PROLIFERATION AND CYTOKINE PRODUCTION IN BOVINE PROLIFERATIVE BLOOD CELLS AS BIOMARKERS OF IMMUNE MEMORY IN OMNIGEN-AF® FED HEIFERS

by

CARLY BARBER

(Under the Direction of David Hurley)

ABSTRACT

The impact of an immunostimulant, OmniGen-AF®, on BRD vaccine response in Holstein heifers was tested. They were fed a standard diet (12 heifers) or also supplemented with OmniGen-AF® (13 heifers) for 170 days. All were vaccinated at the beginning of the trial (day -110), and again on study day 0. Lymphocyte proliferation and cytokine production in response to BHV-1 and BVDV viruses were measured *in vitro*. Neutralizing antibody titers were determined for the same viruses. Initial vaccination induced increased antibody titers against the viruses, and booster vaccination greatly increased titers to BVDV virus; though, no differences were seen between diets. Feeding OmniGen-AF® significantly enhanced proliferation responses to the viruses measured 42 and 60 days following booster vaccination. Significant differences in cytokine production were also observed relative to the diet treatments, but did not show a useful pattern of response relative to booster vaccination.

INDEX WORDS: OmniGen-AF®, BRDC, recall immune response, memory cells

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DEDICATION

I dedicate my thesis work to my family and friends who have always supported me and never left my side. A special feeling of gratitude goes to my mom, who always had words of encouragement through the many twist and turns that my life has taken. I also want to thank you for the many unspoken ways that you share your love in our family, I hope you know that you are loved and appreciated beyond measure. Thanks for hanging in there with me, I could have never gotten this far without you.

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CHAPTER 1

INTRODUCTION

Bovine respiratory disease complex (BRDC), also referred to as pneumonia or shipping fever, is the leading natural cause of death and economic loss in beef and dairy cattle in the world (Schneider et al., 2009). Control of the viral agents that appear to seed multi-agent, polymicrobial respiratory infections is highly variable. Increasing immunity in a group of cattle prior to exposure of the viral agents that cause BRDC will decrease the severity and number of clinically affected cows (Step et al., 2009). Immunity to these upper respiratory pathogens can be accomplished through vaccination; however, a variety of factors have been demonstrated to impact the quality of BRDC viral vaccine response.

Two factors that contribute to vaccine response are the quality and completeness of nutrition and the total energy available for immune processes. Nutrients, such as protein and vitamins, are essential for maintaining and activating the immune system, building amino acids, synthesis of immunoglobulins, and the maintenance and production of white blood cells, natural killer cells, and sustaining a high level of antibody production (Ingvartsen et al., 2013). Energy is required for the replication and function of cells. Limited energy supplies can negatively affect the integrity of the cow's physical barriers, mucus, and other innate immune functions (Chandra, 1997).

Another factor is stress; an increase in the stress level of the animal can negatively impact its immune response to vaccines. Stress causes down regulation of the adaptive

immune system and reduces expression of key mediators of innate immunity (Wang et al., 2007). Stress causes cortisol levels to increase, suppressing physiological responses in innate and adaptive immune cells (Bondy, 1952). Stress also changes the level of glucose and fat circulating in the body, altering available energy for immune activity (Rebuffe-Scrive, 1992). This causes the animal to have higher nutritional requirements, that if not met can further suppress the immune system.

An additional factor impacting the cow's vaccine response is the functional capacity of innate immune cells in the tissues at the site of vaccine delivery. These cells need to optimally process and present the vaccine antigens by recognition of the danger signals carried by invaders, and the indicators of cellular and tissue damage associated with colonization. A component of proper immune response at the tissue level is the generation of signals of danger and context of controlled damage that must be communicated to the lymphocytes in secondary lymphoid tissues. Lack of energy or protein, high levels of unmodulated stress, or poorly regulated tissue innate responses limit the development of vaccine induced immune effectors and the development of memory responses.

Dairy heifer calves are generally comingled between 2 and 3 months of age, and are housed on a pasture in common with consistent sources of feed and water once completely weaned. These heifers should be on a relatively high nutritional regimen to optimize growth and have relatively little social stress, as they have been grouped together for about 3 months. Good priming vaccine responses provide the basis for optimization of recall protection. Dairy heifers should receive booster vaccines before they are moved to a new location for their initial breeding at about 12 months of age. Translocation and the process of breeding are stressful events. Stress has a negative effect on the immune response to

respiratory viral infections, making cows more susceptible to BRDC, like the period of first breeding. Optimizing the vaccine response prior to entering the production herd is a key to long-term management of agents that can cause losses in production due to respiratory disease, reproductive failure, or inflammatory problems.

One key to optimizing vaccine response is to have well primed and tightly regulated innate immunity. Vaccines contain specific antigens key to the pathogenesis of viruses. When the antigen and the other components of the vaccine are given, the innate immune system will be the first to respond to the foreign antigen and danger carried by the vaccine live agent or construct. Further, damage caused by the limited infection with live vaccine agent or the adjuvant-antigen construct, will additionally drive the actions of the innate response. Leukocytes, primarily neutrophils, are a major innate defense against invading pathogens via phagocytosis and intracellular killing. During phagocytosis, various processes act to kill bacteria and prevent bacterial growth and colonization (Ryman et al., 2013). Cytokine production during the initial stages of infection, or vaccination, leads to an influx of neutrophils into the tissues. The interactions between innate and adaptive immunity are complex, with innate immunity being instrumental in the initiation of adaptive responses and controlling the type of adaptive response induced. After the pathogen has been eliminated, memory T cells are generated from activated effector T cells and are distinguished by their ability to survive long-term and to mediate rapid effector responses upon antigenic recall (Ndejembi et al., 2007). In some cases, memory cells can persist over the lifetime of an individual and are able to mediate a more rapid and stronger immune response upon a sequential encounter with the antigen. For a period of time after clearing the antigen from tissues, memory cells circulate in the blood and enter tissues

exhibiting signs of damage and danger to enhance the speed and efficacy of the memory response (Lefrancois, 2006; Schenkel & Masopust, 2014).

While antibiotic therapy has been effective in eliminating existing bacterial respiratory infections in heifers, prevention is the key for reducing new infections. The use of antibiotics in production animals has negative consequences in the mind of consumers. As an alternative to antibiotics, a commercially available feed supplement containing yeast and B-complex vitamins (OmniGen-AF®) has been shown to enhance innate immune function in heifers and to restore innate function in animals under stress (Wang et al., 2007, Ryman et al., 2013). Adding OmniGen-AF® to the diet of developing heifers has been shown to enhance neutrophil and monocyte function (Wang et al., 2009). Two previous studies have failed to demonstrate significant enhancement of antibody titers in OmniGen-AF® fed cows or heifers (Prince Agri, unpublished data), but the effects of OmniGen-AF® feeding on the development of recall memory have never been previously tested. In an attempt to further enhance the protection vaccines provide against BRDC viral agents, this study assessed the impact of OmniGen-AF® on the development of antibody serum neutralizing titers following booster vaccination; and on recall antigen induced clonal expansion of mononuclear cells from blood, and the ability of those mononuclear cells to produce cytokines relevant to the calves' immune response to BVDV and BHV-1 viruses on recall stimulation.

CHAPTER 2

LITERATURE REVIEW

Bovine Respiratory Disease Complex

Bovine respiratory disease complex (BRDC) is a disease associated with polymicrobial viral, bacterial, and mycoplasma infections. This disease has a common pathogenesis and can be associated with many combinations of different agents. The most common pathogenesis of BRDC is believed to be the development of a pro-inflammatory viral infection with BVDV, BHV-1, BRSV, or PI-3 that is rapidly followed by a significant infection with gram negative bacteria that cause serious deep lung inflammatory damage and pneumonia (Ridpath, 2010). Given this model, it is important to understand the economics, biology, and ecology of BRD to have a complete understanding of this project and its objectives.

The economic cost associated with the BRDC has been reported to cost the beef production industry $750 million annually. The average cost of 1 treatment was estimated at $15.57 and is amplified to $92.26 when indirect costs are also considered such as the reduction of average daily gain (ADG) and a decrease in carcass value due to a less desirable quality grade of meat (Schneider et al., 2009).

Bovine respiratory disease is considered a disease complex because many different viral and bacterial pathogens are known to be involved in causing disease symptoms. Viral pathogens associated with BRDC include: infectious bovine rhinotracheitis (IBR), which is

caused by bovine herpesvirus-1 (BHV-1), bovine viral diarrhea virus (BVDV), parainfluenza-3 (PI3), and bovine respiratory syncytial virus (BRSV). The most common bacterial pathogens involved with BRDC include: *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis* (Edwards, 2010). Establishment of upper respiratory viral infection results in immunosuppression, allowing commensal bacterial pathogens to migrate and colonize the lower respiratory track.

A great deal of emphasis has been placed on the role of the major viral pathogens of BRDC. As viral agents appear to be the "lead" infection in most BRDC problems, the study of the virus and its biological management have been deemed critical to control of BRDC losses. The establishment of reasonably reproducible *in vivo* disease models using each viral pathogen as single agents has allowed research workers to study the pathogenesis of preliminary pro-inflammatory acute infections and their biological, immunological and physiological consequences, and how vaccines modulate these aspects of disease.

BVDV is an economically important pathogen that belongs to the genus *Pestivirus* of the family *Flaviviridae* and has been reported worldwide. Two genotypes exist: BVDV type 1 and BVDV type 2. Although acute BVDV infections are often subclinical and produce only mild clinical signs, they induce lymphopenia and stimulate an immune response by infecting a wide variety of cell types such as monocytes, macrophages, dendritic cells, and lymphocytes (Brodersen, 2014). BVDV is an important virus in the BRDC due to its highly suppressive impact on the immune system (Larson, 2015). Clinical signs of acute BVDV infection are fever, diarrhea, ulceration of the muzzle and oral cavity, and leucopenia. Highly virulent strains of BVDV can produce severe and widespread ulceration of the

oropharynx, larynx, and esophagus and hemorrhagic enteritis. Observed clinical signs consist of inappetence, lethargy, reduced milk yield, and abortion (Brodersen, 2014).

BHV-1 is an alphaherpesvirus found only in cattle and has also been reported worldwide. BHV-1 infection induces a variety of clinical signs in the upper respiratory tract. Severe inflammation of the lining of nasal passages and trachea results in fever, coughing, nasal discharge, depression, inappetance, weight loss, and conjunctivitis. BHV-1 is immunosuppressive, inhibiting cell-mediated immunity and CD8+ T-cell recognition of infected cells and inducing apoptosis in CD4+ T cells (Jones et al., 2011).

Infection, by either BVDV or BHV-1, erodes mucosal surfaces of the upper respiratory tract, bolstering establishment of bacterial pathogens in the lower respiratory tract. Many of the bacterial pathogens involved in BRDC are normal inhabitants of the upper respiratory tract; however, damage from the primary viral infections or immune suppression allows these commensal bacterial pathogens to colonize, resulting in pulmonary compromise, inflammation, and gross pathology (Edwards, 2010). A co-infection of the BRD viruses with a bacterial pathogen dramatically increases the severity of clinical illness and increases the chance of morbidity (Tuncer et al., 2015).

Increased Prevalence of Bovine Respiratory Disease

BRDC is a multifactorial disease and develops as a result of complex interactions between environmental factors, host factors, and pathogens. Many viruses are not able to survive for long outside the body, so close contact between cattle is the primary transmission route for respiratory infection to spread. Risk factors associated with increased transmission include contact with or shared air space with older animals,

relative humidity levels greater than 75%, poor air quality, increased stocking density, bedding type, and bedding density (Gorden et al., 2010).

Stressors, especially heat stress, can also have a negative impact on the calves' health. Heat stress occurs when an animal cannot adequately dissipate body heat in order to maintain thermal balance (Bernabucci et al., 2014). Values above 70 on the temperature-humidity index may affect reproductive cycle, feed intake, and growth (Bova et al., 2014). Some studies suggest that the immune system is also affected by heat stress in cows (Salak-Johnson and McGlone, 2007). Cold and fluctuating air temperatures, and excessive wind and moisture are common weather-related thermal stressors contributing to reduced survival of calves (Olson et al., 1980). Moisture and mud increase the effects of cold stress and further decreases animals' feed consumption (Broucek et al., 1991).

Combinations of stressors have a negative effect on the cow's immune response to respiratory viral infections. A study was done by Hodgson and coworkers (Hodgson et al., 2012) seeking to determine if the stress of weaning and maternal separation impacted the calves' susceptibility to a secondary bacterial respiratory infection. A primary BHV-1 respiratory infection was established in all calves followed by a *Mannheimia haemolytica* four-day challenge. Mortality doubled in the calves that had just been weaned when compared to calves pre-adapted to weaning for two weeks prior to the viral respiratory infection.

Stress is an important immunosuppressive event that causes down regulation of the adaptive immune system and reduces expression of key mediators of innate immunity (Wang et al., 2007). Stress can cause active cells to produce altered genes that change their capacity to take up nutrients and to stay activated (Bondy, 1952). This reduces antibody,

cytokine, lipid mediator, and other immune actors and signals, reducing the animals' ability to mount an effective immune response. The stress factors facing calves, such as unfavorable weather conditions, weaning, transportation, commingling of calves from different sources, and feedlot processing, make them more susceptible to the pathogens in the bovine respiratory disease complex (Schneider et al., 2009).

Dairy heifers also face stress when entering breeding production at about 12 months of age. Prior to first calving, heifers are relatively unstressed as they spend little to no time in holding yards and spend most of their time at pasture. On entering the milking herd, they experience more competition for feed and social position in the herd. During the breeding phase there is increased contact and shared air space with older animals and comingling in dense and confined areas. Therefore, breeding and entering the milking herd are other periods during which stress and environmental changes increase the chance of infections with BRD viral agents.

Heifer Growth

Monitoring dairy heifer growth is necessary to determine if the calves are developing normally to reach the appropriate height and weight for optimal future production. From an economic perspective, one of the most important factors for milk production is age at first calving. Because puberty is not reached until the heifer reaches a certain weight, breeding age depends on the growth rate (Windeyer et al., 2014). Daily feed intake is positively correlated with ADG with faster-growing animals having greater intake than slower-growing animals (Green et al., 2013). Dry matter intake can decrease due to

stress and environmental conditions such as hot weather, muddy conditions, weather, handling, and disease.

Calfhood disease is an important problem on many dairy operations. In addition to the cost of treating sick calves, the economic consequences may include increased mortality, delayed estrus and reduced growth. Neonatal calf diarrhea (NCD) and BRDC are the most common causes of morbidity and mortality in young dairy cattle, and calves treated for NCD and BRDC have lower average body weights (Windeyer et al., 2014). Infectious disease in early life has also been associated with delayed calving.

A healthy immune system contributes to proper growth in calves because less energy is expended fighting calfhood diseases. This shows the importance of disease prevention because of the impact that diseases have on mortality, growth, and production in dairy heifers.

Prevention and Control

The focus for prevention and control of BRDC in cattle is to effectively minimize pathogen exposure, stimulate herd immunity, and manage risk factors. The feedlot phase has been identified as the most critical time in the development of BRD. This stage is frequently related to stressors associated with weaning, shipping, nutritional changes, and handling before or shortly after arriving at the feedlot (Edwards, 2010). Some common practices to reduce the spread of disease are frequent cleaning of feed bunks and water troughs, regulation of core body temperature, decreasing heat stress, providing better bedding to reduce mud and dust, and decreasing crowding in holding pens.

Another current practice is to administer antibiotics to all calves upon arrival at the feedlot as a prophylactic treatment to reduce the incidence of BRDC. In a study done by Rerat and coworkers (Rerat et al., 2012), sixty calves with negative antibody titers for BVDV were purchased and directly transported to the research station. They were separated into 3 groups. Group A and B received different antibiotic treatment on arrival whereas group C receive no prophylactic antibiotic treatment. A and B treatment groups in comparison to group C demonstrated significantly lower incidence of respiratory disorders and considerably higher ADG over the entire experimental period.

However, the practice of giving antibiotics is becoming less desirable due to the emergence of drug-resistant microorganisms and consumer concerns about residual antibiotics in the final product. Because of this, there is a growing urgency to find more effective non-antibiotic based alternatives for the prevention and treatment of BRDC in cattle.

Respiratory Immune Defenses: Innate Immunity

The pathogens in the BRDC enter the body through the mucosal surfaces in the respiratory tract. The trachea, bronchi, and bronchioles are lined mainly by epithelial cells, the surfaces of which are covered with ciliated cells (Whitsett et al., 2015). Respiratory epithelial cells create multiple barriers against pathogens because they have very tight junctions that secrete an abundance of fluids, antimicrobial proteins, and mucins. Mucins serve to maintain airway homeostasis and aid in the removal of pathogens and cellular debris during recovery from infection or injury (Whitsett et al., 2015). Respiratory epithelial cells also have an essential role in the initial recognition of pathogens and are

active participants in mucosal defense. They are involved in the aggregation, trapping, and killing of microbes and send cytokine and chemokine signals to underlying mucosal cells, dendritic cells, and macrophages to trigger innate immune responses and promote adaptive immune responses (Neutra et al., 2006).

The surfaces of respiratory epithelial cells are well equipped to respond to pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Pattern recognition receptors, including toll-like receptors (TLRs) and nod-like receptors (NLRs), are widely expressed by respiratory epithelial cells and are able to detect dangerous microbial components (Whitsett et al., 2015). PAMPs and DAMPs regulate signaling cascades that alter epithelial gene expression, cytokine production, and chemokine signals, which then influence the recruitment and activation of professional cells of the immune system to regulate inflammatory responses, barrier function, and the clearance of apoptotic cells and pathogens in the lungs.

Leukocytes, primarily neutrophils, are considered the second line of defense against invading pathogens after the first line provided by epithelial cells lining the respiratory tract is breached. Initial contact between macrophages and epithelial cells with bacterial invaders releases chemoattractants, such as cytokines, complement factors, and prostaglandins in response to the invading pathogens (Kumar et al., 2010). These signals elicit the influx of neutrophils into the area of infection. Neutrophils help contain and clear infectious particles; furthermore, they provide signals to other innate immune cells about an invading foreign threat.

Respiratory Immune Defenses: Adaptive Immunity

An important characteristic of the mucosal adaptive immune response is the local production and secretion of immunoglobulin A (IgA) antibodies that, unlike other antibody isotypes, are resistant to degradation in the protease-rich external environments of mucosal surfaces (Neutra et al., 2006). IgA has multiple roles in mucosal defense, including the ability to bind and trap antigens or microorganisms in the mucus. This prevents the pathogen from having direct contact with the mucosal surface, thereby preventing it from entering the body, this is known as 'immune exclusion'. IgA bound to antigens or microorganisms also tags them for opsonization, a process that targets these pathogens for destruction by phagocytes, such as dendritic cells and macrophages. This increases the uptake of the pathogen by immune cells and phagocytes that transport the antigens or microorganisms to the organized mucosa-associated lymphatic tissue (O-MALT).

IgA production is a very important consequence of natural infection at mucosal surfaces, but is rarely an outcome of conventional subcutaneous or intramuscular vaccination. Recent BRDC vaccines are designed to be given intranasally (IN) and theoretically provide a significant IgA response and likely some level of IgA memory. Only IM vaccines were tested in this study, but it may be prudent to consider IN vaccination in future trials.

The mucosal immune system consists of specialized local inductive sites collectively know as O-MALT, which is characterized by mucosal lymphoid follicles present in the lingual and palatine tonsils and adenoids of the oral and nasopharynx, Peyer's patches in small intestine, and the appendix (Neutra et al., 1996). There are antigen-processing dendritic cells in the epithelial layer, which serve as motile scouts that obtain samples of

luminal antigens and then migrate back to local or distant organized lymphoid tissues. In intestinal and airway epithelia, the intercellular spaces of which are sealed by tight junctions, specialized epithelial M cells deliver samples of foreign material by transepithelial transport from the lumen to organized lymphoid tissues within the mucosa (Neutra et al., 1996). Once antigen sampling occurs at the mucosal surface, phagocytes will engulf the foreign antigen and present them on their surface. They also upregulate the expression of maturation markers and major histocompatibility gene complex (MHC) molecules, which help them migrate to organized mucosal sites. Antigens sampled from the respiratory track are taken to lingual tonsils and adenoids, while antigens in the intestine are taken to Peyer's patches.

B and T cells are activated in the O-MALT. The antigen is presented by class II MHC receptors on the dendritic cells. Both naïve T cells and activated dendritic cells are drawn to the T cell rich zones of the lymph nodes. Antigen recognition is the first signal for lymphocyte activation and the dendritic cells have to find the specific T cell to tightly bind to the antigen, hence activating the T cell. Helper T cells will also be created during this process, which will activate B cells and stimulate production of a humoral response. B and T cells that are activated in O-MALT upregulate the expression of tissue specific adhesion molecules and chemokine receptors that function as 'homing receptors' to guide these lymphocytes back to the mucosa where the microorganism was initially encountered through recognition of endothelial counter-receptors in the mucosal vasculature (Neutra et al., 1996).

When a CD4+ T cell is activated, it becomes a Th1 effector cell, a Th2 effector cell or a Th17 effector cell. Naïve CD4+ T cells acquire lineage specificity by encountering distinct

sets of cytokines and each subset is better equipped to eliminate distinct types of pathogens and microorganisms (Wang et al., 2015). T helper 1 (Th1) cells are the principal cell type involved in cell-mediated inflammation and are important for immunity to intracellular pathogens. They are most often defined by their production of Interlukin (IL)-2) and Interferon (IFN)-gamma. T helper 2 (Th2) cells are recognized for their role in host defense against multi-cellular parasites and their involvement in allergies, and are best known for the production of IL-4, IL-5, and IL-13, as well as IL-9 and IL-10. Th17 cells are potent inflammatory mediators involved in host defense against extracellular bacteria and fungi and are the major source of IL-17 production (Raphael et al., 2015).

IFN-gamma is a potent proinflammatory cytokine which has a number of important roles including increasing the expression of TLRs by innate immune cells, promoting IgG class switching, increasing MHC class I and class II antigen presentation, and induction of chemokine secretion, macrophage activation, and increased phagocytosis (Raphael et al., 2015).

IL-17 initiates the recruitment of neutrophils, activation of innate immune cells, enhances B cell functions, and induces release of proinflammatory cytokine expression, chemokines, and other inflammatory mediators.

Transforming Growth Factor (TGF)-beta is required for the generation of T regulatory cells (Tregs) by inducing the expression of Foxp3. The positive feedback loop between TGF-beta and Foxp3 plays a critical role in maintaining peripheral tolerance and is also key to the maintenance of Tregs.

IL-10 has anti-inflammatory and immunosuppressive effects, which are largely mediated through its impact on antigen-presenting cells. It has been shown to

downregulate the expression of MHC-II and co-stimulatory molecules, and it reduces the release of proinflammatory cytokines by mast cells and macrophages as well as suppressing their function and activation.

IL-4 is an important survival factor for lymphocytes. In B cells, it promotes plasma cell differentiation and induces antibody class switching to IgG1 and IgE. In the innate immune system, IL-4 has been shown to promote the differentiation of dendritic cells from stem cells and to promote their maturation.

Vaccination

In theory, vaccines are designed to stimulate the immune response in almost the same way that a natural infection occurs. This is important because it allows the vaccine to induce a strong memory response on subsequent exposure to the viruses being vaccinated against. Vaccines contain specific antigens key to the pathogenesis of the virus that one is trying to prevent. Many vaccines, particularly killed or subunit vaccines, also contain an adjuvant. Adjuvants are compounds that enhance the efficacy of vaccine magnitude, breadth, and longevity of specific immune responses to antigens, as well as direct the quality of the immune response (Carter et al., 2010). When antigen and adjuvant are given concurrently, the innate immune response will be the first to respond to the foreign antigen, and the adjuvant will drive the danger and damage signals in the innate response. Adjuvants stimulate the natural defense mechanism by triggering signals from inflammatory processes while minimizing tissue damage, which alerts the immune system and guides it towards the most appropriate type of response. A proper innate response is required to stimulate a strong and comprehensive adaptive immune response to the

antigen (Garcon et al., 2011). At the site of injection, monocytes rapidly differentiate into activated macrophages or dendritic cells. The adjuvant "danger signal" must accompany the antigen in order to activate dendritic cells and aids in the transport of information concerning the danger and target antigen to the secondary lymphoid tissues. Just as in a naturally produced infection, the dendritic cells engulf the antigens, in turn activating the dendritic cell. The dendritic cell then presents the antigen on its surface and migrates to the lymph nodes where it activates naïve lymphocytes.

Assessment of Vaccine Immunity

When combined with good management practices, vaccination is considered an effective method of control of BRDC (Peters et al., 2004). The first justification for the use of vaccination to control BRDC infections is to limit the spread of infection within a population, and another is to reduce the extent of clinical disease in an infected animal (Ridpath, 2013). Vaccines stimulate a humoral and cell-mediated immune response against the antigens in the vaccine. Humoral immune responses are easier to measure; the serum neutralization test is commonly used to measure levels of immune response to vaccination and to assess herd immunity (Ridpath, 2013). Even though viral neutralizing titers, resulting from vaccination with modified live vaccine, are generally lower than those resulting from natural exposure, they follow similar development and decay curves and are therefore a good measure of the immune response (Ridpath 2013, Woolums et al., 2013).

Many studies have shown that BRD vaccines provide efficient priming, and therefore vaccinated calves elicit higher memory response when later exposed to viral antigens. A BRDC challenge study by Peters and coworkers (Peters et al., 2004) verified the

efficacy of a BRD vaccine. The study showed there was a dramatic rise in antibody titers in the vaccinated groups when compared to the control group, thus demonstrating clear anamnestic responses for a minimum of 6 months (in their BHV-1, PI3 V, and BVDV trails) and up to 12 months (in their BRSV trail), and also a decrease in viral shedding in the vaccinated animals after challenge.

In another study, done by Martin and Bohac (Martin et al., 1986), calves that were newly received at the feedlot were tested for BRD viral antigens. A total of 322 calves were bled on arrival and then again one month later. Calves presenting with low antibody titers on arrival suggested a lower level of previous exposure to respiratory viruses than the calves that exhibited high antibody titers on arrival. A significantly high titer was likely due to a recent infection. Higher circulating antibody titers on arrival, indicative of previous exposure, was also an indicator of better protection from respiratory disease when undergoing stress and comingling. The general tendency found in this study was that calves that had lower viral titers on arrival were more likely to be treated for BRD symptoms during that first month.

Of the many important factors impacting the efficiency of a vaccine, age is important due to circulating maternal antibodies from colostrum. A study done to determine at what age calves have the best immune response to vaccination without the interference of maternal antibodies was done by Platt and coworkers (Platt et al., 2009). Three groups of 12 Holstein calves 1–2 weeks, 4–5 weeks, and 7–8 weeks of age were used in this study. In each age group, eight calves were vaccinated and four calves served as non-vaccinated controls. In the presence of maternal antibodies, a single dose of pentavalent MLV vaccine failed to induce antibody or T cell responses to BHV-1, BRSV, or PI-3 in calves. However, a

single dose of a MLV vaccine could induce detectable T cell responses to BVDV types 1 and 2, and protected vaccinated calves from virulent BVDV type 2 challenge.

A subsequent (booster) vaccination is also important because it stimulates memory recall immune response. A bovine respiratory syncytial virus (BRSV) vaccine challenge study done by Blodorn and coworkers (Blodorn et al., 2014) indicted good protection in all vaccinated cows; whereas, all control animals developed severe respiratory disease and shed high levels of virus following BRSV challenge. Immunized calves demonstrated almost complete clinical and virological protection five weeks after a single intranasal (IN) vaccination. Although mucosal vaccination failed to induce a detectable immunological response, virus neutralizing antibody and local T cell response were observed following challenge with a virulent strain of BRSV. Calves immunized twice intramuscularly (IM), three weeks apart had increased protection that extended two weeks after booster vaccination. This group also produced significantly higher levels of IFN-gamma compared to those from animals in all other groups.

There are two common types of vaccines that are used to prevent viral and bacterial infections; one is a killed vaccine and the other is a modified live vaccine. Stimulating both a cell mediated response and a humoral response is essential for a vaccine to be highly efficient. Studies indicate that inactivated (killed) vaccines, such as bacterins and toxoids, generate a strong humoral immune response that is characterized by secretion of IL-4 by T helper cells. Studies also indicate that modified live virus vaccines (MLV) establish a limited infection at the site of deposition, mimicking natural infection. This elicits a cell-mediated immune response, characterized by production of IFN-gamma by T helper cells (Reber et al., 2006).

While challenge trials are considered to be the gold standard for the assessment of vaccine protection efficacy, they are difficult to standardize and are quite expensive. Often, a suitable challenge organism cannot be readily obtained for use in such studies. Therefore, *ex vivo* assessment of immune activity following vaccination is a desirable tool for measuring vaccine induced-immune responses. It is generally possible to measure the function of memory cells in circulation after vaccination or infection. A large number of such assessments have been developed and are in general use. However, only rarely do researchers utilize a broad range of these indicator assessments in parallel to study the immune response in vaccinated animals. Yet, it has been documented that the indicators often paint different pictures of the response depending on the focus of the measurement. (Reber et al., 2006)

Another factor that affects immune response is the combination of priming and booster vaccine types. The development of immunity to vaccine antigens was examined in a study by Reber and coworkers (Reber et al., 2006) using three prime/boost strategies, and the progression of immune activities was evaluated over the course of 8 weeks. Calves were vaccinated and multiple immune parameters were evaluated to assess humoral and cellular immunity. Evidence suggests that both humoral and cell mediated immune responses are important in protection against BVDV induced disease in cattle. The MLV/MLV and MLV/killed groups produced significant SN titers against all of the BVDV strains tested relative to control calves. The killed/killed group developed no significant titers above controls at any time after vaccination. Vaccination with MLV/killed or MLV/MLV induced significant SN titers against both the type 1 BVDV. Their results indicated a significant correlation between the SN titers and proliferation assays.

One other important finding of the studies by Reber and coworkers (Reber et al., 2006) was that measurements of cell mediated immunity and the level of serum neutralizing antibody did not strongly correlate after vaccine booster administration. In fact, multiple measures of circulating cell mediated memory cell activity (i.e. recall antigen), proliferation, production of interferon-gamma, and message for IL-4, IL-10, IL-12 and interferon-gamma did not have a strong concordance in reporting the response to booster vaccination; instead, they showed different patterns of response in magnitude and timing following the vaccination given to the cattle involved. This study did not include a challenge, so it is not clear if any of the indicators measured would provide a clear indicator of immune protection.

Importance of Gut Immunity

Epithelial cells line the intestine, where bacteria are abundant. Here, the epithelial cells can modulate and dampen signals to prevent undesirable responses to non-threatening bacteria and nutrients (Neutra et al., 2006). Mammals have evolved to form symbiotic relationships with a variety of microbes, which provide numerous nutritional, developmental, and physiological benefits (Taschuk et al., 2012). This predominantly occurs at the host's mucosal surfaces such as the gut, where a large and diverse community of microorganisms reside, dominated by bacteria. These microbes are known to have a critical role in the intestinal functions and in overall health of the host (Di Mauro et al., 2013).

The microbiome in the ruminant gastrointestinal tract is very specialized. It not only aids digestion of plant material, allowing the use of fermentation products for up to 70% of

total dietary energy, but each compartment of the gastrointestinal tract creates specific environmental conditions for the breakdown and absorption of dietary nutrients (Taschuk et al., 2012). The variability in each compartment creates niches that are selective for microbes tailored to each environment. The early microbial composition of the gastrointestinal tract has long-lasting functional effects because it helps the immune system distinguish potentially dangerous from harmless antigens and to establish oral tolerance (Di Mauro et al., 2013). Oral tolerance is the ability to avoid inflammatory response against food proteins and self aggression against the host's own resident intestinal bacterial microbiota through the establishment of a tolerogenic mechanism on naïve CD4+ T cells that suppress the expression of T effector cells and stimulates the expansion of regulatory T cells (Di Mauro et al., 2013). Surveillance at mucosal surfaces occurs primarily through specialized antigen sampling cells, M-cells, and genetically encoded pattern recognition receptors (PRRs) expressed by mucosal epithelium and leukocytes constituting the mucosa-associated lymphoid tissue (MALT) (Taschuk et al., 2012).

While adaptive immunity at mucosal surfaces has generated substantial interest, there have been studies that show directly or indirectly that innate immune function and priming are impacted by elements of the microbiome. An example of this is innate immune recognition pathways that have evolved to assess changes in the gut and relay information about the local nutrient and metabolite environment to help direct immune response (Kau, 2011). It has also ben demonstrated that changes in mucosal and systemic innate immunity function relative to modulation of the gut microbiome, particularly with killed or live yeast (Rizzetto, 2010). These studies demonstrated changes in macrophage activation with shifts

from M2 resident macrophages to M1 macrophages, enhanced IgA and IgG production at the gut surface and systemically, respectively. Further, they have shown that yeast has the ability to enhance neutrophil priming in circulation.

Keeping a healthy population of gut microbiota is essential to both innate immunity and adaptive immunity. Alterations of gut microbial communities can cause immune disregulation and lead to autoimmune disorders and a weakened immune system (Wu et al., 2012). The greatest influence on gut microbial structure is environmental factors in the gastrointestinal tract, which is directly affected by the diet of individual animals (Taschuk et al., 2012). Many nutrients have been established as required for the immune system to function efficiently, and practically all forms of immunity may be affected by deficiencies in one or more of these essential nutrients (amino acids, B vitamins, the minerals Zn, Ca, Mg, Mn, Se; and specific polyunsaturated fats). Animal and human studies have demonstrated that adding deficient nutrients back to the diet can restore immune function and resistance to infection (Calder et al., 2002).

Nutritional Immunostimmulant

OmniGen-AF® contains a mixture of active dried yeast (*Saccharomyces cerevisiae*), dried *Trichoderma longibrachiatum* fermentation product, niacin, vitamin B12, riboflavin-5-phosphate, d-calcium pantothenate, choline chloride, biotin, thiamine monohydrate, pyridoxine hydrochloride, menadione dimethylpyrimidinol bisulfate, folic acid, calcium aluminosilicate, sodium aluminosilicate, diatomaceous earth, calcium carbonate, rice hulls and mineral oil.

There are different avenues to enhance a robust immune system in cattle; these include good nutrition, decreased levels of stress, and through feed supplementation such as OmniGen-AF®. Yeast and yeast cell wall products have been demonstrated to improve productivity during several periods of cattle production and have the potential to be a viable non-antibiotic alternative feed supplement. Yeast supplementation has been demonstrated to improve dry matter intake and average daily gain while decreasing morbidity in cattle.

In a study done by Burdick-Sanchez and coworkers (Burdick-Sanchez et al., 2013), twenty-four heifers were used to test 2 different yeast cell wall supplement products. Both products were made from strains of *Saccharomyces cerevisiae*. 37 days after starting the yeast supplement, the heifers were challenged with an endotoxin (LPS). They found that vaginal temperatures, blood cortisol levels, and IL-6 responses were significantly lower in all yeast cell wall supplemented heifers compared with control heifers. These preliminary data suggest that yeast cell wall supplementation may enhance the overall health status of heifers, resulting in a reduced response to subsequent immune challenge.

Another study was done by Cole and coworkers (Cole et al., 1992) to determine the effects of yeast culture on the health and performance of stressed feeder calves. The effects of yeast culture on dry matter intake, fever, and weight loss of calves challenged with infectious bovine rhinotracheitis virus (IBRV) were evaluated. The yeast culture used in this study was from *S. cerevisiae*, which was dried to preserve the fermenting activity of the yeast. 101 steer calves were used to test yeast culture on the health and performance, and 30 steer calves were used in the IBRV challenge. Sick calves fed yeast culture responded more favorably to antibiotic therapy and spent fewer days in the hospital pen than did

control calves. Calves fed yeast culture also had higher feed intakes resulting in less weight loss of yeast fed calves compared to control calves that did not receive yeast culture.

A study done by Keyser and coworkers (Keyser et al., 2007) evaluated health and performance of newly received heifer calves when fed a live *S. cerevisiae* supplement. The percentage of newly received heifers treated for BRDC was decreased when yeast was fed. A decrease in the number of heifers treated for BRDC suggests that this yeast supplement boosted the immune system, giving the calves better protection during this transitional period in which they are exposed to higher numbers of foreign pathogens. A second experiment was done by this group to determine the effects of the yeast supplementation when given in conjunction with a prophylactic treatment of the antibiotic florfenicol. An injection of florfenicol in healthy beef steers resulted in a short-term decrease in dry matter intake, and that dry matter intake returned to normal sooner after injection when steers were fed yeast supplement. The more quickly calves return to eating normal amounts after the stress of transportation, the less weight calves lost.

A study was done by Nsereko and coworkers (Nsereko et al., 2002) to investigate the effects of supplementing the diet of dairy cows with a fibrolytic enzyme preparation from *Trichoderma longibrachiatum*. This exogenous fibrolytic enzyme increased the total viable rumen bacteria numbers in the dairy cows by stimulating the cellobiose and glucose-utilizing bacteria. The study indicates that the *T. longibrachiatum* supplement increases fiber digestion, which leads to increased microbial numbers, thus increasing the ability of the rumen to digest feed, increasing the quantity of microbial protein available to the cow.

Although cattle do not make vitamin B12, bacteria present in the rumen synthesize enough vitamin B12 to prevent deficiency symptoms in the lactating dairy cow. A study

done by Preynat and coworkers (Preynat et al., 2009) showed that dairy cattle given Vitamin B12 in early lactation in combination with folic acid, had improved lactational performance and metabolic efficiency, suggesting that rumen microbial synthesis might not always be sufficient to optimize animal performance.

The Effect of OmniGen-AF® on Innate Immunity

Neutrophils are among the most important cells of the innate immune system because they are the first cells to arrive at the site of infection (Forsberg, 2004). Neutrophils contain numerous cytoplasmic granules that provide constituents for killing bacteria and a highly convoluted surface that is used for phagocytosis of bacteria (Paape et al., 2003). L-selectin is an extracellular adhesion molecule on the surface of neutrophils that enables the neutrophil to interact with endothelial selectins and enter sites of infection. IL-1 beta is a potent cytokine secreted by neutrophils when infection is first encountered, and this cytokine increases the ability of these phagocytic cells to gain quick access to sites of infections (Wang et al., 2007).

In a study done by Wang and coworkers (Wang et al., 2007) to evaluate OmniGen-AF® effect on innate immunity, immunosuppression was induced in sheep by a twice daily injection of dexamethasone. The dexamethasone injection reduced neutrophil L-selectin activity by 62% and IL-1 beta activity by 99% compared to the control sheep. They concluded that OmniGen-AF® increased expression of the innate immune markers L-selectin and IL-1 beta in immunosuppressed sheep, and when exposed to a pathogen, the supplemented sheep had even higher expressions of these markers. OmniGen-AF® also increased circulating levels of both neutrophils and lymphocytes in this study.

Another study, done by Ryman and coworkers (Ryman et al., 2013) using a herd of pregnant dairy heifers evaluated the effect of OmniGen-AF® on enhancing blood leukocyte antimicrobial activity. This herd of heifers exhibited a high prevalence of *S. aureus* mastitis during gestation. Their study showed that OmniGen-AF® treatment significantly increased L-selectin and IL-8 expressions, and neutrophils had increased surface-binding of bacteria, phagocytic activity, and ROS production compared with unsupplemented controls. Increases in these markers indicate increased innate immunity in the supplement heifers. A stronger immune response leads to a healthier animal, ensuring less antibiotic use against pathogens, such as the ones that cause mastitis.

These studies show the impact that OmniGen-AF® has on improving the innate immune system by enhancing neutrophil function. The innate immune system represents the cow's first line of defense against a pathogen, and provides the adaptive system the time required to develop an appropriate antibody response against specific antigens. The adaptive immune system has the advantage of flexibility and immunologic memory, but it is completely dependent upon elements of the innate immune system for the initiation and direction of responses (Clark et al., 2005). Appropriate innate and acquired immune system interactions lead to highly efficient recognition and clearance of pathogens.

CHAPTER 3

HYPOTHESIS AND OBJECTIVES

The hypotheses driving this research were:

1) Vaccine responses are dependent on events that occur in the body tissues where they are delivered, and enhanced innate immune function in the tissues will enhance adaptive immune responses (both humoral and cellular),
2) The cell mediated adaptive immune response will be enhanced in OmniGen-AF® fed heifers as demonstrated by the recall antigen response of mononuclear cells from peripheral blood against viral antigens from the vaccines given.

The objectives of this study were to:

A) Determine if long-term (170 days) feeding of OmniGen-AF® will alter the development of T-helper memory response in dairy heifers after vaccination as indicated by the generation of antibody and circulation of memory T cells;
B) Compare the measurement of T-helper memory responses to BHV-1, BVDV type 1, and BVDV type 2 viral recall antigens using several methods:
    a. Evaluating proliferation of mononuclear cells (CD4+ memory cells), and
    b. Measuring induced cytokine activity (INF-gamma, IL-10, IL-17, IL-2, TFG-β, and IL-4) using commercial ELISA kits or commercial matched antibody pairs using in-lab ELISA assays;

C) Determine if there is a concordance between T-helper memory responses and the level of serum neutralizing antibody in circulation after vaccination; and

D) Determine if the magnitude and duration of the adaptive immune response is affected by long-term feeding of OmniGen-AF®.

CHAPTER 4

MATERIALS AND METHODS

Overview

A total of 24 Holstein heifers from the UGA Teaching Dairy were studied over an 11-month period (March 2015 to February 2016) to determine the effect of OmniGen-AF® (Prince Agri Products, Inc., Quincy IL, USA) on vaccine immune responses. Due to the difference in ages, the 2 groups began the trial at two separate times. However, each group was enrolled in the trial for 170 days.

At the start of each trial, groups of 12-14 heifers were allotted to 1- to 2- acre pastures; groups began the trial at an average age of 6 months. Once acclimated to the stanchion head-lock system constructed in each pasture, blood samples were collected from the jugular vein to obtain pre-trial sera for baseline antibody titers (D-110 relative to final booster vaccination). Blood collection continued throughout the trial on days -60, 0, 20, 40 and 60 relative to the booster vaccination. Hip heights and body weights were also recorded monthly throughout the trial.

All heifers were given an IM priming vaccination with 5-mL of Bovi-Sheild Gold 5® (Zoetis, Kalamazoo, MI) before the initiation of the trial. Bovi-Sheild Gold 5® contains modified live virus strains of IBR, BVDV type 1, BVDV type 2, PI_3 and BRSV. After the initial pre-trial bleeding and growth measurements, heifers began receiving the daily supplement (OmniGen-AF®) or control (no OmniGen-AF®) diets. OmniGen-AF® was given at the ration

of 4 grams per 100 pounds of body weight and was mixed into the daily diets. 110 days after trial initiation, heifers received a IM booster vaccination with 5-mL of Bovi-Sheild Gold 5®. Both groups were vaccinated and fed under the same protocol.

Initiation of Trial

The two groups of heifers were started on the trial once they reached an average age of 6 months. Group 2 had a broader range in age (5 -13 months of age) than Group 1, which ranged from 5 to 6 months of age. The initiation date for Group 1 was March 27th 2015 and the initiation date for Group 2 was August 28th 2015. Heifers were allowed an adjustment period before receiving treatment (feed supplement) and before sampling was performed. Each group of heifers was placed in a pasture containing a stanchion head-lock system to enable animals to be secured during feedings, bleedings, growth measurements, and vaccinations. Upon placement in the pasture, heifers were randomly assigned as either receiving the treatment diet or the control diet. The first group contained six treated and six control heifers housed together to allow all heifers to experience the same environmental conditions. This was repeated with seven treated and seven control heifers in the second group. Less than one month into the trial, two control animals were removed from Group 2 for use in a breeding trial leaving seven heifers fed OmniGen-AF® and five non-supplemented controls. During daily feeding, heifers were separated by treatment group to ensure that no "cross-feeding" occurred between treated and control animals. Heifers were fed directly on the concrete slab in front of the head-lock gaits. Approximately 20 pounds of grain was placed directly on the concrete slab in the stanchion head-lock system to prompt the heifers to lock up. Control heifers were each fed an additional 5

pounds of grain first (Figure 1) then released from the head-locks, then all empty head-locks were closed so that the control heifers had no access to the OmniGen-AF® feed supplement. The treated heifers were each fed an additional 5 pounds of grain (mixed with the appropriate amount of OmniGen-AF® per pound of body weight) second (Figure 2). The concrete slab was cleaned of all OmniGen-AF® residue before re-opening the head-lock gates.

Figure 1. Head lock stanchion system used to feed the unsupplemened control heifers.

Figure 2. Head lock stanchion system used to feed the OmniGen-AF® fed heifers.

Growth measurements and feeding

Pre-trial heights and body weights were recorded while heifers were secured in stanchions. Heights were taken (in centimeters) at the hip using a height stick. Weights were taken (in kilograms) around the heart girth using a weigh tape. The determination of heights and weights continued once a month for the remainder of the study.

Figure 3. Heights being measured using a height stick at the top of the heifers hip.

Figure 4. Weights being measured using a measuring tape around the heifers' heart girth.

Blood Collection and Processing

All blood samples were taken via jugular venipuncture. While secured in the head stanchion, heifers were restrained using a halter. The area of the jugular vein was sanitized with a cotton pad soaked in 70% isopropyl alcohol. One 9-mL Tempus Blood RNA Tube (AB Applied Biosystems), seven 8.5-mL yellow top BD vaccutainer® blood tubes with 1.5-mL acid citrate dextrose anticoagulant (Becton, Dickenson and Company, Franklin Lakes NJ, USA) and two 10-mL red top BD vaccutainer® Serum blood tubes (Becton, Dickenson and Company, Franklin Lakes NJ, USA) were pre-labeled with the heifer number and collection day. A plastic sleeve was used to house the 18-G x 1-inch multiple use vacuette® drawing needle (Greiner Bio-One GmBH, Kremsmünster, Austria) during blood collection. After collection, blood samples were placed in a styrofoam container (with 37°C warm packs when outside temperature was below 5°C) to keep warm until transported to the laboratory for immediate processing.

Upon returning to the lab, the seven 8.5-mL yellow top BD vaccutainer® blood tubes with anticoagulant were inverted 10 times to mix the contents. 1-mL of blood was removed from one of the yellow top blood tubes using a sterile pipette and placed in a pre-labeled 1.5-mL microcentrifuge tube for hematology processing. All 7 blood tubes from each heifer were then centrifuged at 2,000 rpm (850xg) for 10 minutes at room temperature. Three-milliliters of plasma were removed using a sterile pipette and placed into three 1-mL portions in pre-labeled 1.5-mL microcentrifuge tubes, which were then stored in an -80°C freezer. The remainder of the plasma was removed and discarded. The buffy coat from each tube was collected, combining the buffy coat from all blood tubes from each animal into two 50-mL centrifuge tubes. Fifty-milliliter centrifuge tubes were brought

to 40-mL with Ca++ and Mg+ free 1 X PBS and mixed well to disperse any clumps. The 40 mL of diluted buffy coat cells were layered on top of 10-mL of Histopaque 1083 (density 1.083 gm/mL) at room temperature using a sterile 60-cc syringe barrel and 18-g x 1.5-inch sterile needle to slowly pour the cell suspension on top the Histopaque. The tubes were then centrifuged at 2,000 rpm for 30 minutes. After centrifugation, the PBS layer above the cell interface was removed and discarded. The mononuclear cell layer was collected and transferred to a 50-mL centrifuge tube, one tube per animal, and QS to 50-mL with 1 X PBS. The centrifuge tubes were then centrifuged at 2,000 rpm for 5 minutes and then the PBS solution was carefully poured off. The cells were suspended in 10-mL of PBS and 50-µL were added to 450-µL of 0.04% Trypan Blue Solution (CellGro, Manassas, VA), 10-µL were added to hemocytometer C-chip (Thermo Fisher Scientific, Asheville, NC) and a viable cell count was performed. The number of live cells, dead cells, and squares were counted, and the number of live cells per mL in the sample was calculated to determine the amount of media (RPMI + 10% gamma-irradiated-FCS + 0.1% (50-µg/ml) gentamicin sulfate) to dilute the cells to get a suspension of $6x10^6$ per mL.

The stock stimulants utilized in these assays were: 1) killed BHV-1 [binary ethyleneimine-inactivated BHV-1 Cooper strain] at a concentration of $6.1x10^7$ tissue culture infectious dose 50 ($TCID_{50}$) equivalent units/mL, 2) live BVDV type 1 virus [strain NADL] at a concentration of $6.6x10^6$ $TCID_{50}$ per mL, 3) live BVDV type 2 virus [strain A125] at a concentration of $6.3x10^6$ $TCID_{50}$ per mL, 4) *Staphylococci enterotoxin* B (SEB) at a concentration of 1.0 µg/mL in phosphate buffered saline), and 5) negative control media (RPMI + 10% GI-FCS + 1% gentamicin sulfate). The working stimulants used for each day were diluted in the media to generate final concentrations of each virus at the desired

multiplicity of infection (MOI) relative to the number of cells in the cultures. BVDV type 1 and BVDV type 2 virus were diluted to give a final working concentration of 0.5 MOI and 0.25 MOI in the cultures. Killed BHV-1 virus was diluted to give a final working concentration of 1.0 MOI and 0.5 MOI equivalents in the final cultures. The SEB stimulant was prepared as a 1:1000 dilution of SEB stock in media as a working stock to yield the desired final working concentration. The negative control stimulant contained only media. Thirty-milliliters of each diluted stimulant were prepared on each collection day.

Using a repeating pipette with a 5-mL dispenser and a 200-µl pipet tip at the end, 100-µL of mononuclear cells were paced in the appropriate well of a round bottom 96-well plate, and 600-µL of mononuclear cells were paced in the appropriate well of a 24-well plate. Using a repeating pipette with a 5-mL dispenser and a 200-µl pipet tip, 100-µL of each stimulant were placed in quadruplicates in the appropriate well of a round bottom 96-well plate, and 600-µL of each stimulant were placed in triplicates of the appropriate well of a 24-well plate.

Both the round bottom 96-well plates and the 24-well plates were placed in a 37˚C with 5% CO_2 humidified chamber incubator. The 24-well plates were incubated for 72 hours, and were removed from the incubator, and supernatants were collected. The 96-well plates were incubated for 5 days, removed from the incubator, and processed for proliferation.

Blood Analysis

Collection of Supernatants

After 72 hours, the 24-well plates were removed from the incubator and centrifuged at 1,200 rpm for 5 minutes. The plates were tipped to a 45-degree angle and 1,000-µL of supernatant was collected form each well and placed into a pre-labeled 1.5-mL microcentrifuge tube. The tubes were stored in an -80˚C freezer until ELISA cytokine assays were preformed.

All cytokines were measured using commercial kits or antibody pairs per the standard protocol. Culture supernatants were tested in duplicate and analyzed with a recombinant cytokine reference standard to allow for quantification.

Proliferation Measurements

After 5 days, the 96-well plates were removed from the incubator. There were 2 cows per plate and each well contained 100-µl of mononuclear cells and 100-µl of stimulant. Each stimulant was plated in quadruplicates. 10-ml of 1:50 (200-µl tritiated thymidine (^{3}H-TdR) stock in 9.8 ml of sterile PBS). TdR working stock was prepared per collection day. The plates were pulsed by adding 10-µl of ^{3}H-TdR working stock into each well using repeating dispenser with a 500-µl dispenser syringe with a 200-µl pipet tip. One plate was pulsed at a time and all plates were pulsed within 10 minutes of each other. The plates were then placed in the radio-isotope incubator at 37˚C with 5% CO_2 for six hours.

Harvesting and Counting - After the six-hour incubation, the contents of each 96-well round bottom plate were harvested onto a glass fiber filter pad in an automated harvester (FilterMate, PerkinElmer, Waltham, MA) to collect the cells from the culture. The

contents were aspirated by vacuum for 10 seconds. This was followed by 12 ten-second distilled water washes of the samples to remove all water soluble (other than double stranded DNA biomolecules) from the paper. The papers were dried under vacuum for 2-3 minutes. The glass fiber pad was removed from the automated harvester. Individual disks cut into the pad by the harvester were transferred to 7-mL counting vials in racks. The disks were dried for 12-24 hours. The vials were transferred to counting racks, filled with 3-mL of counting cocktail (Cytoscint, MP bioproducts, Indianapolis, IN), and capped. The racks for vials were placed in a Beckmann LS6500 liquid scintillation counter (LSC) (Beckmann, Fullerton, CA) and counted using a tritium program for 2 minutes per sample.

The LSC detects and counts the release of electrons from radioactive decay. Decay of tritium produces low energy electrons, which interact with hetrocyclic ring compounds in the cocktail to convert the energy into light. A secondary compound captures light at that wavelength and produces two new photons for each original photon at the wavelength the counter captures. Photomultiplier tubes in the scintillation counter create a current when 2 photons are captured at the same time at 180 degrees separation. Each nuclear decay event is registered and converted into measurable electrical pluses and these data are accumulated over the counting time of the sample. The number of electrons captured is reported in counts per minute. The efficiency of counting was previously determined to be 34% of decays per minute.

To determine how metabolically active the cells are, the count for the control wells (mononuclear cells plus media, no stimulant) are used as the background count. The stimulation index is calculated by dividing the count for the stimulant wells (mononuclear cells plus virus) divided by the count for the control wells.

Cells undergoing division are metabolically active, require thymidine and will use any available thymidine outside of the cell before creating its own. The more metabolically active the cell is, the more thymidine is taken up, and most all of this thymidine in incorporated into new DNA.

Hematology Assessment

A one-ml sample of blood was removed from one of the yellow top tubes (ACD anticoagulant) during blood processing and held at 4˚C until being analyzed on a Heska CBC-Diff analyzer (Fort Collins, CO). Data were collected for total white blood cell (WBC) count, total lymphocyte count, total monocyte count, total neutrophil count, and percentages of lymphocytes, monocytes, and neutrophils were calculated.

Serum Processing for Serum Neutralizing (SN) Titers

Serum was processed from blood that was allowed to clot at 4˚ C for at least 4 hours; the tubes were then centrifuged at 900 rpm for 15 minutes. Serum was stored in four one-mL aliquots in pre-labeled 1.5-mL microcentrifuge tubes. One of the microcentrifuge tubes was heat inactivated at 56˚ C for 30 minutes (to remove complement activity) for SN titer determination. The remaining portions were stored at -20˚C or colder for future assessment. SN titers were measured by the Athens Veterinary Diagnostic Laboratory of the University of Georgia using the standard clinical protocol. (http://www.vet.uga.edu/dlab)

Plasma Processing for Prostaglandin Measurements

Three-mL of plasma were removed using a sterile pipette during blood processing and placed in three one-mL portions in pre-labeled 1.5-mL tubes, which were then stored in an -80˚C freezer. One tube was removed from the freezer and placed in an 37˚C incubator until completely thawed. 100-µl of plasma was removed from the tube, added to 900-uL of methanol in a new 1.5-mL microcentrifuge tube, and vortexed. Once this was done for all the tubes, they were then centrifuged at 12,000 rpm for 5 minutes. Exactly 800-uL of the methanol phase liquid was drawn up and put in a new pre-labeled 1.5-mL microcentrifuge tube and dried down in the Centrivap (Labconco, Kansas City, MO). Approximately 4 hours were required for the tubes to completely dry; the time varied with relative humidity for that day.

Tempus Tube Analysis for L-selectin and IL-8 mRNA Markers

9-mL Tempus Blood RNA Tubes (AB Applied Biosystems) were vigorously shaken at the time of collection for complete mixing of blood and stabilizing reagent. After collection, the Tempus Blood RNA Tubes were brought back to the lab and stored in a -20°C freezer until being sent to Prince Agri for analysis.

ELISA Cytokine Assays

IL-4

Bovine IL-4 Screen Set kit produced by Thermo Scientific (Pierce Biotechnology, Rockford, IL) was used for this assay. 100-ul of IL-4 coating antibody diluted 1:150 in pH 9.7 carbonate bicarbonate buffer was dispensed in each well of a Flat-Bottom Maxisorb 96-

well ELISA plate (Thermo Scientific, Waltham, MA). The plate was covered with a sealer and incubated overnight at room temperature. The plates were then washed 3 times on a plate washer (Dynex Technologies, Waltham, MA) with wash buffer (ultra pure water + 10% 10xPBS + 0.05% tween). 300-ul of blocking buffer (4% Bovine Albumin Serum (BSA) + 5% sucrose in 1xPBS) were added to each well and incubated for 1 hour. The plates were then washed 3 times on the plate washer. Bovine IL-4 standards were diluted to a high concentration of 2,000 pg/mL in regent diluent (4% BSA in 1xPBS) and serially diluted 1:2 for the standard curve. 100-µl of each standard was plated in duplicate in the first 2 columns of the plate. 100-µl of each supernatant sample was plated in duplicate in the rest of the wells. The plates were covered, incubated for 1.5 hours, and then washed 3 times on the plate washer. 100-µl of IL-4 detection antibody diluted 1:200 in reagent diluent was added to each well and incubated for 1 hour. Plates were washed 3 times on the plate washer. Strepavidin-HRP was diluted 1:200 in reagent diluent, 100-ul was added to each well, and the plates were incubated for 30 minutes. Plates were washed 3 times on the plate washer. 100-µl of the provided substrate solution was added to each well and incubated in the dark for 15 minutes. The reaction was stopped by adding 100-µl of the provided stop solution to each well. The plates were then read on the Epoch plate reader (BioTek, Burlingtion, VT) and using ELISA software, the optical density (OD) was read at am absorbance of 450 nm.

IL-10

The mouse anti bovine interleukin-10 capture antibody (Bio-Rad AbD Serotec, Raleigh, NC) was diluted 1:333 in carbonate bicarbonate buffer and 100-µl was dispensed

in each well of a 96-well ELISA plate. This was covered with a plate sealer and incubated overnight at room temperature. The plates were then washed 3 times on the plate washer with wash buffer. 300-µl of blocking buffer (1% BSA in 1xPBS + 0.05% tween) added to each well and the plates were covered and incubated for 2 hours at room temperature. Plates were washed 3 times on the plate washer. Bovine IL-10 standard (Kingfisher, St. Paul, MN) was diluted to 5,000 pg/mL in reagent diluent (1% BSA in 1xPBS + 0.05% tween) and serially diluted 1:2 for the standard curve. 100-µl of each standard was plated in duplicate in the first 2 columns of the plate. 100-µl of each supernatant sample was plated in duplicate in the rest of the wells. The plates were covered, incubated for 1 hour and then washed 3 times on the plate washer. 50-µl of mouse anti bovine interleukin-10: biotin detection antibody (Kingfisher, St. Paul, MN) diluted 1:500 in reagent diluent was added to each well and incubated for 1 hour. Plates were washed 3 times on the plate washer. Strepavidin-HRP was diluted 1:200 in reagent diluent, 100-µl was added to each well, and the plates were incubated for 45 minutes. Plates were washed 3 times on the plate washer. TMB was prepared by mixing equal parts of Reagent A (stabilized hydrogen peroxide) and Reagent B (stabilized tetramethylbenzidine) (R&D Systems, Minneapolis, MN). 100-µl of the TMB was added to each well and incubated in the dark for 15 minutes. The reaction was stopped by adding 100-µl of the stop solution (0.5 M sulfuric acid) to each well. The plates were then read on the Epoch plate reader (BioTek) and using ELISA software, the OD was read at an absorbance of 450 nm.

IL-17

Bovine IL-17A VetSet ELISA Development Kit by (Kingfisher, St. Paul, MN) was used for this assay. Bovine IL-17A standards were diluted to a high concentration of 12 ng/mL in regent diluent (4% BSA in 1xPBS) and serially diluted 1:2 for the standard curve. 100-µl of each standard was plated in duplicate in the first 2 columns of the provided bovine IL-17A coated plates. 100-µl of each supernatant sample was plated in duplicate in the rest of the wells. The plates were covered, incubated for 1 hour and then washed 3 times on the plate washer. 100-µl of IL-17A detection antibody diluted 1:23 in reagent diluent was added to each well and incubated for 2 hours. Plates were washed 3 times on the plate washer. Strepavidin–HRP was diluted 1:200 in reagent diluent, 100µl was added to each well, and the plates were incubated for 30 minutes. Plates were washed 4 times on the plate washer. TMB was prepared as stated above and 100-µl was added to each well and incubated in the dark for 15 minutes. The reaction was stopped by adding 100-µl of stop solution (0.5 M sulfuric acid) to each well. The plates were then read on the Epoch plate reader (BioTek) and using ELISA software, the OD was read at an absorbance of 450 nm.

IFN-gamma

Bovine IFN-gamma ELISA kit provided by R&D Systems was used for this assay. 100-µl of IFN-gamma coating antibody diluted 1:182 in carbonate bicarbonate buffer was dispensed in each well of a 96-well ELISA plate (Thermo Scientific, Waltham, MA). This was covered with a plate sealer and incubated overnight at room temperature. The plates were then washed 3 times on a plate washer with wash buffer. 300-µL of blocking buffer (1% BSA in 1xPBS) was added to each well and incubated for 1 hour. The plates were then

washed 3 times on the plate washer. Bovine IFN-gamma standards were diluted to a high concentration of 10,000 pg/mL in regent diluent (4% BSA in 1xPBS) and serially diluted 1:2 for the standard curve. 100-µL of each standard was plated in duplicate in the first 2 columns of the ELISA plate. 100-µL of each supernatant sample was plated in duplicate in the rest of the wells. The plates were covered, incubated for 2 hours, and then washed 3 times on the plate washer. 100-µL of IFN-gamma detection antibody diluted 1:182 in reagent diluent was added to each well and incubated for 2 hours. Plates were washed 3 times on the plate washer. Strepavidin-HRP was diluted 1:200 in reagent diluent, 100-µL was added to each well, and the plates were incubated for 30 minutes. Plates were washed 3 times on the plate washer. TMB was prepared as stated above and 100-µL was added to each well and incubated in the dark for 15 minutes. The reaction was stopped by adding 100-µL of stop solution (0.5 M sulfuric acid) to each well. The plates were then read on the Epoch plate reader (BioTek) and using ELISA software, the OD was read at an absorbance of 450 nm.

TGF-beta

The bovine TGF-beta capture antibody (R&D Systems, Minneapolis, MN) was diluted 1:500 in coating buffer (1M $Na_2CO_3^{-2}$, 9.7 pH) and 100-µl was dispensed in each well of a 96-well ELISA plate. This was covered with a plate sealer and incubated overnight at room temperature. The plates were then washed 3 times on the plate washer with wash buffer. 300-µl of blocking buffer (0.5% BSA in 1xPBS) were added to each well and the plates were covered and incubated for 1 hour at room temperature. Plates were washed 3 times on the plate washer. Recombinant TGF-beta standard (R&D Systems, Minneapolis, MN) was

diluted to 200 ng/mL in reagent diluent (wash buffer) and serially diluted 1:2 for the standard curve. 100-µl of each standard was plated in duplicates in the first 2 columns of the plate. 100-µl of each supernatant sample was plated in duplicate in the rest of the wells. The plates were covered, incubated for 1 hour and then washed 3 times on the plate washer. 100-µl of Bovine TGF-beta detection antibody (R&D Systems, Minneapolis, MN) diluted 1:500 in reagent diluent was added to each well and incubated for 1 hour. Plates were washed 3 times on the plate washer. Goat anti-mouse IgG HRP was diluted 1:1000 in reagent diluent, 100-µl was added to each well, and the plates were incubated for 30 minutes. Plates were washed 4 times on the plate washer. 15-µl of 30% of hydrogen peroxide is added per 11-mL of 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid **(**ABTS) 15 minutes before use. 100-µl of the ABTS was added to each well and incubated in the dark for 15 minutes. The plates were then immediately read on the Epoch plate reader (BioTek) and using ELISA software, the OD was read at an absorbance of 405 nm.

IL-2

Bovine IL-2 ELISA Kit provided by R&D Systems was used for this assay. 100-µl of IL-2 capture antibody diluted 1:180 in 1xPBS was dispensed in each well of a 96-well ELISA plate (Thermo Scientific, Waltham, MA). This was covered with a plate sealer and incubated overnight at room temperature. The plates were then washed 3 times on a plate washer with wash buffer. 300-µl of blocking buffer (5% Tween20 in PBS with 0.05% NaN_3) were added to each well and incubated for 1 hour. The plates were then washed 3 times on the plate washer. Bovine IL-2 standards were diluted to a high concentration of 10,000 pg/mL in regent diluent (5% Tween20 in 1xPBS) and serially diluted 1:2 for the standard curve.

100-µl of each standard was plated in duplicate in the first 2 columns of the ELISA plate. 100-µl of each supernatant sample was plated in duplicate in the rest of the wells. The plates were covered, incubated for 2 hours, and then washed 3 times on the plate washer. 100-µl of IL-2 detection antibody diluted 1:180 in reagent diluent was added to each well and incubated for 2 hours. Plates were washed 3 times on the plate washer. Strepavidin-HRP was diluted 1:200 in reagent diluent, 100-µl was added to each well, and the plates were incubated for 20 minutes. Plates were washed 3 times on the plate washer. TMB was prepared as stated above and 100-µl was added to each well and incubated in the dark for 15 minutes. The reaction was stopped by adding 100-µl of stop solution (0.5 M sulfuric acid) to each well. The plates were then read on the Epoch plate reader (BioTek) and using ELISA software, the OD was read at an absorbance of 450 nm.

Prostaglandin E_2

Plasma samples were prepared for prostaglandin E_2 analysis by methanol extraction. 100-µL of plasma was removed, added to 900-mL of methanol in pre-labeled 1.5-mL microcentrifuge tubes, and then immediately vortexed. Samples were then centrifuged at 12,000 rpm for 5 minutes. Exactly 800-mL of the methanol phase that were layered over the cell pellet was measured out and placed in a new pre-labeled 1.5-mL microcentrifuge tube. Samples were dried using the Centrivap Console (Labconco, Kansas City, MO) at 35°C for approximately 4-6 hours (depending on humidity levels). Samples were frozen and stored at -80°C.

Prostaglandin E_2 Express EIA Kit (Caymen Chemical Company, Ann Arbor, MI) was used for this assay. Plates pre-coated with goat polycolonal anti-mouse IgG and blocked

with proteins were provided. EIA buffer was prepared by diluting the provided vial of EIA buffer concentrate (10X) in 90ml of UltraPure water. Wash buffer was prepared by diluting the provided 5-mL vial of wash buffer concentrate in 2-liters of UltraPure water plus 1-ml of provided Polysorbate20.

All plating was done according to the instructions that were provided in the Prostaglandin E_2 Express SIA Kit. The contents of the provided PGE_2 Express EIA standard were reconstituted and diluted in EIA buffer to a high concentration of 2,000 pg/mL and serially diluted 1:2 for the standard curve. 50-µL of each standard were plated in duplicate in the specified columns of the provided plates. The methanol extracted plasma samples were diluted 1:2 in EIA buffer and 50-µl of each sample were added to the appropriate wells. 50-µl of provided Prostaglandin E_2 Express Ache Tracer and 50-µL of provided Prostaglandin E_2 Express Monoclonal Antibody were added to the appropriate wells as instructed. Plates were then coved with plastic film and incubated for 60 minutes at room temperature on an orbital shaker. After incubation, the plates were washed on the plate washer with the E_2 wash buffer 5 times. The provided Ellman's Reagent was reconstituted in UltraPure water and 200-µL were immediately added to each well. Plates were then recovered with the plastic film and developed in the dark for 45 minutes. Plates were read on the Epoch plate reader (BioTek) and using ELISA software, the OD was read at an absorbance of 405 nm.

Data Calculations

The known sample concentrations from the diluted standards for each ELISA assay and its corresponding OD are entered into Prism (Version 5, GraphPad Software, La Jolla, CA) to produce a linear regression standard curve. Then, the supernatant sample OD measurement obtained for each assay and the known standard curve was used to interpolate the corresponding unknown substance concentration.

The ELISA calculated concentrations were then imported into JMP (Version 11, SAS software, Cary, NJ) and unpaired t-tests were run for each specific time point to calculate the p-values, which were then reported and used to determine significance. Significance was accepted at a p-value of 0.05 or less.

CHAPTER 5

RESULTS

Overview

This study was designed and run as two parallel trials to facilitate the handling of the samples and optimize the quality of the assessments conducted on immune function. Handling and processing of the blood collected required significant time, and with the limited personnel resources available, we chose to limit the number of heifers in each trial to no more than 14.

The trials were conducted over the course of about 18 months. The second trial group was started as the final sample collection phase of the first group was being undertaken. This sequence worked because the last 60 days of the trial had more intensive sampling than the first 110 days.

The two groups were intended to be run under as close to identical conditions as could be achieved. However, the weather during the second trial period introduced stress of a significant magnitude on that group. The first group was subjected to only 27.65 inches of rainfall over the course of the trial. The second group was subjected to 12 days of severe rain (days that had over 1 inch of rainfall) totaling 35.88 inches over the course of the trial. The housing area for the heifers became saturated, particularly in the area of the feeding station, and made for difficult and unsure footing. The heifers in this study were moved twice after the initiation of the trial. Both groups were fed a standard dairy diet consisting of silage and grain with unlimited access to

hay and water. We observed that some animals in Group 2 were not feeding normally on 92 days that were documented in our records. The quality of silage (main diet of the heifers) declined during the wettest part of the trial (a period of about 70 days). Trench silage preparation is used on this dairy and the heavy rains and saturation decreased the quality.

Thus, the results of Group 1 and Group 2 will be presented separately in this thesis. A comparison of the two trials and impact of the introduction of a high level of natural weather induced stress will be addressed in the discussion of these results. All statistical measurements were calculated in JMP using standard unpaired t-tests.

Table 1: Group 1 and Group 2 - Weather data for the duration of each trial

	Group 1	Group 2
Trial Start Date	April 9th	August 28th
Trail End Date	September 23rd	February 18th
Missed Feeding Days	1	1
Total Rain Fall	27.63 inches	35.88 inches
Days with <1 in Rain Fall	4 days	12 days
Average Humidity	72.8	74.8
High Temperature	100.7°F	94.0°F
Low Temperature	46.3°F	22.9°F
Average Temperature	76.1°F	57.3°F

Table 2: Group 1 and Group 2 – Feeding data for the duration of each trial

Cow	Group	Treatment	Number of Days
4040	Group 1	Control	Did not lock up for over 90% of the trial and had to be fed her portion of the grain on the concrete ledge
4022	Group 1	Control	Did not lock up on 32 recorded days
4050	Group 2	OmniGen	Did not eat well on 13 recorded days
4055	Group 2	OmniGen	Stopped locking up in December and after about 30 days had to be removed from the trial
5001	Group 2	Control	Did not lock up on 20 recorded days
5006	Group 2	Control	Did not lock up on 27 recorded days

Group 1

Body Weight

The heifers grew as expected in both treatment groups during the course of this study (figure 5). The measurements for body weight were consistent for each treatment group throughout the duration of the trial, and we observed little difference among the heifers between groups. The calves gained an average of 67 pounds per month. The OmniGen-AF® fed group weighed an average of 14 more pounds at the end of the trial than the control group. This did not prove to be a statistically significant difference.

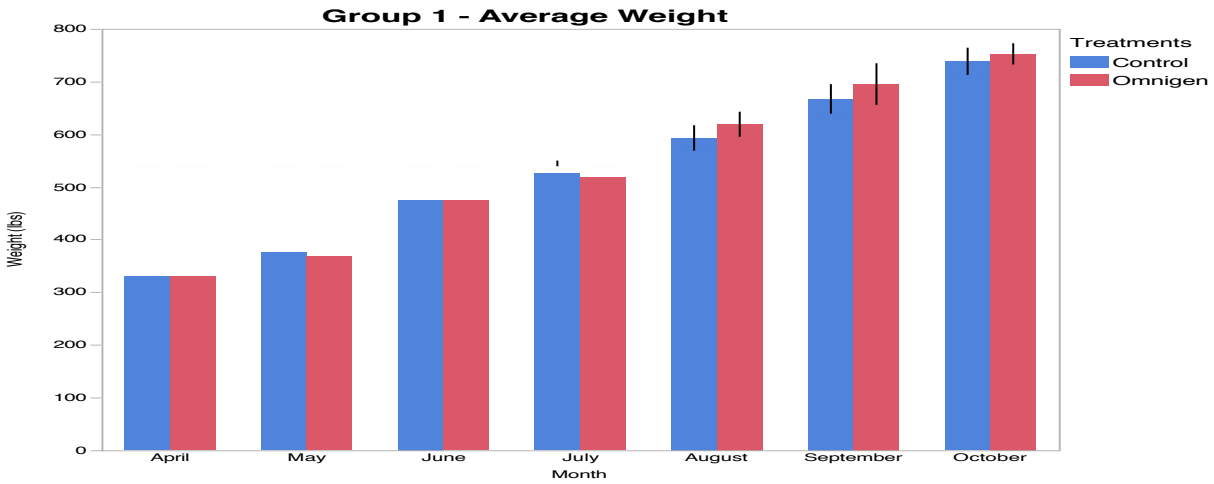

Figure 5. Average weight in pounds for both treatment groups taken at the beginning of each month. Measurements were taken using a measuring tape around the heifers' heart girth.

Hip Heights

The measurements for hip height were consistent for each treatment group throughout the duration of the trial (Figure 6). No differences in growth of the calves during the handling period were observed. The calves grew an average of 4 centimeters per month with no difference in heights between the two treatment groups at the end of the trial.

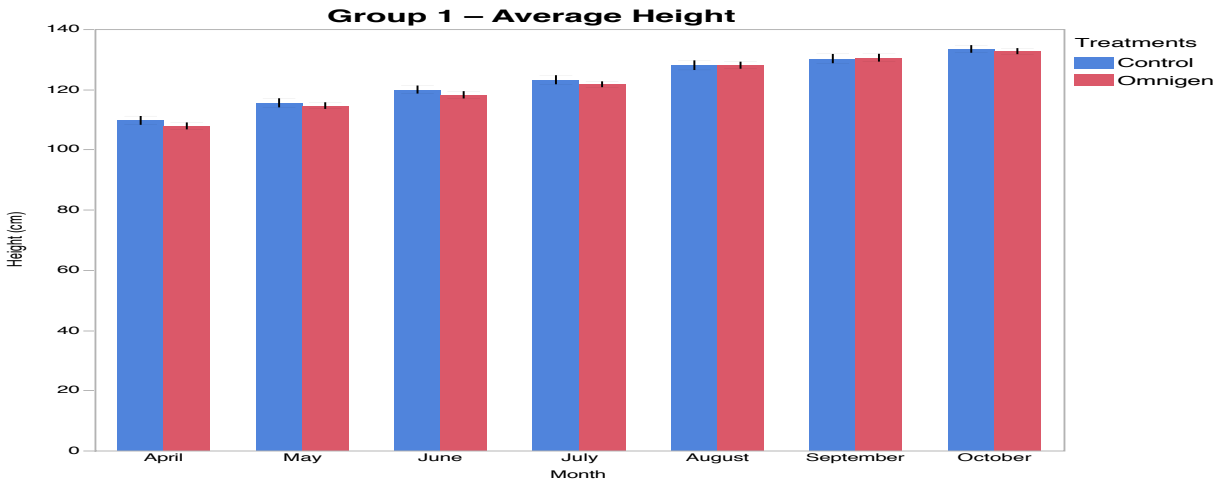

Figure 6. Group 1 - Average height in centimeters for each treatment group taken at the beginning of each month. Measurements were taken using a measuring stick at the heifers' hip.

Hematological Profile

To monitor the general immunological and physiological status of the heifers, a complete blood count (CBC) profile was measured for each heifer each time they were bled. The total white blood cell (WBC) count was relatively consistent between each treatment group with the control group being slightly higher, but did not reach a level of significance until D40 when the control group had a significantly higher WBC than the OmniGen-AF® group ($p=0.0458$) and approached a significance difference in WBC count, with the control group having a higher count, on D60 ($p=0.0988$) (Figure 7A). The total lymphocyte count was higher in the control group compared to the OmniGen-AF® group on all collection days (Figure 7B). There was a significant difference on D-60 ($p=0.0397$), D40 ($p=0.0138$), and approached significance on day D60 ($p=0.0705$). Both the total monocyte count (Figure 7C) and the total neutrophil count (Figure 7D) fluctuated in both treatment groups throughout the trial. Post booster vaccination given on D0, the control group had slightly higher counts of monocytes and neutrophils in the secondary response but this did not prove to be a statistically significant difference.

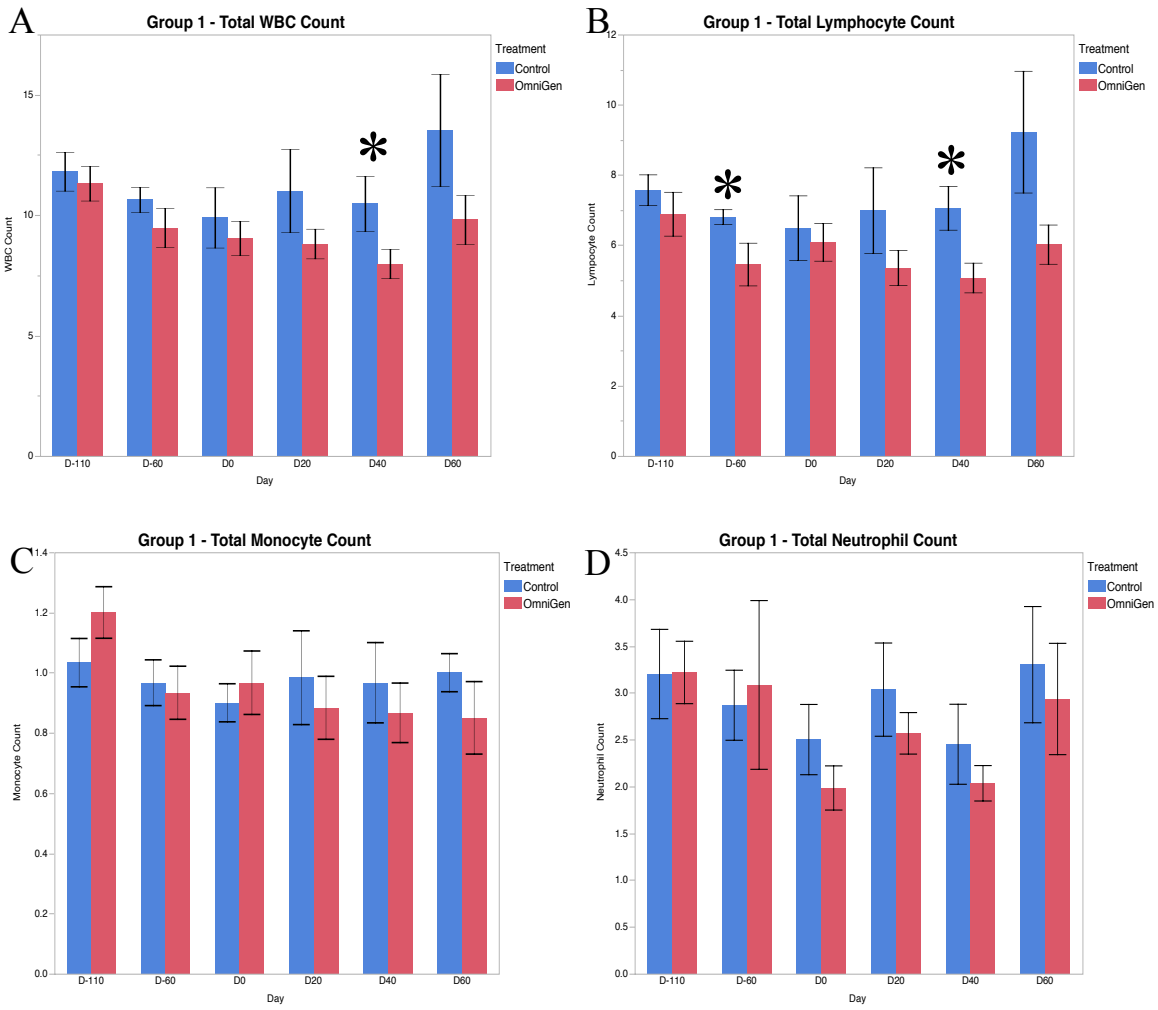

Figure 7. Group 1 – Hematological Profiles. (A) total white blood cell (WBC), (B) total lymphocyte count, (C) total monocyte count, and (D) total neutrophil count for each treatment group on each of the collection days (D-110, D-60, D0, D20, D40, D60).

Proliferation

BVDV1

Following priming vaccination given on D-110 the recall antigen proliferation response of the OmniGen-AF® group was slightly greater for both the 0.25 MOI and the 0.5 MOI exposure of BVDV1 on D-60, but the differences observed did not reach the level of significance (Figure 8). Following the booster vaccination, given on D0, there was a significant increase in proliferation for the OmniGen-AF® treatment group compared to the control group. The OmniGen-AF® group had an increase in proliferation at both the 0.25 MOI exposure of BVDV1 (p=0.0042) and the 0.5 MOI exposure of BVDV1 (p=0.0001) on D20, an increase at both the 0.25 MOI exposure of BVDV1 (p=0.0003) and the 0.5 MOI exposure of BVDV1 (p=0.0009) on D40, and an increase in proliferation for both the 0.25 MOI exposure of BVDV1 (p=0.0034) and the 0.5 MOI exposure of BVDV1 (p=0.0002) at D60.

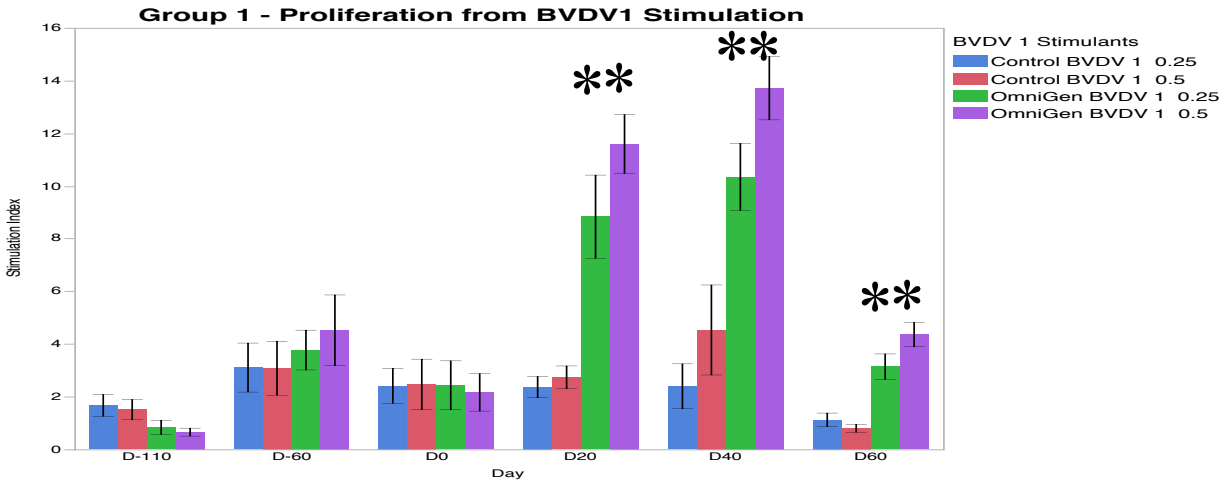

Figure 8: Group 1 - Level of proliferation (as stimulation index) of mononuclear cells from each treatment group when stimulated against two different concentrations of the BVDV1 virus (0.5 MOI and 0.25 MOI).

BVDV2

Following priming vaccination given on D-110, the primary response in the control group had higher levels of proliferation induced by both the 0.25 MOI (p=0.0367) and the 0.5 MOI exposures to BVDV1 (p=0.0320) on D-60 (Figure 9). After receiving the booster vaccine (given on D0) there was a significant increase in proliferation for the OmniGen-AF® treatment group compared to the control group starting on D40. There was a highly variable proliferation response for both of the treatment groups on D20, a significant increase at both the 0.25 MOI (p=0.0283) and the 0.5 MOI exposure of BVDV1 (p=0.0044) at D40, and an increase at both the 0.25 MOI (p=0.0461) and the 0.5 MOI exposure of BVDV1 (p=0.0354) at D60.

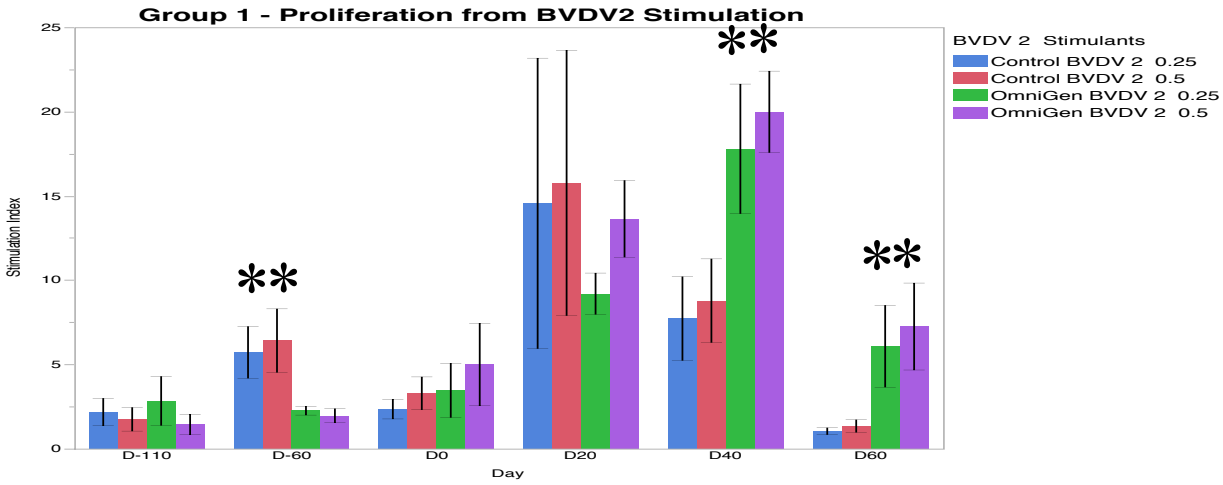

Figure 9: Group 1 - Level of proliferation (as stimulation index) of mononuclear cells from each treatment group when stimulated against two different concentrations of the BVDV2 virus (0.5 MOI and 0.25 MOI).

BHV-1

Following priming vaccination given on D-110, the primary response had relatively consistent levels of proliferation with no significant difference between the two treatment groups between D-110 and D0 (Figure 10). After the booster vaccine was given on D0, there was a significant increase in proliferation for the OmniGen-AF® treatment group compared to the control group starting on D40 for the 0.5 MOI exposure of BHV-1 (p=0.0036) and approached significance for the 1.0 MOI exposure of BHV-1 (p=0.0606). This increase in proliferation for the OmniGen-AF® fed heifers continued through D60 for both the 0.5 MOI (p=0.0025) and for the 1.0 MOI exposure of BHV-1 (p=0.0268).

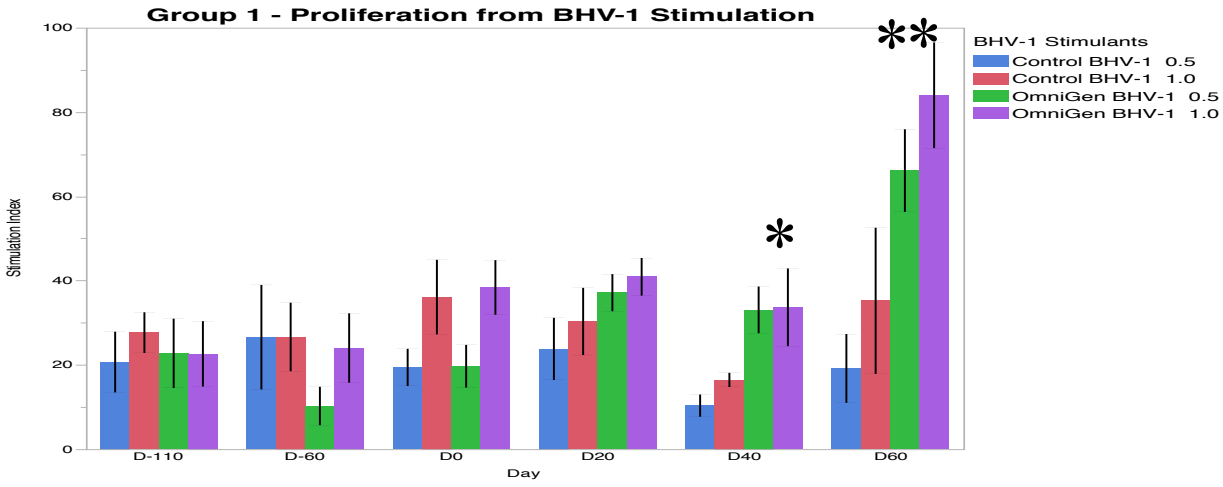

Figure 10: Group 1 - Level of proliferation (as stimulation index) of mononuclear cells from each treatment group when stimulated against two different concentrations of the BHV-1 virus (1.0 MOI and 0.5 MOI).

Serum Neutralizing Titers

BVDV1

The mean antibody levels for BVDV1 for both treatment groups on D-110 were low or negative, and stayed relatively low through the primary immune response with no significant difference in SN titer between the two treatment groups (Figure 11). After the booster vaccine was given on D0, SN antibody levels rose 18-fold between D0 and D20 and slowly declined on D40 and D60. However, no significant difference in SN titers between the two treatment groups were observed on any of the collection days.

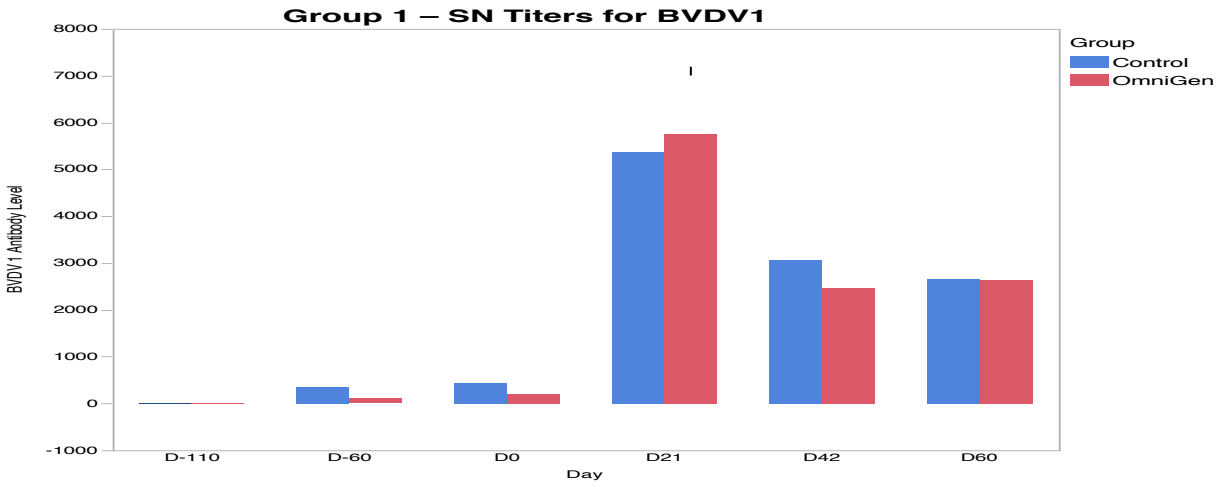

Figure 11: Group 1 - Levels of circulating SN antibody titers in serum against the virus BVDV1 on each of the collection days.

BVDV2

The mean SN titers for BVDV2 for each treatment group on D-110 was low or negative (Figure 12). Between D-110 and D-60, antibody levels rose 22-fold with no significant difference between the treatment groups. On D-60 and D0 the control group had higher antibody levels compared to the OmniGen-AF® group, but this difference was not significant. After the booster vaccine was given on D0, antibody levels rose 14 fold in the OmniGen-AF® fed group and 3-fold in the control group between D0 and D20; however, no significant difference in SN titers between the two treatment groups was observed. There was a slow decline in antibodies on D40 and D60 in both treatment groups.

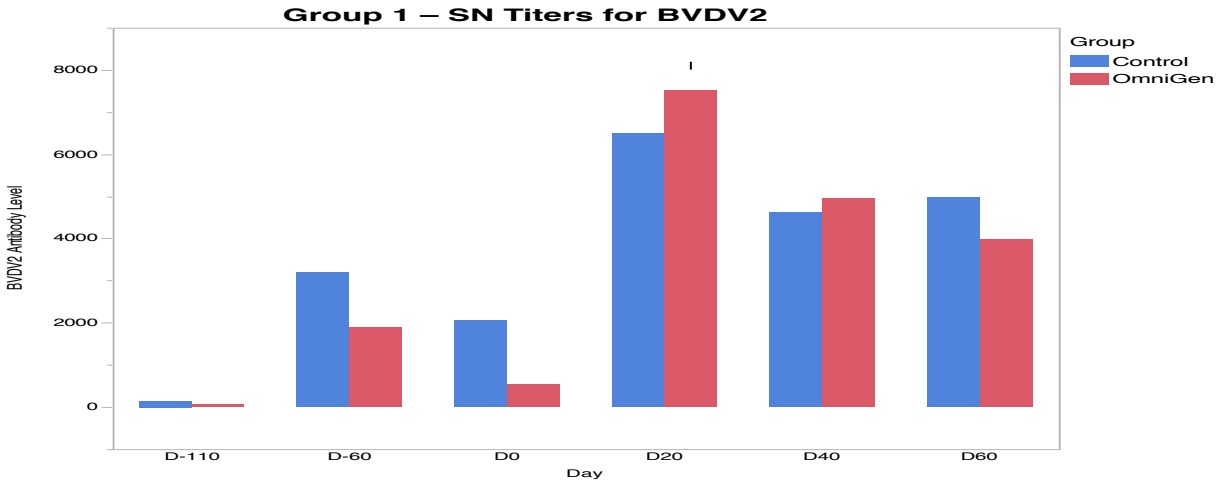

Figure 12: Group 1 - Levels of circulating SN antibody titers in serum against the virus BVDV2 on each of the collection days.

BHV-1

The mean antibody levels for BHV-1 for both treatment groups on D-110 were extremely low (Figure 13). The titers increased significantly on D-60, but there was no significant difference between the treatment groups observed. On D0, the control group had higher antibody levels compared to the OmniGen-AF® group (p=0.0357). After the booster vaccine was given on D0, the control group had a 4-fold increase in antibody titers and the OmniGen-AF® fed heifers had 16-fold increase. The control heifers had slightly higher levels of circulating antibodies for most of the secondary immune response, but the differences observed between the treatment groups did not reach the level of significance.

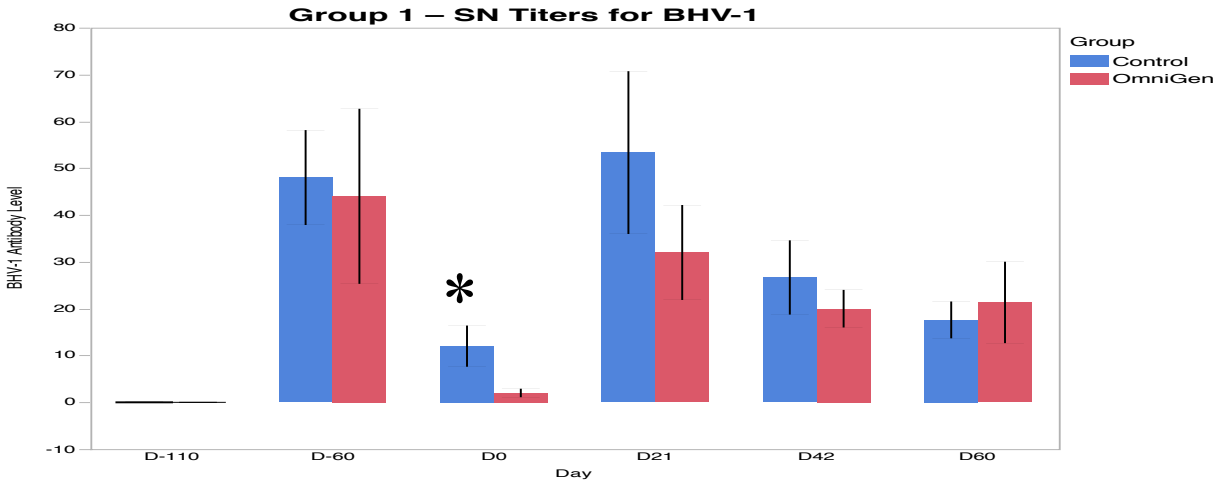

Figure 13: Group 1 - Levels of circulating SN antibody titers in serum against the virus BHV-1 on each of the collection days.

L-selectin and IL-8 mRNA Markers

CD62L and CXCR2 were used as indicator genes for L-selectin and IL-8 mRNA which are immune markers specific for innate immunity. These two markers are used in all OmniGen-AF® trials as an indicator that the supplement was properly fed and that the expected innate immune effects occurred. In this study, the effect of OmniGen-AF® on both CD62L ($p=0.0985$) and on CXCR2 ($p=0.0592$) approached significance (unpublished data by Derek McLean, Prince Agri), which confirmed that feeding OmniGen-AF® enhanced innate immunity.

IL-4

BVDV1

Very low levels of IL-4 were produced when the monocytes were stimulated with BVDV1 (Figure 14). Production was below detectable levels of IL-4 for the primary immune response, and only on D60 in the secondary immune response were there any substantial IL-4 levels produced. The OmniGen-AF® fed heifers produced higher IL-4 levels; however, there was not a significant difference in levels between the control and OmniGen-AF® treatment groups.

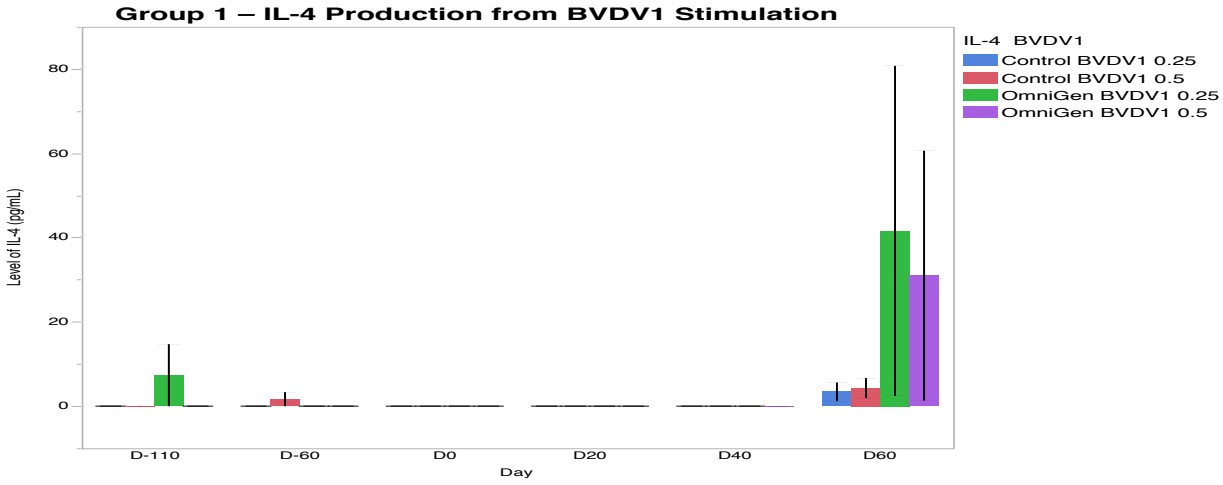

Figure 14: Group 1 - Level of IL-4 (pg/mL) produced when mononuclear cells were stimulated with 0.25 MOI and 0.5 MOI exposure of BVDV1.

BVDV2

Very low levels of IL-4 were produced when the monocytes were stimulated with BVDV2 (Figure 15). Production was below detectable levels of IL-4 for the primary immune response and only on D60 in the secondary immune response were there any substantial IL-4 levels produced but there was not a significant difference in IL-4 levels between the control and OmniGen-AF® treatment groups.

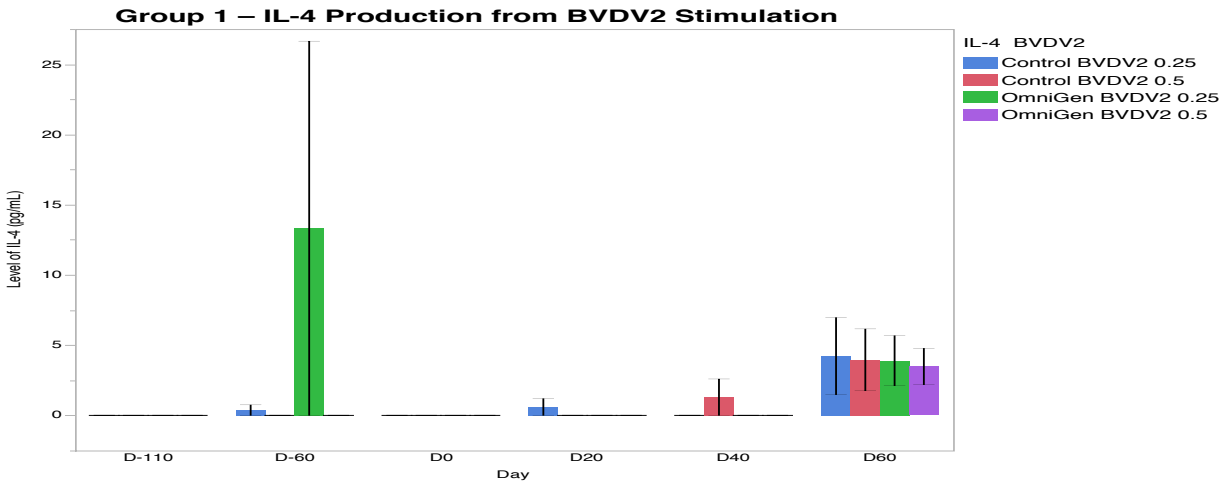

Figure 15: Group 1 - Level of IL-4 (pg/mL) produced when mononuclear cells were stimulated with 0.25 MOI and 0.5 MOI exposure of BVDV2.

BHV-1

All animals had low initial levels of IL-4 production (D-110) (Figure 16). Following priming vaccination, the OmniGen-AF® fed cows produced higher levels of IL-4 on D-60 in the primary response than the control treatment group when stimulated with both the 0.5 MOI (p=0.0184) and the 1.0 MOI (p=0.0446) BHV-1 virus. On D0, the control treatment had higher levels of IL-4 at the 1.0 MOI stimulant of BHV-1 (p=0.0321). For the secondary response to the booster vaccine given on D0, there was an increase in IL-4 production in all treatment groups. The OmniGen-AF® groups had significantly higher levels of IL-4 at the 0.5 MOI exposure of BHV-1 on D40 (p=0.0471) and on D60 (p=0.0146) after booster vaccination.

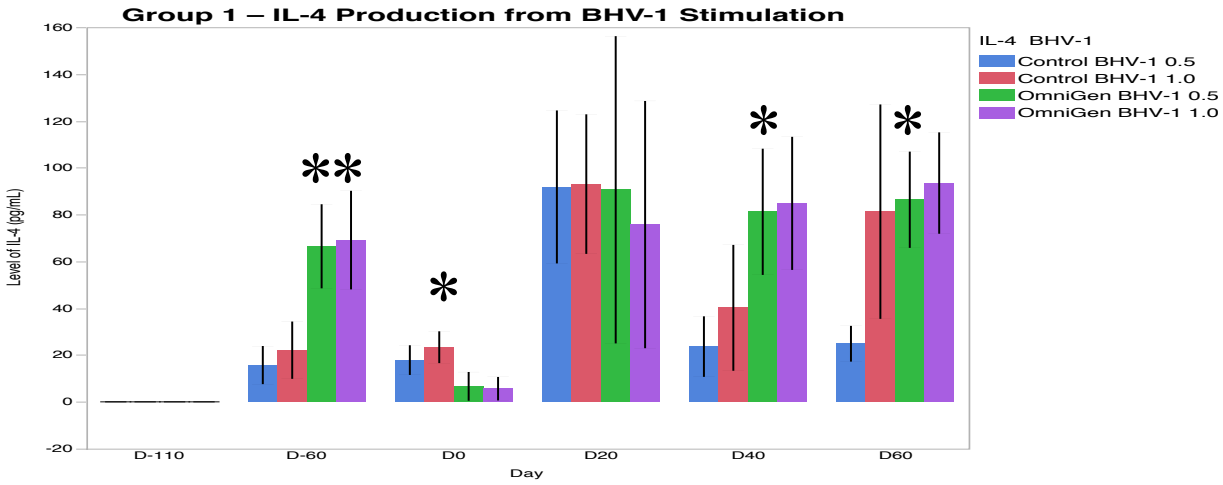

Figure 16: Group 1 - Level of IL-4 (pg/mL) produced when mononuclear cells were stimulated with 0.5 MOI and 1.0 MOI exposure of BHV-1.

IL-10

BVDV1

All animals had low initial levels of IL-10 production (D-110) (Figure 17). Following priming vaccination, the control fed cows produced higher levels of IL-10 on D0 when stimulated with both the 0.25 MOI (p=0.0337) and the 0.5 MOI (p=0.0172) BVDV1 virus. Following the booster vaccine (given on D0), there was an increase in IL-10 production by the control group which approached significance at the 0.25 MOI (p=0.0837) and was statistically significant at the 0.5 MOI (p=0.0400) when exposed to BVDV1 on D20. This trend was also observed on D40, but this did not reach the level of significance.

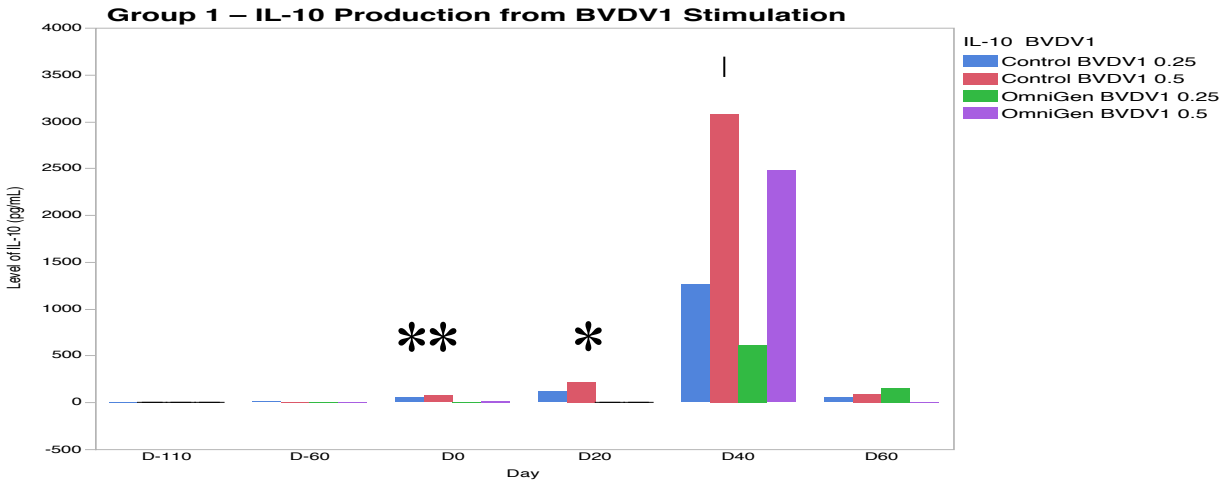

Figure 17: Group 1 - Level of IL-10 (pg/mL) produced when mononuclear cells were stimulated with 0.25 MOI and 0.5 MOI exposure of BVDV1.

BVDV2

All animals had relatively high initial levels of IL-10 production (D-110 and D-60) (Figure 18). By D0, the control heifers produced higher levels of IL-10 when mononuclear cells were stimulated with either the 0.25 MOI (p=0.0185) or the 0.5 MOI (p=0.0084) dose of BVDV2 virus. After the booster vaccine (given on D0), there was an increase in IL-10 production by the control group that approached significance when stimulated with the 0.25 MOI (p=0.0701) and was statistically significant at the 0.5 MOI (p=0.0432) dose of BVDV2 on D20. The control treatment group continued to have higher levels of IL-10 throughout the secondary immune response, but the differences did not reach the level of significance.

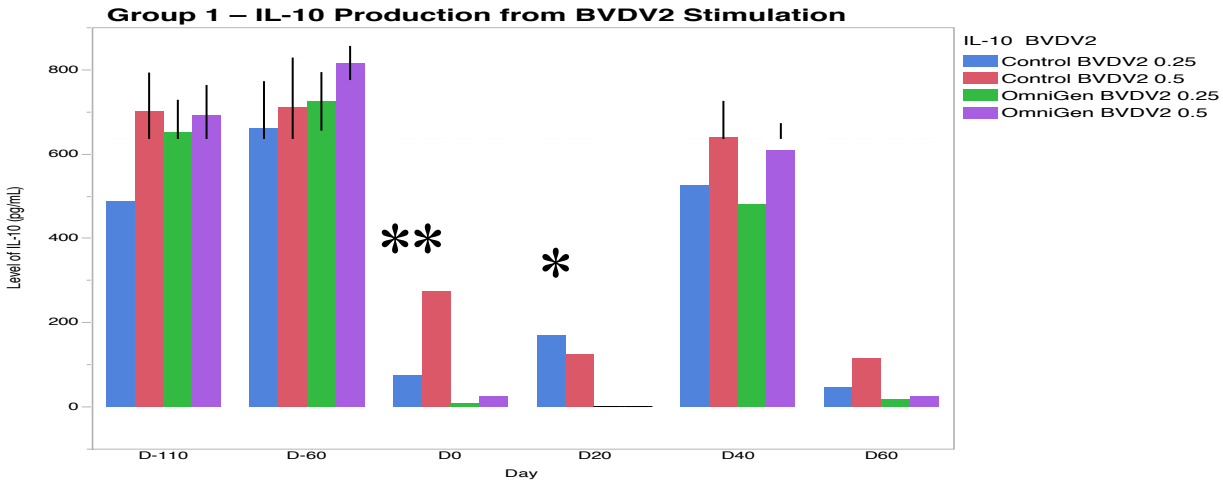

Figure 18: Group 1 - Level of IL-10 (pg/mL) produced when mononuclear cells were stimulated with 0.25 MOI and 0.5 MOI exposure of BVDV2.

BHV-1

Following priming vaccination, the primary response to BHV-1 antigen generated similar levels of IL-10 secretion with no significant difference between the two treatment groups observed (Figure 19). Following the booster vaccine (given on D0), there was a significant increase in IL-10 production in mononuclear cells from the control heifers compared to the OmniGen-AF® treated group starting on D20 for both the 0.5 MOI and the 1.0 MOI (p=0.0039) dose of killed BHV-1 (p=0.0161). The control cows continued through to have higher levels of induced IL-10 through D60 of the secondary immune response, but this did not reach the level of significance.

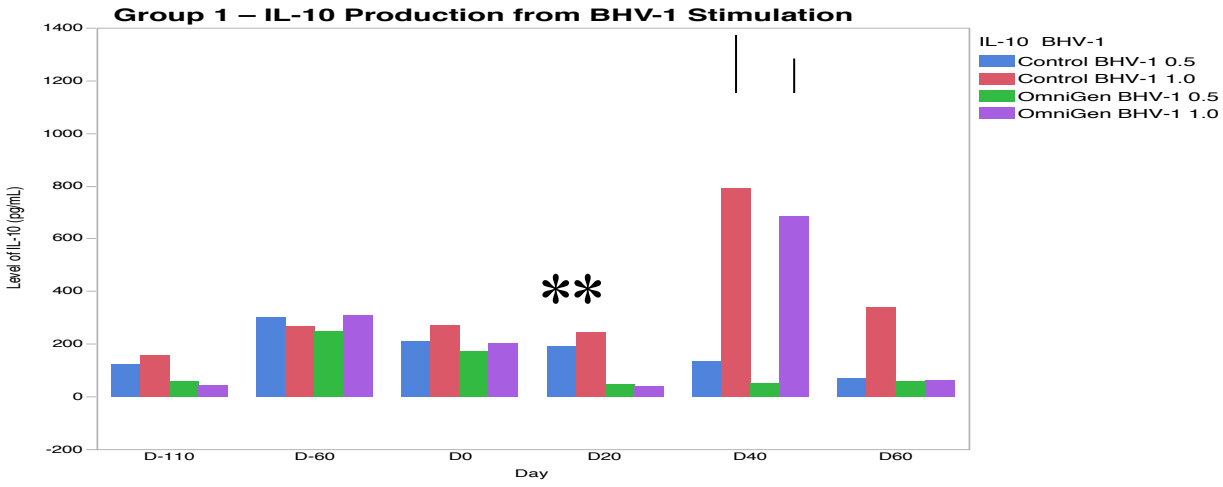

Figure 19: Group 1 - Level of IL-10 (pg/mL) produced when mononuclear cells were stimulated with 0.5 MOI and 1.0 MOI exposure of BHV-1.

IL-17

BVDV1

All animals had low initial levels of IL-17 production when exposed to BVDV type 1 virus (Figure 20). Following priming vaccination, the OmniGen-AF® fed heifers produced higher levels of IL-17 on D-60 in the primary response than the control group. However, this did not reach the level of significance. Following booster vaccination, there was a high level of variability in the response of individual animals and between the two treatment groups. On D60, the OmniGen-AF® treatment group did generate higher levels of IL-17 than the controls, but again this did not reach the level of significance.

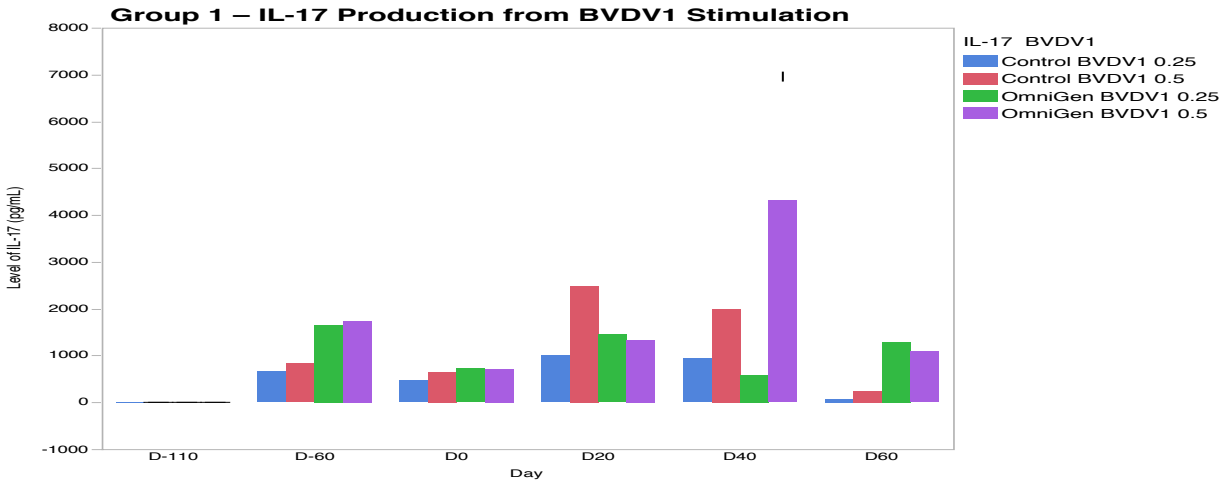

Figure 20: Group 1 - Level of IL-17 (pg/mL) produced when mononuclear cells were stimulated with 0.25 MOI and 0.5 MOI exposure of BVDV1.

BVDV2

Mononuclear cells from most animals had low initial levels of IL-17 production when exposed to BVDV type 2 virus (Figure 21). Following priming vaccination, the control group produced higher levels of IL-17 on D-60 than the OmniGen-AF® fed heifers at either the 0.25 MOI (p=0.0496) or the 0.5 MOI (p=0.0166) dose of BVDV2. Following the booster vaccine (given on D0), both treatment groups generated similar levels of IL-17 in response to BVDV type 2. On D60, the OmniGen-AF® heifers generated higher levels of IL-17 in response to BVDV type 2, but this did not reach the level of significance.

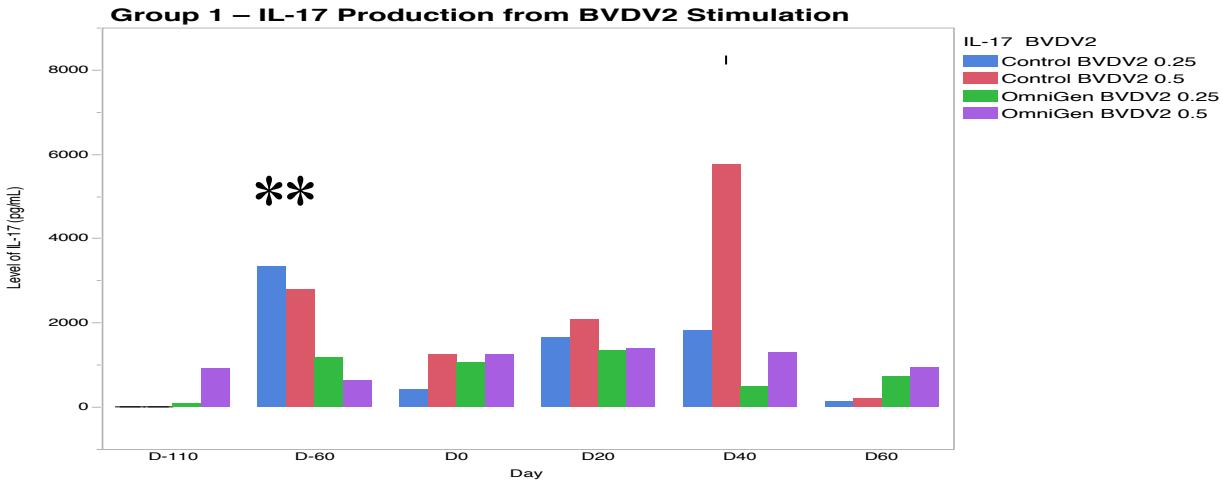

Figure 21: Group 1 - Level of IL-17 (pg/mL) produced when mononuclear cells were stimulated with 0.25 MOI and 0.5 MOI exposure of BVDV2.

BHV-1

Following priming vaccination, the primary response generated very similar levels of Il-17 in each group of heifers (Figure 22). There was no significant difference between the two treatment groups after the priming vaccine was given on D-110. Both treatment groups demonstrated a significant rise in IL-17 levels on D0 before the booster vaccine was given. This suggested a prolonged primary response in IL-17 production. Following booster vaccine given on D0, there was no significant difference in the level of IL-17 produced by mononuclear cells stimulated with killed BHV-1 between the two treatment groups. Induced IL-17 levels slowly declined in both groups during the secondary immune response.

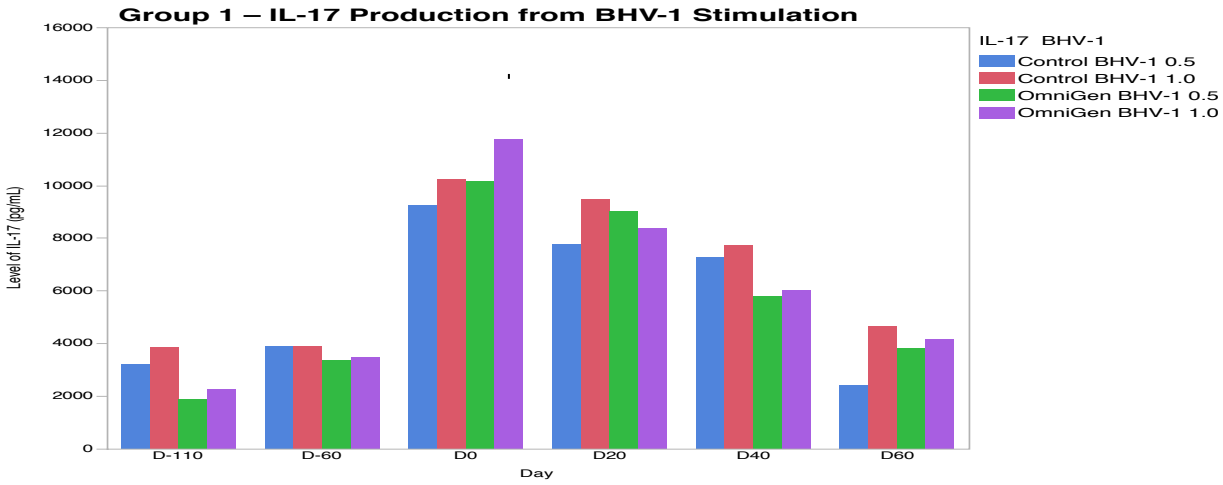

Figure 22: Group 1 - Level of IL-17 (pg/mL) produced when mononuclear cells were stimulated with 0.5 MOI and 1.0 MOI exposure of BHV-1.

IFN-gamma

BVDV1

Following priming vaccination, the primary response the OmniGen-AF® fed heifers yielded higher levels of IFN-gamma from BVDV type 1 stimulated mononuclear cells than was observed for the control group (Figure 23). However, this difference did not reach the level of significance. The level of IFN-gamma induced in mononuclear cells stimulated with BVDV type 1 virus was variable. The variability was observed for both treatment groups, and among heifers within each group during the secondary immune response. No significant difference in IFN-gamma production was observed between the two treatment groups after booster vaccination.

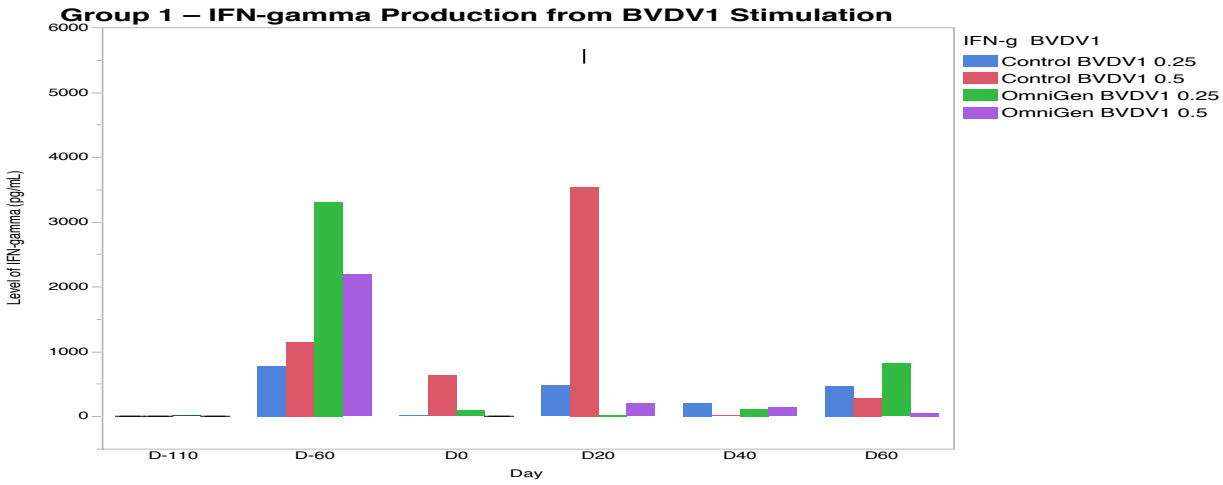

Figure 23: Group 1 - Level of IFN-gamma (pg/mL) produced when mononuclear cells were stimulated with 0.25 MOI and 0.5 MOI exposure of BVDV1.

BVDV2

The level of IFN-gamma induced in mononuclear cells stimulated with BVDV type 2 virus was variabile (Figure 24). The variability was observed for both treatment groups, and among heifers within each group. This was observed during both the primary and secondary immune response with no significant difference in IFN-gamma induction of mononuclear cells with BVDV type 2 virus between the two treatment groups.

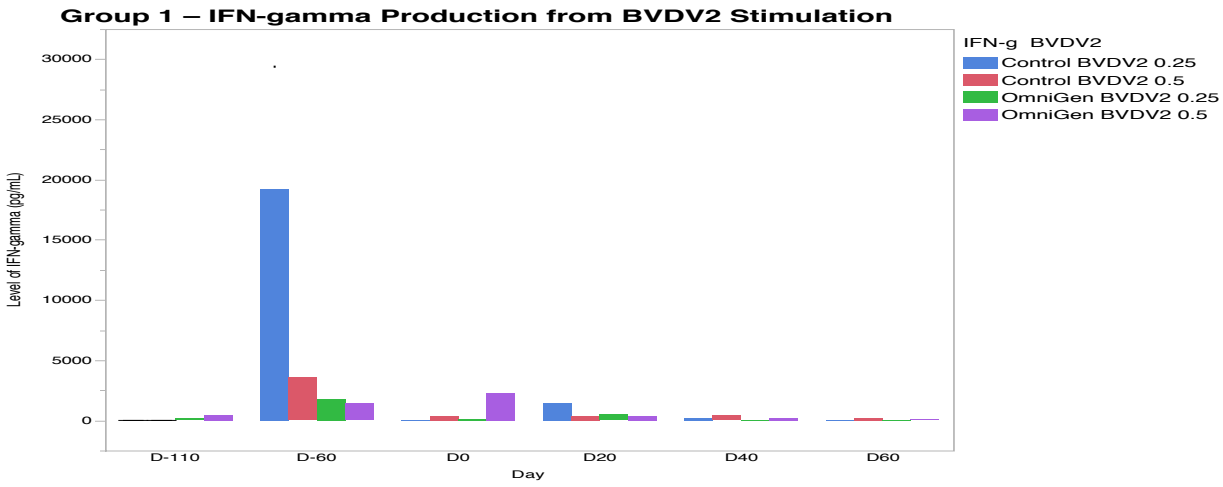

Figure 24: Group 1 - Level of IFN-gamma (pg/mL) produced when mononuclear cells were stimulated with 0.25 MOI and 0.5 MOI exposure of BVDV2.

BHV-1

Mononuclear cells from all animals had low initial levels of IFN-gamma production in response to killed BHV-1 (Figure 25). Following priming vaccination, both treatment groups showed a significant increase in IFN-gamma production. Mononuclear cells from the OmniGen-AF® fed group produced higher levels of IFN-gamma on D-60 than the mononuclear cells of the control group at both doses of killed BHV-1, but this difference did not reach the level of significance. Mononuclear cells from the OmniGen-AF® fed group produced higher levels of IFN-gamma on D0 at both the 0.5 MOI (p=0.0275) and the 1.0 MOI (p=0.0186) dose of killed BHV-1 than was observed for controls. Following the booster vaccine (given on D0), mononuclear cells from the OmniGen-AF® fed group produced higher levels of IFN-gamma on D20 at the 0.5 MOI dose of killed BHV-1 (p=0.0468). Mononuclear cells from the OmniGen-AF® heifers continued to produce slightly higher levels of IFN-gamma during the rest of the secondary immune response (D40 and D60), but the difference from control mononuclear cells did not reach the level of significance.

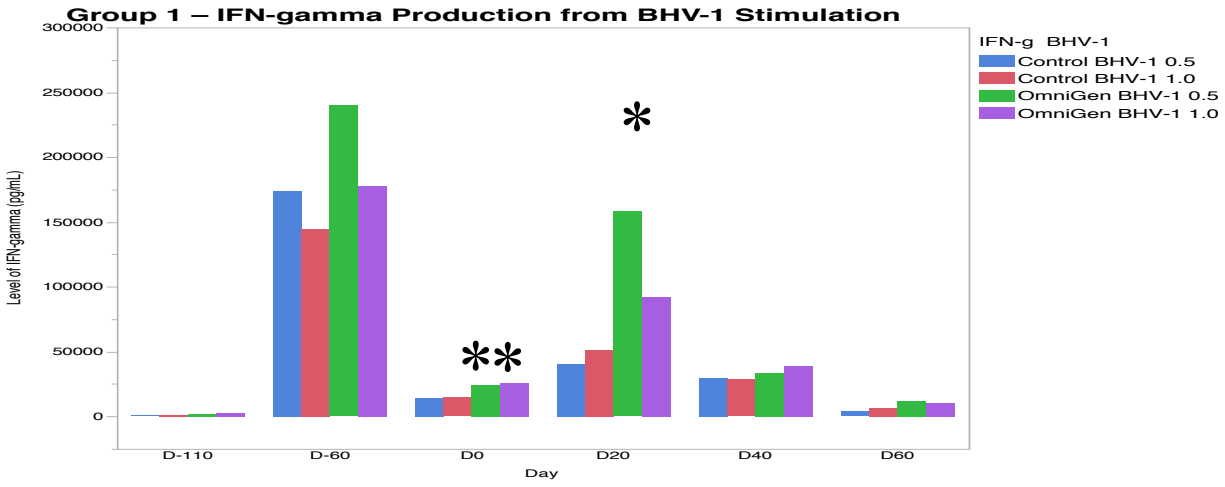

Figure 25: Group 1 – Level of IFN-gamma (pg/mL) produced when mononuclear cells were stimulated with 0.5 MOI and 1.0 MOI exposure of BHV-1.

Prostaglandin E_2

Following priming vaccination, the heifers in each group produced similar circulating levels of prostaglandin E_2 on D-110 and D-60 (Figure 26). We observed no significant difference between the two treatment groups. On D0, the control group had a higher level of PGE_2 in serum than the OmniGen-AF® fed heifers, but this did not reach the level of significance. Following the booster vaccine (given on D0), the control group had higher levels of prostaglandin E_2 than the OmniGen-AF® fed heifers on most of the collection days; however, there was no significant difference between the two treatment groups.

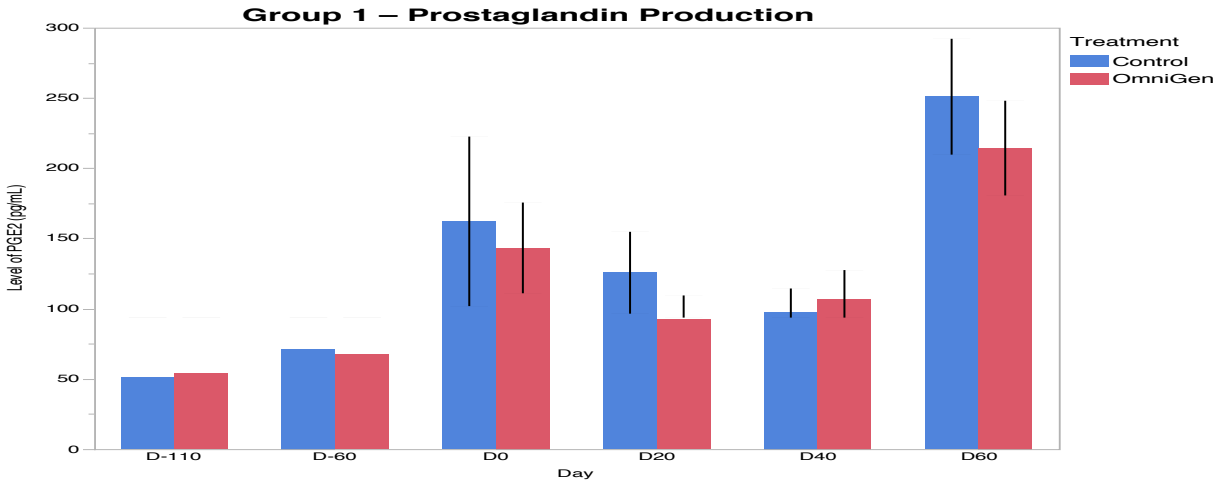

Figure 26: Group 1 - Level of prostaglandin E_2 (pg/mL) produced from plasma samples.

TGF-beta and IL-2

The assays for IL-2 and TGF-beta were not sensitive enough or the supernatants were collected from incubation at the wrong time and did not produce useful ELISA assay data; therefore, both were discontinued halfway through processing Group 1 data and the results were not reported in this thesis. Because different cytokines are produced at specific times during an immune response, depending on when the mononuclear cells were harvested there can be a fluctuation in the levels of the target cytokines. In previous unpublished studies by this laboratory, we established the level of production of IFN-gamma, IL-17, IL-10 and IL-4 in cultures stimulated with SEB and whole cell bacterial antigens. While the optimal level of cytokine protein we could measure in culture differed among these cytokines from 2-5 days, we found that all were present in representative quantities on day 3 of incubation with either SEB or the bacterial antigens. (Hurley, unpublished) Harvesting of supernatants on multiple collections days is needed to measure peak levels of these cytokines in the immune response.

Group 2

Average Daily Gain

Body Weight

The heifers grew as expected in both treatment groups during the course of this study. The measurements for body weight were very consistent for both treatment groups with little differences among the heifers at the beginning of the trial (Figure 27). Starting in November, the OmniGen-AF® fed heifers were 80 lbs heavier than the control treatment group, which proved to be a significant difference (p=0.0235). This trend continued through the end of the trial. December, they were 130 lbs heavier (p=0.0033), in January, they were 70 lbs heavier (p=0.058), and by February, they were 120 lbs heavier (p=0.008). The OmniGen-AF® heifers grew an average of 86 lbs a month whereas the control heifers grew an average of 66 lbs a month.

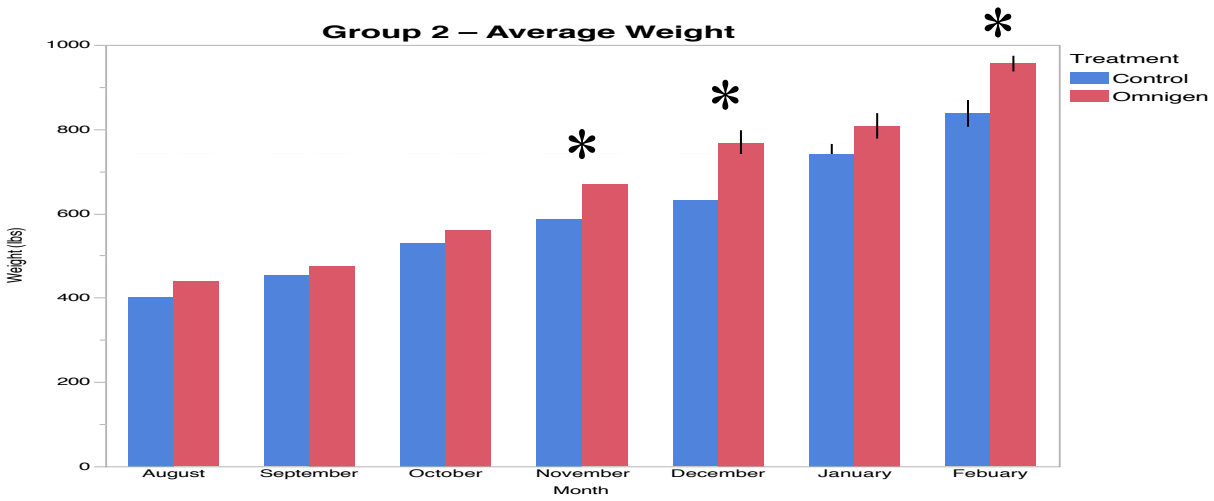

Figure 27: Group 2 - Average weight in pounds for both treatment groups taken at the beginning of each month. Measurements were taken using a measuring tape around the heifers' heart girth.

Hip Heights

The measurements for hip height were very consistent for each treatment group throughout the duration of the trial. The OmniGen-AF® fed heifers were slightly taller than the control heifers but this only reached the level of significance in December (p=0.0114) (Figure 28). The calves grew and average of 3.4 centimeters per month with little difference between the two treatment groups at the end of the trial.

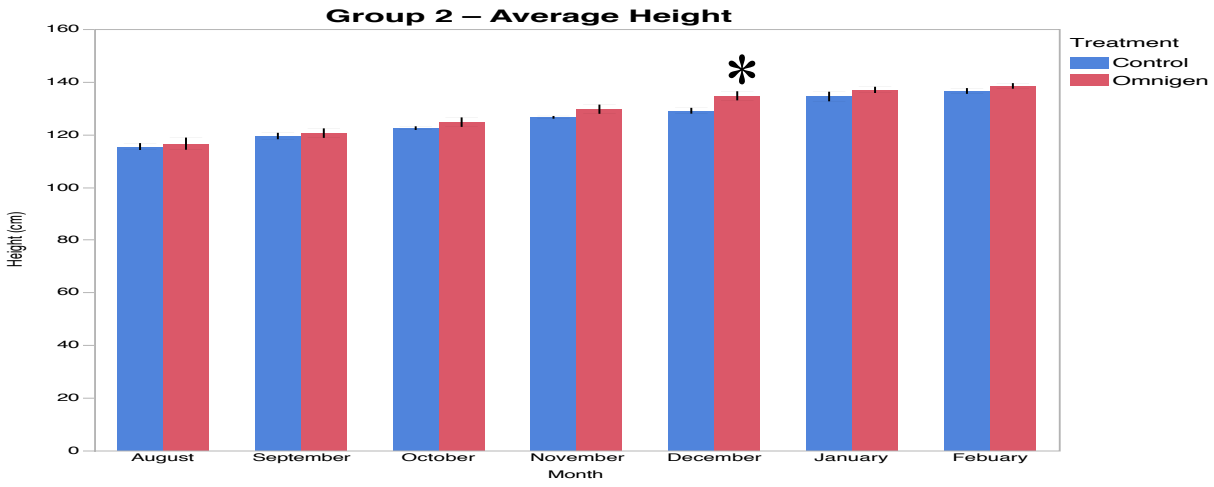

Figure 28: Group 2 - Average height in centimeters for each treatment group taken at the beginning of each month. Measurements were taken using a measuring stick at the heifers' hip.

Hematological Profile

To monitor the general immunological and physiological status of the heifers, a CBC profile was measured for each heifer each time they were bled. The total WBC count was relatively consistent between each treatment group with the control group being slightly higher, but did not reach the level of significance (Figure 29A). The total lymphocyte count was higher in the control group compared to the OmniGen-AF® group on most collection days but did not reach the level of significance (Figure 29B). The total monocyte count was higher in the control group compared to the OmniGen-AF® group on most collection days but did not reach the level of significance (Figure 29C). The total neutrophil count fluctuated in both treatment groups throughout the trial (Figure 29C). The OmniGen-AF® fed heifers had slightly higher neutrophil counts in the primary response, whereas the control treatment group had slightly higher neutrophil counts in the secondary response, but this did not prove to be a statistically significant difference.

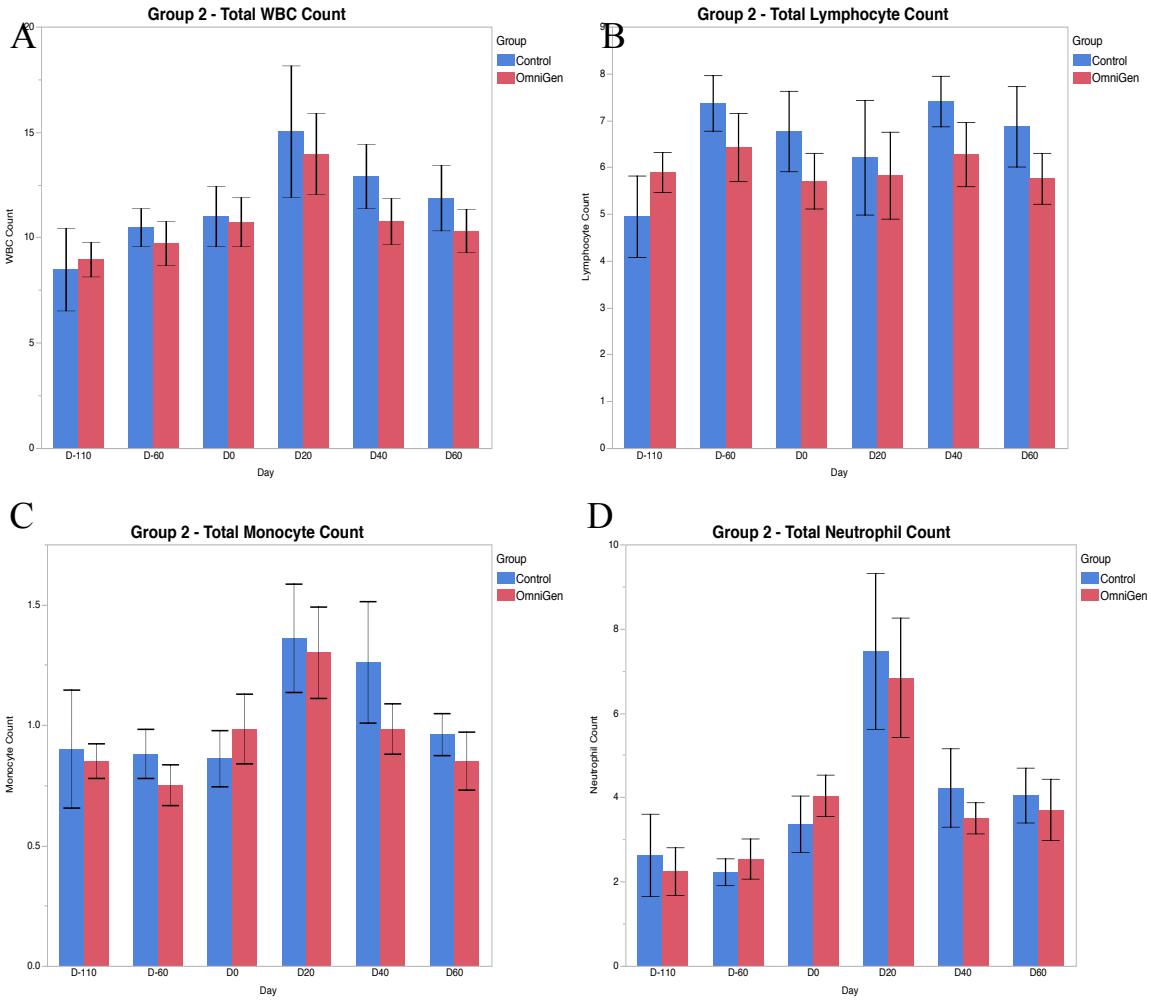

Figure 29: Group 2 – Hematological Profiles. (A) Total white blood cell (WBC), (B) total lymphocyte count, (C) total monocyte count, and (D) total neutrophil count for each treatment group on each of the collection days (D-110, D-60, D0, D20, D40, D60).

Proliferation

BVDV1

Following priming vaccination, the recall antigen proliferation responses for both treatment groups were relatively the same throughout the primary immune response when stimulated with BVDV1 (Figure 30). On D0, the heifers received a booster vaccine. It wasn't until D42 that a significant difference in secondary immune response was found between the two treatment groups. The OmniGen-AF® fed group had increased proliferation on D42 for both the 0.25 MOI (p=0.0404) and the 0.5 MOI (p=0.0114) stimulant of BVDV1 virus, and on D60 for both the 0.25 MOI (p=0.0482) and the 0.5 MOI (p=0.0021) stimulant of BVDV1 virus.

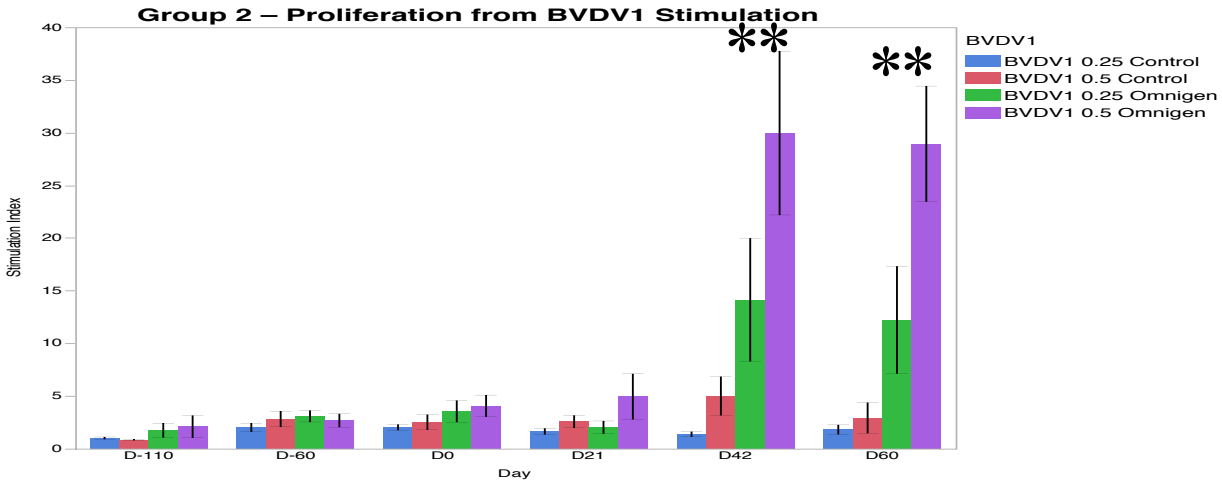

Figure 30: Group 2 - Level of proliferation (as stimulation index) of mononuclear cells from each treatment group when stimulated against two different concentrations of the BVDV1 virus (0.5 MOI and 0.25 MOI).

BVDV2

Following the priming vaccination, the recall antigen proliferation responses for both treatment groups were relatively low throughout the primary response when stimulated with BVDV2 (Figure 31). The OmniGen-AF® fed group had increased proliferation compared to the control group on D-110, but this did not reach the level of significance. More proliferation occurred from the OmniGen-AF® fed heifer's mononuclear cells than from the control treatment group on D-60 at the 0.25 MOI (p=0.0444) stimulant of BVDV2 and on D0 at the 0.5 MOI (p=0.0378) stimulant of BVDV2. On D0, the heifers received a booster vaccine. It wasn't until D42 that a significant difference in secondary immune response was found between the two treatment groups. The OmniGen-AF® fed group had increased proliferation on D42 for both the 0.25 MOI (p=0.0157) and the 0.5 MOI (p=0.0428) stimulant of BVDV2 virus, and on D60 at the 0.5 MOI (p=0.0195) stimulant of BVDV2 virus.

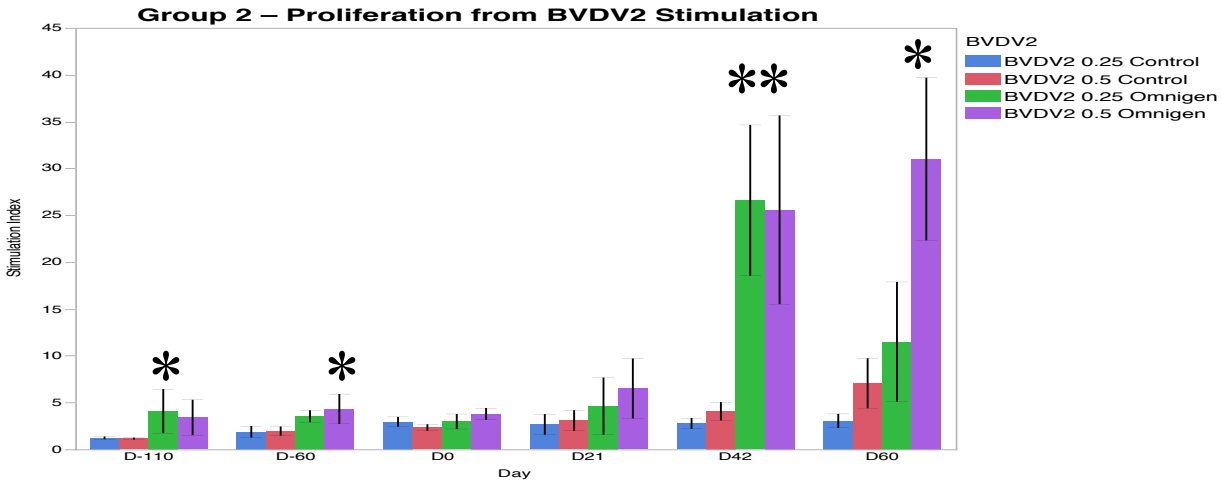

Figure 31: Group 2 - Level of proliferation (as stimulation index) of mononuclear cells from each treatment group when stimulated against two different concentrations of the BVDV2 virus (0.5 MOI and 0.25 MOI).

BHV-1

Following the priming vaccination, the recall antigen proliferation responses for both treatment groups at the same MOI were equivalent on D-110 when stimulated with BHV-1 (Figure 32). On D-60, the OmniGen-AF® fed heifers had increased proliferation compared to the control treatment group but this did not reach the level of significance until D0 at the 0.5 MOI stimulant of BHV-1 virus (p=0.0265). On D0 the heifers received a booster vaccine. More proliferation occurred from the OmniGen-AF® fed heifer's mononuclear cells than from the control treatment group on D21 at the 0.5 MOI stimulant of BHV-1 (p=0.0225). This increase in proliferation in the OmniGen-AF® fed heifers continued though the rest of the trial. The OmniGen-AF® fed group had increased proliferation on D42 for both the 0.5 MOI (p=0.0145) and the 1.0 MOI (p=0.0386) stimulant of BHV-1 virus and on D60 at the 0.5 MOI (p=0.0328) and the 1.0 MOI (p=0.0077) stimulant of BHV-1 virus.

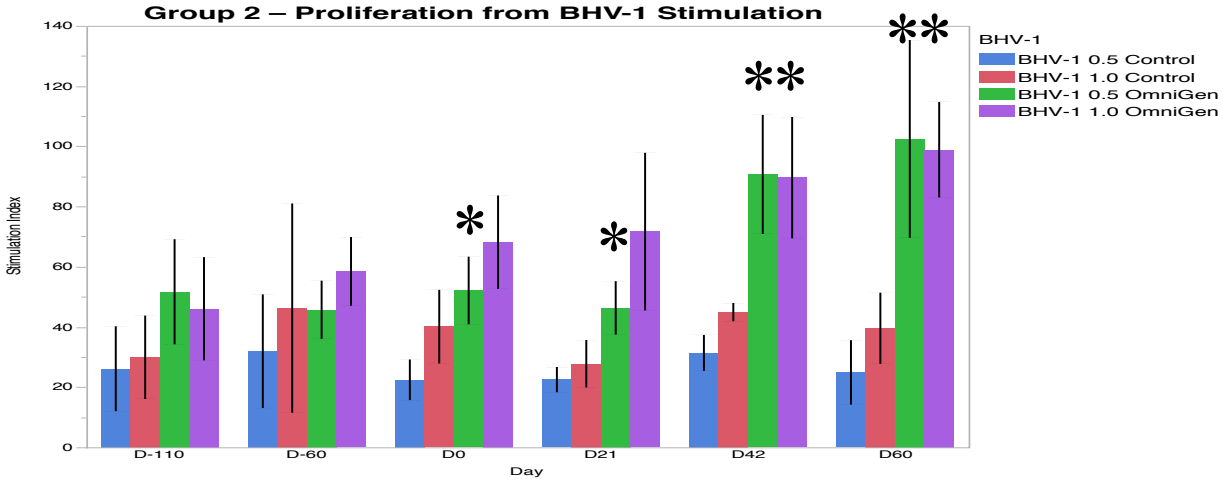

Figure 32: Group 2 - Level of proliferation (as stimulation index) of Mononuclear cells from each treatment group when stimulated against two different concentrations of the BHV-1 virus (1.0 MOI and 0.5 MOI).

Serum Neutralizing Titers

BVDV1

The mean antibody levels for BVDV1 for both treatment groups on D-110 were extremely low and stayed relatively low through the primary immune response (Figure 33). After the booster vaccine was given on D0, antibody levels rose 30-fold between D20 and D40 in both treatment groups. The control group had slightly higher levels of circulating antibodies for most of the primary and secondary immune responses. This approached significance on D60 (p=0.0627); however, there was very little difference in SN titers between the two treatment groups on any of the other collection days.

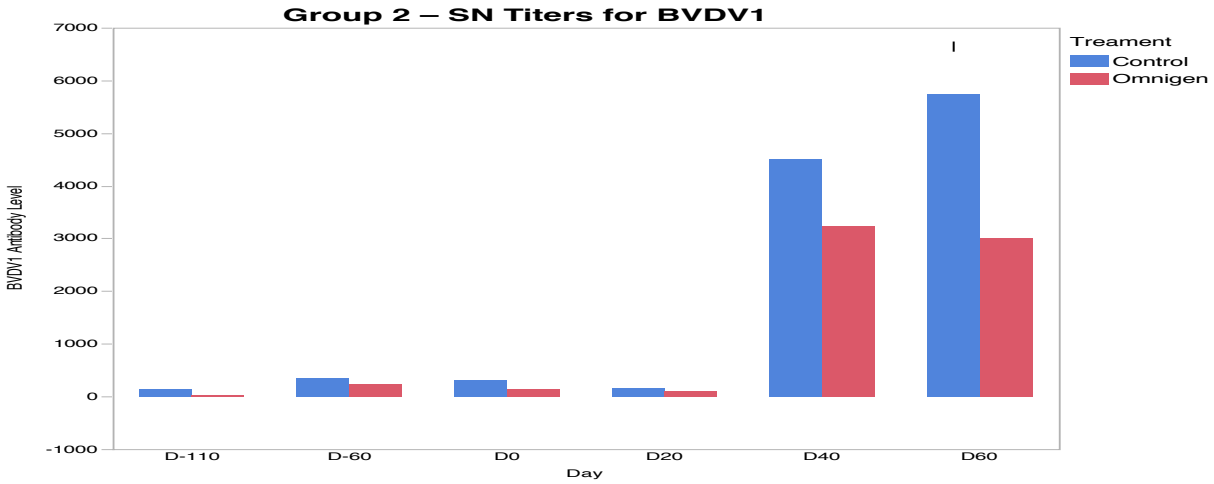

Figure 33: Group 2 - Levels of circulating antibodies in serum against the virus BVDV1 on each of the collection days.

BVDV2

The mean antibody level for BVDV2 for both treatment groups on D-110 were low; the control group had higher levels of circulating antibodies at the start of the trial, but this did not reach the level of significance (Figure 34). After the booster vaccine was given on D0, there was a delayed immune response observed relative to what was observed in Group 1. The SN titers did not rise until D40 against BVDV2. There was a 9-fold increase in antibody levels in the control treatment group from D40 to D0, but only a 3-fold increase for the OmniGen-AF® fed group between D0 and D40. The control heifers had significantly higher SN titer levels on D40 ($p=0.0378$) than the OmniGen-AF® fed heifers. Antibody levels for both treatment groups remained high on D60 after the booster vaccine, but the differences observed did not reach the level of significance.

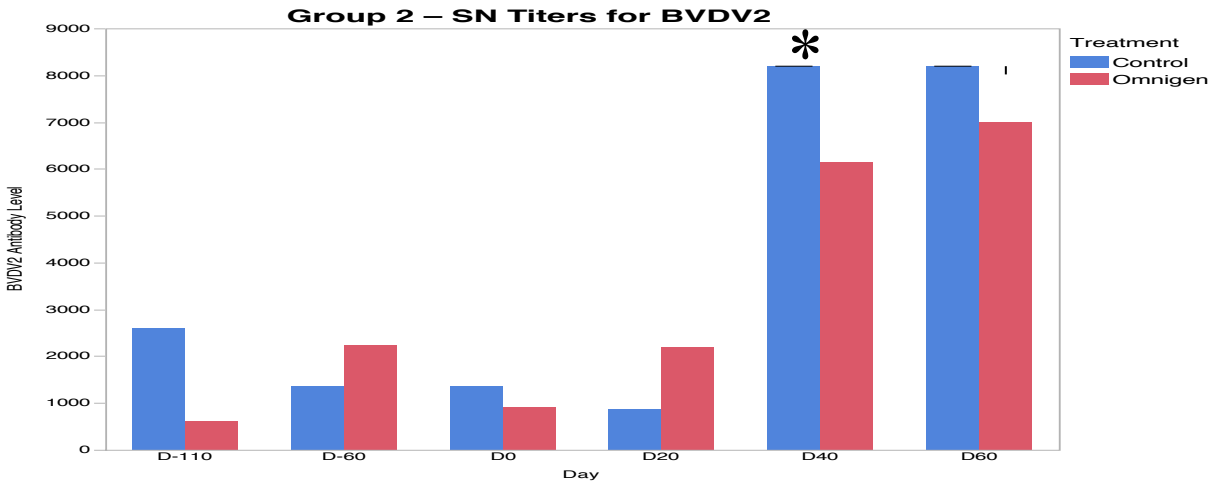

Figure 34: Group 2 - Levels of circulating antibodies in serum against the virus BVDV2 on each of the collection days.

BHV-1

The mean antibody levels for BHV-1 for both treatment groups on D-110 were extremely low until after the booster vaccine was given on D0 when SN antibody levels increased over 50 fold on D40 relative to D20 (Figure 35). The control group had significantly higher antibody levels compared to the OmniGen-AF® group on D0 (p=0.0307), on D20 (p=0.0067) and approached significance on D60 (p=0.0653).

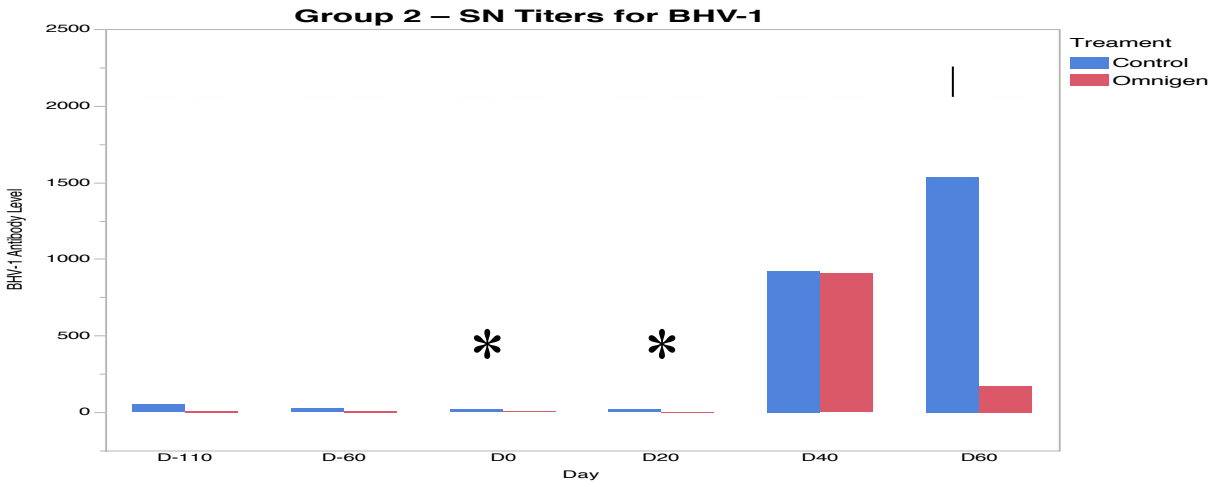

Figure 35: Group 2 - Levels of circulating antibodies in serum against the virus BHV-1 on each of the collection days.

IL-4

BVDV1

Very low levels of IL-4 were produced when the mononuclear cells were stimulated with BVDV1 (Figure 36). Production was below detectable levels of IL-4 for D-110 of the primary immune response. On D-60, there was a slight increase in IL-4 production with little difference between the treatment groups. Booster vaccination was given on D0; however, there was no production of IL-4 in the beginning of the secondary immune response. On days D40 and D60, all treatment groups had increased IL-4 production with no significant difference between the groups.

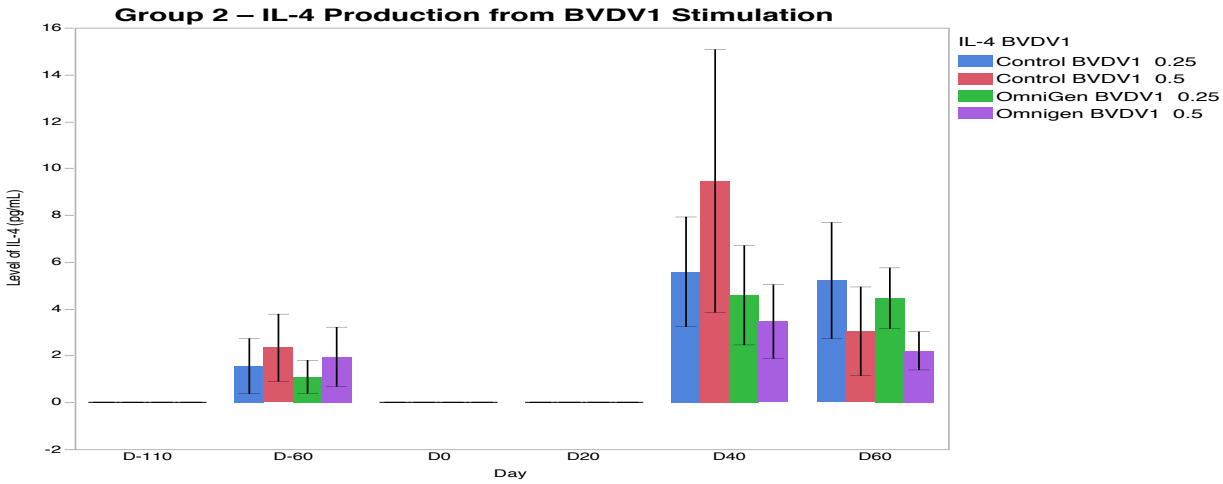

Figure 36: Group 2 - Level of IL-4 (pg/mL) produced when mononuclear cells were stimulated with 0.25 MOI and 0.5 MOI exposure of BVDV1.

BVDV2

Very low levels of IL-4 were produced when the mononuclear cells were stimulated with BVDV2 (Figure 37). Production was below detectable levels of IL-4 for D-110 of the primary immune response. On D-60, there was a slight increase in IL-4 production with little difference between the treatment groups. Booster vaccination was given on D0; however, there was no production of IL-4 in the beginning of the secondary immune response. On days D40 and D60, all treatment groups had increased IL-4 production with no significant difference between the groups.

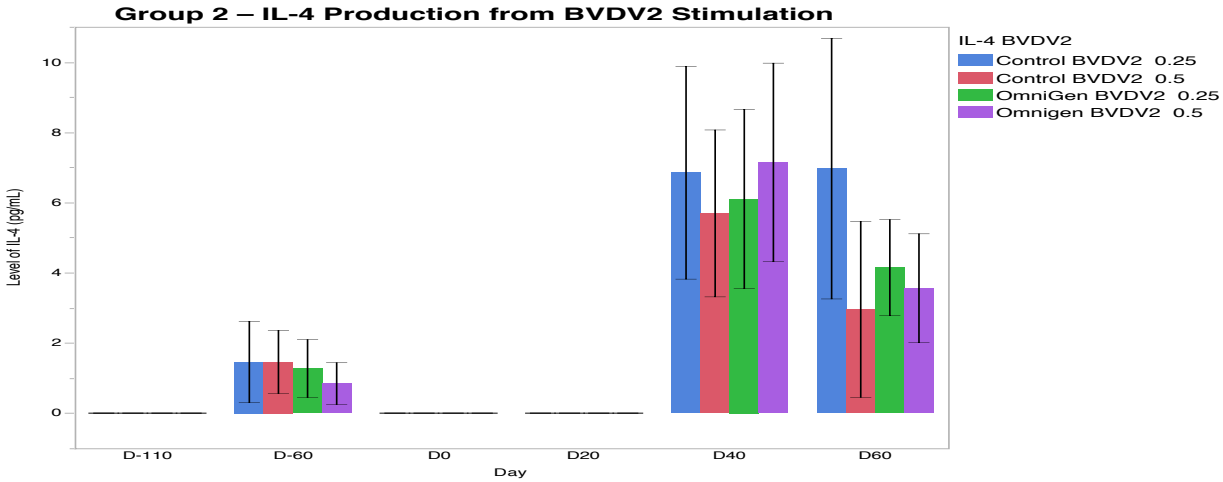

Figure 37: Group 2 - Level of IL-4 (pg/mL) produced when mononuclear cells were stimulated with 0.25 MOI and 0.5 MOI exposure of BVDV2.

BHV-1

Following priming vaccination, the heifers in each group produced similar circulating levels of IL-4 on D-110 and D-60 (Figure 38). No significant difference between the two treatment groups were observed. On D0, the OmniGen-AF® fed group had a higher level of IL-4 than the control heifers, but this did not reach the level of significance. The booster vaccine was given on D0 and the OmniGen-AF® fed heifers had higher levels of IL-4 than the control group on all of the collection days post booster vaccination; however, there was no significant differences between the two treatment groups.

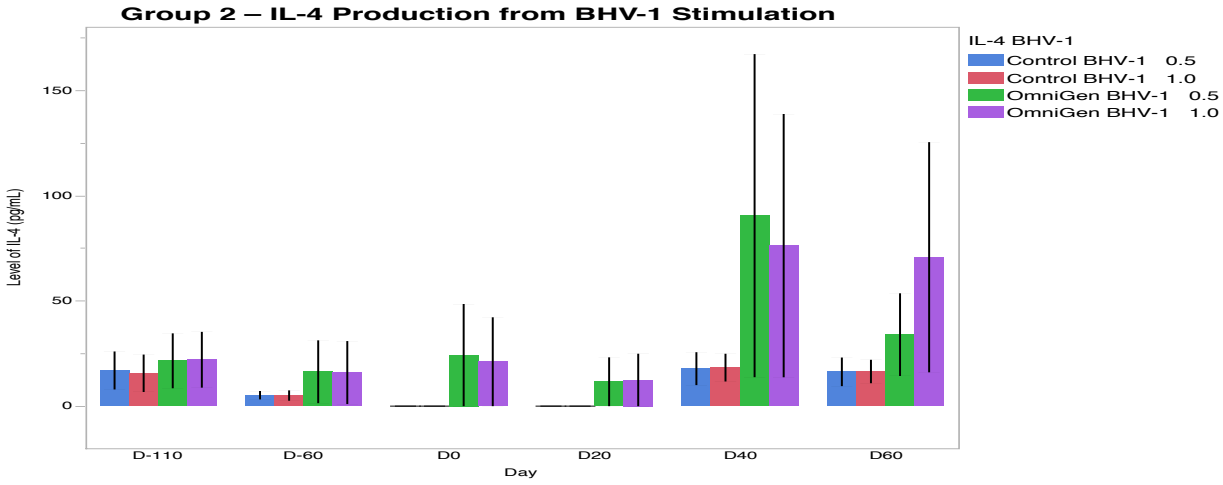

Figure 38: Group 2 - Level of IL-4 (pg/mL) produced when mononuclear cells were stimulated with 0.5 MOI and 1.0 MOI exposure of BHV-1.

IL-10

BVDV1

After the priming vaccine was given, the OmniGen-AF® fed heifers produced higher initial levels of IL-10, which approached significance, on both D-110 (p=0.085 and 0.088) and on D-60 (p=0.068 and 0.06) when mononuclear cells were stimulated with either the 0.25 MOI or the 0.5 MOI dose of BVDV1 virus, respectively (Figure 39). By D0, the OmniGen-AF® heifers were producing only slightly higher levels of IL-10. After the booster vaccine given on D0, there was an increase in IL-10 production by the OmniGen-AF® groups at both concentrations of BVDV1 on D20 and this reached the level of significance when stimulated with the 0.25 MOI (p=0.0327) dose. On D40 and D60 of the secondary immune response, the IL-10 levels between the 2 groups were relatively similar with the control groups being slightly higher than the OmniGen-AF® fed heifers.

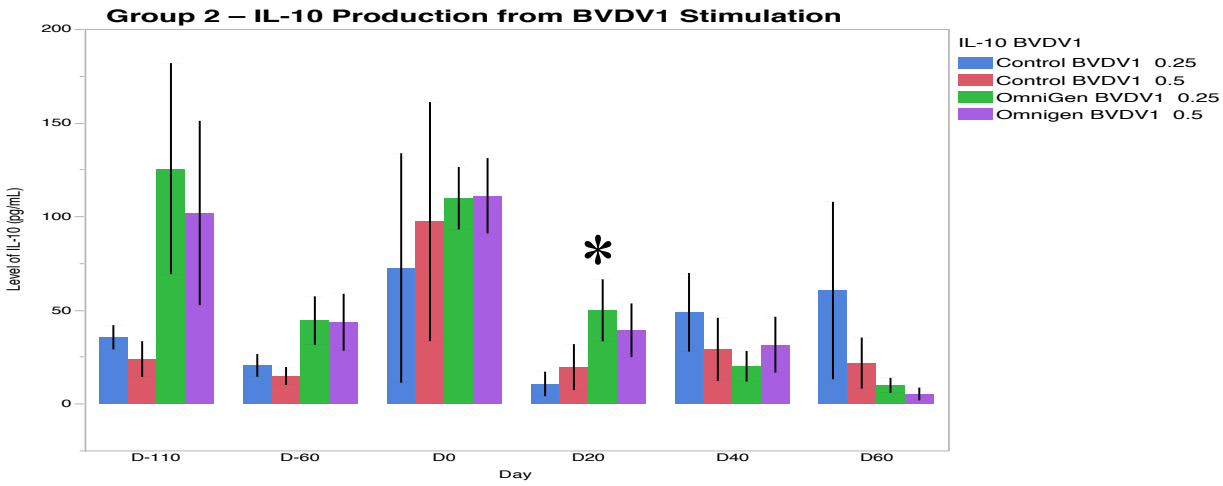

Figure 39: Group 2 - Level of IL-10 (pg/mL) produced when mononuclear cells were stimulated with 0.25 MOI and 0.5 MOI exposure of BVDV1.

BVDV2

After the priming vaccine was given, both treatment groups produced high initial levels of IL-10 on both D-110 (Figure 40). Levels of circulating IL-10 quickly declined by D-60 in both groups. On D0, there was a 2-fold increase in IL-10 compared to levels on D-60 in both treatment groups. Levels of IL-10 quickly declined by D20, and levels for both treatment groups stayed low through the remainder of the trial. There were no significant differences in IL-10 production between the treatment groups on any of the collection days when stimulated with BVDV2.

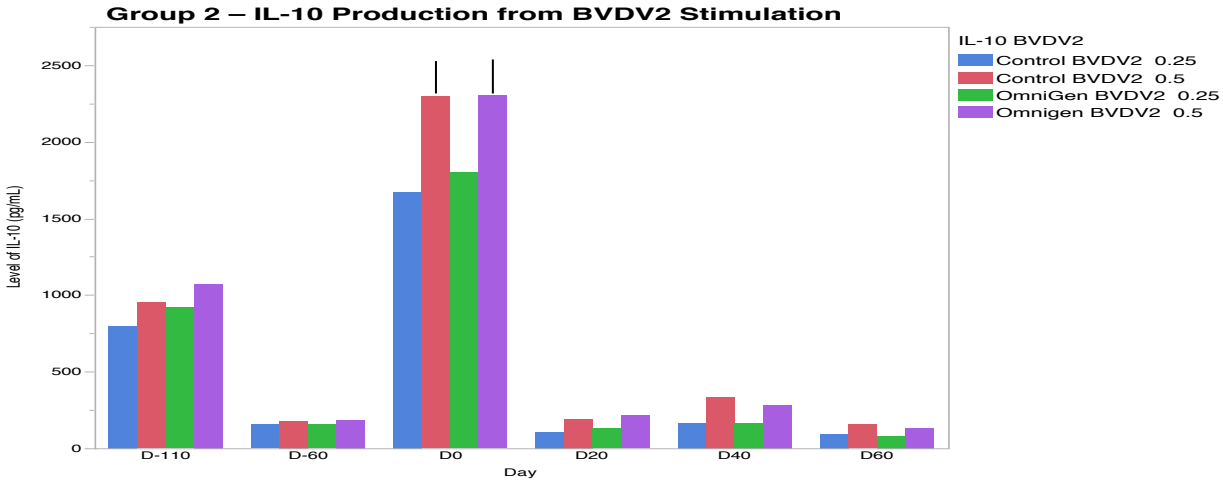

Figure 40: Group 2 - Level of IL-10 (pg/mL) produced when mononuclear cells were stimulated with 0.25 MOI and 0.5 MOI exposure of BVDV2.

BHV-1

After the priming vaccine was given, the OmniGen-AF® fed heifers produced higher levels of IL-10, which approached significance, on D-110 when the mononuclear cells were stimulated with the 0.5 MOI (p=0.073) and with the 1.0 MOI (p=0.074) dose of BHV-1 virus (Figure 41). IL-10 Levels for both treatment groups decreased by D-60 of the primary immune response. After booster vaccination was given on D0, IL-10 levels in both groups increased and then slowly declined over the course of the trial. The OmniGen-AF® fed heifers at the had higher levels of IL-when stimulated with the 1.0 MOI dose of BHV-1, this difference approached the level of significance (p=0.0746); however, there was little difference in IL-10 levels between the two treatment groups on any of the other days during the secondary immune response.

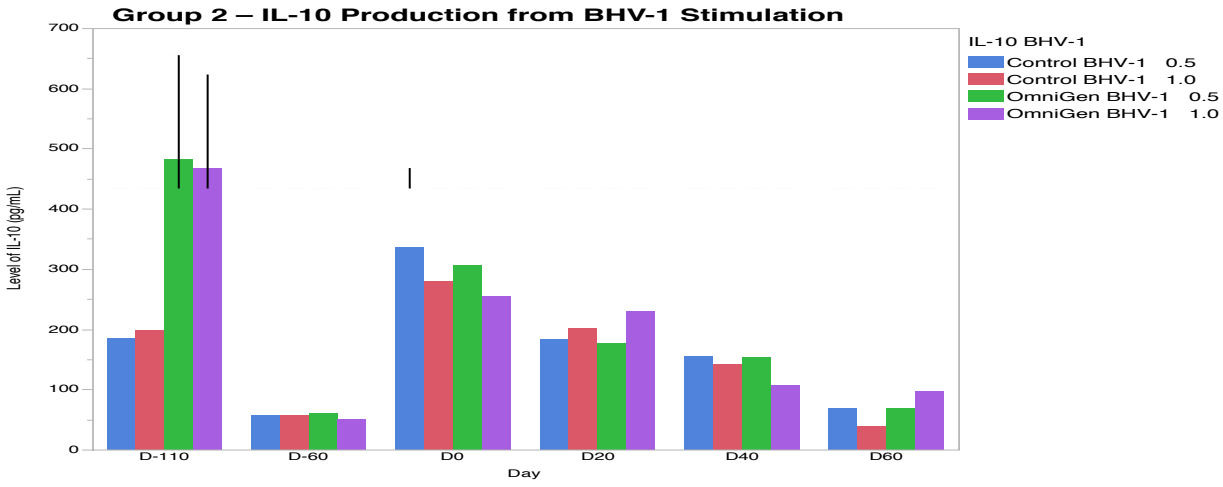

Figure 41: Group 2 - Level of IL-10 (pg/mL) produced when mononuclear cells were stimulated with 0.5 MOI and 1.0 MOI exposure of BVDV1.

IL-17

BVDV1

After the priming vaccine was given, IL-17 levels remained low throughout the primary immune response (Figure 42). Booster vaccination was given on D0 and a delay in immune response was seen. It wasn't until D40 that a significant boost in IL-17 production during the secondary immune response occurred in the OmniGen-AF® fed heifers when the mononuclear cells were stimulated with both the 0.25 MOI (p=0.0012) and the 0.5 MOI (p=0.0078) dose of BVDV1 virus. On D60, the significance at 0.25 MOI was (p=0.0027) and at 0.5 MOI was (p=0.0031) when exposed to the BVDV1 virus.

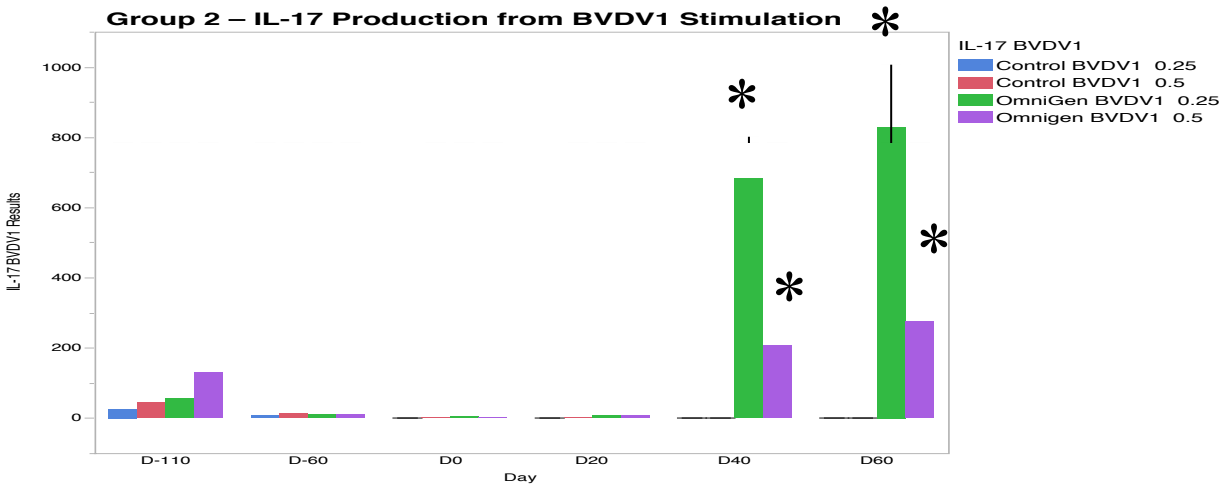

Figure 42: Group 2 - Level of IL-17 (pg/mL) produced when mononuclear cells were stimulated with 0.25 MOI and 0.5 MOI exposure of BVDV1.

BVDV2

After the priming vaccine was given, high levels of IL-17 were seen on D-110 but remained low throughout the rest of the primary immune response. Booster vaccination was given on D0 and a delay in immune response was seen. It wasn't until D40 that a significant boost in IL-17 production during the secondary immune response occurred in the OmniGen-AF® fed heifers when the mononuclear cells were stimulated with both the 0.25 MOI (p=0.0231) and the 0.5 MOI (p=0.0106) dose of BVDV2 virus. The OmniGen-AF® fed heifers continued to produce significantly higher levels of IL-17 on D60 at the 0.25 MOI (p=0.0123) and at 0.5 MOI (p=0.0227) dose of BVDV2.

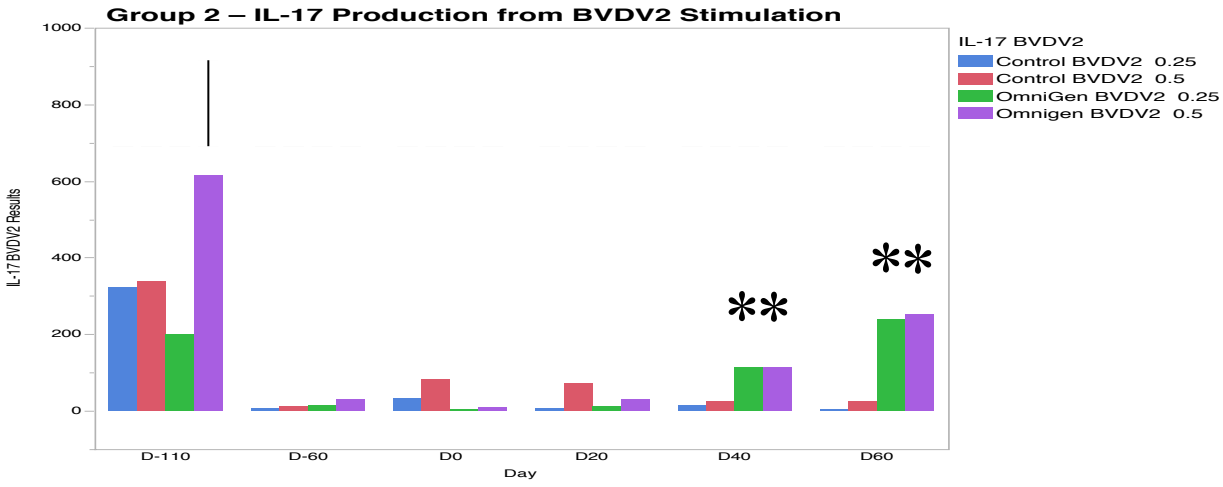

Figure 43: Group 2 - Level of IL-17 (pg/mL) produced when mononuclear cells were stimulated with 0.25 MOI and 0.5 MOI exposure of BVDV2.

BHV-1

After the priming vaccine was given, high levels of IL-17 were seen on D-110 but decreased throughout the rest of the primary immune response (Figure 44). After booster vaccination was given on D0, the control heifers had higher levels of Il-17 on D20 but this did not reach the level of significance. On D40, the OmniGen-AF® fed heifers produced significantly higher levels of IL-17 when the mononuclear cells were stimulated with the 0.5 MOI (p=0.0015) and the 1.0 MOI (p=0.005) doses of BHV-1 virus. The OmniGen-AF® fed heifers continued to produce significantly higher levels of IL-17 on D60 at the 0.5 MOI (p=0.0033) and the 1.0 MOI (p=0.0272) dose of BHV-1.

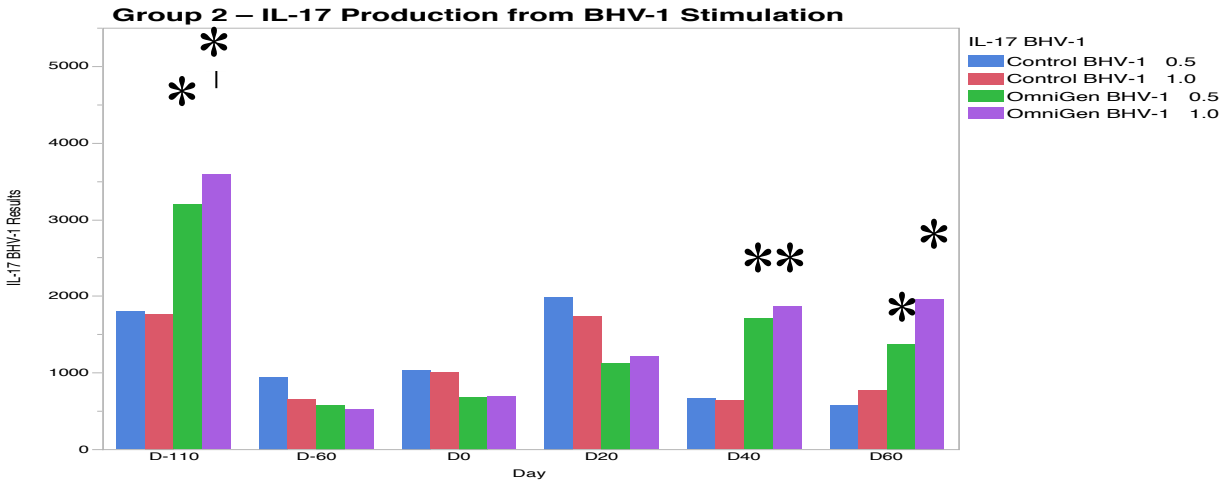

Figure 44: Group 2 - Level of IL-17 (pg/mL) produced when mononuclear cells were stimulated with 0.5 MOI and 1.0 MOI exposure of BHV-1.

IFN-gamma

BVDV1

After the priming vaccine was given, there was low initial IFN-gamma levels on D-110 through D0 (Figure 45). The OmniGen-AF® fed heifers had higher levels of IFN-gamma on D-110 approached significance on D-60 (p=0.053) at the 0.5 MOI stimulation with BVDV1. Booster vaccination was given on D0 and had no effect on the IFN-gamma production until D40. Only the OmniGen-AF® fed heifers had an increase in IFN-gamma production during the secondary immune response but this did not reach the level of significance.

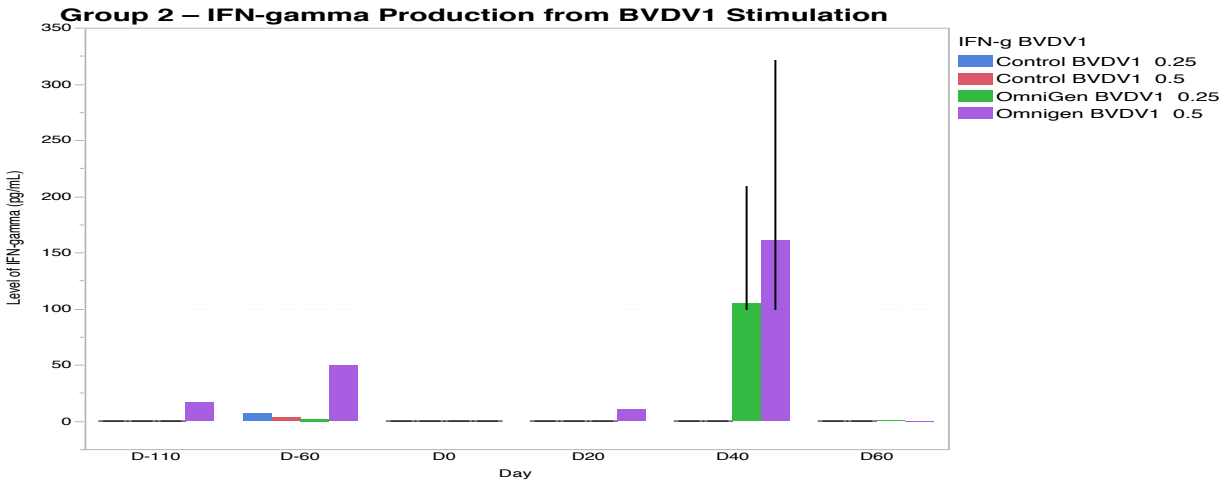

Figure 45: Group 2 - Level of IFN-gamma (pg/mL) produced when mononuclear cells were stimulated with 0.25 MOI and 0.5 MOI exposure of BVDV1.

BVDV2

After the priming vaccine was given, the OmniGen-AF® fed heifers had higher initial levels of IFN-gamma on D-110. This continued until D-60 when it reached the level of significance at the 0.25 MOI (p=0.0434) and approached significance at the 0.5 MOI (p=0.0775) stimulation of BVDV2. The booster vaccination given on D0 did not stimulate the production of IFN-gamma to BVDV2 during the secondary immune response in either treatment group.

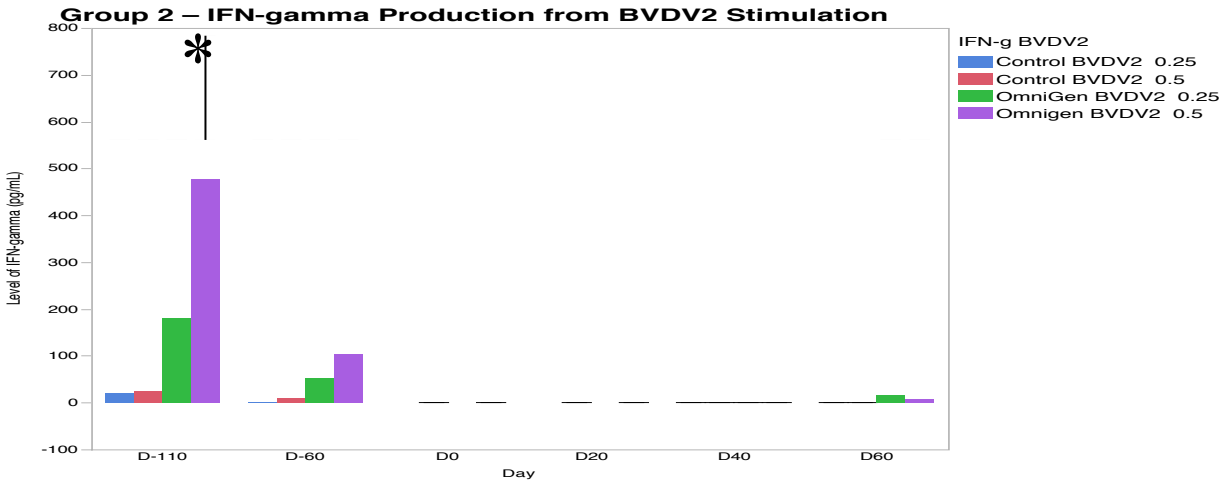

Figure 46: Group 2 - Level of IFN-gamma (pg/mL) produced when mononuclear cells were stimulated with 0.25 MOI and 0.5 MOI exposure of BVDV2.

BHV-1

After the priming vaccine was given, the control treatment group had higher initial levels of IFN-gamma but this did not reach the level of significance (Figure 47). On D-60 through D20, the two treatment groups had relatively equal levels of IGN-gamma production. After booster vaccination on D0, the OmniGen-AF® heifers had higher IFN-gamma production to BHV-1 on D40 and D60 though this did not reach the level of significance.

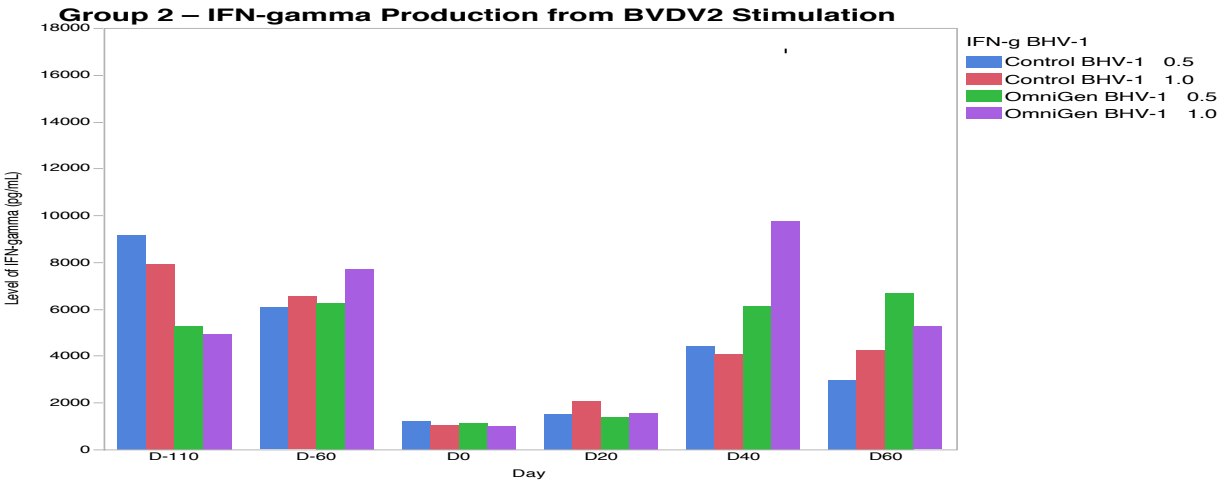

Figure 47: Group 2 - Level of IFN-gamma (pg/mL) produced when mononuclear cells were stimulated with 0.5 MOI and 1.0 MOI exposure of BHV-1.

Prostaglandin E_2

After the priming vaccine was given, the control treatment group had higher initial levels of prostaglandin E_2 but this did not reach the level of significance (Figure 48). On D-60 through D0, the two treatment groups had relatively equal levels of prostaglandin E_2 production. After booster vaccination was given on D0, the OmniGen-AF® heifers had higher prostaglandin E_2 production on D20 and the control treatment group had higher prostaglandin E_2 levels on D60; however, neither reached the level of significance.

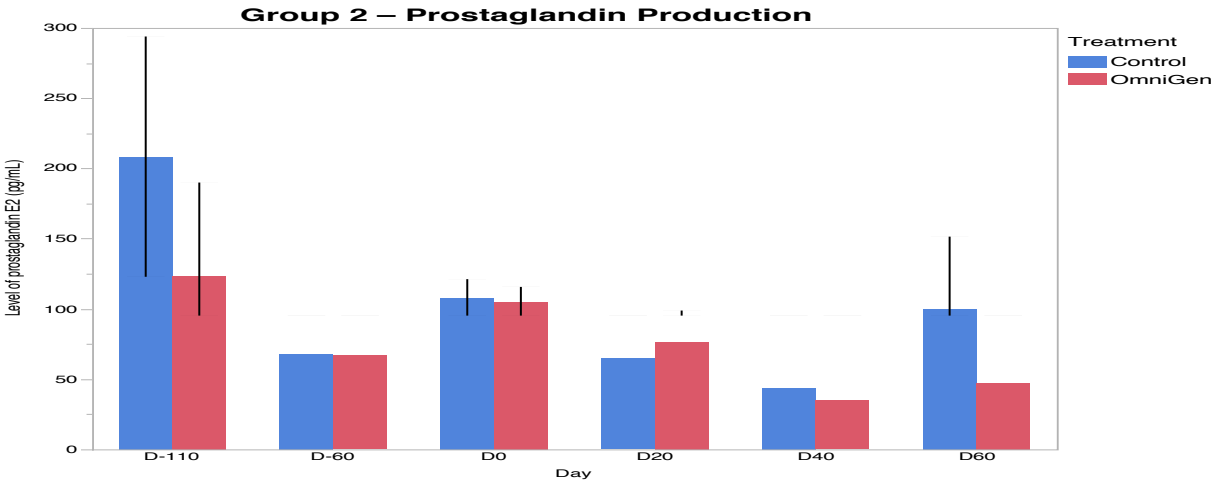

Figure 48: Level of prostaglandin E_2 (pg/mL) produced from plasma samples.

CHAPTER 6

DISCUSSION

A Summary of What We Learned

The results of these experiments demonstrated that feeding of OmniGen-AF® to heifers had a clear enhancing impact on the capacity of circulating leukocytes to respond to viral antigens similar to those in the vaccine given. This was particularly true of the response to the booster vaccine given on D0 after maternal immunity had waned.

Specifically, the number of circulating memory cells capable of proliferating in response to both BVDV and BHV-1 in culture was enhanced for heifers in Group 1 and 2. The response was more clearly different between the controls and OmniGen-AF® fed heifers in Group 2. These heifers were subjected to much greater weather stress and OmniGen-AF® has been shown to ameliorate the negative effects of stress (Nace et al., 2014).

Differences in vaccine antigen induced cytokines were also observed. Cytokine production did not demonstrate a pattern strongly associated with vaccination, nor was it as tightly linked to the treatments across the range of viral stimulation as proliferation. Generally, OmniGen-AF® feeding resulted in enhanced IL-4 and IL-17 production and reduced IL-10 production by cultured cells. More refinement of the cytokine production cultures (in culture duration and viral dose) are likely needed to fully optimize those measurements.

Overall, it appears that OmniGen-AF® enhancement of adaptive immune response to vaccines occurs. Enhanced innate responses were also seen in the OmniGen-AF® fed heifers using indicator genes for L-selectin and IL-8 which are specific markers for innate immunity. We speculate that enhanced innate immune regulation and response may play a contributing role in the enhanced adaptive immune response to the vaccine.

Average Daily Gain

Feeding OmniGen-AF® daily did not significantly affect the average growth in height or weight gain in Group 1 over the 170 days of the trial. The immune stimulation associated with enhanced innate, and now adaptive, immune function induced by feeding OmniGen-AF® did not appear to have a significant energy cost to the heifers. No loss of growth in either height or weight was observed. Thus, this appears to be an energy neutral immune modulator in heifers raised in close to ideal conditions.

Inflammation and adaptive immunity are expensive functions of mammals due to a combination of profound recruitment of inflammatory cells (neutrophils and monocytes) and high proliferation rates among lymphocyte populations. These functions cause a depletion of nutrients and increased oxygen consumption, resulting in a shift in energy supply and demand, which causes a change in tissue metabolism (Kominsky et al., 2010).

In a study done by Cuterra and coworkers (Cutrera et al., 2010), rodents were exposed to a non-pathogenic antigen to measure the effect this antigen had on the energetic expenditure used by the immune system. They measured a significant increase in oxygen consumption in the treatment group that was exposed to the antigen compared with control animals. This study indicated that mounting an immune response entailed

significant energetic costs, leading to trade-off decisions during energetically demanding periods.

Feeding OmniGen-AF® daily to young heifers enhanced their immune response to the upper-respiratory viral vaccine. Though this increased immune response requires a higher demand in resources, the OmniGen-AF® fed heifers in this trial did not experience any negative impacts on growth or body weight compared to the control treatment group. This is important in the cattle industry because is proves that OmniGen-AF® has a positive impact on the protection that vaccination gives without negatively impacting growth and production.

The two treatment groups of heifers in Group 1 grew at a similar pace with relatively the same starting and finishing weights. However, the two treatments groups of heifers in Group 2 gained weight at different rates. The OmniGen-AF® heifers grew significantly faster than the heifers in the control treatment group. The data collected from Group 2 not only supports the fact that feeding OmniGen-AF® enhances the immune response to the upper respiratory vaccine, it indicates that feeding OmniGen-AF® might actually enhance growth and production in heifers under heavy stress.

Hematological Profile

WBCs or leukocytes play an essential role in immune defense. Leukocytosis, an increase in the number of white cells in the blood, can develop as a consequence of inflammation, stress, injury, and infections (Alvarez et al., 2013).

For Group 1, the total WBC count was higher in the control group compared to the OmniGen-AF® fed treatment group on all collections days, and for Group 2, the total white

blood cell count was higher in the control group compared to the OmniGen-AF® fed treatment group on almost all collections days (though for both groups it did not always reach the level of significance). The higher levels of WBCs in the control heifers indicated more uncontrolled inflammation in the animals that did not receive daily OmniGen-AF® supplementation.

Lymphocytes can be divided into two major types by their functions: B cells and T cells. Though they are unable to engulf organisms, they fulfill their function of defending the body in several other ways and are essential in fighting an infection. An increase in the number of lymphocytes can indicate a prolonged illness.

For Group 1, the total lymphocyte count was higher in the control group compared to the OmniGen-AF® group on all collection days, and for Group 2, the total lymphocyte count was higher in the control group compared to the OmniGen-AF® group on almost all collection days (though in both groups it did not always reach the level of significance). This indicates that the control group had more prolonged chronic inflammation when fighting endogenous viral or fungal antigens from the environment compared to the OmniGen-AF® fed treatment group.

Neutrophils function by actually engulfing disease-causing bacteria and other small particles. In the presence of an infection, their number in the peripheral blood increases. Monocytes also have the ability to engulf foreign material; additionally, they secrete various protein molecules that help heal inflamed and irritated tissue.

For Group 1 and Group 2, there was a lot of variability in both treatment groups for the primary response to the priming vaccine. Following the booster vaccine in both groups, the control group had slightly higher numbers of monocytes and neutrophils in both study

groups. These differences did not reach the level of significance. The slightly higher levels of monocytes and neutrophils along with the higher numbers of circulating WBC and lymphocytes all indicate that the non-supplemented heifers had a higher level of uncontrolled inflammation compared to the OmniGen-AF® fed treatment group.

The dietary supplement utilized in this study contains yeast and fungal cell wall components, which have been demonstrated in other studies to enhance inflammatory activation and enhance the migration of leukocytes (Ryman et al., 2013). Lymphocytes use cell surface markers, such as L-selection, to migrate out of circulation and into the tissue at the site of infection and inflammation. OmniGen-AF®'s ability to upregulate these surface markers that leukocytes use to migrate into the tissue increases the number of leukocytes available to fight and control infection at the tissue level.

The enhanced quantity of proinflammatory cytokines needed to fight an infection often leads to increased inflammation. A better-controlled response resolves the infection more quickly and leads to less non-specific inflammation and tissue damage. The OmniGen-AF® fed heifers in this study had decreased numbers of leukocytes in circulation. From this, we inferred that it is possible these heifers were more effective at getting these cells out of circulation and into the tissues to the site of infection. Therefore, they were better able to respond to the viral antigens in the upper-respiratory vaccine.

Proliferation

Lymphocytes undergo essential rounds of proliferation to allow for expansion of the number of molecular specific cells required to provide adaptive immunity. Lymphocytes are produced by random rearrangement of the immunoglobulin (B cells) or T cell receptor

(T cell) genes. After screening out the self-responsive cells, the remaining cells with functional immunoglobulin or T cell receptor genes are released to home to organized lymphoid tissue. When the right combination of signs of damage, danger and molecular target are brought to the organized lymphoid tissue, these lymphocytes can be activated. Only single cells are produced with each rearrangement of the genes, so a mechanism to make a functional number is required. Activated cells are first induced to divide as the initial step in mounting an adaptive immune response.

Proliferation is the rapid reproduction and division of cells to create a large pool of identical cells. Next, these cells are stimulated by different combinations of cytokines and chemokines that determine what type of cells they differentiate into and what their effector functions is. Both proliferation and differentiation are essential to mount an effective immune response against a pathogen.

In the first study group, when the mononuclear cells were stimulated with either BVDV1 or BVDV2, or with BHV-1, the heifers that received OmniGen-AF® daily had higher levels of proliferation following the booster vaccine. In the second study group, when the mononuclear cells were stimulated with BVDV1 the heifers that received OmniGen-AF® daily had higher levels of proliferation following the booster vaccine. The second study group after mononuclear cell stimulation with BVDV2 or BHV-1 had higher levels of proliferation following both the primary and booster vaccine for heifers that received OmniGen-AF® daily than the controls.

Higher levels of proliferation in the fixed sided population of PBMC indicates an increased size of the pool of memory cells capable of establishing an effective immune response against each virus. When the antigens in the vaccine are placed in culture, they

interact and are processed by monocytes or B cells, and the antigen fragments are placed in association with MHC II receptors that present non-self antigens. CD4+ T-cells have the ability to bind to these MHC II receptors that are loaded with antigen fragment and become activated. The larger the pool of memory CD4+ cells induced by the vaccine, the higher the level of proliferation that will be observed in the cultures. More proliferating cells indicated an increased capacity for clonal expansion in the host heifer on re-exposure to the antigen *in vivo*. Therefore, indicating the capacity for a faster and stronger immune response. Based on the limits in stringency of the presentation of antigen (monocytes and B cells can activate memory CD4+ cells, but not CD8+ cells or naïve CD4+ cells), the increased proliferation observed was occurring in CD4+ memory T-cells.

In both study groups of heifers, when the mononuclear cells were stimulated with the viruses following booster vaccination, the immune response in the OmniGen-AF® fed heifers was both higher in magnitude and longer in duration than that observed for the control heifers. This indicated that OmniGen-AF® treatment enhanced the number of circulating memory cells following the primary vaccination that quickly and effectively responded to the antigens in the booster vaccine. This increase in clonal expansion of CD4+ T-cells indicated the capacity for a stronger and more lasting protection against the three upper respiratory viruses represented in the vaccine.

SN Titers

Serum Neutralizing antibody titers were measured to determine the induction of specific antibodies to vaccine antigens in the serum of all the heifers in the study. It has been reported that virus neutralizing antibody titers greater than 1:250 to 1:1024 against

BVDV are associated with immunoprotection against infection (DesCoteaux et al., 2003, Swan et al, 2003). A bovine ephemeral fever (BEF) challenge study was done by Barigye and coworkers (Barigye et al., 2015) that reported that higher antibody titers were associated with a shorter period of viremia in heifers, indicating that neutralizing antibodies play an important role in the development of anti-viral immunity. In our study, we analyzed SN titers for BVDV1, BVDV2, and BHV-1 to see if OmniGen-AF® supplementation increased the level neutralizing antibodies produced that would indicate greater immunoprotection against the viral antigens after vaccination.

For the Group 1 heifers following the priming vaccination, BVDV1 SN titers were not increased. However, there was a large increase in BVDV1 SN antibody following booster vaccination. Following priming vaccination, the SN titers for both BVDV2 and BHV-1 increased significantly, and after booster vaccination, another significant increase in SN antibody titers was observed. No significant differences in the SN titers to BVDV1, BVDV2, and BHV-1 were observed between the treatment groups following the primary or booster vaccination.

No increase in SN titers was observed in the Group 2 heifers following the priming vaccine against BVDV1, BVDV2, or BHV-1. However, all viruses had a significant increase in SN antibodies following the booster vaccination on D40. There were no significant differences between the treatment groups for BVDV1 or BVDV2, though on most days, the control treatment group had slightly higher antibody levels. There were multiple collection days that the control treatment group had significantly higher SN antibody titers against BHV-1 (see Figure 35).

Although the control heifers had slightly higher SN antibodies levels at the conclusion of the trial, they also had higher SN titers at the initiation of the study. This was common to all three viruses. Both treatment groups had an enhanced antibody response to the vaccines, but higher post-vaccine SN titers in controls suggests that the higher initial SN antibody titers of the control group provided initial conditions leading to higher ultimate titers at the end of the trial.

Previous studies by our group found no significant difference in serum antibody titers between the OmniGen-AF® fed cows and the unsupplemented control heifers. (Ryman et al., 2013) evaluated dairy heifers on a continuous feeding program with OmniGen-AF® that were vaccinated against *Staphylococcus aureus*. The trial was conducted to determine if daily feeding of OmniGen-AF® would enhance the antibody titer response to this vaccine in these young heifers; however, they found that OmniGen-AF® fed heifers had no improvement in antibody titers during calfhood or throughout the first pregnancy compared to the control group.

The titers in our study do show a significant increase in antibodies levels against BVDV in both treatment groups following the booster vaccine. The antibodies to BHV-1 increased during the priming vaccine response and then again after the booster vaccine for Group 1, but only rose after the booster vaccination in Group 2. The common increase in SN antibody titers in both in the OmniGen-AF® and control heifers after upper-respiratory vaccination indicates that functional systemic antibodies are being produced by a mechanism largely independent of the effects that OmniGen-AF® has on innate and adaptive responses. Although the vaccinations are successfully stimulating and enhancing antibody production that are specific for these viruses, feeding OmniGen-AF® did not seem

to have a direct effect on the activity of B-cells secreting antibodies in response to the vaccines. This is in contrast to our findings that indicate OmniGen-AF® enhances T-cell memory development that can be measured among circulating mononuclear cells.

IL-4

IL-4 is the main cytokine secreted by TH2 cells and is very important to their expansion because it has a positive feedback mechanism on these cells by further amplifying their growth and division. IL-4 produced by these TH2 cells also inhibits the development of TH1 and TH17 cells and inhibits activated macrophages. This increase in TH2 response attempts to clear the infection and control exacerbated inflammatory reaction and collateral damage (Palomares et al., 2014). Il-4 has an important role in suppressing pro-inflammatory cytokine expression as well as preventing tissue damage through the regulation of type I cytokine production (Widdison et al., 2006).

In a previous study done by Norbiron and coworkers (Nobiron et al., 2003), they found that a highly polarized Th2 type CD4+ response was dominant, with IL-4 being the dominant cytokine secreted by CD4+ T-cells following *in vitro* stimulation with BVDV infected mononuclear cells. However, in both of our study groups of heifers when mononuclear cells were stimulated with either BVDV1 or BVDV2 there were low initial levels of IL-4 production during most of the trial and then a slight increase in IL-4 production was seen on D40 and D60. In Group 1, there was an increase of IL-4 in some of the OmniGen-AF® treatment groups during the secondary immune response to the booster vaccine. Very little differences between the treatment groups were seen in Group 2 when stimulated with either BVDV1 or BVDV2.

Low initial levels of Il-4 production suggest that the upper respiratory vaccine produces primarily a TH1 response instead of a TH2 primary immune response against BVDV 1 and BVDV 2. Low levels of IL-4 were also seen in a challenge study that used the bacteria *Mycobacterium avium*. IL-4 was not differentially expressed in tissues from infected and control cows; they concluded that the immune response to the infection was predominantly a proinflammatory or Th1-like response (Coussens et al., 2004). IL-4 may be actively down-regulated in an immune response to allow maintenance of the pro-inflammatory response that is necessary for the killing of pathogens (Widdison et al., 2006).

In Group 1, when mononuclear cells were stimulated with BHV-1, there were low initial levels of IL-4 production. All treatment groups had an increase in IL-4 levels in response to the priming and booster vaccine, with the OmniGen-AF® fed treatment group having significantly higher IL-4 levels than the unsupplemented control heifers. In Group 2, when mononuclear cells were stimulated with BHV-1 the OmniGen-AF® fed heifers had higher amounts of IL-4 production at both concentrations of BHV-1 virus in both the primary and secondary immune response.

The OmniGen-AF® fed heifers had an increase in duration and magnitude of IL-4 production in the secondary immune response when stimulated with BHV-1. Group 1 and Group 2 provided consistent evidence that feeding OmniGen-AF® daily does increases the heifer's ability to mount an effective TH2 immune response against the vaccine antigens for BHV-1.

IL-10

Memory Treg cells are activated and proliferate in recall vaccination and inhibit the expansion of TH1 and TH2 memory cells. IL-10 and TGF-beta are produced as the primary regulatory products causing a suppression of both innate and adaptive immune responses. IL-10 and TGF-beta directly prevent B cells from differentiating, and inhibit the function of effector T cells. They also indirectly regulate antigen-presenting cells (APC), which in turn, limits T cell proliferation and cytokine production.

In Group 1, when mononuclear cells were stimulated with both BVDV1 and BVDV2 as well as to BHV-1, the controls treatment group consistently had higher levels of IL-10 during the secondary immune response to the booster vaccine. In Group 2, when mononuclear cells were stimulated with the three viruses, there was little difference between treatment groups in IL-10 production in the secondary immune response to the booster vaccine.

A study found that cows with subclinical endometritis had significantly higher levels of IL-10 than those in healthy cows. They found a higher activity of induced immune cells particularly in peripheral blood, which resulted in an increase in the concentration of inflammatory mediators (Brodzki et al., 2015). Another study had a dramatic increase in IL-10 gene expression in PBMCs from subclinically infected cows following exposure to *M. avium* subsp. *paratuberculosis in vitro* (Coussens et al., 2004).

Enhanced expression of IL-10 helps control local inflammatory responses by suppressing pro-inflammatory cytokines (Barigye et al., 2015). The treatment cows in the challenge studies exhibited more inflammation due to infection and therefore needed higher levels of IL-10 to help balance the amount of inflammatory cytokines produced.

The higher levels of IL-10 that the unsupplemented control heifers in Group 1 produced in this study indicated that these animals had increased amounts of uncontrolled inflammation in response to the vaccine compared to the OmniGen-AF® fed heifers. An increase in the amount of memory T cells that the OmniGen-AF® heifers created from the primary response allow them to mount a higher and more efficient secondary response, reducing the amount of time it takes to fight off an infection. This shorted response time decreases the amount of inflammation created as a byproduct of fighting a prolonged infection. A better primed immune system leads to a better controlled immune response.

In Group 2, there was little difference in IL-10 production between the treatment groups when the mononuclear cells were stimulated against each of the viruses, and this could be due to the amount of increased environmental stress that this group encountered over the course of the trial. Stress can be a strong immunosuppressant and could inhibit the ability of the calves to mount an effective immune response to the vaccines; therefore, decreasing the need of IL-10 in the immune response.

IL-17

IL-17 is the main cytokine produced by Th17 T cells. IL-17 promotes plasma cell development in tissues, IgA to mucosal surfaces, and increased circulating IgG. IL-17 also recruits neutrophils to the site of injection and enhances neutrophil, macrophage, epithelial cell, and lymphocyte interaction in recall responses. It is a major pro-inflammatory cytokine that plays an important role in conjunction with IFN-gamma in controlling infection (Flynn et al., 2011).

In Group 1, when mononuclear cells were stimulated with either BVDV1 or BVDV2, the OmniGen-AF® treatment produced higher levels of IL-17 during the primary and secondary immune responses. When stimulated with BHV-1, there was no difference between the two treatment groups for IL-17 production. In Group 2 when the mononuclear cells were stimulated with either BVDV1 or BVDV2 there was a difference between the treatment groups in IL-17 production in the secondary immune response, with the OmniGen-AF® fed calves producing significantly more IL-17. When stimulated with BHV-1s there was a difference between the treatment groups in IL-17 production in both the primary and the secondary immune response, with the OmniGen-AF® fed calves producing significantly more IL-17.

A study was done to provide evidence that the production of a second major inflammatory cytokine, IL-17A, may also play a role in tandem with IFN-gamma in controlling infection (Flynn et al., 2011). They identified the production of a second yet distinct inflammatory cytokine, IL-17A, during infection with *Neospora caninum*. Another study was done that demonstrated the production of IL-17 in response to PPDB stimulation was higher in vaccinated animals than in unvaccinated animals, though after *M. bovis* challenge, the expression of IL-17 was comparable between both groups (Blanco et al., 2011).

Th17 cells are good at fighting extracellular pathogens and help maintain the barrier functions, which is key in combating upper respiratory infections. The OmniGen-AF® fed heifers in both study groups, having a stronger IL-17 response to the vaccine for both the primary and secondary immune response, indicates an increased pro-inflammatory response and better protection against BVDV1 and BVDV2. The second group of heifers,

having a better immune response to BHV-1 from the booster vaccine, indicates that IL-17 may play an importation role in protection against BHV-1 as well.

IFN-gamma

IFN-gamma is the core cytokine associated with Th1 response. It is produced by a variety of immune cells and has a positive feedback mechanism; it further amplifies and activates the expansion of many essential immune cells including those that secrete it. In recent years, IFN-gamma and especially IFN-gamma-secreting CD4+ T cells have been proposed as key elements required for protection, as their levels tend to be inversely correlated with severity of disease (Sacchini et al., 2012). IFN-gamma stimulates immunoglobulin isotype switching, promotes activation and enhanced killing power of macrophages, and is important in the activation of NK and cytotoxic T-cells, which are crucial in antiviral immunity (Barigye et al., 2015).

In Group 1, when mononuclear cells were stimulated with BHV-1, the OmniGen-AF® fed heifers had higher levels of IFN-gamma production than the control group on most collection days, and reached a significant difference on D0 and D20 of the secondary response. In Group 2, when mononuclear cells were stimulated with BHV-1, the OmniGen-AF® fed heifers had higher levels of IFN-gamma production than the control group during the secondary immune response to the booster vaccine.

In both groups of heifers, there was a lot of variability in IFN-gamma production to BVDV in both treatment groups throughout the duration of the trial. In Group 1, there was no difference in IFN-gamma production levels when mononuclear cells were stimulated with either BVDV1 or BVDV2. In Group 2, there were multiple days during both the

primary and secondary immune response the OmniGen-AF® heifers had increased production of IFN-gamma.

In a challenge study, IFN-gamma levels were increased only after inoculation with a virulent strain of BVDV2 and not other strains (Palomares et al., 2014). This indicated the activation of a Th1 immune response in an attempt to control the highly virulent BVDV2 infection. This type of immune response would evoke a strong inflammatory response in the respiratory tract (cell mediated immunity). Another study found that increased production of IL-2 and IFN-gamma may shorten the duration of viraemia in Bovine Ephemeral Fever Virus infected cattle (Barigye et al., 2015).

There is a lot of evidence of IFN-gamma production being significantly increased when the immune system is faced with foreign pathogens, as it is the main cytokine produced in a classical TH1 immune response. A study done by Burdick-Sanchez and coworkers (Burdick-Sanchez et al., 2013) measured the effect that yeast cell wall supplementation had on the immune response of heifers when challenged with an endotoxin. They reported that concentrations of IFN-gamma increased significantly post challenge; however, IFN-gamma levels were not affected by treatment. Based on previous research we expected to see a strong IFN-gamma response to all three viral agents, so why we did not see a strong IFN-gamma response in any of the treatment groups to either BVDV1 or BVDV2 is unclear.

Natural Killer cells, memory TH-1 cells, memory T-cytotoxic cells, and memory gamma-delta cells can be activated in local lymphoid tissue to produce IFN-gamma. The OmniGen-AF® fed heifers having a significantly stronger IFN-gamma response to BHV-1 for

both the primary and secondary immune response to the vaccine indicates an increased pro-inflammatory response and possibly better protection against BHV-1.

PG E2

Prostaglandin E2, a key mediator of inflammation, regulates a variety of immune responses and is most commonly recognized as a proinflammatory, but in some aspects, PGE_2 can play an anti-inflammatory role (Przybysz et al., 2016). It has been reported that high concentrations of PGE_2 inhibit the production of the Th1 cytokines IFN-gamma and IL-12, whereas lower concentrations of PGE_2 have been shown to enhance Th1 function (Connor et al., 2009). A study was done to assess the influence of PGE_2 on the production of IFN-gamma by bovine CD4+, CD8+ T cells (Procario et al., 2016). They reported that exposure of bovine peripheral blood mononuclear cells (PBMCs) to PGE_2 significantly decreased the percentage of IFN-gamma-producing CD4+ and CD8+ T cells; therefore, indicating that PGE_2 is a proinflammatory lipid mediator with immunosuppressive activity.

In Group 1, the PGE_2 concentrations for both treatment groups were very similar throughout the trial. Following the booster vaccine given on D0, the control group had slightly higher levels of prostaglandin E_2 than the OmniGen-AF® fed heifers on most of the collection days; however, there was no significant difference between the two treatment groups.

In Group 2, there was a lot of variability in the the PGE_2 levels. On most collection days both treatment groups had very similar levels of PGE_2 secretion throughout the trial. On D-110 and D60, the control heifers had higher PGE_2 levels than the OmniGen-AF® fed heifers, though this did not reach the level of significance.

PGE_2 is a suppressive modulator at the tissue level and is expressed by monocytes and epithelial cells. In this study, we measured the overall amount of PGE_2 in plasma instead of inducing it by stimulating the cells with virus. There were slightly higher PGE_2 levels in the nonsupplemented control groups, and this indicates there is suppressive activity being induced by the monocytes and epithelial cells in the plasma. Feeding OmniGen-AF® supports a stronger and better-controlled immune response and this is supported by the OmniGen-AF® fed heifers producing slightly less PGE2, a suppressive mediator.

Group Comparisons

An examination of the extremely different conditions that the Group 1 and Group 2 heifers faced (environmental conditions such as mud and rain, food quality, and number of times the calves were relocated), lead us to the realization that the heifers in Group 2 were under severe stress during most of the experiment compared to the heifers in Group 1. Therefore, we decided that combining the data from the two groups of heifers in these two trials would not yield a fair representation of the data collected. The two groups were analyzed separately and the results between the two groups by treatment were compared to determine if there was an observable effect of the stress conditions on the effects of the OmniGen-AF® diet or the vaccine response overall. As expected, there were significant differences found between the two study groups for multiple immune markers that were measured in this study.

The biggest difference was found in the mononuclear cell proliferation rates in both groups of the OmniGen-AF® fed calves after booster vaccination when stimulated with the

three upper-respiratory viruses. When comparing the mononuclear cells of the unsupplemented control groups stimulated with the three viruses, Group 2 had a slight increase in proliferation rates. The OmniGen-AF® fed heifers in Group 2 had an average of 146% more proliferation than the OmniGen-AF® fed heifers in Group 1 when stimulated with BVDV1, an average of 65% more proliferation when stimulated with BVDV2, and an average of 85% more proliferation when stimulated with BHV-1 (see appendix 3 for table). The OmniGen-AF® fed heifers in Group 2 had both an increase in magnitude and duration in proliferation rates during the secondary immune response. This substantial increase indicates that OmniGen-AF® supplementation might have a greater effect on the immune system of animals that might be suppressed due to factors such as high levels of stress.

Another difference that was seen between the two study groups was that the OmniGen-AF® calves in Group 2 gained significantly more weight than the nonsupplemented heifers. In Group 1, the OmniGen-AF® and control heifers gained weight at the same rate. An increased immune response requires a higher demand in resources, which should result in energy costs. However, feeding OmniGen-AF® to the highly stressed calves in Group 2 facilitated an increase in weight gain that demonstrates a positive impact on future production.

The stressed cows in Group 2 took longer to respond to the booster vaccine. This is clearly demonstrated in the serum neutralizing titers measured after booster vaccination. There was a significant rise in antibody levels on D20 following the booster vaccine given on D0 in Group 1; whereas in Group 2, an amnestic antibody response for all three viruses was not seen until D40. Similarly, the proliferation response in Group 2, even for the OmniGen-AF® heifers, did not rise until the D40 sampling relative to booster vaccination,

and the control heifers never mounted a significant recall proliferation response. In Group 1, heifers on both diets showed a significant increase in proliferation and titer response in the D20 samples.

BRDC infections in calves cause severe morbidity and mortality. Previous studies have demonstrated the ability of OmniGen-AF® to enhance innate immunity. One such study induced immunosuppression in sheep, and by measuring innate markers, neutrophil L-selectin and IL-1b**,** found that the immunosuppressive effects were countered by adding OmniGen-AF® to their diets (Wang et al., 2007). The significance of our study is that in addition to the previously demonstrated enhancement in innate response, we have found evidence that feeding the OmniGen-AF® supplement boosts the adaptive immune response to vaccination, which could provide the the cattle with better protection against natural infection. Further, these studies provide new evidence that OmniGen-AF® has a stronger effect on animals that are faced with stressors. This is an import finding because calves go through a lot of stressful events at a young age: weaning, comingling, transportation, changes in weather, and changes in diet. A boost in innate and adaptive immune function, without any significant energy cost to the calves, makes giving this supplement well worth the cost and time it takes to add it to their daily diets.

CHAPTER 7

CONCLUSION

A small number of heifers were used in this trial because it was a proof of concept study. The two study groups were analyzed separately due to extremely differential level of stress that Group 2 was subjected to relative to Group 1. Even with the small numbers of heifers in each group, there were significant patterns of immune enhancement seen in the OmniGen-AF® fed heifers relative to the unsupplemented heifers in response to the primary and booster vaccinations.

Significantly increased proliferation responses of circulating memory cells among OmniGen-AF® fed heifers suggest that the dietary supplement stimulated the adaptive immune system in response to booster vaccination. Likewise, increased production of IL-4, IL-17, and IFN-gamma, and evidence of suppressed production of IL-10 cytokine activity in the OmniGen-AF® fed heifers compared with unsupplemented controls offers insight into the mechanisms by which OmniGen-AF® enhances adaptive immunity. A stronger innate and adaptive immune response leads to a healthier animal; thus, feed supplements, such as OmniGen-AF®, may be instrumental in enhancing the immune response against the pathogens that cause bovine respiratory disease. Further, better vaccine responses and healthier cattle should help to reduce the need for the use of antibiotics in cattle production.

Measuring the adaptive immune markers for more than 60 days post booster vaccination might be helpful in the future to look at the full effect that OmniGen-AF® has, not only on increasing the magnitude, but also on the duration of secondary immune responses. Some of the markers examined, like proliferation, SN titers, and cytokine activity, especially in the stressed study group, had a significant increase in immune response during the last few collection days and still had amplified levels of response during the conclusion of this study. Also, adjusting the timing of some of the cytokine measurements to better comprehend how OmniGen-AF® enhances the adaptive immune response would be beneficial in future studies.

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APPENDICES

Appendix A: Study group, treatment group and date of birth for all calves in this study.

Cow Number	Group	Treatment	Birthday	Starting Weights
4038	Group 1	Control	10.11.14	355
4039	Group 1	Control	10.11.14	360
4040	Group 1	Control	10.12.14	*Did not lock up*
4041	Group 1	OmniGen	10.14.14	334
4042	Group 1	OmniGen	10.18.14	300
4043	Group 1	OmniGen	10.22.14	314
4044	Group 1	Control	10.27.14	277
4045	Group 1	Control	10.28.14	275
4046	Group 1	Control	10.31.14	*Did not lock up*
4047	Group 1	OmniGen	11.03.14	260
4048	Group 1	OmniGen	11.03.14	300
4049	Group 1	OmniGen	11.13.14	300
4022	Group 2	Control	08.19.14	495
4050	Group 2	OmniGen	11.23.14	481.8
4051	Group 2	OmniGen	11.28.14	462
4052	Group 2	OmniGen	12.16.14	481.8
4053	Group 2	Control	12.17.14	356.4
4054	Group 2	OmniGen	12.20.14	462
4056	Group 2	Control	12.31.14	424.6
5001	Group 2	Control	01.07.15	444.4
5004	Group 2	OmniGen	02.01.15	371.8
5006	Group 2	Control	02.20.15	356.4
5008	Group 2	OmniGen	2.21.15	371.8

The heifers in both study groups had to reach an average of 6-months in age before entering the trial. The calves were then blocked and separated into the two treatment groups at the start of the trial to make the average starting weight of the treatment groups equivalent. Both treatment groups in Group 1 had starting weights of about 310 lbs. Both treatment groups in Group 2 had starting weights of exactly 257 lbs.

Appendix B: Amount of OmniGen-AF® fed daily to the OmniGen-AF® treatment groups.

Group 1							
Collection Dates	4.03.15	5.01.15	6.01.15	7.01.15	8.03.15	8.28.15	9.24.15
Average Weight (lbs)	338	372.64	475.73	522.76	607.3	683.72	747.12
OmniGen Fed (grams)	13.52	14.9	19	21	24.3	27.34	
Group 2							
Collection Dates	8.03.15	8.28.15	10.01.15	11.04.15	12.01.15	1.08.15	2.19.16
Average Weight (lbs)	440.51	494.38	551.42	639	710.6	779.02	901.8
OmniGen Fed (grams)	17	19.7	22.05	25.6	28.4	31.16	

The amount of OmniGen-AF® supplement that the OmniGen-AF® treatment group received every day with their grain increased at the being of each month based on average monthly weights that were calculated using a measuring tape around the heart girth of the calves. OmniGen-AF® was fed at 4 grams/100 lbs of body weight.

Appendix C: Average proliferation rates during the secondary immune response for Group 1 and Group 2.

Study Group	Treatment Group	Stimulant	D20	D40	D60
Group 1	Control	BHV-1 0.5	23.8	10.3	19.2
Group 1	Control	BHV-1 1.0	30.3	16.4	35.2
Group 1	OmniGen	BHV-1 0.5	37.1	33.1	66.1
Group 1	OmniGen	BHV-1 1.0	40.9	33.7	84
Group 2	Control	BHV-1 0.5	22.5	31.4	24.9
Group 2	Control	BHV-1 1.0	27.8	44.9	39.5
Group 2	OmniGen	BHV-1 0.5	46.3	90.7	102
Group 2	OmniGen	BHV-1 1.0	71.7	89.6	98.9
Group 1	Control	BVDV1 0.25	2.36	2.39	1.12
Group 1	Control	BVDV1 0.5	2.73	4.53	0.797
Group 1	OmniGen	BVDV1 0.25	8.83	10.3	3.14
Group 1	OmniGen	BVDV1 0.5	11.6	13.7	4.35
Group 2	Control	BVDV1 0.25	1.64	1.37	1.81
Group 2	Control	BVDV1 0.5	2.57	5	2.91
Group 2	OmniGen	BVDV1 0.25	2.03	14.1	12.2
Group 2	OmniGen	BVDV1 0.5	4.94	30	28.9
Group 1	Control	BVDV2 0.25	14.6	7.72	1.04
Group 1	Control	BVDV2 0.5	15.8	8.78	1.34
Group 1	OmniGen	BVDV2 0.25	9.19	17.8	6.08
Group 1	OmniGen	BVDV2 0.5	13.6	20	7.25
Group 2	Control	BVDV2 0.25	2.05	2.76	3.03
Group 2	Control	BVDV2 0.5	3.07	4.06	7.05
Group 2	OmniGen	BVDV2 0.25	4.6	26.6	11.5
Group 2	OmniGen	BVDV2 0.5	6.5	25.6	31

This table shows the average proliferation rates during the secondary immune response to the booster vaccination given on D0 for Group 1 and Group 2. The percent age increase was calculated for both MOIs for all three viruses and then averaged. The nononuclear cells from Group 2 had significantly more proliferation compared to Group 1 when stimulated with each of the three viruses (BHV-1, BVDV1, and BVDV2).