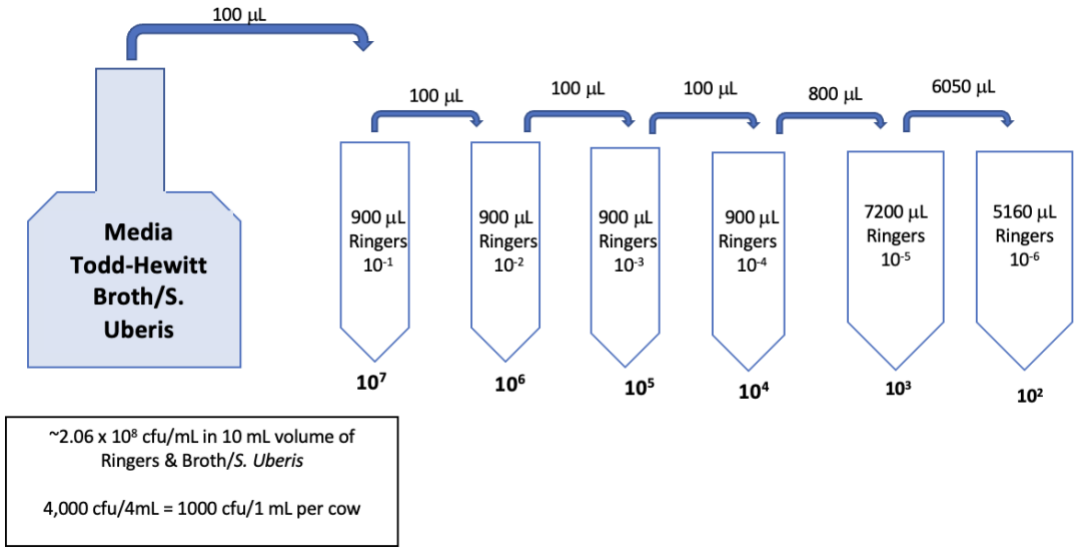

Figure 1.2: Dilution calculation for *S. uberis* and Ringer's Solution inoculum concentration

This calculation was based on the bacteria growth prior to the inoculation day. Each of the eight cows chosen for infection received a sterile syringe containing 4 mL of the inoculum that was inserted into the teat using the partial insertion method in the parlor.

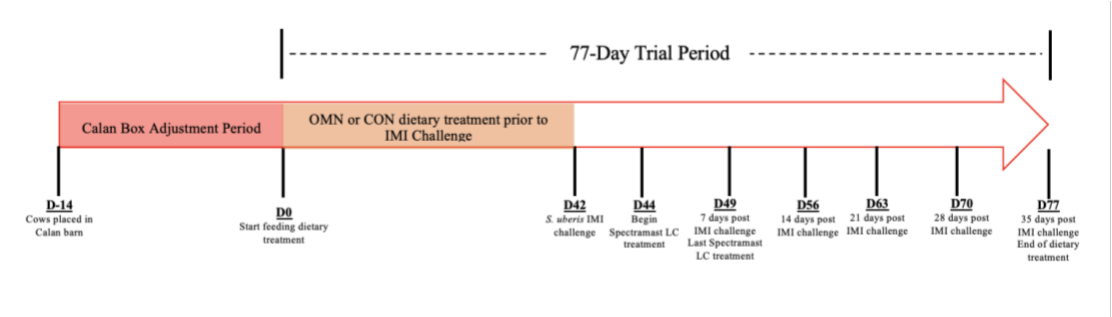

Figure 1.3: Timeline of dietary treatment administration of OmniGen-AF® (OMN) or Control (CON) and *Streptococcus uberis* intramammary infection (IMI) challenge over trial period

The remaining ml of inoculum was plated on tryptic soy agar with 5% sheep's blood in triplicate to calculate the concentration of bacteria inoculated in the cattle. Once the inoculum dried, the plates were inverted and incubated at 37°C for 18h to confirm pathogenic bacteria concentration. Colonies of *S. uberis* were counted and bacterial concentration in ringer's solution were calculated based on the triplicate plates.

Intramammary Treatment

Intramammary antibiotic therapy was administered to challenged cows starting at 48 hours (day 2) after intramammary infection challenge (peak in clinical mastitis signs) until cured (~7days). Spectramast LC (Zoetis, Parsippany, New Jersey), a common mastitis therapy for lactating cattle, was used for intramammary antibiotic treatment. Spectramast LC is a ceftiofur hydrochloride suspension used for treating staphylococci and streptococci and *E. Coli* associated with clinical and subclinical mastitis. Before administration of antibiotics, the infected teat was rigorously cleaned with cotton balls coated with 70% ethanol. Spectramast LC was administered using the partial insertion method. Immediately following treatment, teats were coated in a teat dip cup post-

milking teat disinfectant containing 1% iodine with lanolin. Quarter foremilk was aseptically collected and analyzed for SCC and shedding of *S. uberis* after intramammary infection. Cows that were selected to receive the *S. uberis* inoculum instead of PBS solution in one selected rear quarter were monitored daily to ensure clinical mastitis symptoms did not progress to severe symptoms (i.e., gangrenous mastitis, inability to stand due to udder pain or inflammation, extreme dehydration, or refusal of feed). If Spectramast LC intramammary antibiotic therapy alone is not sufficient in treating the mammary infection and severe symptoms are observed additional therapies were implemented to ensure the health and well-being of the animal and administered by the veterinarian (i.e., intravenous Banamine and Penicillin). Animals expressing distress or severe clinical symptoms needing additional therapies for treatment were removed from the study to allow them to recover from the infection properly with extended antibiotic treatment.

Milk Component and SCC Sampling

Quarter foremilk samples were collected aseptically from all quarters during morning milking on d -14, 0 and 42 prior to inoculation to identify and monitor bacterial status. Following intramammary inoculation challenge (d 42.5), foremilk samples were collected from the selected rear quarter aseptically every 12 hours (prior to morning and afternoon milking) for 7 consecutive days (until d 48.5) and then once weekly until 35 days post-inoculation. Quarter foremilk samples were collected aseptically from all mammary quarters on d 49, 56, 63, 70, and 77 post inoculation to monitor bacterial status and confirm antibiotic treatment cured the mastitis infection. Differences in response to

inoculant dose of *S. uberis* administered were evaluated based on duration of inflammatory response and establishment of infection in quarters infected determined by milk abnormalities, somatic cell count, and change in milk yield. Milk sampling occurred during morning milking when teats were dipped in foam germicide containing hydrogen peroxide, stripped, and wiped dry with single service cloth towels and teat ends were rigorously cleaned with cotton balls containing 70% ethanol. Approximately 150 mL of milk was sampled aseptically into sterile tubes for milk component determination, whey isolation and bacteriological status. Milk was stored on ice and then immediately refrigerated after collection. Approximately 30-20 mL of sampled milk was poured into vails from Lancaster Dairy Herd Improvement Association (DHIA) that were shipped to Manheim, PA for analysis and measurements of somatic cells, protein and fat percentage were taken. SCC values for each cow were given as a logarithm with base 10 with the lowest available count that the Lancaster DHIA can determine from a given milk sample being 13 (13,000 SCC) and the highest count 9701 (9,701,000 SCC).

Health Examinations

Body weight was recorded weekly for each cow upon exiting the parlor following morning milking. Daily milk yield and feed intake was recorded throughout the study period. Physical exams (including heart rate, respiration rate, and rectal temperature, dehydration, appetite, lethargy, posture, and diarrhea), mammary palpation exams (including udder redness, soreness, and swelling) and clinical score of mastitis signs were performed for 7 consecutive days post-inoculation, then once a week for 35 days. Clinical

scored were recorded on a 5-point scale (listed below) and any other health observations will be recorded:

1 = normal milk and normal quarter,

2 = normal quarter but milk was questionable,

3 = normal quarter but abnormal milk,

4 = a swollen quarter and abnormal milk, and

5 = swollen quarter, abnormal milk, and systemic signs of infection.

Forage Sampling

Rations, refusals, and individual feed ingredients were collected from day -14 until day 77 once a week and pooled once a month for analysis by Cumberland Valley Analytical Services (CVAS) in Waynesboro, PA. Samples were stored at -20°C until pooling (thaw in -4°C). The forage samples collected were alfalfa hay, corn silage, and alfalfa haylage. The concentrate samples collected were corn, soybean meal, University of Maryland lactating cow premix, energy booster, top-dress treatment (OmniGen-AF®) and control.

Blood Sampling for cortisol and serum amyloid

Directly after milking, blood samples (50 mL) were collected on day 0, once daily for 7 consecutive days post-inoculation, then once a week for 35 days total (day 56, 63, 70, 77). The cows were placed in a chute inside the lactating free stall barn at the time of blood sampling and blood was collected via coccygeal venipuncture into evacuated serum separator tubes for serum. The area of skin used for blood collection was cleaned

with rubbing alcohol and then immediately after, pressure was applied to the puncture and massaged to stop the bleeding. Blood was placed on ice directly after taken and then stored in the fridge until transport.

Serum tubes were allowed to clot for 60 minutes at room temperature before being spun in centrifuge at 1,300 x g for 15 minutes at 22°C. The blood serum was then aliquoted into microcentrifuge tubes and then stored at -80°C at University of Maryland, College Park until sent to Phibro Animal Health in Corvallis, Oregon for cortisol and serum amyloid assay analysis.

Whey Isolation and Milk Culture for cytokine TNF-alpha and IL-10

After returning from the CMREC facility, whey was isolated from milk within three days of collection using the previously described procedure (Moyes et al., 2009). Initially, 40 mL of inverted milk was transferred into ultracentrifuge polycarbonate tubes then spun at 44,000 x g for 30 minutes at 4°C in ultracentrifuge. To prepare for the second spin, the whey layer was transferred to a clean ultracentrifuge tube and was spun again at 44,000 x g for 30 minutes at 4°C. After the second spin, the layer of whey was decanted and then filtered through 0.45 µm mixed cellulose esters syringe filter (33 mm) into a pre-labeled 1.5 mL microcentrifuge tubes. Whey samples placed in microcentrifuge tubes were stored at -80°C at the University of Maryland, College Park until sent to Phibro Animal Health in Corvallis, Oregon for cytokine TNF-alpha and IL-10 analyses.

Quarter foremilk samples for culture were collected aseptically in compliance to National Mastitis Council (2004) recommendations. A 100 µL subculture from all bovine

milk was plated onto tryptic soy agar with 5% sheep's blood using the swirl plate method using a sterile, disposable L-shaped cell spreader. Samples that produced overgrowth (or there were too many cells to count) were serial diluted with sterile, endotoxin-free PBS to facilitate colony counting and replated. Once the milk dried the plates were inverted and incubated at 37°C for 18h to confirm pathogenic bacteria infection and were plated in triplicate. Colonies of *S. uberis* were counted and bacterial concentration in milk was calculated based on the triplicate plates. A positive isolation for any one pathogen was determined when a minimum of three colony forming units (CFUs) were identified on a plate; three or more types of pathogens isolated from the same milk culture as determined to be a contamination.

Statistical Analysis

Statistical analyses were conducted using JMP Pro 15 (JMP®, Version *15*. SAS Institute Inc., Cary, NC, 1989–2021). A fit model was used: $y = \mu + OMN + INOC + OMN*INOC$ for response variables by each repeated measure (e.g., day). Each sampling over time was used as a by variable. This model was fit to each time point individually or to the means of the four weeks post challenge. For this model Y includes LogSCC, average LogSCC, fat percentage, protein percentage, average milk yield, and average daily feed intake, μ is the mean of the population, OMN is the effect of the administration of OmniGen-AF® or control to daily feed, INOC is the effect of inoculation of *Streptococcus uberis* or no inoculation, OMN*INOC is the effect of the interaction between administration of OmniGen-AF® and inoculation of *S. uberis*. This model measured the effect of feeding OmniGen-AF® and *S. uberis* inoculation on total somatic

cell count, fat percentage, protein percentage, milk yield, and feed intake over time. Significance was set at $P < 0.05$, and tendencies were determined if $P < 0.10$. Results are reported according to treatment effects.

Trial Round I Results

Table 1.1: Effects of OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on LogSCC least squared means

	No Challenge		**With Challenge**		**Challenge**	**OMN**	**Interaction**
Day	OMN +	OMN -	OMN +	OMN -	P<	P<	P<
0	1.2	1.4	1.3	1.5	NS	NS	NS
0.5	1.2	1.7	1.3	1.6	NS	0.1**	NS
1	1.1	1.7	3.1	2.7	0.0002*	NS	0.09**
1.5	1.2	1.8	3.5	2.8	<.0001*	NS	0.0477*
2	1.1	1.7	3.7	3.3	<.0001*	NS	0.09**
2.5	1.4	2.1	3.5	3.6	<.0001*	NS	NS
3	1.2	1.8	3.5	3.5	<.0001*	NS	NS
3.5	1.7	2.0	3.8	3.4	<.0001*	NS	0.0106*
4	1.9	2.2	2.7	2.1	NS	0.05**	NS
4.5	1.6	2.3	3.0	2.1	NS	NS	NS
5	1.7	2.1	2.6	2.0	NS	NS	NS
5.5	1.8	1.7	2.4	2.0	NS	NS	NS
6	1.7	2.0	2.4	1.9	NS	NS	NS
6.5	1.8	2.0	2.6	2.0	NS	NS	NS
7	1.2	1.4	2.4	2.4	<.0001*	NS	NS
14	1.4	1.4	2.7	1.7	0.0002*	0.0231*	0.0067*
21	1.4	1.6	1.3	1.9	NS	NS	NS
28	1.4	1.5	2.3	2.0	0.0006*	NS	NS
35	1.4	1.5	1.5	1.4	NS	NS	NS

* Indicating significance at $P < 0.05$
** Indicating a trend at $P < 0.10$

Log SCC was higher ($P<0.05$) for the Challenge groups (*S. uberis*) compared to unchallenged groups on days 1-3.5, 7, 14, and 28 and tended ($P <0.10$) to be higher on Days 0.5 and 4 (Table 1.1). There was an interaction ($P <0.05$) between OmniGen-AF and Challenge (*S. uberis*) on Days 1.5, 3.5, and 14, and a tendency ($P <0.10$) for an interaction on Days 1 and 2. The OmniGen treated cows had higher Log SCC during challenge than the cows not receiving OmniGen that were challenged, but the OmniGen

treated cows had lower Log SCC when not challenged compared to the cows not receiving OmniGen. This interaction suggests a stronger immune response raised somatic cell counts during challenge for the OmniGen treated cows.

SCC values for least squared means for cows inoculated with *S. uberis* (With Challenge) while administered OmniGen-AF (OMN+) increased from ~20,000 SCC on Day 0.5 to ~1,259,000 SCC on Day 1, with a peak on Day 3.5 of a SCC of ~6,310,000 and declined after Day 4. Values for cows inoculated with *S. uberis* (With Challenge) while not administered OmniGen-AF (OMN-) displayed increased Log SCC LS-means on Day 1 (~501,000 SCC compared to ~40,000 SCC on Day 0.5) with a peak of ~3,981,000 SCC on Day 2.5 and steadily declined thereafter.

Table 1.2: Average LogSCC of the selected mammary quarter least squared means for the week of inoculation compared to the four weeks post-inoculation

	No Challenge		**With Challenge**		**Challenge**	**OMN**	**Interaction**
Day	OMN +	OMN -	OMN +	OMN -	P<	P<	P<
0-7	1.4	1.9	2.8	2.4	0.0006*	NS	0.09**
14-35	1.2	1.5	2.1	1.7	0.03*	NS	0.1**

* Indicating significance at $P < 0.05$
** Indicating a trend at $P < 0.10$

Challenge groups had higher (P<0.05) Log SCC during Days 0-7 and 14-35 (Table 1.2). There was no main effect of OmniGen treatment, but there was a tendency (P<0.1) for an interaction of OmniGen by Challenge during both aggregated time periods. As for the data described previously by twice per day for all quarters combined, the quarter data showed OmniGen treated cows had higher Log SCC when challenged than the non-OmniGen cows, but had lower Log SCC when not challenged.

Table 1.3: Effects of OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on least squared means of average daily feed intake for the week of inoculation and the average feed intake of the four weeks post inoculation

	No Challenge		**With Challenge**		**Challenge**	**OMN**	**Interaction**
Day	OMN +	OMN -	OMN +	OMN -	P<	P<	P<
0	78.5	73.5	76.7	76.0	NS	NS	NS
1	69.2	68.4	74.4	73.7	NS	NS	NS
2	71.0	61.6	63.1	64.8	NS	NS	NS
3	77.6	70.0	72.6	80.6	NS	NS	0.03*
4	70.0	72.2	68.4	73.5	NS	NS	NS
5	71.0	65.6	71.0	64.4	NS	NS	NS
6	70.4	69.6	70.1	68.9	NS	NS	NS
7	72.6	64.8	71.7	61.9	NS	0.008*	NS
14	70.3	68.2	62.2	62.0	0.08**	NS	NS
21	64.7	69.4	69.6	65.2	NS	NS	NS
28	59.3	58.2	66.2	61.2	NS	NS	NS
35	68.5	64.1	65.2	66.5	NS	NS	NS

* Indicating significance at $P < 0.05$
** Indicating a trend at $P < 0.10$

Least squared means (LS-mean) of average daily feed intake for the week of inoculation were compared to the average weekly feed intake for the four weeks post inoculation. Cows that were not inoculated with *Streptococcus uberis* (No Challenge) and administered OmniGen-AF® (OMN+) typically had a higher LS-mean values for average daily and weekly feed intake compared to cows not administered OmniGen-AF (OMN-) throughout the trial period. Cows inoculated with *S. uberis* (With Challenge) and administered OmniGen-AF® (OMN+) typically had higher LS-mean values for average daily and weekly feed intake compared to cows not administered OmniGen-AF® (OMN-) over the entire trial period.

Significance was indicated ($P < 0.05$) for OmniGen-AF® groups on Day 7 and for the interaction between Challenge and OmniGen-AF on Day 3. A trend was indicated ($P < 0.10$) for Challenge groups on Day 14.

Table 1.4: Effects of OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on least squared means of average daily fluid milk yield for the week of inoculation and the average weekly milk yield of the four weeks post inoculation

	No Challenge		**With Challenge**		**Challenge**	**OMN**	**Interaction**
Day	OMN +	OMN -	OMN +	OMN -	P<	P<	P<
0	41.2	33.6	38.4	37.6	NS	NS	NS
1	43.6	39.7	38.0	34.4	NS	NS	NS
2	42.6	40.5	37.0	30.5	0.09**	NS	NS
3	43.4	42.1	38.8	31.8	0.03*	NS	NS
4	43.9	40.8	39.2	34.0	NS	NS	NS
5	42.0	39.1	38.4	36.2	NS	NS	NS
6	45.5	40.0	38.9	36.5	NS	NS	NS
7	43.1	40.0	37.5	34.4	NS	NS	NS
14	38.2	41.1	29.2	33.6	0.05**	NS	NS
21	39.2	41.8	36.2	30.2	NS	NS	NS
28	39.3	39.2	32.4	32.7	0.09	NS	NS
35	41.0	33.5	26.7	30.5	0.1**	NS	NS

* Indicating significance at $P < 0.05$
** Indicating a trend at $P < 0.10$

Least squared means (LS-mean) of average daily fluid milk yield for the week of inoculation were compared to the average weekly fluid milk yield for the four weeks post inoculation. Cows that were not inoculated with *Streptococcus uberis* (No Challenge) and were administered OmniGen-AF® (OMN+) displayed consistently similar average fluid milk yield values for the week of inoculation and in comparison, a slight decrease for the three weeks post-inoculation, with values returning to 41.0 lbs on Day 35. Cows that were not administered OmniGen-AF® (OMN-) displayed an increase in average daily fluid milk yield from Day 1 until Day 3, with values tending to be approximately 40.0 ± 1 lbs until Day 35 where the lowest average milk yield is reported. Cows that were inoculated with *S. uberis* (With Challenge) and were administered OmniGen-AF® (OMN+) displayed consistently similar average daily fluid milk yield values of 38.0 ± 1 lbs for the week of inoculation and a noticeable decrease post-inoculation during Days 14, 28, and 35, with the lowest average fluid milk yield value being 26.7 lbs. Cows that were not administered OmniGen-AF® (OMN-) displayed a decrease in average daily milk

yield from Day 0 (37.6 lbs) until Day 4 (34.0 lbs). On Day 5 and 6 average daily fluid milk yields are the closest to the values at the beginning of the week of inoculation, however the average weekly fluid milk yield values seen in the post-inoculation weeks (Day 14-35) indicate that the cows continued to have decreased fluid milk yields.

No significance was displayed for OmniGen-AF® or Interaction between variables; however, significance was indicated ($P < 0.05$) for Challenge groups on Day 3 with trends ($P < 0.10$) seen on Day 2, 14, and 35.

Table 1.5: Effects of OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on least squared means of fat percentage

	No Challenge		**With Challenge**		**Challenge**	**OMN**	**Interaction**
Day	OMN +	OMN -	OMN +	OMN -	P<	P<	P<
0	1.1	1.4	1.1	1.6	NS	0.03*	NS
0.5	3.2	3.1	3.8	3.6	NS	NS	NS
1	1.3	1.7	2.0	2.1	0.08**	NS	NS
1.5	3.5	3.4	4.6	4.2	0.09**	NS	NS
2	1.2	1.3	2.1	2.1	0.0037*	NS	NS
2.5	4.1	3.5	2.9	3.6	NS	NS	NS
3	1.1	1.3	1.8	2.4	0.0002*	0.0415*	NS
3.5	5.0	3.8	3.7	4.7	NS	NS	NS
4	1.8	1.7	1.8	1.6	NS	NS	NS
4.5	2.0	3.8	3.3	3.1	NS	NS	0.09**
5	1.7	2.0	2.1	1.4	NS	NS	NS
5.5	2.5	3.8	2.8	2.5	NS	NS	0.09**
6	1.7	1.8	1.6	1.4	NS	NS	NS
6.5	2.4	3.7	3.0	2.9	NS	NS	0.09**
7	1.1	1.5	1.3	1.5	NS	NS	NS
14	1.4	1.5	1.7	1.4	NS	NS	NS
21	2.3	2.9	1.5	3.2	NS	NS	NS
28	2.2	3.0	2.8	3.2	NS	NS	NS
35	4.4	4.2	4.5	5.0	NS	NS	NS

* Indicating significance at $P < 0.05$
** Indicating a trend at $P < 0.10$

Least squared means (LS-mean) values of milk fat percentage for cows not inoculated with *Streptococcus uberis* (No Challenge) and administered OmniGen-AF® (OMN+) showed a peak at Day 3.5 of 5.0% fat with not clear increase or decrease over the trial period, and for cows not administered OmniGen-AF® (OMN-) a similar fluctuation of percentages was seen over the trial period with a peak at Day 35 of 4.2% fat. For animals inoculated with *S. uberis* (With Challenge) and administered OmniGen-AF® (OM +) LS-mean values of milk percentage peaked on Day 1.5 at 4.6%, while for cows not administered OmniGen-AF® (OMN-) the peak was indicated on Day 35 at 5.0%. For both groups of inoculated animals there was no clear increase or decrease in fat percentages over the trial period.

For the Challenge groups significance was indicated ($P < 0.05$) on Day 2 and 3, while a trend was indicated ($P < 0.10$) on Day 1 and 1.5. Significance was indicated ($P < 0.05$) for OmniGen-AF® groups on Day 0 and 3, with only a trend ($P < 0.10$) for the interaction between Challenge and OmniGen-AF® seen on Day 4.5, 5.5 and 6.5.

Table 1.6: Least squared means by treatment for OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on milk protein percentage

	No Challenge		**With Challenge**		**Challenge**	**OMN**	**Interaction**
Day	OMN +	OMN -	OMN +	OMN -	P<	P<	P<
0	3.0	3.1	3.1	3.1	NS	NS	NS
0.5	3.2	3.3	3.3	3.2	NS	NS	NS
1	3.1	3.3	3.0	3.2	NS	0.1**	NS
1.5	3.1	3.3	3.3	3.1	NS	NS	NS
2	3.0	3.3	3.3	3.3	NS	NS	NS
2.5	3.1	3.3	2.9	3.1	NS	NS	NS
3	3.1	3.3	3.4	3.4	NS	NS	NS
3.5	3.1	3.2	3.6	3.4	0.05**	NS	NS
4	3.3	3.3	3.2	3.2	NS	NS	NS
4.5	3.4	3.3	3.2	3.2	NS	NS	NS
5	3.3	3.4	3.1	3.1	0.09**	NS	NS
5.5	3.3	3.3	3.2	3.2	NS	NS	NS
6	3.3	3.2	3.1	3.1	NS	NS	NS
6.5	3.3	3.3	3.1	3.2	NS	NS	NS
7	3.1	3.2	3.2	3.3	NS	NS	NS
14	3.1	3.3	3.5	3.4	0.03*	NS	0.09**
21	3.1	3.2	3.3	3.5	0.08**	0.009**	NS
28	3.2	3.2	3.2	3.4	NS	NS	NS
35	3.2	3.2	3.1	3.0	NS	NS	NS

* Indicating significance at $P < 0.05$
** Indicating a trend at $P < 0.10$

Least squared means (LS-mean) values of milk protein percentage for cows not inoculated with *Streptococcus uberis* (No Challenge) and administered OmniGen-AF® (OMN+) along with non-administered OmniGen-AF® (OMN-) both had consistently similar protein percentage values (3.0 ± 0.4) throughout the trial period. Cows that were inoculated with *S. uberis* and administered OmniGen-AF® (OMN+) displayed a notable decrease in protein percentage on Day 2.5 of 2.9% from 3.3% on Day 2 and a notable

peak on Day 3.5 of 3.6% protein. Cows that were not administered OmniGen-AF® (OMN-) had consistently similar protein percentages throughout the entire trial period with values at 3.0 ± 0.4.

Significance was indicated ($P < 0.05$) for Challenge groups on Day 14, while no other significance was found for OmniGen-AF® or Interaction. A trend was indicated for Challenge groups on Day 3.5, 5 and 21, OmniGen-AF® groups on Day 1 and 21, along with Day 14 for the Interaction between the variables.

Trial Round II Results

Figure 2.1: Round two cow list detailing treatment keys and therapy treatments administered for mastitis control

Cow	Treatment: OmniGen-AF (OMN+) vs (OMN-) No OmniGen *Strep. uberis* (With Challenge) vs No *Strep. uberis* (No Challenge)
17	OMN-/With Challenge
18	OMN+/With Challenge
19	OMN-/With Challenge
20	OMN+/No Challnege
21	OMN-/With Challenge
22	OMN+/With Challenge
23	OMN-/No Challenge
24	OMN-/No Challenge
25	OMN+/No Challenge
26	OMN+/With Challenge
27	OMN+/No Challenge
28	OMN-/No Challenge
29	OMN+/With Challenge
30	OMN+/No Challenge
31	OMN-/With Challenge
32	OMN-/No Challenge

KEY
Challenged with *Streptococcus uberis* and treated with antibiotics intrammmary Spectramast LC along with intravenous Penicillin and Banamine
Challenged with *Streptococcus uberis* and treated with Spectramast LC only
Euthenized

The call for discovering and confirming the efficacy of potential antimicrobial alternative strategies for controlling mastitis is evident. This was demonstrated further in the second round of sixteen lactating Holstein cows used in this study round, when antibiotics that are commonly used in human medicine were used to aid in treating a severe clinical mastitis infection in five lactating Holstein dairy cows. A mastitis infection was initiated on Day 0 in eight cows were challenged with *Streptococcus uberis* and Ringer's Solution (With Challenge) inoculum after morning milking (Figure 2.1). Preliminary milk samples were taken before the intramammary challenge with *S. uberis*. Four cows were supplemented with OmniGen-AF (OMN+), and four cows were supplemented with a control dried distillers grain (OMN-) top dressed daily on a total mixed ration (Figure 2.1). The remaining 8 cows on study were given a sterile phosphate-buffered saline (PBS) solution intramammarily (No Challenge), four of which were

supplemented with OmniGen (OMN+) and four supplemented with the control (OMN-) as seen in Figure 2.1. Although protocols set in place to ensure sterile procedure were implemented throughout the mammary inoculation procedure after aseptic milk samples were taken on day 0, an unknown contamination occurred in the animals challenged with the inoculum (With Challenge). To determine the source of the contamination, milk samples from the day after inoculation prior to antibiotic treatment (Day 1) were sent to IDEXX Bioanalytics in Columbia, Missouri for bacterial species identification done by maldi-tof analysis. The two cows (19 and 21) that were given the dried distillers grain (OMN-) and *S. uberis* (With Challenge) treatments displayed severe signs of gangrenous mastitis, lethargy, reduced milk yield, udder inflammation, mammary redness, decreased appetite, along with bloody and abnormal milk. A contamination was confirmed in these two cows (19 and 21) through extensive analyses which concluded severe mastitis in these animals was caused by a mixed bacterial infection of *Bacillus cereus* with *Streptococcus uberis* and an unidentified gram-negative coccus. *Bacillus cereus* is gram-positive bacteria frequently seen in cattle with the capability to infect the bovine udder that causes an acute septicemic infection, that has the potential to develop into gangrenous mastitis (Carter, 1990). Gangrenous mastitis develops when there is severe inflammation with signs of swelling, redness and pain that progresses to necrosis of the affected tissue by decreasing blood flow which can result in the area looking blue or black in color (Islam et. al., 2008). This type of mastitis is extremely difficult to treat and can be caused by *Staphylococcus spp.*, *E. coli*, *Clostridia*, or *Bacillus spp*. bacteria species (Islam et. al., 2008). *Bacillus* bacterium species are reported to be the most common laboratory contaminants that are known to be found in air, water, and soil

(Carter, 1990). Laboratory materials used to prepare the inoculum at the University of Maryland were analyzed and no contamination was detected. There was speculation that the cryovial containing the *S. uberis* strain contained a foreign bacterium, however, when analyzed and it was confirmed to only contain *S. uberis*. We hypnotize that the contamination happened in the parlor during morning milking but there is no way to determine the true cause. Cows challenged with *S. uberis* were noticeably lethargic at evening milking (Day 0.5) and noticeably displaying severe symptoms of clinical mastitis by morning milking 24 hours post inoculation (Day 1). Animals were treated with Spectramast LC starting on Day 1 with the addition of intravenous Banamine and Penicillin beginning on Day 2. Ultimately, a veterinarian from Mid-Maryland Dairy Veterinarians determined the animals were under severe stress which in accordance with IACUC protocol were euthanized humanely to discontinue anymore prolonged stress on Day 5.

Cultures of quarter milk samples were taken from the six remaining animals inoculated with *Streptococcus uberis* (With Challenge) and analyzed by Antech Diagnostics (Parkville, MD) to determine the scope of the contamination. Of those cows, two samples (17 that was given OMN-/With Challenge treatment and 29 that was given OMN+/With Challenge treatment seen in Figure 2.1) yielded *Enterococcus spp.* with a wide range of sensitivity to a variety of different antibiotics. It was determined that Spectramast LC was having some effect on combating the infection, however, the sample from cow 17 (OMN-/With Challenge) showed resistance to Streptomycin and Gentamicin but was sensitive to all other drugs tested which are commonly used to treat mastitis. Unfortunately, *S. uberis* and *Enterococcus spp* are structurally and

biochemically similar considering they are both common environmental contaminants which made it difficult to determine if that specific contaminant originated before inoculation. Furthermore, of the remaining animals, three cows (17, 18 and 29) displayed more severe signs of mastitis compared to three other cows (22, 26, and 31) that were also challenged with *S. uberis* (With Challenge). The cows displayed lethargy, decreased appetite, mammary inflammation, bloody and abnormal milk. Cows #17, #18, and #29 that displayed severe signs of mastitis were treated with Spectramast LC starting on Day 1 due to the extreme abnormal milk observed at time of milking and ultimately given additional antibiotics on Day 5 administered by a veterinarian intravenously, Banamine once per day for three days and Penicillin once per day for five days. Cows 22, 26, and 31 that displayed less severe mastitis symptoms were given Spectramast LC as the sole antibiotic treatment and recovered from the mastitis infection without further issue. Cows 17, 18, and 29 (severe mastitis symptom animals) regained appetite and began to develop normal milk qualities after intravenous antibiotic treatments. The cows experienced decreased milk yields at the beginning of the challenge and slowly improved after treatment post-inoculation. Without supplemental antibiotic treatment with Banamine and Penicillin the likelihood of these cows developing severe gangrenous mastitis is high.

Although clear conclusions cannot be made from trial round II, we hypothesize that supplementation of OmniGen-AF may have played a role in the survival of the three cows that experienced severe clinical mastitis symptoms caused by a foreign bacterial contamination. Considering the data from this round is incomplete due to the two euthanized cows, key observations from the animals fed OMN while fighting a severe mastitis infection are of interest. The results from the incomplete round are as follows.

Table 2.1: Effects of OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on LogSCC least squared means

	No Challenge		**With Challenge**		**Challenge**	**OMN**	**Interaction**
Day	OMN +	OMN -	OMN +	OMN -	P<	P<	P<
0	1.2	1.2	1.1	1.1	NA	NA	NA
0.5	1.4	1.8	1.6	1.2	NA	NA	NA
1	1.7	1.3	3.8	3.8	0.0039*	NA	NA
1.5	2.0	1.8	4.0	4.0	0.0028*	NA	NA
2	1.7	1.5	4.0	4.0	0.0006*	NA	NA
2.5	2.8	1.5	2.0	2.4	NA	NA	NA
3	1.6	2.3	2.0	1.1	NA	NA	NA
3.5	1.6	1.7	3.7	3.5	0.0015*	NA	NA
4	1.4	1.6	3.6	3.2	<.0001*	NA	NA
4.5	1.5	1.5	3.3	3.3	0.0003*	NA	NA
5	1.4	1.3	2.1	2.9	0.0419*	NA	NA
5.5	1.6	1.6	3.4	2.9	0.0017*	NA	NA
6	1.5	1.4	2.9	2.6	0.005*	NA	NA
6.5	1.7	2.6	2.8	2.6	0.0328*	NA	NA
7	1.6	1.6	3.1	2.5	0.0157*	NA	NA
14	1.2	1.4	2.2	1.1	NA	0.0444*	0.0108*
21	1.5	1.5	2.7	1.4	NA	NA	NA
28	1.3	1.4	2.0	1.3	NA	NA	NA
35	1.3	1.5	4.0	1.4	0.0002*	<.0001*	<.0001*

* Indicating significance at $P < 0.05$
** Indicating a trend at $P < 0.10$

Log somatic cell count (SCC) was higher (P<0.05) for the Challenge groups (S. uberis) compared to unchallenged groups on Days 1, 1.5, 2, 3.5, 4, 4.5, 5, 6, 6.5, 7, 35. Log SCC was higher (P<0.05) for OmniGen-AF groups (OMN) on Days 14 and 35. There was an interaction (P<0.05) between OmniGen-AF and Challenge (*S. uberis*) on Days 14 and 35. The OmniGen treated cows had slightly higher Log SCC during challenge days 0.5, 2.5, 3, 3.5, 4, 5.5, 6, 6.5, 7, 14, 21, 28, and 35 than the cows not receiving OmniGen that were challenged, but the OmniGen treated cows had lower Log SCC when not challenged compared to the cows not receiving OmniGen. This interaction suggests a stronger immune response raised somatic cells counts during challenge for the OmniGen treated cows.

SCC values for least squared means for cows administered OmniGen-AF (OMN-) on Day 6.5 of ~398,000 SCC. Cows who were inoculated with *S. uberis* (With Challenge) while administered OmniGen-AF (OMN+) and not (OMN-) both displayed peaks in SCC values for least squared means starting on Day 1 with a slight decreased observed on Day 2.5 and 3. On Day 3.5 another increase in SCC values for least squared means is seen, with values of ~5,011,000 for OMN+ and ~3,162,000 for OMN- from the morning of Day 3 values ~100,000 SCC for OMN+ and ~12,000 for OMN-. For cows inoculated with *S. uberis* (With Challenge) and administered OMN (+) there was another spike in SCC values for least squared mean on Day 35 after a fluctuating decrease in values is seen starting from Day 5.5 (Table 2.1). Cows that were inoculated with S. uberis (With Challenge) and not administered OmniGen-AF (OMN-) displayed a decrease in SCC values for least squared means starting on Day 5.5 that fluctuated on Days 21, 28, and 35, however, the value on the last day of the trial (Day 35) was lower compared to cows administered OmniGen-AF (OMN+).

Table 2.2: Effects of OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on least squared means of average daily feed intake for the week of inoculation and the average feed intake of the four weeks post inoculation

	No Challenge		**With Challenge**		**Challenge**	**OMN**	**Interaction**
Day	OMN +	OMN -	OMN +	OMN -	P<	P<	P<
0	121.3	119.8	112.8	122.0	NA	NA	NA
1	123.2	119.4	98.0	118.4	NA	NA	NA
2	121.8	118.0	36.0	40.9	0.0025*	NA	NA
3	111.0	122.7	70.0	76.0	0.1**	NA	NA
4	118.0	114.6	76.4	79.2	NA	NA	NA
5	115.9	120.0	97.0	88.4	NA	NA	NA
6	118.1	119.0	108.0	118.3	NA	NA	NA
7	110.8	109.0	103.0	93.2	NA	NA	NA
14	120.7	118.0	112.0	103.2	NA	NA	NA
21	120.2	116.0	112.0	114.0	NA	NA	NA
28	116.5	118.0	116.0	117.0	NA	NA	NA
35	118.6	121.4	116.6	116.2	NA	NA	NA

* Indicating significance at $P < 0.05$
** Indicating a trend at $P < 0.10$

Challenge groups (*S. uberis*) had higher ($P<0.05$) average daily feed intakes values for Day 2 and tended to have higher values ($P<0.10$) on Day 3. There was no discernable significance in OmniGen-AF groups or Interaction groups. Average daily feed intake values for least squared means for the week of inoculation were compared to the average weekly feed intake for the four weeks post inoculation. Cows inoculated with *S. uberis* (With Challenge) displayed decreased average daily feed intakes during the week of inoculation compared to cows that were not challenged (No Challenge). Average daily feed intake for Challenge groups displayed a steady increase beginning on Day 2 (Table 2.2). Cows that were not inoculated with S. uberis (No Challenge) displayed no treatment effects during the entire trial period.

Table 2.3: Effects of OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on least squared means of average daily fluid milk yield for the week of inoculation and the average weekly fluid milk yield of the four weeks post inoculation

	No Challenge		**With Challenge**		**Challenge**	**OMN**	**Interaction**
Day	OMN +	OMN -	OMN +	OMN -	P<	P<	P<
0	36.5	38.2	38.1	43.8	NA	NA	NA
1	40.0	41.2	22.2	17.2	0.0020*	NA	NA
2	40.1	41.5	16.1	14.7	0.0023*	NA	NA
3	39.5	42.7	20.1	21.3	0.0324*	NA	NA
4	39.1	40.6	21.1	21.0	NA	NA	NA
5	40.0	44.0	25.0	26.4	0.1**	NA	NA
6	39.2	42.0	27.0	28.5	NA	NA	NA
7	40.2	41.1	28.0	28.3	0.1**	NA	NA
14	40.0	40.6	35.0	32.0	NA	NA	NA
21	37.0	38.3	28.0	30.0	NA	NA	NA
28	40.4	40.0	34.0	38.1	NA	NA	NA
35	38.0	36.1	31.1	39.0	NA	NA	NA

* Indicating significance at $P < 0.05$
** Indicating a trend at $P < 0.10$

Least squared means (LS-mean) of average daily fluid milk yield for the week of inoculation were compared to the average weekly fluid milk yield for the four weeks post inoculation. Cows that were not inoculated with *Streptococcus uberis* (No Challenge) and were administered OmniGen-AF® (OMN+) displayed consistently similar average fluid milk yield values for the entire trial length with the lowest value of 36.5 lbs seen on Day 0. Cows that were not administered OmniGen-AF® (OMN-) displayed an increase in average daily fluid milk yield from 38.1 lbs on Day 0 to 42.7 lbs on Day 3, with a peak in fluid milk yield seen on Day 5 of 44 lbs. Cows that were inoculated with *S. uberis* (With Challenge) and were administered OmniGen-AF® (OMN+) had decreased average daily fluid milk yields after Day 0 with a fluctuating increase seen to start on Day 4 of 21.1 lbs, although average fluid milk yields never returned to values seen on Day 0 of 38.1 lbs throughout the trial period. Cows that were inoculated with *S. uberis* (With Challenge) and were not administered OmniGen-AF® (OMN-) also displayed decreased average daily fluid milk yields starting after Day 0 with a value of 43.8 lbs as fluctuating

increased values were seen starting on Day 3 of 21.3 lbs. Neither group of challenged cows returned to the average daily fluid milk yield value that was seen at the start of the trial period. Significance was indicated (P<0.05) on Day 1, 2 and 3 with a trend is indicated (P<0.10) on Day 5 and 7 between Challenge groups (*S. uberis*).

Table 2.4: Least squared means by treatment for OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on fat percentage

	No Challenge		**With Challenge**		**Challenge**	**OMN**	**Interaction**
Day	OMN +	OMN -	OMN +	OMN -	P<	P<	P<
0	1.3	1.5	1.3	1.4	NA	NA	NA
0.5	4.2	3.8	3.1	3.8	NA	NA	NA
1	1.8	1.8	1.4	1.9	NA	NA	NA
1.5	4.0	3.8	2.8	8.2	0.1**	0.0198*	0.0152*
2	1.6	1.5	2.7	4.3	0.0011*	NA	0.0444*
2.5	4.2	3.3	4.0	3.0	NA	NA	NA
3	1.8	1.6	1.6	2.1	NA	NA	NA
3.5	3.5	3.7	3.3	4.5	NA	NA	NA
4	1.8	1.8	2.3	1.9	NA	NA	NA
4.5	3.6	3.6	2.9	3.6	NA	NA	NA
5	1.6	1.5	1.3	2.0	NA	NA	NA
5.5	3.2	3.6	3.6	4.4	NA	NA	NA
6	1.6	1.6	1.9	2.1	NA	NA	NA
6.5	3.8	3.5	2.6	4.4	NA	NA	NA
7	2.1	2.1	1.8	2.2	NA	NA	NA
14	1.5	1.7	1.5	1.5	NA	NA	NA
21	1.8	1.8	2.4	1.9	NA	NA	NA
28	1.5	1.6	1.2	1.2	NA	NA	NA
35	1.8	1.6	1.8	1.4	NA	NA	NA

* Indicating significance at $P < 0.05$
** Indicating a trend at $P < 0.10$

Significance was indicated (P<0.05) on Day 2 and a trend is indicated (P<0.10) on Day 1.5 between Challenge groups (*S. uberis*). Significance was indicated ($P < 0.05$) for OmniGen-AF® groups on Day 1.5 and for the interaction between Challenge and OmniGen-AF seen on Day 1.5 and 2. The challenge significance seen is possibly due to decreased amounts of useable milk samples to calculate fat percentage for the Challenge and OMN– group specifically, which possibly caused fat percentage least squared means

to increase. No discernable conclusions can be made from least squared means of fat percentage for Trial II.

Least squared means (LS-mean) values of milk fat percentage for cows not inoculated with *S. uberis* and administered OmniGen-AF (OMN+) showed a peak on Days 0.5 and 2.5 with values of 4.2% fat with fluctuating increases and decreases throughout the trial period. Cows who were not inoculated with S. uberis and not administered OmniGen-AF (OMN-) showed a peak on Day 0.5 and 1.5 with a value of 3.8% fat, and fluctuating increases and decreases mirrored the days seen in cows administered OMN as seen on Days 1.5, 2.5, 3.5, 4.5, 5.5 and 6.5. For animals inoculated with *S. uberis* (With Challenge) and administered OmniGen-AF® (OMN +) LS-mean values of milk fat percentage peaked on Day 2.5 with a value of 4.0% fat, with other increased values seen on Day 0.5 of 3.1%, Day 3.5 of 3.3% and Day 5.5 of 3.6% fat. Interestingly, the highest least squared mean of fat percentage is seen on Day 1.5 in cows who were inoculated with *S. uberis* (With Challenge) and were not administered OmniGen-AF (OMN-) with a value of 8.2% fat. Other increased values are seen on Day 0.5 of 3.8%, Day 2 of 4.3%, Day 3.5 of 4.5%, Day 5.5 and 6.5 of 4.4% fat.

Table 2.5: Least squared means by treatment for OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on milk protein percentage

	No Challenge		**With Challenge**		**Challenge**	**OMN**	**Interaction**
Day	OMN +	OMN -	OMN +	OMN -	P<	P<	P<
0	3.3	3.2	3.1	3.2	NA	NA	NA
0.5	3.4	3.4	3.1	3.3	NA	NA	NA
1	3.5	3.1	2.9	3.1	NA	NA	NA
1.5	3.5	3.3	3.1	3.6	NA	NA	NA
2	3.5	3.5	3.2	3.5	NA	NA	NA
2.5	3.3	3.3	3.3	3.6	NA	NA	NA
3	3.4	3.3	3.0	3.9	NA	0.0343*	0.0148*
3.5	3.5	3.3	3.5	3.3	NA	NA	NA
4	3.5	3.3	3.4	3.2	NA	NA	Na
4.5	3.5	3.2	3.0	3.3	NA	NA	NA
5	3.5	3.3	3.0	3.2	NA	NA	NA
5.5	3.5	3.3	3.8	3.4	NA	NA	NA
6	3.5	3.3	3.7	3.2	NA	NA	NA
6.5	3.5	3.2	3.1	3.4	NA	NA	NA
7	3.4	3.3	3.6	3.3	NA	NA	NA
14	3.5	3.3	3.3	3.2	NA	NA	NA
21	3.6	3.4	3.5	3.3	NA	NA	NA
28	3.5	3.4	3.3	3.3	NA	NA	NA
35	3.4	3.3	3.0	3.2	NA	NA	NA

* Indicating significance at $P < 0.05$
** Indicating a trend at $P < 0.10$

Least squared means (LS-mean) values of milk protein percentage for cows not inoculated with *S. uberis* and administered OmniGen-AF (OMN+) along with non-administered OmniGen-AF® (OMN-) both had consistently similar protein percentage values (3.1 ± 0.5) throughout the trial period. Cows that were inoculated with *S. uberis* and administered OmniGen-AF® (OMN+) displayed fluctuating values (3.1± 0.6) with the highest percentage seen on Day 5.5 of 3.8% protein. Cows that were inoculated with *S. uberis* and not administered OmniGen-AF® (OMN-) also displayed fluctuating values (3.1± 0.5) with the highest percentage seen on Day 3 of 3.9% protein. Significance was indicated ($P < 0.05$) for OmniGen-AF® and Interaction on Day 3, while no other significance was found for Challenge groups.

Discussion

In the trial round I, our goal was to monitor the period of extreme stress dairy cows experience from the beginning of mastitis infection until post-infection with antibiotic treatment. We wished to determine if supplementation of OmniGen-AF® for 42 d before challenging cows with *S. uberis* and 35 d post challenge would help boost innate immune activity, reduce recovery time, and reduce the prevalence of other typical effects from mastitis infections (i.e., decreased milk yield, increased SCC, decreased appetite). Our goal for trial round II was the same as the first, however, the immunomodulatory ability of OMN was of strong interest for trial round II due to the severe clinical and gangrenous mastitis development which placed cows under a period of extreme stress.

OmniGen-AF® has demonstrated immunomodulatory effects and benefits across many applications, pathogens, and animal models. Daily feeding of an immunomodulatory feed additive has been reported to enhance immunity, stimulate PMN antibacterial activity, enhance expression of cell surface trafficking proteins, and increase *S. uberis* phagocytosis (Wang et al., 2007, 2009; Rowson et al., 2011; Ryman et al., 2013; Nace et al., 2014). Research has shown that Holstein cows that were supplemented with OMN 60 d prior to dry-off, during the dry period, and through the first 30 DIM for a total of 150 d exhibited less mastitis, lower SCC, and greater fluid milk yield the treated animal's first DHIA test compared with control cows (Nickerson et al., 2019).

These results were similarly observed in trial round I, wherein cows not administered OMN and not challenged with *S. uberis* displayed fluctuating increased SCC least squared means compared to cows supplemented with OMN, with means on d 2.5 increasing to ~125,000 SCC compared to ~25,000 SCC seen on d 0 and d 7 (Chart

1.1). However, cows that were supplemented OMN (+) and challenged with *S. uberis* (With Challenge) had significantly higher SCC compared to cows supplemented with the control (OMN-) and challenged with *S. uberis*. Normal migration of bovine PMN to the sight of infection causes increased SCC (Paape et al., 1991). The ability of OmniGen-AF® to improve influx of PMNs to the sight of infection and upregulation of important membrane receptors associated with binding to an invading pathogen has been shown to aid in innate immune function and pathogen phagocytosis in animals fed this immunomodulator (Rowman et al., 2011; Nace et al., 2014; Nickerson et al., 2019). This ability is displayed in the increase in log SCC increase in the cows fed OMN and challenged with *S. uberis* from d 0.5 to d 1; approximately 20,000 SCC to 1,259,000 SCC respectively. These animals continued to maintain a high log SCC from d 1 until d 3.5 when on d 4 a noticeable decrease was observed; approximately 501,000 SCC. In comparison, cows that were not supplemented with OMN and were challenged with *S. uberis* demonstrated a lower progression of increased log SCC with approximately 40,000 SCC on d 0.5 and ~500,000 SCC by d 1. The log SCC peaks at d 2.5 but does not return to levels seen on d 0 until d 35. This is interesting since animals supplemented with OMN and challenged with *S. uberis* displayed a peak on d 3.5 and values fluctuate in varying decreasing log SCC least squared means until d 21, where the value returns to that seen on d 0. This suggests that PMN response to the sight of infection was higher than animals not supplemented with OMN and challenged with *S. uberis*. It can be assumed that the challenged and immunomodulatory supplemented animals had an improved innate response.

Trial round II cows that were inoculated with *S. uberis* (With Challenge) and supplemented with OmniGen-AF (OMN+) typically had higher Log SCC values than cows that were not supplemented with OmniGen-AF (OMN-). This can be attributed to the ability of OmniGen-AF® to improve migration and influx of PMNs to the sight of infection and upregulation of important membrane receptors associated with binding to an invading pathogen have been proven to aid in innate immune function and pathogen phagocytosis in animals fed this immunomodulator (Rowman et al., 2011; Nace et al., 2014; Nickerson et al., 2019). Cows that were challenged with S. uberis and given OMN (+) typically had numerically higher Log SCC least squared means compared to OMN (-) cows, and this held true throughout the entire 35-day challenge trial. This suggests that PMN response to the sight of infection was higher than animals not supplemented with OMN and challenged with *S. uberis*. It can be assumed that the challenged and immunomodulatory supplemented cows had an improved initial innate immune response. Cows who were not challenged with S. uberis but were given OmniGen-AF (OMN+) displayed more irregular LogSCC least squared mean values compared to OMN- supplemented cows. OmniGen-AF supplemented cows had more consistent LogSCC values compared to OMN- cows, except during the first few days of the challenge trial period when they had numerically higher values on Days 1, 1.5, 2 and 2.5. Although the bacteria which caused the contamination and the elevated SCC in trial round II was determined, the true method of the contamination was never determined. Bacterial load could be a contributing factor in the severity of the gangrenous mastitis each cow developed. The contamination of a *Bacillus* species while a cow already is inoculated with *S. uberis*, a strong environmental pathogen, may have contributed to the severe

mastitis infection (Jayarao et al., 1999; Phuektes et al., 2001). An interesting observation that should be noted, two of the three cows that developed severe clinical mastitis symptoms were administered OmniGen-AF (OMN+) throughout the entire trial period, while the third cow was supplemented with the control (OMN-). Although clear conclusions cannot be made the results found in trial round II, the results found could prove helpful in future research.

Lactating Holstein cows have shown benefits from continuous OMN supplementation in diet rations according to Nickerson et al. (2019) and in another study fluid milk levels during the start of the lactation cycle improved compared to control animals (Hurley et al., 2019). The cows fed OMN (+) in both Challenge groups typically had a numerically higher average daily feed intake the week of inoculation and average weekly feed intake the four weeks post inoculation compared to animals not fed OMN (OMN-). However, results were not significant owing perhaps to the small sample size.

Results found in milk fat and protein percentages throughout the trial round I and II for all groups of animals also suggests interesting findings. In trail round I, milk fat percentage for cows challenged with S. uberis (With Challenge) and supplemented with OMN (OMN+) showed a peak value of 4.6% on d 1.5 along with fluctuating increases and decreases in fat percentages throughout the trial until the last day, d 35, where the second highest value is observed (4.5% fat). Cows that were challenged with *S. uberis* (With Challenge) and not supplemented OMN (-) had a peak milk fat percentage at d 35 of 5.0% and the second highest value on d 3.5 at 4.7%, again with fluctuating increases and decreases in fat percentage throughout the trial. Although the milk fat percentages are not consistent there is a trend towards effects caused by the challenge and from the

interaction of the immunomodulator with *S. uberis*. Less conclusive results were seen in milk protein percentage where trends were seen in challenge, OMN, and interaction effects even though protein percentages remained between 2.9 and 3.6 in all treatment groups. In trail round II, milk fat and protein percentages for cows challenged with S. uberis and supplemented with OmniGen-AF (OMN+) or the control (OMN-) fluctuated in least squared means values throughout the entire trial period. There were no clear trends to protein percentages for any treatment group but there was an indication of significance ($P < 0.05$) on d 3 for OMN group effects and the interaction between OmniGen-AF and *S. uberis.* Milk fat percentage had fluctuating least squared means values for all treatment groups as well, although a trend ($P < 0.10$) was indicated for challenge groups on d 1.5. Significance ($P < 0.05$) was indicated on d 1.5 for OMN and Interaction groups, along with significance indicated on d 2 for challenge and interaction groups. One hypothesized cause of these results is the observation of the ability of yeast, *Saccharomyces cerevisiae*, to increase in milk fat percentage which can be attributed to increased flow of microbial protein in the small intestine from the addition of yeast in the diet. Furthermore, it was noted that an increase in protein could be attributed to a protein deficient diet which in which yeast would also benefit the host by increasing microbial protein flow (Putman et al. 1997; Chiquette, 2009). Although these composition results can be variable it is an interesting benchmark to monitor when supplementing OMN in a lactating dairy diet and may be beneficial for producers looking to improve milk composite composition.

Further analysis of milk and blood samples using ELISA immunoassays to monitor the effects OMN has on cytokine and cortisol levels throughout mastitis infection

is needed. These analyzes have the potential to add data supporting the results establishing OmniGen-AF® as immunomodulator that improve innate immune system activity and with continuous addition in the diet has the potential to play a role in prevention of dairy cattle environmental mastitis.

Conclusion

This study has demonstrated that, using OmniGen-AF® improved immune response when combating a mastitis infection. Although OmniGen-AF® did not completely eliminate mastitis infection completely in either trail round, there were promising results that show a tendency to reduce *Strep. uberis* infection.

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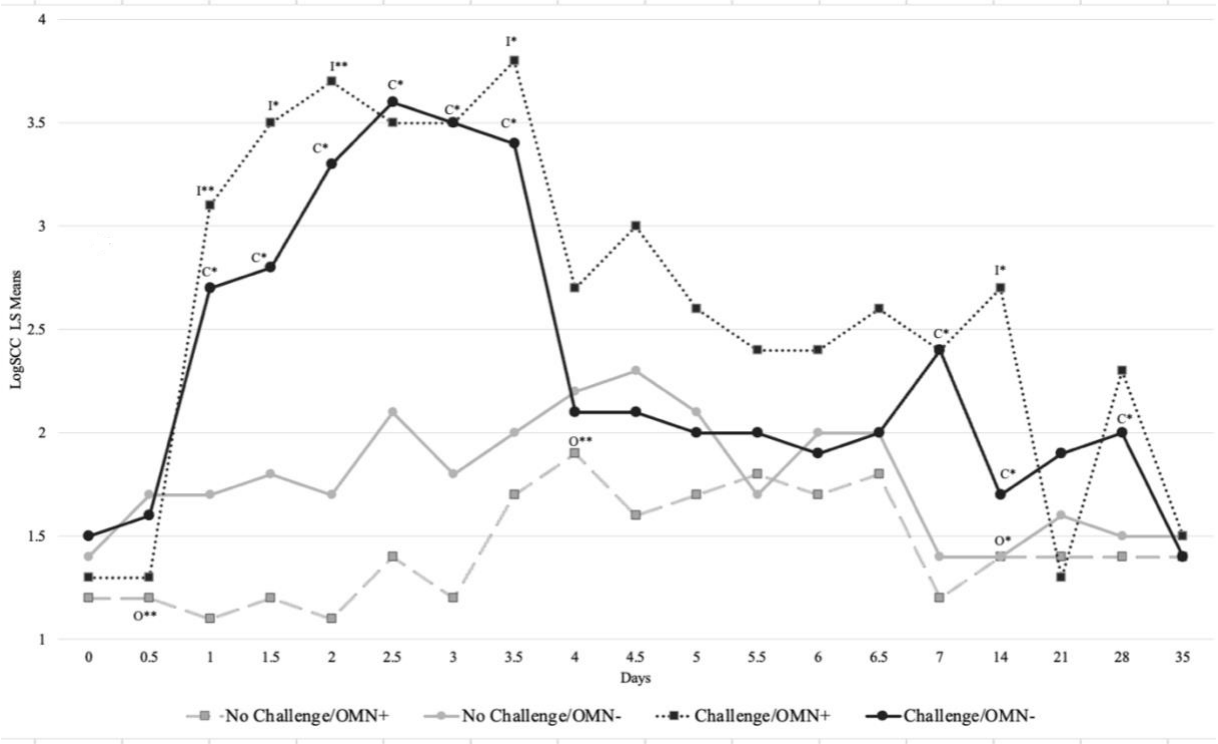
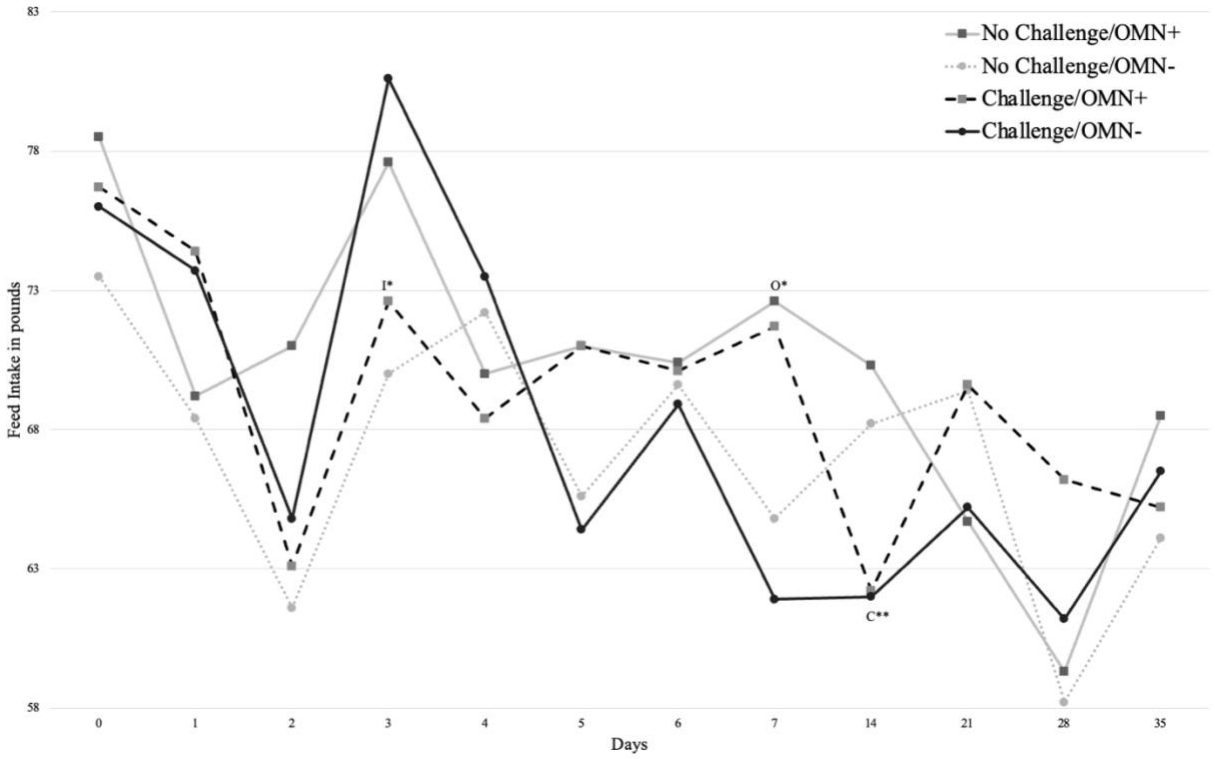
Appendices

Chart 1.1: Effects of OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on LogSCC least squared means



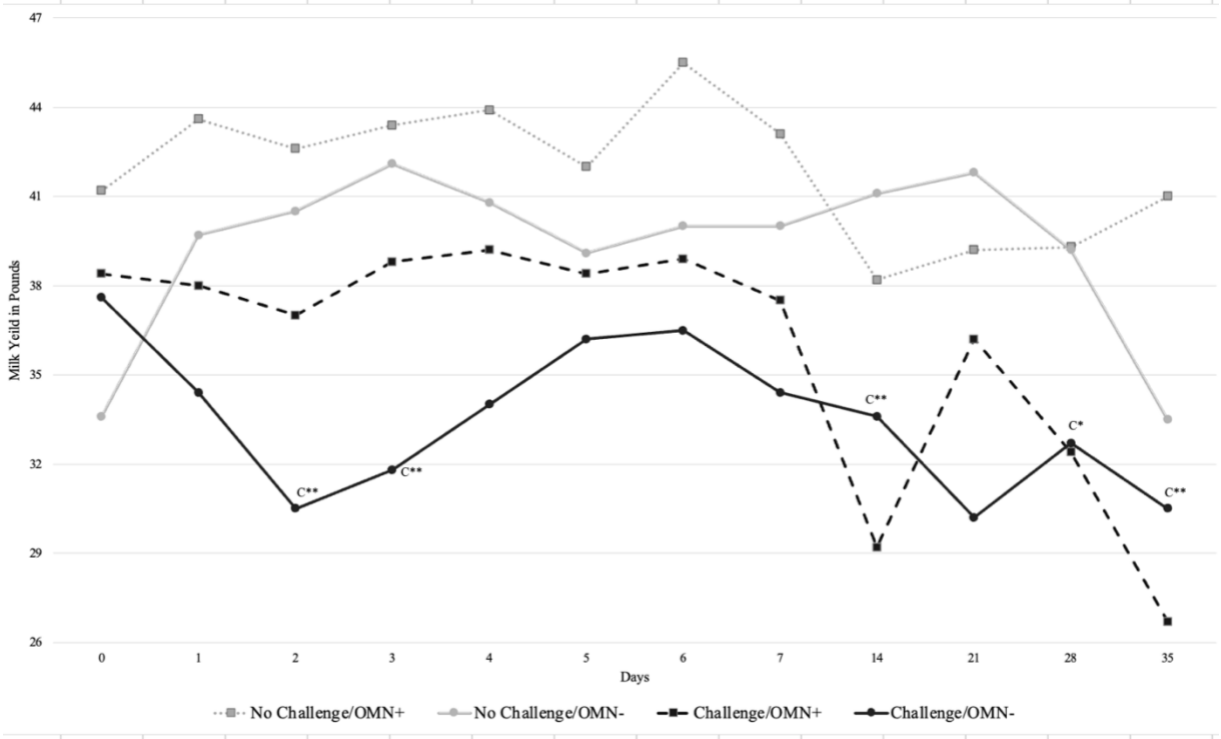
* Indicating significance at $P < 0.05$
** Indicating a trend at $P < 0.10$
(C) Indicating Challenge Group *P* value
(O) Indicating OmniGen-AF Group *P* value
(I) Indicating Interaction between Challenge and OmniGen-AF *P* value
Day 0.5 – Inoculation occurred
Day 3 – Antibiotic treatment begins

Chart 1.2: Effects of OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on least squared means of average daily feed intake for the week of inoculation and the average feed intake of the four weeks post inoculation



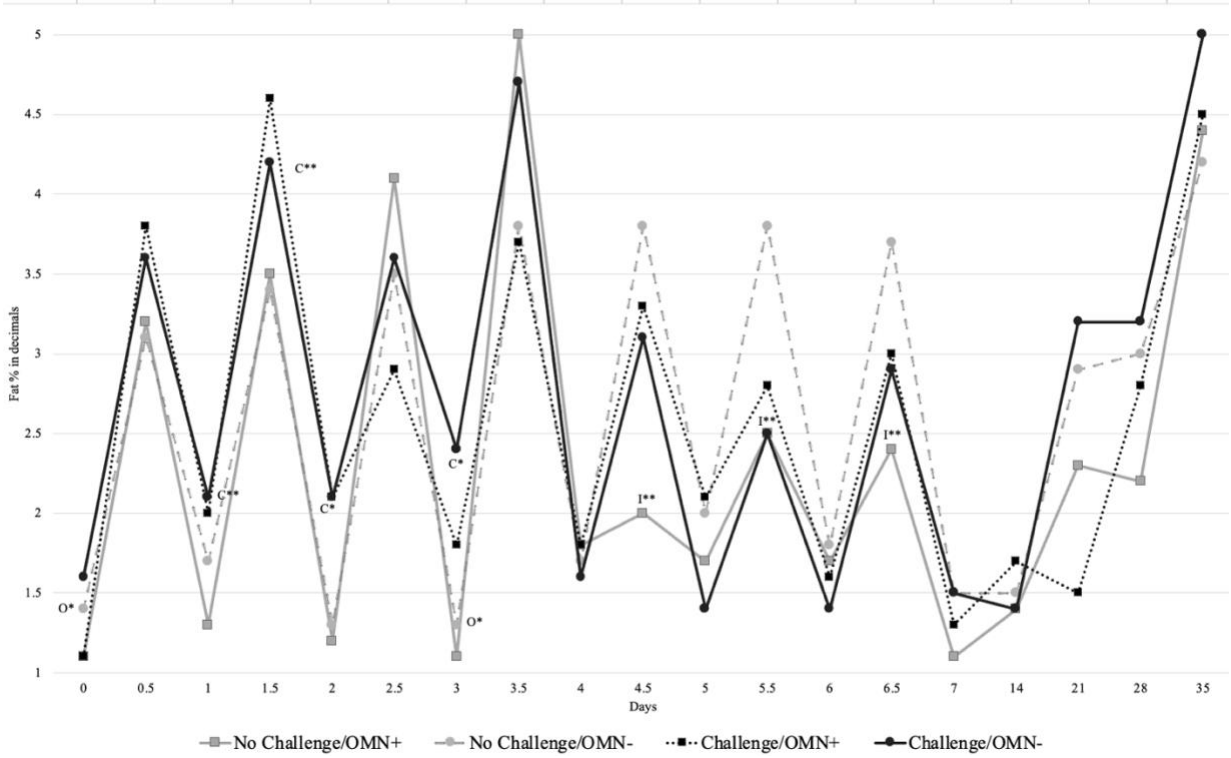
* Indicating significance at $P < 0.05$
** Indicating a trend at $P < 0.10$
(C) Indicating Challenge Group *P* value
(O) Indicating OmniGen-AF Group *P* value
(I) Indicating Interaction between Challenge and OmniGen-AF *P* value
Day 0.5 – Inoculation occurred
Day 3 – Antibiotic treatment begin

Chart 1.3: Effects of OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on least squared means of average daily fluid milk yield for the week of inoculation and the average weekly milk yield of the four weeks post inoculation



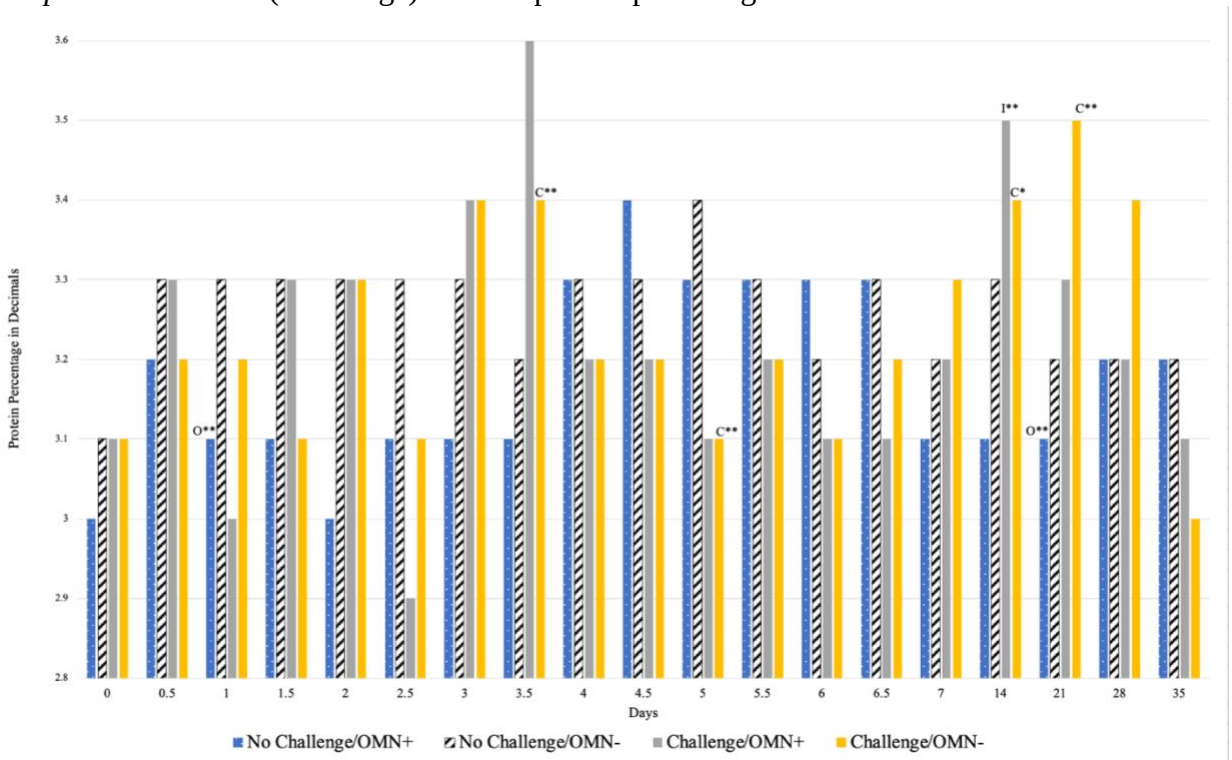
* Indicating significance at $P < 0.05$
** Indicating a trend at $P < 0.10$
(C) Indicating Challenge Group *P* value
Day 0.5 – Inoculation occurred
Day 3 – Antibiotic treatment begins

Chart 1.4: Effects of OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on least squared means of fat percentage



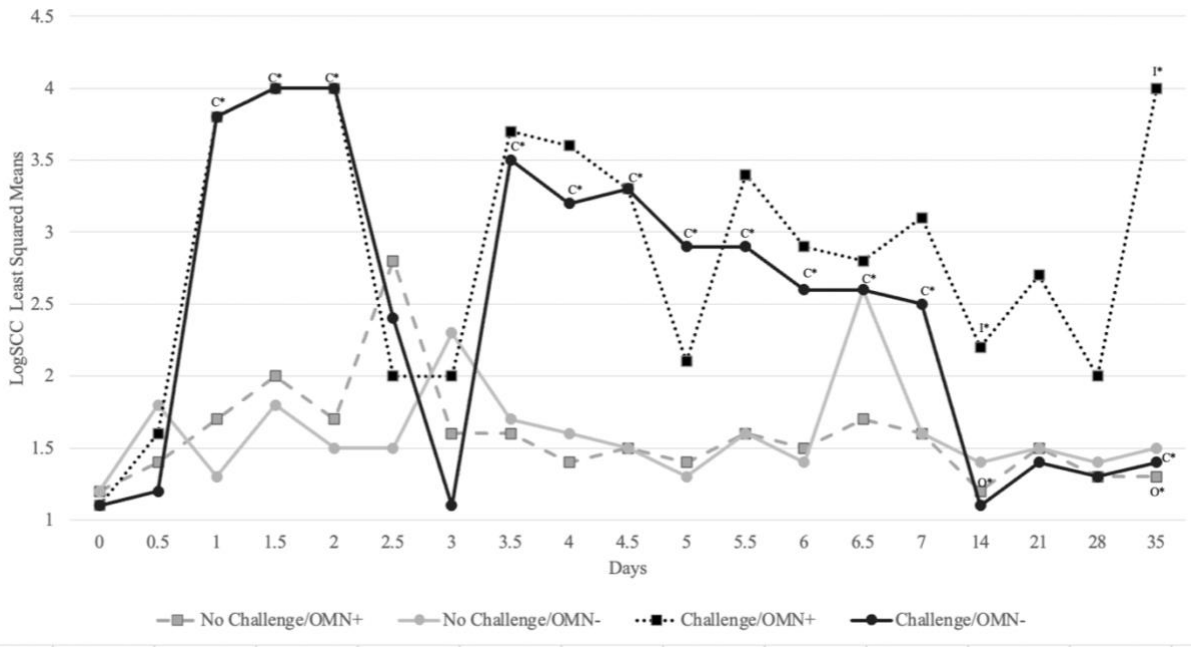
* Indicating significance at $P < 0.05$
** Indicating a trend at $P < 0.10$
(C) Indicating Challenge Group *P* value
(O) Indicating OmniGen-AF Group *P* value
(I) Indicating Interaction between Challenge and OmniGen-AF *P* value
Day 0.5 – Inoculation occurred
Day 3 – Antibiotic treatment begins

Chart 1.5: Least squared means by treatment for OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on milk protein percentage



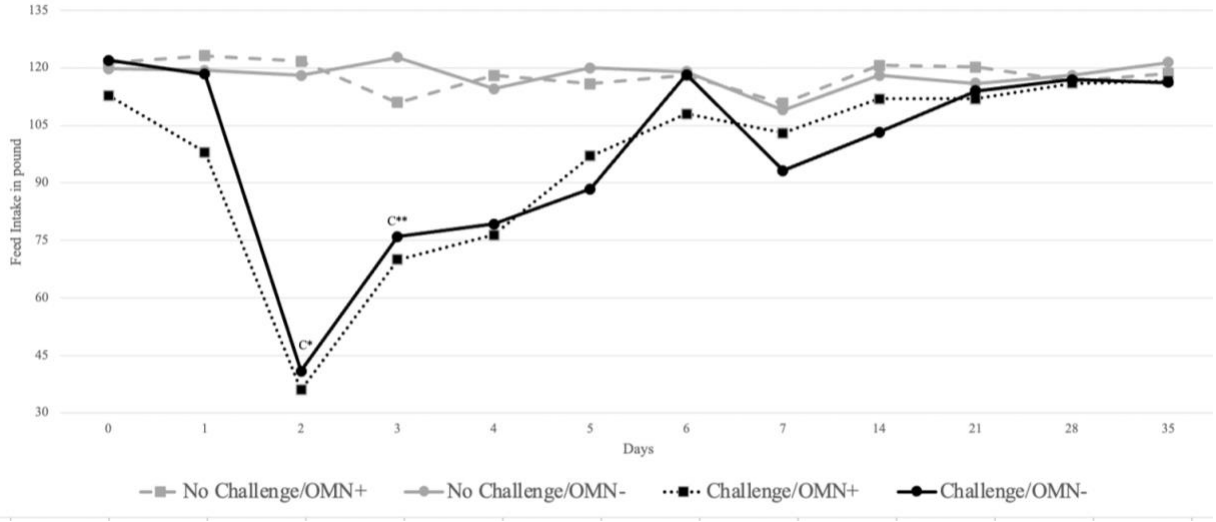
* Indicating significance at $P < 0.05$
** Indicating a trend at $P < 0.10$
(C) Indicating Challenge Group *P* value
(O) Indicating OmniGen-AF Group *P* value
(I) Indicating Interaction between Challenge and OmniGen-AF *P* value
Day 0.5 – Inoculation occurred
Day 3 – Antibiotic treatment begins

Chart 2.1: Effects of OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on LogSCC least squared means



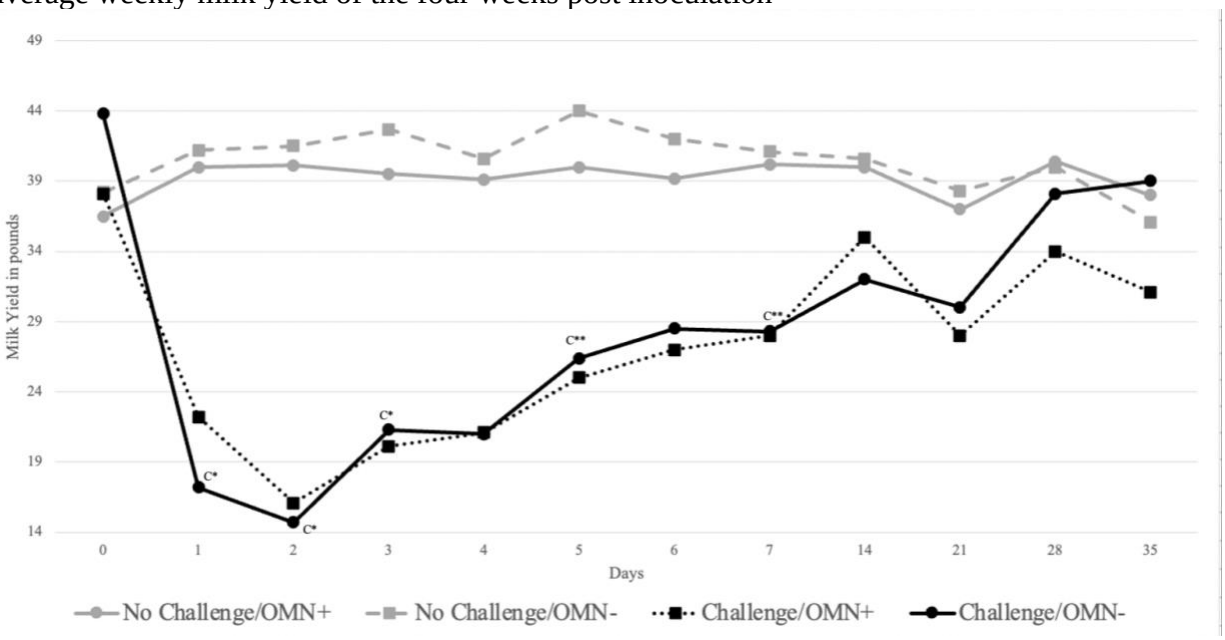
* Indicating significance at $P < 0.05$
(C) Indicating Challenge Group *P* value
(O) Indicating OmniGen-AF Group *P* value
(I) Indicating Interaction between Challenge and OmniGen-AF *P* value
Day 0.5 – Inoculation occurred
Day 3 – Antibiotic treatment begins

Chart 2.2: Effects of OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on least squared means of average daily feed intake for the week of inoculation and the average feed intake of the four weeks post inoculation



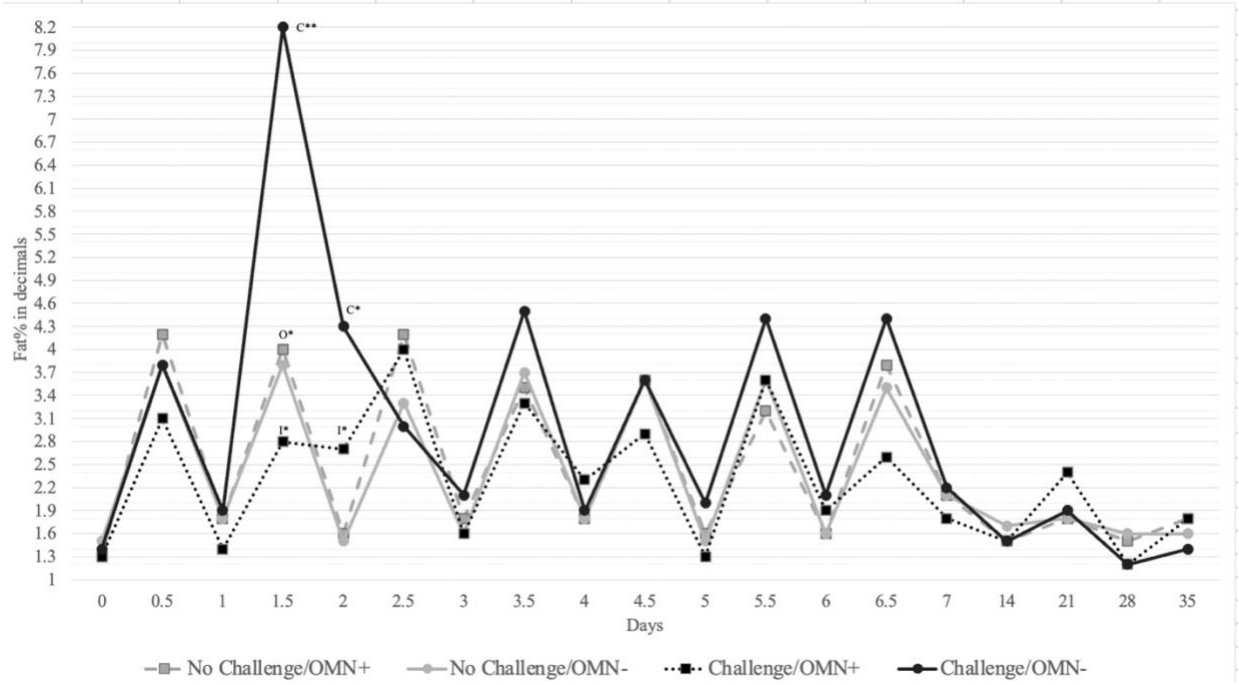
* Indicating significance at $P < 0.05$
** Indicating a trend at $P < 0.10$
(C) Indicating Challenge Group *P* value
Day 0.5 – Inoculation occurred
Day 3 – Antibiotic treatment begins

Chart 2.3: Effects of OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on least squared means of average daily milk yield for the week of inoculation and the average weekly milk yield of the four weeks post inoculation



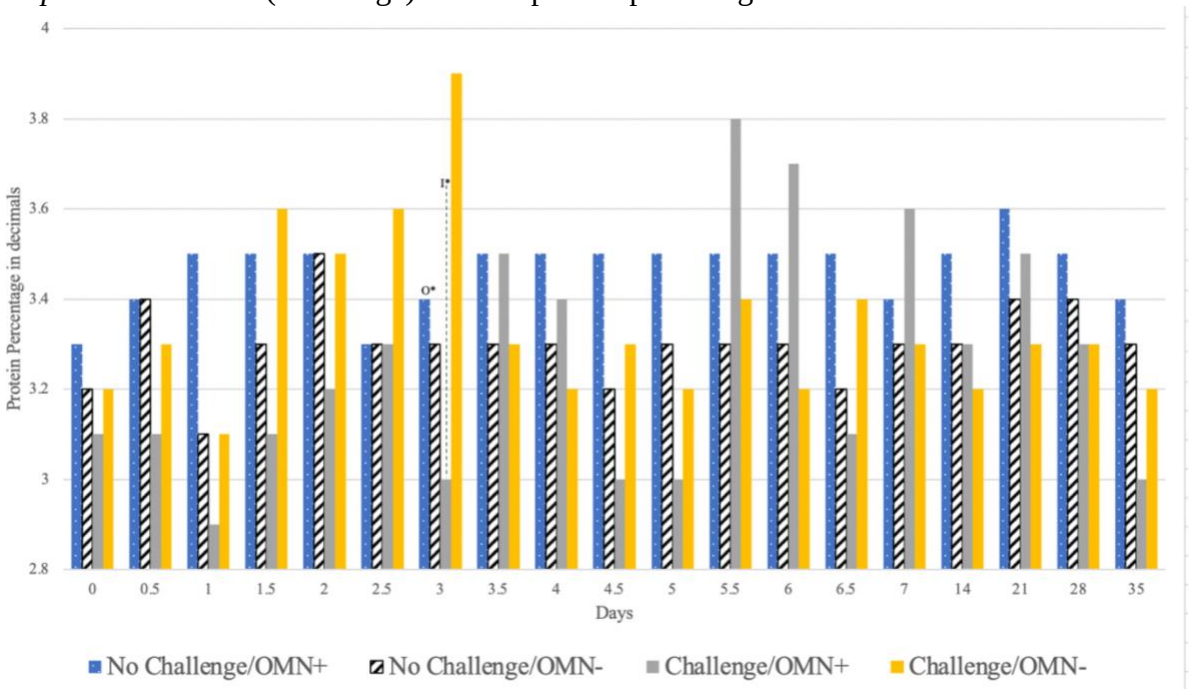
* Indicating significance at $P < 0.05$
** Indicating a trend at $P < 0.10$
(C) Indicating Challenge Group *P* value
Day 0.5 – Inoculation occurred
Day 3 – Antibiotic treatment begins

Chart 2.4: Least squared means by treatment for OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on fat percentage



* Indicating significance at $P < 0.05$
** Indicating a trend at $P < 0.10$
(C) Indicating Challenge Group *P* value
(O) Indicating OmniGen-AF Group *P* value
(I) Indicating Interaction between Challenge and OmniGen-AF *P* value
Day 0.5 – Inoculation occurred
Day 3 – Antibiotic treatment begins

Chart 2.5: Least squared means by treatment for OmniGen-AF® (OMN) and *Streptococcus uberis* (Challenge) on milk protein percentage



* Indicating significance at $P < 0.05$
(O) Indicating OmniGen-AF Group *P* value
(I) Indicating Interaction between Challenge and OmniGen-AF *P* value
Day 0.5 – Inoculation occurred
Day 3 – Antibiotic treatment begins

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