EFFECTS OF INJECTABLE TRACE MINERAL SUPPLEMENTATION ON THE IMMUNE RESPONSE AND PROTECTION ELICITED BY VACCINATION AGAINST BOVINE RESPIRATORY DISEASE

by

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(Under the Direction of Roberto A. Palomares Naveda)

ABSTRACT

Bovine respiratory disease (BRD) is one of the most economically important diseases affecting cattle. It is highly detrimental to young stock. This disease has a worldwide impact with a dramatic effect on the North American cattle industry because of the types of production systems employed that submit calves to a wide range of stressors increasing BRD risk in calves. Treatment of cattle affected by BRD often requires the use of antibiotics. The most costly component of BRD is secondary bacterial infections that complicate the pathogenesis of the disease. The use of minerals, more specifically trace minerals, is crucial to cattle production because of their role in a wide range of physiological functions in cattle (health, growth, and fertility). However, the current social and political pressure to enhance cattle health and the broad social concerns about antimicrobial over-usage and the development of microbial resistance, call for a new approach to research that evaluates strategies to improve the immune response to vaccines, promotes the overall health status, and enhances the performance of cattle in combination to minimize the use of

antimicrobials in cattle production. This dissertation investigates the effects of administration of a commercial injectable trace mineral supplementation product that contains Copper (Cu), Zinc (Zn), Selenium (Se) and Manganese (Mn) on the development of vaccine immune responses, and on the associated post-vaccinal protection elicited by modified-live virus and attenuated-live bacterial vaccines in dairy and beef calves. The first study evaluated the effects of the injectable trace minerals (ITM) used concurrently with live-attenuated bacterin vaccines in dairy calves. The second study evaluated the long-term protection induced by a modified-live virus (MLV) vaccine combined with the injectable trace minerals (ITM) in bull dairy calves that were challenged with *Bovine viral diarrhea virus* (BVDV) five months after vaccination. The last study was performed using beef calves. It evaluated if ITM impacted the rapid onset of protection elicited by an MLV BVDV vaccine that was given concurrently with ITM in weaned calves that were challenged with BVDV five days following vaccination and the impact of ITM treatment. The results of these studies provide both new strategies and insights for ranchers and veterinarians with respect to the value of using the ITM product in conjunction with BRD MLV or bacterin vaccines, and additional beneficial information about how to enhance cattle health. These studies also generated new questions about how the use of injectable trace minerals in young cattle can enhance the efficacy of the cattle production system to provide potential gains to the whole of society.

INDEX WORDS: Bovine respiratory disease complex; injectable trace minerals; MLV and live attenuated bacterial vaccines; vaccine immune response; protection; BVDV challenge.

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DEDICATION

I want to dedicate this work first to all my family, and especially to my dear wife Cassandra, my daughters Ana-Tereza and Maria-Laura, my parents Elias and Oneida, my brothers Jarbas and Lindomar, and my sister Elisa and their families, my mother in law - Ms. Miriam. Additionally, to all my friends and colleagues who gave me the inspiration and fuel to persist in this journey to successfully conclude my doctorate graduate program at the University of Georgia. I highly honor my grandparents who are not more physically present in my life, but influenced me very early in life to pursue professional development in the veterinary medicine field. Thank you very much for all the support, prayers, understanding and love to me in this interesting educational phase of my life. You all are and always will be in my heart.

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CHAPTER 1

INTRODUCTION AND OVERVIEW

Bovine respiratory disease (BRD) is one of the most important diseases affecting cattle. It has its largest detrimental effect on young stock. The disease has a worldwide economic impact, with a dramatic effect on the North American cattle industry. This is due to the specific type of production systems employed in North America that submit calves to a wide range of stressors increasing calves' BRD risk (Irwin et al., 1979; Griffin, 1997; Cusack et al., 2003). Initially, this illness affects the upper respiratory tract of cattle. It subsequently leads to the colonization and damage of the bronchia and lungs (Lillie, 1974). This is a multifactorial disease involving a number of etiological agents and a very complex pathobiology. The complex nature of the disease is due to the interrelation of environmental stressors and host factors (Cusack et al., 2003; Earley et al., 2017). A wide range of pathogens (primarily viruses and bacteria) have been identified as etiological agents associated with BRD. Some of these infectious agents can cause immunosuppression. It is believed that the immunosuppression potentiates the pathogenesis of the complex we see as BRD (Cusack et al., 2003; Derek, 2014).

Bovine respiratory disease complex affects both dairy and beef cattle, and leads to substantial losses to producers. Income losses result from cattle deaths, the cost of prevention, treatment of sick animals, and reduced performance of affected animals (Griffin, 1997; Irsik et al., 2006; USDA, 2013a). Treatment of affected cattle with BRD

often requires the use of antibiotics, because bacterial pathogens are often involved as part of the disease complex (Cusack et al., 2003; Thomas, 2009). Therefore, the judicious use of antimicrobial agents in the face of efforts to reduce their use is a concern for producers. The concerns are driven by the general perceptions of society and regulations issued by federal agencies (e.g. FDA and USDA). This is pressuring veterinarians and cattle producers to change their practices in cattle rearing. Among the many pathogens involved in this complex disease (Lillie, 1974; Fulton et al., 2000; Dabo et al., 2007; Griffin et al., 2010; Confer, 2009; Fulton, 2009), *Bovine viral diarrhea virus* (BVDV) is known to induce subclinical infections with an associated immunosuppression. Moreover, BVDV also causes mild to severe clinical disease and death when infections with a highly virulent strains occur (Brock et al., 2007; Walz et al., 2010; Palomares et al., 2012; Ridpath, 2013). The current methods to control the cost of BVDV infection, and to prevent transmission of the virus rely on a combination of biosecurity, effective diagnosis (with identification and removal of BVDV-persistently infected animals) and the use of commercial vaccines (Larson et al., 2002; Kapil et al., 2005; Walz et al., 2010).

Vaccination for the prevention and control of BVDV has two goals. The first is the reduction of BVDV transmission within the herd, and the second is to provide immune protection to susceptible cattle to reduce the impact of clinical disease during acute infections with BVDV (Lindberg and Houe, 2005; Newcomer et al., 2017). There are more than 160 commercially available vaccines in the US (Compendium of Veterinary Products, 2003; Ridpath, 2013). Yet, the level of vaccine compliance among farmers is still questionable, as many do not use BRD vaccines in their herds (USDA,

2010). Cattle should be immunized before going to the sale barns, backgrounder, or feedlot. It has been demonstrated that better calf performance at feedlot is observed when high serum neutralizing (SN) antibody titers against BVDV are present on entry to the feedlot (Fulton et al, 2002). Moreover, even though commercial vaccines are known to generally induce adequate levels of SN antibodies to modulate disease (Fulton, 2005a; Kelling et al., 2005 and 2007; Xue et al., 2010; Ridpath, 2013; Newcomer et al., 2017; Platt et al., 2017), vaccine failure is still a possibility. Poor vaccine response, or outright failure, can be caused by interference from maternal antibodies in young calves (Ellis et al., 2001; Chamorro et al., 2015), poor immunological status in cattle subjected to stress, a mismatch between vaccine and field antigen, errors in vaccine handling, and impairment of vaccine efficacy by pathogen infectious pressure (Blecha et al., 1984; Heininger et al., 2012). In addition, nutritional deficiencies prior to or during vaccination (Rice et al., 1986; Thomas, 2009) may hamper vaccine response in cattle.

The use of minerals, and more specifically trace minerals, is crucial to cattle production because of their role in a wide range of physiological functions in cattle, such as health, growth, and fertility (Underwood and Suttle, 1999; Stewart, 2017). As a result, producers striving for proper nutrition to ensure adequate minerals levels in cattle and avoid deficiencies, find supplementation to be necessary. Further, the current social and political pressure to enhance cattle health and the broad social concerns about antimicrobial over-usage and the development of microbial resistance, call for a new approach of research that evaluates strategies to improve the immune response, promotes the health status and enhances the performance of cattle. The combination of

these novel strategies potentially can lead to minimize the use of antimicrobials in cattle production. Therefore, the use of trace minerals in cattle that already have normal mineral levels represents a potential tool for enhancement of immunity and the physiology of cattle. The use of trace minerals as an adjuvant to production has been the subject of multiple studies over the last decade (Arthington and Havenga, 2012; Teixeira et al., 2014; Roberts et al., 2016; Palomares et al., 2016).

This dissertation investigates the effects of administration of a commercial injectable trace mineral supplementation product that contains copper (Cu), zinc (Zn), selenium (Se) and manganese (Mn) on the development of the vaccine immune response, and on the associated post-vaccinal protection elicited by modified-live virus and attenuated-live bacterial vaccines in dairy and beef calves. The data presented in this dissertation is sourced from three randomized clinical trials done at the University of Georgia with dairy or beef calves utilizing three distinct designs to answer the questions: what are the effects of ITM co-administered with BRD-vaccines on the induction of the immune response to bacterial vaccines, and what are the effects over long-term, and rapid onset of protection against BVDV challenge in dairy and beef calves, respectively. This dissertation is divided in six chapters. Each chapter is designed to stand alone. Each chapter includes its own figures, tables and references that specifically support the presentation of the respective chapter.

Chapter Two is a brief overview of the massive and extensive published literature related to Bovine Respiratory Disease (BRD) with focus on topics directly related to this dissertation. These include: bovine virus diarrhea virus (BVDV) and the use of trace minerals (TM), specifically on the use of injectable formulation of trace

minerals. Chapter Three is a published manuscript in which the use of ITM concurrent with live-attenuated bacterial vaccines was evaluated in dairy calves. Chapter Four is also a published manuscript in which the long-term protection provided by a modified-live virus (MLV) vaccine combined with injectable trace minerals (ITM) was evaluated in bull dairy calves. In Chapter Five, a study performed in beef calves is described. This study provides new insights for ranchers and veterinarians regarding the impact of ITM use on rapid onset of protection from BRD MLV vaccines. The work done in Chapter Five was compiled into a manuscript that will be submitted to the *Veterinary Immunology and Immunopathology Journal*. Finally, this dissertation contains an overall conclusion presented in Chapter Six. This integrates the findings of all three research studies with an interpretation of the impact of ITM use concurrent with vaccination in calves. This discussion also generates new questions about whether the use of ITM in young cattle is able to enhance the efficacy of the cattle production system in a way that is beneficial to the whole society.

CHAPTER 2

REVIEW OF THE LITERATURE

Bovine respiratory disease (BRD)

Definition

Bovine respiratory disease (BRD), also known as shipping fever, is a poly-microbial infection mediated illness of cattle. Bovine respiratory disease complex causes significant inflammation and damage to the respiratory tract. This results in substantial economic losses to the cattle industry. The infectious agents involved generally initially affect the upper respiratory tract. This is followed by colonization of the tissues of the lower respiratory tract, causing varying degrees of inflammation and cellular damage. The amount of damage depends on several host factors and the identity of the pathogens involved (Lillie, 1974). The most severely affected animals are dairy and beef calves when they have undergone a stressful event, such as castration, dehorning, branding, severe weather and weather changes, weaning, transportation, feedlot arrival, comingling, and diet changes (Irwin et al., 1979; Cusack et al., 2003). This disease complex occurs days to few weeks after the calf is subjected to the stressors. The classical clinical signs of BRD include: fever of over 40^{o}C, depression, ocular and nasal discharge, cough, respiratory distress (rapid or shallow breathing), reduced appetite, lack of rumen fill, and self-isolation from herd-mates (Buhman et al., 2000; Smith et al., 2001; Radostits et al., 2007).

Importance in the livestock industry

Bovine respiratory disease (BRD) is one of the most significant causes of calf morbidity and mortality in North America (USDA, 2010). It is also the most costly disease of beef cattle in the United States and Canada (Griffin, 1997; Irsik et al., 2006). Bovine respiratory disease's deaths alone cost an estimate of over $643 million a year in the USA (NASS, 2010). Surveys done in the US confirm that BRD is the leading cause of mortality among dairy heifers (USDA, 2011). It has also been reported that BRD was determined to be the highest single cause of morbidity among weaned dairy heifers (11.2%), and the second highest cause of morbidity among pre-weaned dairy heifers (18.1%), behind digestive problems (diarrhea, bloat), that accounted for 25.3% (USDA, 2011).

In another report (USDA, 2013a), it was shown that nearly every feedlot in the US has some cattle affected by shipping fever at any given time, and that 16.2% of all cattle placed in feedlots were affected by BRD during their stay. In the evaluation based on feedlot capacity, it was a common practice among feedlots with a capacity of 8,000 or more head, to use prophylactic antibiotics to attempt to prevent clinical BRD cases, and almost 14% of all feedlot cattle received injectable antibiotic as part of the receiving treatment. When evaluating cattle of less than 700 pounds, the survey showed that almost 40% of the total fed cattle received some type of injectable antibiotic to prevent or reduce the severity of shipping fever outbreaks. In an effort to evaluate the herd-level BRD risk factors in nursing calves in cow-calf operations, another survey evaluated 2,600 US cow-calf producers covering two important calf production areas of the US (including states in the Eastern region and the Plains States; Woolums et al.,

2013). About 17% of questionnaires were returned that contained BRD data matching the inclusion criteria for analysis. It was reported that 21% of the operations had at least one calf with BRD at the time of the survey. In 89.2% of these operations, at least one calf was treated for BRD, and in 46.4% of the operations with at least one calf diagnosed with BRD, at least one calf died because of BRD (from the production group of calves born during the 2010 calendar year. Woolums et al., 2013). In a subsequent survey done by the same research group, it was reported that 18% of the cow-calf operations identified BRD cases on the previous year (2010 to 2011 production year), and mortality attributed to BRD in at least one calf occurred in 14% of the operations from August 2011 to July 2012 (Woolums et al., 2014).

In addition to the direct costs of BRD, there is a growing concern about the development of antimicrobial resistance among BRD associated pathogens by citizens of the US and other major livestock producing countries (USDA, 2012). Therefore, programs and policies that foster antibiotic stewardship, and policies that require reduction in the widespread use of antibiotics in food animals are having an impact on today's agriculture. Examples can be seen in the recently enacted laws - the veterinary feed directive (VFD. Food and Drug Administration, 2015) and the senate bill no. 27 in the state of California (California Legislative Information, 2015) that tightly regulate the purchase and use of antibiotics for cattle production. Extensive efforts are being made by researchers and other livestock production professionals to not only reduce antibiotic use, but also to develop guidelines for their judicious use and novel strategies to improve cattle health that reduce the need for antimicrobials use in food animal production.

Epidemiology

The BRD is a complex disease of multifactorial etiology. It results from the combination of factors and their interactions, arising from the host animal, the pathogens involved, and the environment. A simplified view of the pathogenesis of BRD comprises three stages: first, BRD appears to be preceded by a stress event (often a viral infection or stress situation); the second stage is the suppression of innate or adaptive immune function relative to stress, and the third stage is the overgrowth of bacteria in the respiratory tract that exploits the reduced innate and adaptive immune response, often leading to pneumonia and damage to the respiratory capacity (Cusack et al., 2003; Fulton, 2009; Earley et al., 2017). Calves, beginning at an early age until after weaning, are affected by this complex disease in ways that detrimentally impact normal development and performance (Irsik et al, 2006). In the worst case, the disease can be life threating to cattle. Affected calves experience a reduction in dry matter intake, with associated poor growth performance. In the BRD pathogenesis, the bacterial component of the disease often requires support and antibiotic therapy, with the aim to treat bacterial infections (if already present) or preventing the rise of secondary bacterial infections (reviewed in Cusack et al., 2003; Fulton, 2009; Derek, 2014).

The disease occurs throughout the US and Canada with similar outcomes. It affects both dairy and beef cattle. In dairy cattle, BRDC often occurs in younger calves due to factors of the production system that enhance host susceptibility to the infections and the risk of disease exposure. These factors include: failure or low levels of passive immune transfer, crowding, confinement and indoors rearing systems, manure or high

organic matter load in the rearing environment coupled with poor ventilation, early weaning, dry dusty feed (such as dry hay, concentrated ration with relative dusty characteristics), and multiple stressors (disbudding and dehorning and castration of bull calves) all increase the risk a calf will develop BRD (Cusack et al., 2003; Earley et al., 2017). These stress factors result in physiological challenges and limitations on the dairy calves that already have an underdeveloped immune system and are struggling to maintain homeostasis (Bolin, 2002). The common respiratory viral pathogens take advantage of the stressed immune system to disrupt the homeostasis between commensal organisms (that can function as pathogens) and host (Hodgins et al., 2002, Risco and Melendez, 2011). Weaning is one of the most stressful events calves undergo. Dairy calf weaning is commonly done within two months of age. By this time, the levels of maternally-derived antibodies start to drop (Chamorro et al., 2014), increasing the disease susceptibility of these calves. The major goal of the individual housing raising system of dairy calves is to prevent nose to nose contact among calves while on a milk only diet. However, once calves are weaned, they are subsequently grouped and comingled in a common area with shared feed throughs and waterers. The competition inside the groups adds another risk factor for disease development.

Beef calves face different types of situations and risk factors that induce challenges to their homeostasis and the function of their immune system. These can lead to similar risk of BRD as is seen in dairy calves. The beef cattle industry in North America is highly dependent on transporting calves over long distances to deliver them to stocker facilities or feedlots as a component of the production cycle (Tucker et al., 2015; Drouillard, 2018). Beef cattle ranchers like to maximize cattle working activities

into as few handling sessions in the corrals as possible. Although this management system makes sense in terms of cost and labor, it does not align with the current scientific recommendations for improvement of cattle health (Lekeux, 1995; Tucker et al., 2015; Schumaher et al., 2017). In other words, calves are managed, processed and vaccinated at the producers' convenience rather than by pursuing a schedule that would maximize a successful health outcome. That is, management is commonly aimed to reduce cattle handling frequency (especially during hot weather), to facilitate cattle gathering in large ranges, and maximize available human resources. Ideally immunizations should be given to non-stressed cattle, before anticipated stress events such as dehorning, castration, or weaning. Those practices common to beef rearing lead to an increase in high-risk conditions that increase susceptibility of calves to the common diseases, especially to BRD. One of the most common situations exemplified in this paradox is when the weaning of beef calves takes place at the same time as priming-vaccination, with immediate shipping to the auction market or background facility. In this situation, the calves undergo a number of major stressors - weaning, vaccination, and transportation, plus a major change in environment, nutrition plan, crowding, entry to the feedlot, and new sources of water, different flooring and mixing with new cattle. This compromises the vaccine immune response and reduces the probability of disease protection, as well as exposing the calves to several risk factors of BRD development. Among these stressors, weaning and feedlot entry are the major stressors cattle undergo (Cooke, 2017). While it is not reasonable, there are still situations in which the calves are not vaccinated at all (USDA, 2010). Under these conditions, the susceptibility of calves to BRD increases dramatically. Therefore, when

we consider the large number of small to middle scale facilities within the US that produce beef calves but do not have the resources to background them, there is additional risk in moving their beef calves to local auctions and to the sale barns. In addition to transportation to these facilities, the level of stress of these calves cascades once they are moved to sale barns or auctions facilities. This cascade of risk includes: commingling, hierarchal social re-arrangement, boarding in pens instead of on pastures, crowding, poor ventilation, abrupt changes in diets, and even lack of or reduced access to water, and others specific to the location involved (Tucker et al., 2015; Drouillard, 2018).

In summary, the problem of BRD in North American beef and dairy cattle is primarily mediated by the interactions of the function and activity of the immune system in governing homeostasis, the density and activity of several causative microbial agents acting in a cascade, and the elements of the environment in which the cattle are placed. The disease is complex in its etiology and pathogenesis, caused by a multifactorial array of factors, generally has a high prevalence in cattle, and impacts the overall cattle production.

Etiology and Pathogenesis

Bovine respiratory disease is a polymicrobial illness. This complex disease almost always involves multiple pathogens within an individual host. The interactions of the organisms with the host immune system leads to the development of disease. The microbes can interact serially in the host respiratory system, with early infection potentiating subsequent infection by microbes in a fashion that enhances the induced

damage of the host's tissues. The combinations of pathogenic microbes in the respiratory tract establish this complex pathogenesis and disease development (Hodgins et al., 2002; Panciera and Confer, 2010; Derek, 2014).

Among the viruses involved in BRD etiology, those most frequently identified are *Bovine viral diarrhea virus* (BVDV), *Bovine herpes virus-1* (BHV1), *Bovine parainlfuenza-3 virus* (PI3), *Bovine respiratory syncytial virus* (BRSV), and recently the family of bovine adenoviruruses A-D. *Bovine coronavirus* may also be involved. In addition, bacteria, many of which are part of the upper respiratory flora, (*Trueperella pyogenes, Mannheimia haemolytica, Pasteurella multocida, Histophilus somni*, and *Mycoplasma spp*.) are the most common secondary pathogens involved in BRD (Confer, 2009; Fulton et al., 2009). Of these bacteria, *Mannheimia haemolytica* and *Pasteurella multocida* are typically found as commensals in the upper respiratory tract of cattle (Hodgins et al., 2002; Griffin et al., 2010). This makes it more difficult to develop effective biological methods for BRD prevention and control (Dabo et al., 2007; Rice et al., 2007; Confer, 2009). When a breach of the respiratory tract defenses occurs, particularly when immunosuppression is induced as part of the breach, secondary infections are facilitated in the lower respiratory tract usually by these commensal bacteria leading to acute clinical BRD (Griffin et al., 2010).

Viruses, particularly BVDV and BHV-1, not only induce suppression of both humoral and cell mediated immunity, but they also interfere with the innate immune function by reducing the cilial function and muco-ciliary clearance of upper respiratory tract, and reduce tracheal antimicrobial peptide production, thus making cattle more susceptible to secondary bacterial infection (reviewed in Earley et al., 2017). Quite

often there is direct interplay between the pathogens involved (virus with virus, virus with bacteria and bacteria with bacteria), and this yields complex co-infections that potentiate the development and impact the severity of BRD (Potgieter et al., 1984, 1985; Pollreisz et al., 1997). Among the viruses involved in BRD, BHV-1 and BVDV are the most clearly immunosuppressive. This favors the enhanced colonization by other pathogens (both viruses and bacteria). This is a direct promoter of expanded growth and colonization of the whole respiratory tract by commensal bacteria that contribute to the severity of BRD (Campbell, 2004; Kapil et al., 2005). *Bovine viral diarrhea virus* is recognized as the most important mediator of the immunosuppression that helps to initiate microbial infection promoting BRD in cattle. In addition, BVDV infection is often related to failures in reproduction. BVDV can cause embryonic death, abortion, stillbirths, and if the calf survives to term, it can cause congenital defects (Muñoz-Zanzi et al., 2003) or if the infection occurs within a certain window during the first trimester of gestation, induce the development of a persistently infected (PI) calf. Persistently infected cattle are the most important mechanism for transmission of BVDV because they continuously shed virus into the environment, resulting in exposure of susceptible cattle within herds (Campbell, 2004; Walz et al., 2010). If a pregnant cow or heifer becomes infected between 45 to 125 days of gestation by a low virulence non-cytopathic (ncp) BVDV strain, then it is highly likely that she will have a PI calf (Harding et al., 2002; Ellsworth et al., 2006). Another way that PI calves are generated is if a PI-cow or heifer conceives and carries the pregnancy to term, it is 100% likely that her offspring will be a BVDV-PI (Thurmond, 2005; McClurkin et al., 1979). The immune system of these calves recognize the PI virus as a self-antigen, and

never mounts an immune response against that virus (or its close relatives). That immune blindness allows the calf to maintain the virus for life and to continue to shed into the environment (Liebler-Tenorio, 2005). The BVDV-PI calf represents the most important source of BVDV transmission to domestic cattle. This is due to the constant shedding of large amounts of virus to naïve calves (Campbell, 2004; Walz et al., 2010). However, non-PI calves during acute BVDV infection can shed virus at significant levels. The quantity of virus is related to BVDV strain virulence (Thurmond, 2005). However, shedding of virus from acute infection is at low to moderate levels compared to typical shedding by PI-calves.

The acute BVDV infection is often asymptomatic. Some strains of BVDV cause serious impairment of the innate and adaptive immune responses. This can lead to immunosuppression of the affected cattle (Chase et al., 2004; Walz et al., 2010; Chase, 2013). A transient leukopenia, occurring within 3 to 10 days post infection, is observed in many acute infections. The severity and duration of the decrease in leukocyte number is associated with the BVDV strain virulence (Bolin and Ridpath, 1992; Liebler-Tenorio, 2005). The innate immune function of cattle is often impacted by BVDV infections. It can cause impairment of microbicidal, chemotactic and antibody-dependent cell mediated cytotoxicity activities of cattle neutrophils (Barkema et al., 2001). In monocytes, the virus can alter the expression of proteins directed at mediating cellular adhesion, regulating apoptosis, and participating in antigen uptake, processing and presentation (Antonis et al., 2004; Bruschke et al., 2001; Studer et al., 2002). A clear detrimental effect on the stability of monocytes *in-vivo* was also shown by a 30 to 70% decrease in monocyte numbers in circulation in an infection trial with

a virulent BVDV field strain (Archambault et al., 2000). Further, macrophages appear to have decreased phagocytosis capacity during BVDV infection. This appears to be mediated by the decreased expression of Fc receptors and complement receptors, in conjunction with impaired microbicidal activity and production of chemotactic factors (Welsh et al., 1995; Giangaspero et al., 2001; Falcone et al., 2003; Balint et al., 2005). *Bovine viral diarrhea virus* infection also alters the production of IFN-α in a biotype dependent fashion. It appears that cytopathic (cp) strains induce IFN-α production but that ncp strains inhibit its production and release (reviewed in Chase et al., 2004 and 2013). The function of adaptive immunity is also impacted by BVDV infection. Lymphopenia is seen and is strain-dependent. In two studies conducted using a mild ncp type 1b field strain (NY-1) to challenge cattle, the infection yielded a 10 to 20% reduction in lymphocyte number (Ellis et al., 1988; Brodersen and Kelling, 1999). In another study using a different ncp type 1b strain (R2360), a 40% to 50% decrease in lymphocyte number was observed (Ridpath et al., 2007). In contrast, when ncp type 2 field isolate (24515) (Archambault et al., 2000) and ncp type 1b strain (CA0401186) Ridpath et al., 2007) were used, a more severe decline in lymphocyte number was observed (50% and 70%, respectively). The effect of BVDV on the subsets of T-cells after infection has been analyzed (Ellis et al., 1988; Brodersen and Kelling, 1999). Those studies documented a great impact, with severe reduction in $CD8^+$ followed by $CD4^+$ subpopulations during acute infection.

The antigen presentation cells (APC) stimulation of T-cells in the organized lymphoid tissues promotes activation of T-cells that express IL-2α receptor ($CD25^+$). These activated cells return to the circulation and enter the local area of infection

(Abbas et al., 2012). An abrupt increase in activated T-cells in the lungs (300- to 150-fold increase for $CD4^+$ and $CD8^+$, respectively) was observed by 10 days post infection with BVDV. This was followed by evidence of enhanced memory function (Silflow et al., 2005). This increase and associated influx of cells into the lungs indicated that memory T-cells were entering the lungs as part of the cell mediated immune (CMI) response to BVDV. Activated T-cells are also important for viral clearance (Chase, 2013). The role of specific T-lymphocyte subsets was evaluated in a study that used mouse monoclonal antibodies specific to each T-cells subset ($CD4^+$, $CD8^+$ and $\gamma\delta^+$ T-cells) to remove each subset individually from cattle. The T-cell depleted cattle were evaluated for virus shedding in nasal secretion, and for BVDV viremia after a BVDV challenge (Howard et al., 1992). Only the depletion of $CD4^+$ cells was associated with an increase in virus shedding duration, and the total quantity of virus shed (they shed an extra 7-10 days and at 0.5-1.5 $\log_{10}$ higher level compared to control). $CD4^+$ T-cells are important in coordinating the humoral and CMI responses in the early stages of the infection, and in managing the duration and focus of their responses. They contribute to the activity of other immune cells by releasing cytokines that promote the expansion, differentiation and arming of responsible cells, and regulate the activity of the immune response (Abbas et al., 2012). $CD4^+$ T-cells target not only the BVDV immunodominant proteins, NS3 and E2 (Howard et al., 1992; Liang et al., 2008) but the capsid protein (C), glycoprotein E^{rns}, amino-terminal proteinase (N^{pro}), and to some extent the nonstructural protein 2-3 (NS2-3) (Collen et al., 2000).

Cytotoxic T lymphocytes, mostly $CD8^+$ T-cells, have important effector functions. They directly kill virus-infected cells under MHC class I restriction, through

the release of several cytotoxins, perforins, granzymes, and granulysin (Abbas et al., 2012). Memory $CD8^+$ T lymphocytes can be observed in circulation even after 9 months following BVDV infection in cattle (Howard et al., 1992; Beer et al., 1997). The production of IL-2 and IFN-γ is induced in proliferating $CD8^+$ T-cells following BVDV infection. These cells facilitate type 1 immune memory responses (Rhodes et al., 2000).

In cattle, gamma delta T-cells, many expressing the WC1 phenotype, accounts for up to 60 % of the total circulating lymphocytes. These T-cells have been described as possessing major regulatory functions in cattle, as they spontaneously secrete IL-10 (Hoek et al., 2009; Guzman et al., 2014). Therefore, increasing activation and trafficking of gamma delta T lymphocytes may be one of the mechanisms used by BVDV to down-regulate the immune response causing the immunosuppression that has been previously reported (Palomares et al., 2015).

Lymphoid organs are a direct target for BVDV. Secondary lymphoid tissue also is depleted of T-cells during BVDV infection. The depletion of T-cells occurs in the thymus, Peyer's patches and regional lymph nodes. Lymphocyte depletion also includes the B-cells (Teichmann et al., 2000; Stoffregen et al., 2000). The exact pattern of changes of lymphocytes in lymphoid tissue occurs in a BVDV-strain dependent fashion. There are examples of virus strains that induce no changes in B-cells or T-cells, and others that induce a severe B-cells reduction. The more virulent strains appear to have a greater capacity to deplete lymphocytes in general (Ellis et al., 1988; Teichmann et al., 2000; Liebler-Tenorio et al., 2003; Pedrera et al., 2009a and 2009b).

Prevention and Control to BVDV

Biological prevention and immune protection methods for BVDV are the central pillars of biosecurity, diagnosis and identification of PI calves (for removal from the herd) and vaccination. The low prevalence (less than 1%) of PI calves in US beef herds (Wittum et al., 2001; Loneragan et al., 2005) makes it difficult to conduct surveillance and identification of PI calves. This is due the large number of animals which need to be tested, and the difficulty in justifying for cost of testing for such a small return. This limitation is an economic challenge to an eradication program in the US (Larson et al., 2002). The PI calves can survive after weaning, and even through to slaughter in the feedlot setting (Taylor et al., 1997; Wittum et al., 2001; Loneragan et al., 2005).

Vaccination is an important tool for the reduction in transmission of BVDV within and between herds, and the protection of susceptible cattle in herds by reducing prevalence and impact of clinical disease in newly infected cattle (Lindberg and Houe, 2005; Newcomer et al., 2017). There are more than 160 licensed BVDV vaccines for sale (Compendium of Veterinary Products, 2003) in North America, including both single pathogen, and combination vaccines, packaged with other BRD pathogens (BHV-1, BRSV, PI-3, *Mannheimia haemolytica, Pasteurella multocida, Histophilus somn*i, leptospirosis, and others). The BVDV vaccines generally contain either one or both genotypes of BVDV (1 or 2) and may contain cp (more common) or ncp strains that induce cross protection including for heterologous strains (Fulton, 2005a; Ridpath, 2013; Newcomer et al., 2017).

There are two major forms of BVDV vaccines commonly available containing killed virus (KV) with adjuvant or modified live virus (MLV). There are pros and cons to the use of each (Fulton, 2005a). Killed vaccines are safer in general. There are no live agents in these vaccines, so there is no chance of virus reversion to virulence. Therefore, they are safe for use in pregnant or immuno-compromised animals. However, there is less cell mediated immune response induced compared to MLV vaccines (Platt et al., 2008). Moreover, multiple doses of KV are required to reach functional immunity and a level of memory. An adjuvant in the formulation of KV is needed to enhance the presentation and response to vaccine antigen (Ridpath et al., 2010). Due the large amount of virus needed to prepare a KV vaccine, the product is expensive to make. It also has a lower risk of contamination because all components are killed (Fulton, 2005a). In a study evaluating killed BVDV vaccine (Platt et al., 2008), it was demonstrated that 2 doses of a killed BVDV vaccine were able to induce high levels of SN antibodies and some level of memory T-cells, but not BVDV specific $CD8^+$ memory cells. Another recently study (Platt et al., 2017) evaluated the immune response of a new live double deletion BVDV mutant vaccine to induce protection upon challenge with a field BVDV strain in 5-months old colostrum deprived calves. Calves from the vaccinated group developed SN antibodies to both BVDV types 1 and 2 while control calves remained seronegative during the study. Further, there was a numerically higher numbers of T-cells (with no statistical difference) in vaccine group compared to field strain BVDV infected calves.

MLV vaccines require less virus per dose to induce an immune response. This is because the vaccine virus is able to replicate in limited sites and for a limited period

in the host. MLV vaccines also stimulate both arms of the adaptive immune response. MLV vaccines induce the humoral and CMI responses because both intra-cellular and extra-cellular antigens are presented following vaccine viral replication. Therefore, no adjuvant is needed. The MLV vaccines also induce more rapid onset of immunity in most cases compared to killed virus vaccine (Newcomer et al., 2017). This contributes to the choice of the type of vaccine needed for the situation at hand (i.e. feedlot entry vs. preconditioning, at birth vs. weaning). The downside of MLV vaccines is the risk of reversion to virulence that may be the source of disease to cohort animals. Further, contaminating microbes may induce disease after the use of the vaccine, as all components are live (Falcone et al., 2003; Palomares et al., 2013). A transient immunosuppression may be seen following MLV vaccine delivery. Some MLV vaccine result in mild lymphopenia and transient viremia in cattle after vaccination. Some shedding of BVDV is a potential problem, but seldom reaches the levels of virus required to infect naïve calves (Ridpath, 2013). Another disadvantage of MLV vaccine is the risk of causing abortion in pregnant animals as shown by Orban et al. (1983) and Liess et al. (1984). They demonstrated abortions, stillbirths and calves with developmental defects after the use of commercial MLV BRD vaccines in pregnant cattle. The use of MLV vaccines in pregnant cattle is recommended only in cattle vaccinated with approved MLV vaccines at least 12 months previously based on the label claim (Animalytix, 2017). In a long-term study done by a group from Auburn University (Givens et al., 2016), researchers followed heifers for more than 3 years to assess the risk of reproductive related losses associated with the use of MLV vaccines in pregnant cattle. They found that properly MLV vaccinated heifers had minimal

additional risk of pregnancy related problems when given MLV vaccines during later pregnancies.

A study evaluating the use of MLV BVDV vaccine in calves 5 to 7 months old demonstrated protection in vaccinated calves after BVDV intranasal challenge compared to non-vaccinated calves (Kelling et al., 2005). Protection in this study was documented as lower viremia and prevention of acute infection clinical signs in vaccinated calves, and specifically sparing of target lymphoid cells (Kelling et al., 2005). Several other studies demonstrated protection by BVDV MLV vaccines using intranasal vaccination route followed by BVDV challenge in calves within one week of age (Xue et al., 2010), or using exposure to BVDV-PI calves as source of infection (Fulton et al., 2006). A study using radioimmune precipitation assays was able to demonstrate that both BVDV natural infection, and vaccination using MLV vaccines directed the immune response targeting primarily at the E2 and NS2/3 proteins, with less emphasis on the response against the E^{rns} and E1 proteins (Bolin and Ridpath, 1989; Donis 1995). The same evaluation done following killed BVDV-vaccine showed a preferential response to E2 protein, and very low or absent antibody response against NS2/3, E^{rns} and E1 proteins. This may explain some of the differences in immune responses between these two types of BVDV-vaccines and their associated protection capacity.

The maternal antibodies circulating in blood of young calves can reduce vaccine efficacy (Zimmerman et al., 2009; Newcomer et al., 2017). In addition, due to the immunosuppressive capacity of BVDV MLV vaccines (Ridpath, 2013), BVDV vaccination is not generally recommended for cattle before 3 months of age. A study

evaluated the efficacy of MLV vaccines given to calves with maternal antibodies and challenged with a BVDV type 2 strain (Zimmerman et al., 2009). In that study, researchers demonstrated some protection induced by the MLV vaccine used in calves with maternal antibodies after challenge with a virulent BVDV strain (strain 1373). In another study, Chamorro et al. (2014) showed BVDV maternal antibodies lasted for up to 6 months in calves. A subsequent study by the same research group (Chamorro et al., 2015) evaluated the immune response of 3 commercially available BRD-vaccines in early weaned calves (two to three months old). This trial demonstrated efficacy of vaccination in young calves. There were few differences between the commercial products. These results reject the hypothesis that maternal antibodies in serum from passive transfer interfere with vaccine response by blocking the production of antibodies. Although most of the vaccinated calves did not develop higher SN antibody titers compared to control (no BVDV maternal antibody) calves following BVDV challenge with a virulent strain (BVDV-2 strain 1373), the vaccinated calves appeared to have mounted a CMI response to the primary vaccination that impacted the development of disease. Following the BVDV challenge, the vaccinated animals developed a stronger anamnestic response, producing higher SN antibody titers, than controls. This anamnestic response resulted in protection against challenge, leading to lower number of calves shedding and less viremia compared to control group.

Long term vaccine protection is important to minimize the disease risk if a calf encounters a BVDV-PI animal during grouping and comingling at the sale barns, backgrounding or transfer to the feedlot. In the study by Zimmerman et al. (2009) calves were challenged with BVDV seven months after vaccination and shown to be

fully protected. In a more recent study, calves were vaccinated twice with an MLV BRD vaccine at about 3 months of age (Palomares et al., 2016), and a subgroup of these were used in another study that challenged the calves with a BVDV 2 strain five months after that prime-vaccination (Bittar et al., 2018b). This study demonstrated protection in vaccinated groups, where vaccinated calves had reduced or no lymphopenia or thrombocytopenia, lower rectal temperatures, no BVDV detected by virus isolation from buffy coat compared to the non-vaccinated control calves.

The onset of protection elicited by vaccines is also an important consideration given the high number of calves going to feedlots that have not been previously vaccinated. Under these conditions, the risk of BRD is increased due to the stressors these calves face during shipping and handling, and the possibilities of encountering a BVDV-PI calf at the receiving site. Brock et al. (2007) and Palomares et al. (2012) evaluated the onset of protection elicited by MLV BVDV vaccines in calves vaccinated 3, 5 or 7 days before challenge with a high virulence (BVDV 2 strain, 1373) or low virulence (BVDV 1a strain, NY-1), respectively. These authors demonstrated that at least 5 days between vaccination and BVDV challenge is needed to ensure an effective development of protection. In Brock et al. (2007), the more virulent BVDV strain caused not only severe clinical disease, but an increased mortality in control non-vaccinated calves. This challenge associated mortality was not observed in the other study that used the mild BVDV strain (Palomares et al., 2012).

Vaccination before feedlot entry, or weaning, is recommended. These vaccines play an important role in feedlot performance. In a recent study (Schumaher et al., 2017) the timing of vaccination related to weaning and feedlot entry was evaluated.

Calves were assigned to three groups. They received two doses of a commercial MLV vaccine. Vaccination was done either 15 days prior weaning, and 15 days before feedlot arrival; at the time of weaning, and at feedlot arrival, or 15 days after weaning, and 15 days after feedlot arrival. Calves vaccinated before weaning and feedlot entry had higher SN antibody titers and lower BRD incidence during the feeding period. Another study in beef cattle evaluated the humoral and CMI responses with respect to the calf age and route of initial multivalent MLV vaccine delivery (Woolums et al., 2013). Calves were vaccinated either 2 or 70 days of age, intranasally (IN) or subcutaneously (SC), and then all calves were boostered with SC vaccine at 217 (weaning) and 231 days of age. Calves vaccinated subcutaneously at 70 days of age had higher SN titers to BVDV 1 at the median ages of 140 and 217 days than the other groups. All of the calves had increased SN titers and evidence of CMI response after booster vaccination at weaning, with no differences observed among the groups.

In spite of the availability of large numbers of both inactivated and MLV BRD vaccines in the marketplace (Compendium of Veterinary Products, 2003; Ridpath, 2013) and their relatively common use, cattle still can succumb to disease (Fulton et al., 2005b; Kelling et al. 2005). The failure to mount immune responses and to develop protection after vaccination can be attributed to many factors. Commonly, interference by transferred maternal antibodies to calves less than 60 days of age inhibits good antibody development (Ellis et al., 2001; Chamorro et al., 2015), immunosuppressed cattle, socially stressed cattle, weather stressed cattle (heat or cold), antigenic mismatch between vaccine and field viruses, failure in vaccine handling, and high levels of pathogen exposure all mediate vaccine failure (Blecha et al., 1984; Heininger et al.,

2012). Nutritional deficiencies may also contribute to poor vaccine response in cattle (Rice et al., 1986; Thomas, 2009).

It is known that virus is directly involved in the infection and subsequent development of BRD. However, the participation of opportunistic bacteria in the disease often calls for antimicrobial use as major part of treatment of BRD in affected cattle. Supportive therapy, anti-inflammatory drugs, and others medications are also used. Yet antibiotics are the most commonly used drugs in management of BRD (USDA, 2013b). In this report, showed that antibiotics were used to treat 100% of the cattle that succumbed to BRD, and that in US feedlots, only approximately 30% of these affected calves received anti-inflammatory medication (second most used medication). Other common medications used in BRD were Vitamin-C in 34.1%, oral electrolytes, fluids and drenches in 6.8%.

Trace Minerals and BRD

Supplementation with trace (TM) minerals, more specifically injectable trace minerals (ITM), may help to optimize cattle performance and health (Underwood and Suttle, 1999). Recently, ITM has been used in cattle with normal mineral status generating a growing body of new research studies performed to develop novel protocols for the use of ITM to enhance cattle production (Richeson and Kegley, 2011; Arthington and Havenga, 2012; Teixeira et al., 2014; Roberts et al., 2016; Palomares et al., 2016; Bittar et al., 2018a). Among the essential trace minerals, several are frequently found to be severely deficient in production animals (Stewart, 2017). These include: copper (Cu), zinc (Zn), and selenium (Se). Trace minerals are involved in a

number of physiological functions in cattle including: growth (Spears and Kegley, 2002), maintaining enzyme structures and function, replication of nucleic acids, and a critical role in the function of the immune system. Among the enzymes of importance to health, superoxide dismutase (SOD) is considered to be of crucial. It functions by converting highly toxic oxygen free radicals to hydrogen peroxide that will be further broken down into water and stable oxygen by glutathione peroxidase enzyme (GPx, Michiels et al., 1994). As some trace minerals play a significant role in the structure of important enzymes (SOD and GPx), minerals including Se, Cu and Zn are considered as indirect components of the antioxidant system (Andrieu, 2008). Copper is important to the production of energy by mitochondria and plays an important role in the process of neutralizing reactive oxygen species (ROS) as part of the superoxidase dismutase (SOD) enzyme complex. Phagocyte killing activity, and other neutrophil and monocyte functions, are also dependent on the presence of copper. Therefore, copper has an important role in the innate immune response of cattle (Linder, 1991). In the adaptive immune system, deficiency of copper in the diet of mice leads to defective antibody production and reduced cell-mediated immune function (Sherman, 1992). Calves supplemented with injectable trace minerals have been shown to have improved antibody production and enhanced leukocyte proliferation in response to mitogens or antigens (Arthington and Havenga, 2012; Palomares et al., 2016; Bittar et al., 2018a).

Multiple factors affect trace mineral levels in cattle. These include considerable variability in the specific requirement base on forage and soil mineral, mineral intake by cattle (based on production stage, feeding protocol, and diet formulation), low mineral content of forages, and feedstuffs, and the presence of mineral antagonists in

feed, water, and forages, which limit their absorption. The use of ITM has several advantages in the production system. These included: delivery of a known and well controlled quantity of mineral that is rapidly and efficiently absorbed with predictable storage in the tissues following treatment (Pogge et al., 2012). This is relevant to the rearing of cattle under conditions of reduced dry matter intake (i.e. during transportation and receiving periods, weaning and vaccination). Thus, the use of ITM reduces the considerable variability in trace mineral levels that is commonly observed in cattle given free choice mineral intake (Arthington and Swenson, 2004). An ITM product available in the US market (containing Se, Cu, Zn and Mn. Multimin 90®. Multimin USA, Fort Collins, CO) has been proven to be absorbed quickly, to increase blood levels of the minerals within 8 to 10 hours after administration, and to last for approximately 24 hours in the body, except for Se that lasts longer in the blood stream (Pogge et al., 2012; Genther and Hansen. 2014). An increase in the activity of liver enzymes, following treatment with trace minerals that function as co-factors, can be observed by 24 hours following administration and the effect continues for 90-120 days (Genther and Hansen. 2014; Machado et al., 2014).

Selenium deficiency is the major cause of nutritional myodegeneration ("white muscle disease") in cattle. Even with significant efforts to prevent the problem, this disease still causes significant losses to cattle producers. Selenium deficiency can be subclinical in cattle, but even at these levels strongly influences the innate immune system with impact on cattle health and performance. Cows experiencing Se deficiency normally have weak calves. These calves have increased susceptibility to diseases, poor reproductive performance as they mature, and a higher incidence of retained fetal

membrane. The function of Se in the immune system is based on its participation in the structure and function of several enzymes that are directly linked to neutralization of ROS. Glutathione peroxidase is a major antioxidant reducing enzyme that contains Se. It neutralizes ROS and minimizes cell damage due to oxidative stress (Herdt and Hoff, 2011). There is evidence that Se deficiency in cattle increases the pathogenicity of some viruses (Beck, 2007), and negatively affects neutrophil migration into tissue that experiences inflammation (Maddox et al., 1999). In contrast, Se supplementation enhances both arms of the adaptive immune system. It promotes antibody production and enhances circulating T-cell numbers and *ex-vivo* function (Maggini et al., 2007).

Zinc is a trace mineral that has a wide range of activities in the animal body. It participates in the structure and function of more than 2500 enzymes. Zn is involved in metabolism and DNA replication (Cousins and King, 2004). Zn plays an important role in the immune system by facilitating proliferation and differentiation of lymphocytes, neutrophils and macrophages, enhancing enzyme function and protein transit, enhancing pro-inflammatory cytokine production, regulation and secretion (specifically in the case of IL-2), enhances T-cells clonal expansion to mitogens and antigens, and B-cell activation and antibody production (Pinna et al., 2002; Tomlinson et al., 2008; Bonaventura et al., 2015).

With the intention to enhance udder health during the transition period in lactating, high producing dairy cows that undergo severe metabolic limitations and a high rate of mammary disease, a study was done evaluating the effect of ITM on peripheral blood leukocyte activity and serum superoxide dismutase (Machado et al., 2014). In that study, the cows were treated twice with ITM during the dry period, and

once at 35 days post-partum. The cows used were high producing Holstein cows. Trace mineral supplemented cows had greater overall serum SOD activity than control cows (16.01 and 12.71 U/mL, respectively. $P < 0.0001$), and a tendency toward lower beta-hydroxybutyric acid (BHBA) during the transition period (in third or greater lactation cows) compared to controls. However, the authors could not explain the direct effect of trace minerals on the decreased BHBA concentration in serum, and its association with oxidative stress as a consequence of altered metabolic status (Bernabucci et al, 2005, Pedernera et al, 2010). In agreement with the previous studies, an increase in SOD activity in milk was also reported in dairy calves supplemented with Zn and Cu (Prasad and Kundu, 1995). This was also seen in ewes fed increased Zn and Cu enhanced diets compared to basal mineral levels diets (Pal et al., 2010).

Another study assessed ITM effects on beef cattle with adequate to mildly deficient mineral levels at the feedlots (Genther and Hansen, 2014). After a period of 88 days of feeding insufficient levels of trace mineral, steers were transported for 20 hours, and either administered ITM or not. The cattle were then returned to adequate trace mineral content diets for the finishing phase. The group receiving ITM had increased red blood cells lysate (RBCL) Mn-SOD activity over the reconstitution period. However, there was no difference between groups with respect to the activity of Se-dependent GPx. The authors speculated that the heat stress experienced over the four-week mineral reconstitution period accounted for the lack of the expected effect (Genther and Hansen, 2014). In contrast, Pogge et al. (2012) showed elevated RBCL GPx activity following ITM treatment at two weeks after trace mineral administration (Pogge et al., 2012).

There are several studies using ITM with intent to enhance the immune response of cattle to BRD, and to reduce the detrimental effects of stress in calves. In a randomized trial, Arthington and Havenga (2012) evaluated the response to BRD vaccination with the concurrent use of a commercial ITM supplement product containing Se, Mn, Cu and Zn in beef calves. In that study, ITM treated calves had higher neutralizing antibodies titers for BHV-1 following vaccination compared to control calves. In addition, there were no injection site reactions presented following ITM administration, indicating that the product was safe to be used. In another beef calf study that utilized newly received feedlot cattle (Roberts et al., 2016), a similar increase in serum neutralizing antibody titers was observed in the ITM treated calves. These calves had higher BVDV antibody titers when compared with saline-treated calves. However, calf performance and morbidity in that study were similar among all experimental groups. The low disease incidence was likely to be the explanation for the similar performance between ITM treated and control cattle. In an effort to compare efficiency of two TM products, Richeson and Kegley (2011) found that TM injected during initial processing of highly stressed, newly received heifers improved average daily gain (ADG), feed efficiency, reduced BRD morbidity, and reduced antibiotic treatment cost compared to calves that were not TM treated. This agrees with other studies presented here, suggesting the use of ITM to promote cattle health is justified.

In the dairy cattle production setting, ITM was also used concurrently with vaccination (Palomares et al., 2016; Bittar et al., 2018a and 2018b) or not (Teixeira et al., 2014). Injectable trace mineral was evaluated for additional benefits to the calf immune response and overall health. Under a recommendation for two ITM doses 30

days apart to newborn dairy heifer calves, a research group from Cornell University (Teixeira et al., 2014) demonstrated increased neutrophil function and glutathione peroxidase activity and a reduction in the incidence of health problems in weaned dairy heifers treated with ITM. In a controlled trial, focused on the adaptive immune response, BVDV naïve calves were vaccinated twice with MLV BRD vaccines and also treated with ITM at the time of vaccination. These treated calves had a more rapid development of humoral immune response against BVDV 1 antigen, higher rate of BVDV seroconversion, a greater level of peripheral blood mononuclear cells proliferation when stimulated with BVDV1 and BRSV recall antigen compared to control calves (Palomares et al., 2016). Similarly, Bittar et al. (2018a) demonstrated an improved immune response in ITM treated calves to a vaccination containing a bacterin of *Mannheimia haemolytica* and *Pasteurella multocida*. In an effort to evaluate long-term vaccine protection, Bittar et al. (2018b) challenged these dairy calves (treated or not with ITM) with a ncp BVDV2 strain. They failed to demonstrated differences in BRD-vaccinated calves with respect to ITM treatment. The authors (Bittar et al., 2018b) believed that under different conditions than those of their study (low infectious pressure and moderate virulence of virus strain given in a reduced stress setting) would be better for evaluation of the enhancement of vaccine protection that ITM can provide when used concurrently with vaccines as shown in previous studies (Arthington and Havenga, 2012; Teixeira et al., 2014; Palomares et al., 2016; Roberts et al., 2016; Bittar et al., 2018a).

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CHAPTER 3

EFFECTS OF INJECTABLE TRACE MINERALS ON THE IMMUNE RESPONSE TO *MANNHEIMIA HAEMOLYTICA* AND *PASTEURELLA MULTOCIDA* FOLLOWING VACCINATION OF DAIRY CALVES WITH A COMMERCIAL ATTENUATED-LIVE BACTERIN VACCINE[1]

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Abstract

The objective was to evaluate the effects of an injectable trace mineral (ITM) supplement containing Zn, Mn, Se, and Cu on the humoral and cell mediated immune responses to vaccine antigens in dairy calves receiving an attenuated-live bacterin vaccine containing *Mannheimia haemolytica* and *Pasteurella multocida*. Thirty 3-mo-old dairy calves received 2 doses (21 d apart) of an attenuated-live *M. haemolytica* and *P. multocida* bacterin vaccine (Once PMH, Merck Animal Health, Summit, NJ), and a 5-way modified-live-virus vaccine (Express 5, Boehringer Ingelheim, Vetmedica, St. Joseph, MO). On the day of primary vaccination, animals were randomly assigned to 1 of the 2 treatment groups (n = 15 per group): ITM (ITM administration) or control (sterile saline injection). Treatments were administered concurrently with vaccinations. Blood samples were collected for determination of antibody titers against *M. haemolytica* and *P. multocida* and of antigen-induced proliferation and interferon-γ secretion by peripheral blood mononuclear cells. Serum Se and Mn concentrations were greater ($P < 0.05$) in the ITM group than the control group after ITM use. Serum end-point antibody titers against both bacteria and interferon- γ secretion by peripheral blood mononuclear cells were not different ($P > 0.05$) between groups. The use of ITM with bovine respiratory disease vaccines enhanced ($P < 0.01$) antibody titer fold-change to *M. haemolytica*. Proliferation of peripheral blood mononuclear cells after *P. multocida* stimulation was increased ($P = 0.03$) in the ITM group on d 21 relative to baseline value. In conclusion, ITM administration concurrently with bacterin vaccination improved the immune response to *M. haemolytica* and *P. multocida* and

might be a valuable tool to enhance dairy calves' response to bovine respiratory disease vaccination.

Key words: trace minerals, dairy calves, bovine respiratory disease, attenuated-live bacterin vaccine, immune response

Introduction

Bovine respiratory disease complex (BRDC) is considered a major illness that affects North America's cattle industry resulting in substantial economic loses (>$1 billion/yr; Griffin, 1997; McVey, 2009). The complexity of BRDC can be attributed to several factors that potentiate its pathogenesis, including its poly-microbial etiology, stress, immune suppression, failure of passive transfer, weather extremes, or poor biosecurity. The infectious agents commonly involved in BRDC include bovine viral diarrhea virus (BVDV), bovine herpes virus-1, bovine respiratory syncytial virus (BRSV), parainfluenza 3 virus, *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis*. The typically commensal relationship of *M. haemolytica* and *P. multocida* with cattle makes prevention and control of BRDC more difficult (Filion et al., 1984; Confer, 2009). These 2 bacteria are considered normal flora of the cattle upper respiratory tract and on occasion, when animals are immunosuppressed (especially during stress), they colonize the bronchi and lungs causing mild to fatal illness (Dabo et al., 2007; Rice et al., 2007; Confer, 2009). The damage caused by these bacteria in the lower respiratory tract is usually due to the excessive influx of neutrophils and accumulation of fibrin in the lungs, which result in

acute respiratory disease and occasionally in death (Dabo et al., 2007; Rice et al., 2007; Confer, 2009).

Appropriate biosecurity measures and vaccination programs are crucial to prevent and control BRDC. The use of bacterins against *M. haemolytica* and *P. multocida* plays an important role in the prevention of BRDC by reducing the level of colonization and decreasing the likelihood of negative effects on cattle health and performance (Aubry et al., 2001; Larson and Step, 2012). Serum antibodies against bacterial leukotoxin and to specific bacterial surface antigens are considered core in the defense against these pathogens (Shewen and Wilkie, 1988; Crouch et al., 2012), and high concentration of *P. multocida* antibodies in serum at feedlot entry has been correlated with improved beef cattle performance (Fulton et al., 2002).

Trace minerals play an important role in adequate health, performance, and immune response to viral BRDC pathogens in dairy calves (Teixeira et al., 2014; Palomares et al., 2016). Some trace minerals, including Se, Zn, Cu, and Mn, are fundamental elements in the structure and function of several proteins that participate in general homeostatic processes essential for an adequate immune function. These include the pathways regulating energy production, DNA replication and transcription, and modulators of reactive oxygen species (ROS; Failla, 2003; Genther and Hansen, 2014). The use of injectable trace minerals (ITM) combined with vaccination demonstrated a positive effect in beef cattle by increasing serum neutralizing antibodies against bovine herpes virus-1 (Arthington and Havenga, 2012; Arthington et al., 2014), reducing morbidity and antibiotic treatment and costs, and increasing ADG in shipping-stressed calves (Richeson and Kegley, 2011). Additionally, in dairy calves with

adequate mineral status, administration of ITM has been proven to enhance health status by increasing neutrophil function, glutathione peroxidase activity (Teixeira et al., 2014), as well as serum neutralizing antibody titers and leukocyte proliferation against common bovine respiratory disease (BRD) pathogens (Palomares et al., 2016).

In a previous study, we reported that administration of ITM concurrent with a modified-live-virus (MLV) vaccine in dairy calves resulted in earlier and more robust antibody titers against BVDV1 and in more robust peripheral blood mononuclear cell (PBMC) proliferation after stimulation with BVDV1 and BRSV antigen than for the control group (Palomares et al., 2016). In the present study, we tested the hypothesis that administration of ITM can also improve the antibody and cell mediated immune responses to vaccine bacterial antigens in dairy calves receiving an attenuated-live bacterin vaccine containing *M. haemolytica* and *P. multocida*.

Materials and Methods

This trial is part of a larger study performed at the University of Georgia, where we investigated the effects of ITM on the immune response to viral antigens following MLV vaccination (Palomares et al., 2016). The calves were cared for in accordance with acceptable practices as for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010). The research protocol was approved by the University of Georgia Institutional Animal Care and Use Committee.

Experimental Design, Animals, and Treatments

A total of 30 weaned dairy bull calves (3.5 mo of age) received 2 mL of an attenuated-live *M. haemolytica* and *P. multocida* bacterin (Once PMH, Merck Animal Health, Summit, NJ) and 5 mL of a MLV vaccine containing bovine herpes virus-1, BVDV1 and 2, BRSV, and parainfluenza 3 virus (Express 5, Boehringer Ingelheim, Vetmedica, St. Joseph, MO). Both vaccines were given subcutaneously as recommended by the manufacturer. On the day of primary vaccination, calves were randomly assigned to 1 of 2 groups (15 calves per group) using a software (Research randomizer, V3.0, Social Psychology Network, Middletown, CT): (1) ITM, administration of injectable trace minerals (1 mL/45 kg of BW; Multimin 90, Multimin USA Inc., Fort Collins, CO) subcutaneously concurrently with vaccination, or (2) control, injection of sterile saline (1 mL/45 kg) subcutaneously at the time of vaccination. Three weeks after initial vaccination, calves received a booster of the same vaccines and a second dose of ITM or saline according to previous group assignment. Administration of ITM provided 15 mg/mL of Cu, 60 mg/mL of Zn, 5 mg/mL of Se, and 10 mg/mL of Mn. Calves were grazing in bermudagrass (*Cynodon dactylon*) and fescue (*Festuca arundinacea*) with ad libitum access to hay and water. A commercial cattle ration (Bulk Cattleman's Special; Godfrey's Warehouse, Madison, GA) containing energy, protein, minerals, and vitamins was offered twice daily as a supplement (around 2.7 kg/d per calf).

Sample Collection

Blood was collected from all the calves via jugular venipuncture into vacuum tubes (Vacutainer, BD Diagnosis, Franklin Lakes, NJ) with and without anticoagulant (acid citrate dextrose) to obtain whole blood and serum, respectively. Blood was collected on d −7, 0 (enrollment), 7, 14, 21, 28, 42, 56, and 90 relative to primary vaccine and ITM administration, for determination of serum trace mineral concentration, serum neutralizing antibody titers to *M. haemolytica* and *P. multocida*, antigen-induced PBMC proliferation, and interferon (**IFN**)-γ production upon stimulation with *P. multocida*. Additionally, trace mineral status was assessed in liver biopsy samples collected from each calf on d −7, 21, and 56 relative to the day of primary vaccination (d 0) as previously described (Palomares et al., 2016).

Blood Sample Processing and PBMC Preparation

Within 2 h after collection, blood samples were transported on ice to the laboratory and tubes without anticoagulant (Vacutainer, BD Diagnosis) were centrifuged (650 x *g* for 12 min at room temperature). The serum was collected and stored in microcentrifuge-tube (Fisherbrand Premium Microcentrifuge Tubes, Fisher Scientific, Waltham, MA) aliquots at –80°C until analysis for antibody titers against *M. haemolytica* and *P. multocida.* Mineral concentrations in liver and serum were determined at the Diagnostic Center for Population and Animal Health (Michigan State University, Lansing, MI). Blood samples with anticoagulant were used to obtain PBMC for antigen-induced proliferation and IFN-γ production assays. Peripheral

blood mononuclear cells were isolated and suspended as previously described (Palomares et al., 2016).

Serum Antibody Titers Against M. haemolytica and P. multocida

Serum IgG antibodies binding to *M. haemolytica* and *P. multocida* antigens were determined via an in-house ELISA at the research laboratory of the Department of Large Animal Medicine of the College of Veterinary Medicine, University of Georgia (building 11). Briefly, 96-well plates coated with *M. haemolytica* antigen (1:200 dilution in coat buffer of a clinical isolate from the University of Georgia Athens Diagnostic Laboratory, Athens, GA) were incubated at 4°C for 18 to 24 h and washed 3 times with wash buffer (phosphate buffered saline with 0.5% Tween-20 added) before use. Serial dilutions in wash buffer of each calves' serum and a 1:40 dilution of positive control serum and 1:100 dilution of negative control serum in wash buffer were plated in quadruplet wells and incubated at room temperature for 1 h. The plates were washed 3 times with wash buffer, and then 100 µL of a commercial anti-bovine IgG (heavy and light chain specific whole serum from rabbit) conjugated with horseradish peroxidase (1:1000 dilution in wash buffer; Sigma-Aldrich, St. Louis, MO) was added to each well. The plates were incubated for 30 min at room temperature and then washed 4 times. Next, 100 µL of ABTS (2,2′-azino-bis[3-ethylbenzothiazoline-6-sulphonic acid], Sigma-Aldrich) substrate solution containing saturating amounts of hydrogen peroxide was added to each well. The plates were incubated at room temperature in the dark for 15 to 60 min (until the positive control serum reached saturation color) to then be read at 405 nm wavelengths (GEN5 Epoch Microplate Spectrophotometer, BioTek

Instruments Inc., Highland Park, Winooski, VT), and mean optical density (**OD**) for each sample was recorded. A similar ELISA protocol was used for detection of *P. multocida* IgG antibody in the calf serum, except the plates were coated with *P. multocida* antigen at 1:500 dilution (using a clinical isolate from the University of Georgia Athens Diagnostic Laboratory, Athens, GA). The endpoint titers for both bacteria were determined by finding the highest dilution of serum that had a mean OD that was twice or greater than the mean OD of the negative control sample on each plate, and titer was expressed as the inverse of the dilution of the serum. Plates were considered valid if the positive control samples yielded an average OD of 0.8 to 1.1 and the negative control yielded an average OD of - 0.6 to 0.01.

PBMC Proliferation in Response to P. multocida

Procedures for PBMC proliferation were performed as previously described (Palomares et al., 2016). For PBMC proliferation assay, 100 µL of the PBMC suspension (6 x 10^6 cells/mL) from each calf was aliquoted into wells of a 96-well round bottom plate in triplicate. To each set of triplicate wells, 100 µL of *P. multocida* killed antigen suspension (prepared from a clinical isolate of the University of Georgia Athens Diagnostic Laboratory, Athens, GA, using 2.9 x 10^{10} CFU killed at 60°C for 1 h before freezing) and staphylococci enterotoxin SEB (Toxin Technology Inc., Sarasota, FL; 0.5 µg/mL as positive control), or Roswell Park Memorial Institute medium + 10% gamma-irradiated fetal calf serum + 2 m*M* of l-glutamine and 1% penicillin-streptomycin (negative control), were added. Results were expressed as the specific 3H-thymidine (7.4 x 10^3 Bq per well, stock 2.48 x 10^{11} Bq per m*M*, from MP

Inc., Indianapolis, IN) incorporation calculated as the mean counts per minute for PBMC cultured with *P. multocida*, or mitogen, divided by the mean counts per minute for PBMC cultured in cell culture media alone.

IFN-γ Secretion by PBMC in Response to P. multocida

A total of 600 µL of PBMC suspension (6 x 10^6 cells/mL) was aliquoted into each well of a 24-well flat bottom plate. To each set of triplicate wells, 600 µL of *P. multocida* killed antigen was added as previously described for other pathogens (Palomares et al., 2016). Secretion of IFN-γ by PBMC in response to stimulation with *P. multocida* was measured by ELISA using a commercially available antibody reagent pair and recombinant standard (R&D Systems, Minneapolis, MN) at dilutions optimized in our laboratory as previously described (Palomares et al., 2016). All PBMC supernatant samples designated for the IFN-γ assay were stored at -80°C until sample collection was completed, so that all the samples could be analyzed at the same time.

Statistical Analysis

Data were analyzed using SAS (SAS version 9.3; SAS Institute Inc., Cary, NC). Statistical assumptions of normality and constant variance were tested through Shapiro Wilk's and Levene's tests, respectively. A logarithmic base 2 transformation was applied to the antibody titer and stimulation index values. For calculation and comparison of geometric mean antibody titers, back-transformed antibody titers were calculated for each group at d 0, 7, 14, 21, 28, 42, 56, and 90 after vaccination.

Stimulation index triplicates for PBMC proliferation were Q-tested (n = 3, CI = 0.941) for outlier identification and elimination.

A 2-independent-sample t-test was performed to compare antibody titers, PBMC proliferation, IFN-γ secretion, and trace mineral concentrations between treatment groups for each day. The model was defined as follows:

$$y_i = \mu + \tau_i + \varepsilon_i,$$

where y_i is the respective response (antibody titers, IFN-γ secretion, or stimulation index) of subject j to treatment i (ITM or saline) at 0, 7, 14, 21, 28, 42, 56, and 90 d after treatment; μ is the mean; τ_i is the effect of treatment i (ITM or saline); and ε_i is the random effect error. Additionally, a repeated measure analysis was done to compare the antibody titers, IFN-γ secretion, and PBMC proliferation during the experimental period (7, 14, 21, 28, 42, 56, and 90 d) with the baseline on d 0. The mixed generalized linear model was defined by the following equation:

$$y_{ij} = \mu + \tau_i + \beta_j + \varepsilon_{ij},$$

where y_{ij} is the response (antibody titer, IFN-γ secretion or stimulation index) of subject j on day i for each treatment group; μ is the mean; τ_i is the effect of day i after ITM or saline treatment (d 7, 14, 21, 28, 42, 56, and 90), compared with the effect on d 0; β_j is a variable associated with subject j on d 0 (baseline); and ε_{ij} is the error. We assumed that days after treatment were fixed and that the calves used were a random sample of calves from a larger population of calves. Thus, the calves collectively represented a random effect, so we assumed that the mean of β_j was zero and that the variance of β_j was σ^2_β. Because the term β_j was common on all days after inoculation of the same calf, the covariance between y_{ij} and $y_{i'j}$ was not zero but

constant across all days and subjects. Nonparametric analysis was also applied using MannWhitney U and Friedman tests for comparisons between treatment groups and over time, respectively. For all analyses, values of $P \leq 0.05$ were considered significant, and $0.05 < P \leq 0.10$ was considered a tendency.

Results and discussion

Trace Mineral Concentration in Serum and Liver

Calves in both groups had similar liver concentrations of Se, Zn, Cu, and Mn on d −7 ($P > 0.05$; Palomares et al., 2016), which were within adequate reference values (Herdt and Hoff, 2011) during the experimental period, indicating that there were no Se, Zn, Cu, and Mn deficiencies in the animals used in this study. Administration of ITM resulted in increased concentrations of liver Se (on d 21 and 56; $P < 0.001$), Cu (on d 56; $P < 0.01$), and Mn (on d 56; $P < 0.05$) compared with saline injected calves (Palomares et al., 2016). In the present study, supplementation with injectable trace minerals in dairy calves was able to increase or mitigate the abrupt decay in hepatic concentrations of the studied trace minerals after vaccination compared with the control calves, which agrees with previous reports (Droke and Loerch, 1989; Arthington and Havenga, 2012; Teixeira et al., 2014). Serum Se, Cu, Zn, and Mn concentrations were also similar in both groups before vaccination and treatment administration (d −7; $P > 0.05$). Selenium concentration in serum increased dramatically between d 14 and 90 after primary vaccination and ITM administration ($P < 0.05$). These values were significantly greater than those observed in the control group during the same time points ($P < 0.05$; Figure 3.1A). Serum concentration of zinc, copper, and manganese

increased in both groups after primary and secondary vaccination ($P < 0.05$; Figure 3.1B, 3.1C, and 3.1D). Zinc concentration was numerally greater in the ITM calves on d 21 and 42 after vaccination compared with the saline-injected calves (Figure 3.1B) but without statistical significance ($P > 0.05$). There was no significant difference in serum copper levels between groups during the experimental period ($P > 0.05$; Figure 3.1C). The injectable trace mineral group had greater manganese concentration on d 21 compared with control group ($P < 0.05$; Figure 3.1D). Previous studies have shown an increase in serum copper and zinc concentrations in newly received beef heifers after ITM administration (Richeson and Kegley, 2011). It is possible that in the present study, the Zn and Cu levels did not show an accurate indication of mineral status; these concentrations might have been augmented due to physiological stress (Richeson and Kegley, 2011).

Antibody Titers Against M. haemolytica and P. multocida

The antibody response was evaluated through absolute values (end point antibody titers) and normalized values relative to the day of the primary vaccinations (fold change antibody titers). Group assignment was not stratified based on the levels of antibody titers against *P. multocida* and *M. haemolytica*. Therefore, there were numerical differences in antibody titers against *M. haemolytica* between groups before vaccination. Fold change antibody titer was calculated to normalize the antibody titers to the baseline values on d 0 in an attempt to better understand the effects of treatments on the dynamics of antibody production. A strong humoral immune response to *M. haemolytica* or *P. multocida* was not observed after the primary vaccination. However,

both the ITM and control group had a significantly increased antibody response (measured as end point antibody titer) to both antigens after secondary vaccination ($P < 0.01$; Figure 3.2A and 3.2C). In this study, the low antibody response (evaluated either as end point antibody titer or as fold change titer) observed after primary vaccination might be associated with the presence of passive (maternally derived antibodies) and active humoral immune responses interfering with vaccine stimulation (Brar et al., 1978; Aubry et al., 2001). The initial dose of vaccine apparently primed the immune system; therefore, the animals were able to respond more effectively to secondary vaccination, even in the presence of maternally derived antibodies (Aubry et al., 2001). Similar results were previously observed in another study using a similar *M. haemolytica* or *P. multocida* vaccine, in which antibody titers were the greatest 2 wk after the second dose of vaccine was administered (Srinand et al., 1996). Significant differences were not observed for the end point serum antibody titers against *M. haemolytica* between groups at any time point ($P > 0.05$; Figure 3.2A). The antibody titers to *M. haemolytica* changed differentially in both groups during the experimental period. In the control group, antibody titers decreased after primary vaccination and rose on d 28, 42, and 56, but a significant increase relative to the baseline levels was only observed on d 90 after primary vaccination ($P < 0.01$). In contrast, in the ITM group, the end point antibody titers significantly increased earlier and more consistently (from d 28 to 90) compared with the baseline titers on d 0 ($P < 0.01$). Accordingly, the ITM group had a greater fold change of antibody titers on d 14 ($P = 0.06$) and 56 ($P = 0.02$) compared with the control group (Figure 3.2B). Antibody titers against *P. multocida* increased similarly in both groups during the experimental period relative to

d 0 ($P < 0.01$; Figure 3.2C). However, there were no differences in fold change antibody titers against *P. multocida* between groups ($P > 0.05$) at any time point (Figure 3.2D). Although a slight increase was observed after the primary vaccination (approximately 1-fold change), the secondary vaccination induced a strong anamnestic immune response with almost 3-fold increase in titers in both treatment groups (Figure 3.2D). The poor antibody response after primary vaccination observed in this study reinforces the necessity of booster vaccination by 2 to 3 wk after primary vaccination to reach a significant humoral immunity to *P. multocida* and *M. haemolytica*, which is particularly important before commingling with other calves (Aubry et al., 2001; Fulton et al., 2002; Larson and Step, 2012). The presence of neutralizing antibodies against *P. multocida* is considered a major arm of defense that contributes to the prevention of BRD and has been positively correlated with enhanced performance at feedlot entry (Fulton et al., 2002). However, a previous study to evaluate the health and performance of young dairy calves vaccinated with a commercial *M. haemolytica* and *P. multocida* vaccine revealed that the vaccine given twice 2 wk apart increased antibody titers against *M. haemolytica* but did not improve growth rate or decrease the need for treatment of calfhood diseases (Aubry et al., 2001). Differences in the type of animals (beef vs. dairy), breeds, management, age, presence of maternally derived antibodies, and infectious pressure may have an influence on the magnitude of the response to vaccination and the expected animal health and performance. Therefore, a study to evaluate the performance of vaccinated calves in a scenario of greater natural disease challenge or submitted to *M. haemolytica* and *P. multocida* challenge is warranted.

Proliferation and IFN-γ Secretion by PBMC to P. multocida Stimulation

There was an augmented PBMC proliferation upon *P. multocida* stimulation only in the ITM group on d 21 after primary vaccination compared with d 0 ($P = 0.03$; Figure 3.3). Conversely, control calves did not show any significant change in PBMC proliferation to *P. multocida* over time when compared with d 0 ($P > 0.05$). The increased PBMC proliferation in the ITM group on d 21 to *P. multocida* stimulation might indicate that ITM enhanced the immune priming induced by the initial vaccine dose, so that PBMC in this group were more responsive to ex vivo bacterial antigen stimulation, suggesting a potential improvement in the anamnestic response after re-exposure to this pathogen. Significant differences were not found in the production of IFN-γ by PBMC upon stimulation with *P. multocida* between calves treated or not with ITM concurrently with bacterin administration at any time point (data not shown). The benefit of Se supplementation at the time of *M. haemolytica* vaccination has been previously documented (Droke and Loerch, 1989). Recent studies have revealed positive effects of ITM supplementation concurrent to BRD virus vaccination on humoral and cell mediated immune responses in beef (Arthington and Havenga, 2012; Roberts et al., 2015; Bittar et al., 2016a) and dairy calves (Bittar et al., 2016b; Palomares et al., 2016) with adequate trace mineral status. In our previous work using the same calves and experimental model, we demonstrated that administration of ITM concurrently with MLV vaccination resulted in earlier and increased antibody titer against BVDV1 ($P = 0.03$; on d 28 after vaccination) and a greater and more consistent PBMC proliferation upon BVDV1 ($P = 0.08$; on d 14 after vaccination) and BRSV stimulation ($P = 0.01$; on d 7 post vaccination) compared with the control group.

Because *P. multocida* and *M. haemolytica* are the bacteria most commonly isolated from the respiratory tract of calves suffering BRD (Filion et al., 1984; Confer, 2009), strategies to improve the immune response to *Mannheimia–Pasteurella* bacterin may have a positive effect and reduce BRD morbidity and antibiotic treatment costs. The increase in hepatic Se and Cu concentrations and serum Se concentration after ITM administration could have contributed to enhance the immune response via reduction of ROS produced during oxidative stress. Previous studies have shown negative effects of ROS on the immune cells (Li et al., 2017). Trace minerals such as Cu, Zn, and Se are structural components of enzymes involved in ROS neutralization (e.g., superoxide dismutase and glutathione peroxidase; Failla, 2003). Increased antioxidant activity related to ITM administration might have led to an augmented innate and subsequent adaptive immune responses as previously reported (Arthington and Havenga, 2012; Arthington et al., 2014; Teixeira et al., 2014; Palomares et al., 2016). That is enhancing antigen presentation, B-cell proliferation and differentiation, and successive antibody production against these specific bacteria. Nonetheless, in the present study we did not measure ROS or antioxidant enzyme levels and activity, which deserves further investigation.

Implications

Administration of ITM concurrently with vaccination in dairy calves resulted in increased fold change in antibody titer against *M. haemolytica* (between d 21 and 56 after primary vaccination; $P = 0.02$) compared with the control group and an augmented PBMC proliferation upon *P. multocida* stimulation on d 21 compared with d 0 ($P =$

0.03). Administration of ITM concomitant with vaccination appears to be a favorable strategy to enhance the immune response after vaccination in an attempt to prevent respiratory disease in the dairy cattle raising system.

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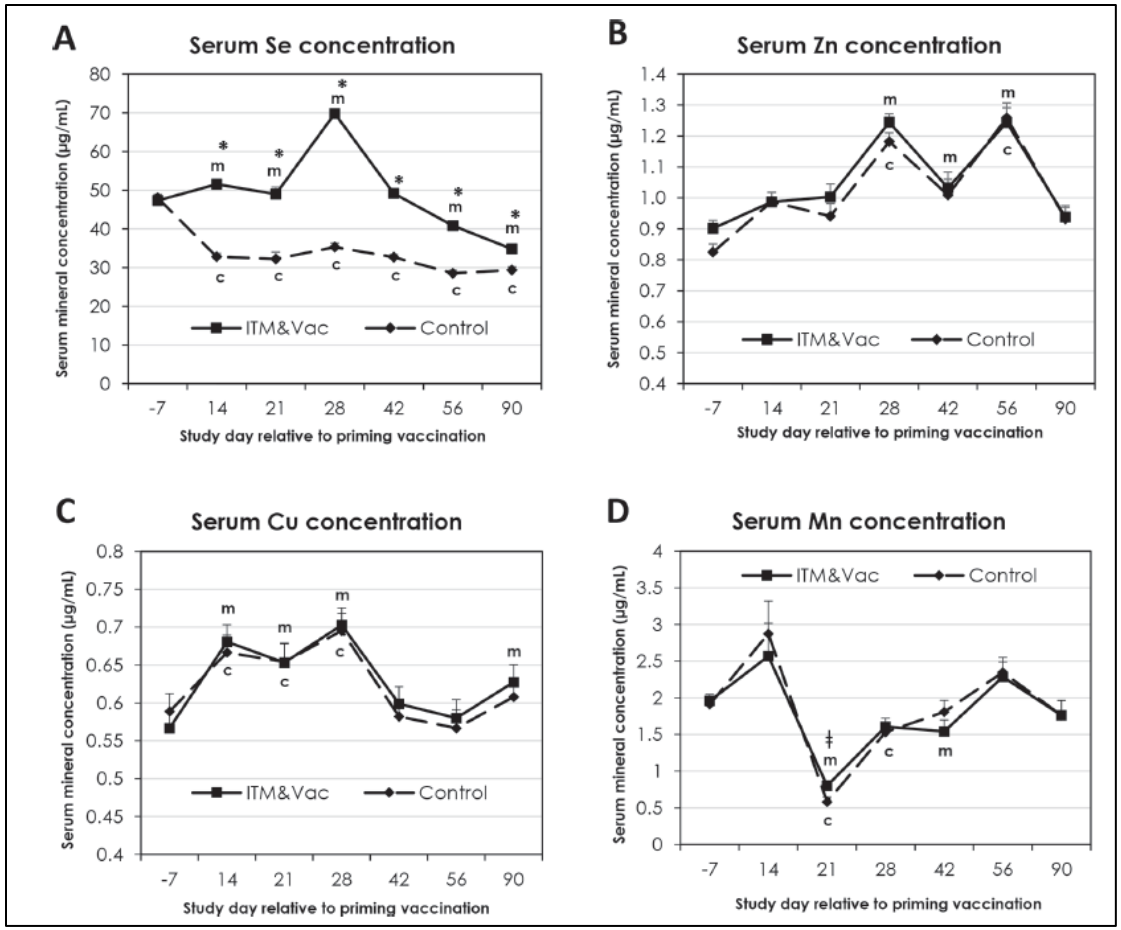

Figure 3.1A-D. Serum trace mineral concentrations for Se (A), Zn (B), Cu (C), and Mn (D) for dairy calves treated or not with injectable trace minerals (ITM) concurrently with attenuated-live bacterin vaccine (Vac). Errors bars represent the SEM. [c]Significant difference compared with d 0 for the control group ($P < 0.01$). [m]Significant difference compared

with d 0 for the ITM group ($P < 0.01$). *Significant difference between groups ($P < 0.001$). ‡Significant difference between groups on d 21 for serum Mn ($P = 0.03$).

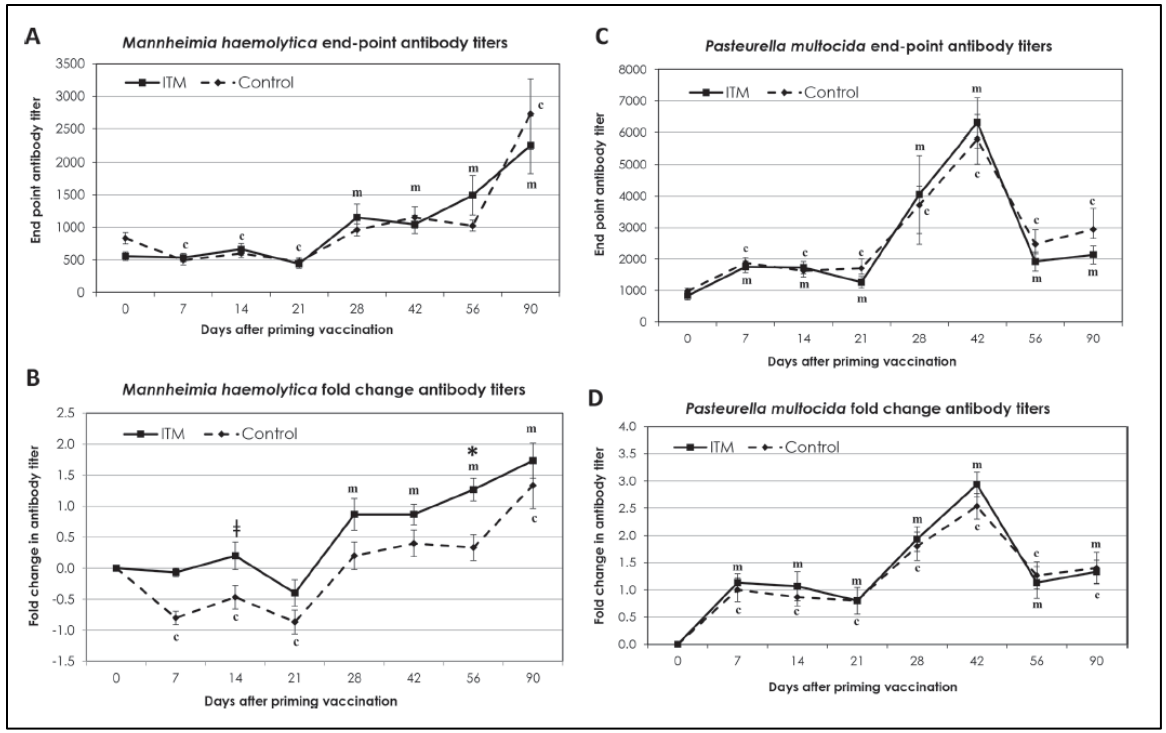

Figure 3.2A-D. End point (A and C) and fold change (B and D) antibody titer to *Mannheimia haemolytica* (A and B) and to *Pasteurella multocida* (C and D), respectively, in dairy calves treated or not with injectable trace minerals (ITM) concurrently with attenuated live bacterin vaccine. Errors bars represent the SEM. [c]Significant difference compared with d 0 for the control group ($P < 0.01$). [m]Significant difference compared with d 0 for the ITM group ($P < 0.01$). *Significant difference between groups on d 56 for *M. haemolytica* fold change antibody titers ($P = 0.02$). [‡]Groups tended to be different on d 14 for *M. haemolytica* fold change antibody titers ($P = 0.06$).

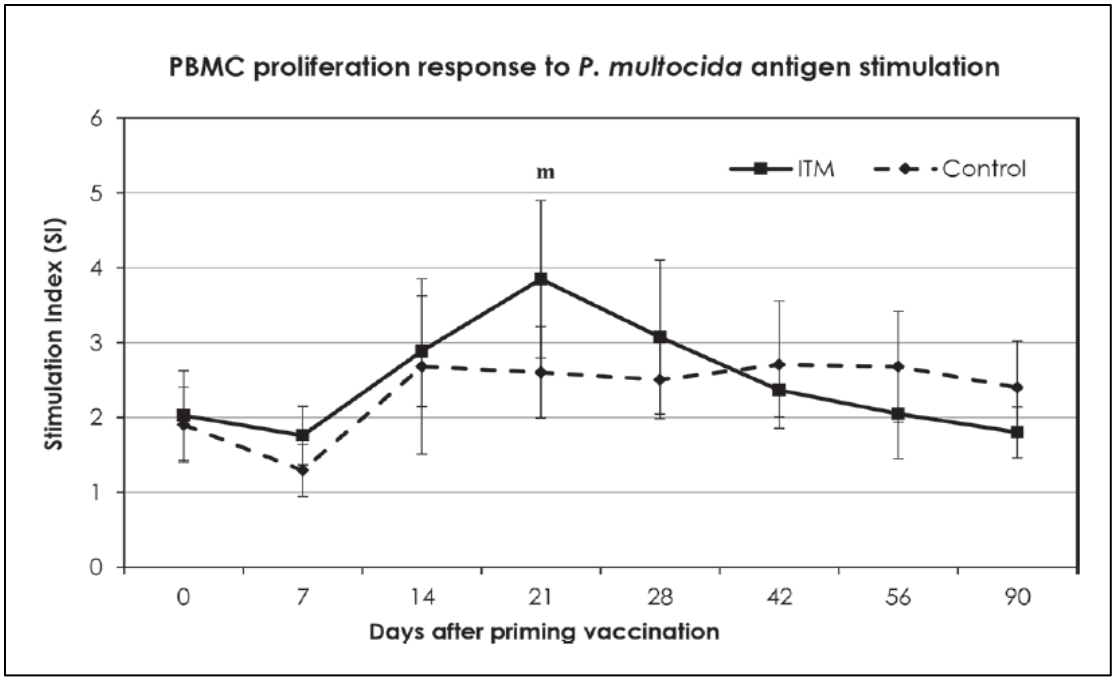

Figure 3.3. Peripheral blood mononuclear cell (PBMC) proliferation response to *Pasteurella multocida* in dairy calves treated or not with injectable trace minerals (ITM) concurrently with an attenuated-live bacterin vaccine. Errors bars represent the SEM. No significant difference existed between groups in any day ($P > 0.05$), and no difference from d 0 existed on any day for the control group ($P > 0.05$). [m]Significant difference compared with d 0 for the ITM group ($P = 0.03$).

CHAPTER 4

EFFECTS OF INJECTABLE TRACE MINERALS ADMINISTERED CONCURRENTLY WITH A MODIFIED LIVE VIRUS VACCINE ON LONG-TERM PROTECTION AGAINST BOVINE VIRAL DIARRHEA VIRUS ACUTE INFECTION IN DAIRY CALVES[2]

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Abstract

The objective was to evaluate the effects of injectable trace minerals (ITM) concurrent with modified-live virus (MLV) vaccination on protection from bovine viral diarrhea virus (BVDV) infection in dairy calves. In a previous study (Palomares et al., 2016), thirty dairy calves received two doses of a MLV vaccine subcutaneously (SC), concurrently with ITM (n=15) or saline (n=15), SC. Five months later, 20 of these calves received ITM (G1, n=10) or saline (G2, n=10) according to their previous groups and were challenged intranasally with BVDV2. Five unvaccinated calves were also challenged with BVDV2 (G3). Blood samples were collected on days 0 (BVDV challenge), 3, 5, 6, 7, 8, 9, 11, 14, 18, 21, 32 and 61 for leukocyte count, virus isolation and BVDV serum neutralizing antibodies (SNA). Mild-moderate clinical signs were observed in G3 after BVDV challenge. Group 1 showed lower sum health score and nasal score on d5 and fecal score on d8 compared to G2. Rectal temperature and leukocyte counts were not different between G1 and G2. In contrast, G3 calves had significant leukopenia and lymphopenia from d3 to d7 ($P < 0.05$) and higher rectal temperatures on d6 to d8, compared to values on d0 ($P < 0.05$). All unvaccinated calves became viremic, while viremia was not detected in G1 or G2. Average daily gain was not different between vaccinated groups, however, only G1 calves had significantly greater (P = 0.04) ADG compared to non-vaccinated calves during the first 14 days post challenge. Vaccinated calves treated or not with ITM were protected from BVDV2 infection five months post-vaccination.

Key words: Trace minerals, Dairy calves, BVDV, Modified-live virus vaccine, Protection

1. Introduction

Bovine respiratory disease complex (BRDC) is one of the most important health issues of young livestock in the United States (USDAAPHIS. Dairy Heifer Raiser, 2011). Moreover, BRDC was the second most common disease in pre-weaned dairy heifers and also a major illness affecting weaned dairy heifers. Prevalence of BRDC has been reported to be almost 6 times higher than all other common diseases affecting post-weaned heifers (digestive problems, lameness/injury and navel infection; USDA APHIS. Dairy Heifer Raiser, 2011). The pathogens most commonly involved in BRDC include bovine viral diarrhea virus (BVDV), bovine herpes virus 1 (BHV1), bovine respiratory syncytial virus (BRSV), parainfluenza 3 virus (PI3V), *Mannheimia haemolytica*, *Pasteurella multocida, Histophilus somni*, and *Mycoplasma bovis*. Bovine viral diarrhea virus is known to cause immunosuppression potentiating infection by other pathogens (e.g. *Pasteurella multocida* and *Mannheimia haemolytica*), which contributes to disease severity. Cattle persistently infected with BVDV are the main source of BVDV transmission in domestic cattle, and play an important role in the dissemination and pathogenesis of BVDV (Moennig and Becher, 2015). Intensive efforts have been made to reduce the incidence BVDV acute infections and to eliminate persistent infections among cattle populations. Vaccination is a powerful tool to prevent BVDV infections, and modified-live virus (MLV) vaccines have the benefit of stimulating both arms of the immune system (antibody production and cell mediated immunity) enhancing all the means for the control of virus replication, viremia and shedding (Rhodes et al., 1999). Nonetheless, even with the extensive use of both inactivated and live attenuated vaccines to manage BVDV, cattle

can succumb to disease (Fulton et al., 2005a; Kelling et al., 2005). Factors such as interference by transferred maternal antibodies in calves < 60 days of age (Ellis et al., 2001; Chamorro et al., 2015), the general immunological status of cattle, weather stress, antigenic mismatch between different vaccine and field viruses, vaccine handling, and level of pathogen exposure, have been documented to affect the immune response and protection after vaccination (Heininger et al., 2012). Nutritional deficiencies may also contribute to poor vaccine response in cattle (Rice et al., 1986; Thomas, 2009). Several studies have demonstrated the impact of trace minerals on cattle health and performance (Enjalbert et al., 2006; Galyean et al., 1999; Underwood and Suttle, 1999). Trace minerals such as zinc (Zn), manganese (Mn), copper (Cu), and selenium (Se) are essential for optimal immune function (Chirase et al., 1994; Percival, 1998; Underwood and Suttle, 1999), health status and growth in cattle (Spears and Kegley, 2002), particularly in highly stressed cattle, such as newly received feeder calves (Duffand Galyean, 2007). Multiple factors affect trace minerals levels in cattle, including high variability in requirements and mineral intake among cattle accordingly to their production stage, inadequate trace mineral levels in soil, forages, and feedstuffs, as well as the presence of mineral antagonists in feed, water, and forages, which limit their absorption. The use of injectable trace minerals (ITM) has advantages such as delivery of a known and controlled amount of mineral and rapid and efficient absorption and storage following treatment (Pogge et al., 2012). This might be relevant for cattle having reduced dry matter intake (i.e. during transportation and receiving periods, weaning and vaccination). Thus, the use of ITM reduces the variability in trace minerals levels observed in most cattle given free choice mineral intake (Arthington and

Swenson, 2004). Previous studies have shown positive effects of ITM administration on humoral and cell mediated immune response to BRDC vaccines in dairy (Palomares et al., 2016; Bittar et al., 2018) and beef cattle (Arthington and Havenga, 2012; Roberts et al., 2016; Bittar et al.,2016). We previously demonstrated that the use of ITM concurrently with a BRDC-MLV vaccine in dairy calves resulted in earlier and more robust antibody titers to BVDV1, and stronger mononuclear cell proliferation after stimulation with BVDV1 and BRSV antigen than the control calves (Palomares et al., 2016). Additionally, ITM enhanced antibody titers to *Mannheimia haemolytica* and proliferation of mono-nuclear cells after *Pasteurella multocida* stimulation (Bittar et al., 2018). Given the available data indicating that ITM can impact immune responses to vaccination, studies to determine the efficacy of the concomitant use of ITM with MLV vaccines on protection against pathogens involved in the BRDC are warranted. In the present study, we hypothesized that administration of ITM at the time of MLV vaccination and again 5 months later improves the immune response and long term protection against experimental BVDV2 acute infection in dairy bull calves. Therefore, our objective was to assess the effects of injectable trace minerals supplementation (containing Cu, Zn, Mn and Se) used concurrently with a BRDC-MLV vaccine, and again 5 months later, on the humoral immune response and protection from a non-cytopathic (ncp) BVDV2 experimental infection five months after priming vaccination in dairy bull calves

2. Materials and methods

2.1. Experimental design, vaccination, and treatments

The study was performed at the University of Georgia (UGA) Oconee Farm (Watkinsville, GA) from October 2014 through January2015. The research protocol was conducted as approved by the University of Georgia, Institutional Animal Care and Use Committee (UGA-IACUC# A201402-005-Y2-A5). This study was performed using 25 weaned intact Holstein bull calves (from the commercial farm BrooksCo Dairy, Quitman, GA) that were previously used in another study (Palomares et al., 2016). The experimental design of the previously reported study (Palomares et al., 2016) and the current trial are shown in Fig. 4.1. The animals averaged eight months of age at enrolment. During the study, calves grazed fescue grass (*Festuca arundinacea*) with free access to Bermuda grass hay (*Cynodon dactylon*), and water ad libitum. In addition, calves received daily supplementation (2.5 Kgs per calf) of a commercial ration (Cattleman's special beef; Godfreys Warehouse; Madison-GA) offered in two meals. No additional mineral supplementation was provided. In the previous study, an initial group of thirty calves (3.5 months of age) were administered 2 mL of a 5-way MLV vaccine containing BHV1, BVDV1 and 2, BRSV, PI3V (Express 5®, Boehringer Ingelheim, Vetmedica, St. Joseph, MO), and 2 mL of an attenuated-live *Mannheimia haemolytica* and *Pasteurella multocida* bacterin (Once PMH, Merck Animal Health, Summit, NJ) subcutaneously (SC). Theses calves were randomly assigned to one of two treatment groups as follows: (1) ITM (n = 15): subcutaneous administration of injectable trace minerals (1 mL/45 Kg BW; Multimin®90, Multimin USA Inc., Fort Collins, CO) or (2) Control (n = 15):

subcutaneous injection of sterile saline (1 mL/45Kg). Administration of ITM provided 15, 60, 10 and 5 mg/mL of Cu, Zn, Mn, and Se respectively. Three weeks after initial vaccination, calves received boosters with the same 5-way MLV vaccine, and the same attenuated-live bacterial vaccine by the SC route. Concurrent with the vaccine booster, a second subcutaneous administration of injectable trace minerals (1 mL/45 Kg; Multimin®90) or sterile saline (1 mL/45Kg) was given to calves in ITM and control group, respectively. Five calves did not receive vaccine or treatment (ITM or saline) and served as sentinel animals to verify that no field virus exposure occurred during the study. These calves were separated from the vaccinated calves for 14 days following priming and booster vaccination in order to prevent infection with shed vaccine virus. The overall schematic of the study activities is shown in Fig. 4.1. Five months after priming vaccination, ten calves belonging to either ITM and control groups were randomly selected from each group and received ITM (1 mL/45 Kg; Multimin® 90; G1, n = 10) or saline (1 mL/45 Kg; G2, n = 10) respectively, according to their previous group assignment. Additionally, the five additional herd-mate calves of the same age that did not receive vaccine or treatment and served as sentinel animals during the previous study, were used as unvaccinated BVDV infected group (G3, n = 5). The injectable trace minerals (Multimin®90) were administered on day −14 to the G1 calves following the manufacturer's recommendations (1 mL/45 Kg of body weight, SC). Animals in G2 received an equivalent volume (1 mL/45 Kg of body weight, SC) of a saline solution (Vetone Sterile Saline®; Nova-Tech Inc., Grand Island, NE) on day -14. All injections followed guidelines of the Beef Quality Assurance Program (Beef Quality Assurance 2010®; Centennial, CO).

2.2. Bovine viral diarrhea virus challenge

All 25 calves (10, 10 and 5 from groups G1, G2 and G3, respectively) were experimentally inoculated with an ncp type 2 BVDV isolate (strain 890). The BVDV 890 strain was originally obtained from the APHIS Center for Veterinary Biologics in 1989 (Ames, IA). It has been in frozen storage at -80 °C and is subjected to propagation in MDBK cells at least every other year to maintain viable stock. It has been produced in quantity to be available as a challenge BVDV virus. The stock BVDV2 strain 890 used in the present study was biologically cloned via successive passages by use of limiting dilutions with subsequent minimal propagation to produce an adequate amount of stock virus for characterization and animal challenge exposure studies during the six-month period prior to the challenge study. This strain has been previously characterized and causes a significant decrease in leukocyte counts (lymphocytes and neutrophils) with a mild to moderate development of classical clinical disease (Walz et al., 2001a, and b). The inoculum consisted of an infected cell culture supernatant containing 1×10^5 50% cell culture infectious dose ($CCID_{50}$) per mL of noncytopathic BVDV2 strain 890. The BVDV isolate was propagated in monolayers of Madin-Darby bovine kidney (MDBK) cells using Dulbecco's Modified eagle's medium (DMEM®; Cellgro, Manassas, VA) supplemented with 10% equine serum (HyClone™ Donor Equine Serum U.S.; Fischer Scientific; Pittsburgh, PA), 1% L-alanyl-L-glutamine (Corning® glutagro 100× Liquid™, 200 mM; Corning Cellgro; Manassas, VA), 0.2% anti-fungal (Amphotericin B Liquid 250µg/mL®;Corning; Manassas, VA) and 0.4% antibiotic (Penicillin 10,000 I.U.-Streptomycin 10,000µg/mL Solution 100×®; Corning; Manassas, VA). After incubation for 72 h at 37 °C and 5% CO_2, the BVDV2

infected cell culture was frozen at -80ºC and thawed to disrupt the cells and release the viral particles. Once thawed, the infected cell culture was aliquoted in 50 mL conical tubes and a 1.5 mL sample was collected for virus titration, and both were further frozen at -80 °C. The 1.5 mL sample was thawed and titrated using immuno-peroxidase staining of MDBK cell monolayers in 96-wells plates. The Reed–Muench method was used to determine the final $CCID_{50}$/mL for inoculation. The inocula remained frozen at -80 °C until the day of inoculation. One hour prior to inoculation, the frozen inocula were thawed. Then, 5 mL of the BVDV2 inoculum were aliquoted into individual 12 mL sterile syringes and held in ice until use. The inoculation was performed by intranasal aerosolization of 5 mL of inoculum (2.5 mL in each nostril) with 10 cm long tip-fenestrated cannula coupled to a 12 mL syringe for each calf. After inoculation all calves were comingled in an 8-acre pasture with adequate shade during the whole experimental period. One sample of the inoculum was transported on ice to the lab to determine the $CCID_{50}$/mL after all inoculations were completed.

2.3. Clinical evaluation

Health status including rectal temperature, hydration, attitude, nasal discharge and fecal consistency of the calves was evaluated using the scoring system developed at the University of Wisconsin (http://www.vetmed.wisc.edu/dms/fapm/fapmtools/8calf/calf_health_scoring_chart.pdf). Clinical examination was performed on each calf on study days −14, 0, 3, 5, 6, 7, 8, 9, 11, 14 and 18 relative to the day of BVDV challenge. Clinical signs were assessed by evaluation of six variables evaluated individually (hydration status, nasal secretion,

ears and attitude, feces, eyes, and cough) on a scale from 0 to 3, with 0 representing a lack of clinical signs and 3 representing severe clinical signs. The daily sum health scores were the total sum of each individual clinical sign variable, and the daily sum health score was generated by adding all individual scores of the six variables evaluated, to the extent that the minimum sum daily health score being equal to 0 (zero) and the maximum equal to 18 (i.e. if the score of all six variables that compose the daily sum health score were the maximum value, in this case a score of 3, then, 3 times 6 is equal 18, that is the maximum score obtained). Clinical signs and health scores were assessed by three experienced veterinarians, who were not aware of calves' treatment allocation. Additionally, body weight was measured on days 0, 8, 14, 21, and 32 relative to challenge (day 0).

2.4. Sample collection and processing

From the calves in each group, blood samples were collected via jugular venipuncture using an 18 gauge × 2.5-cm single sample needle (Vacuette®; Nipro Medical Industries Ltd., Gunma, Japan) into vacuum tubes (Vacutainer®, BD Diagnosis, Franklin Lakes, NJ) with and without anticoagulant to obtain whole blood and serum, respectively. Blood samples were collected on study days −14, 0, 3, 5, 6, 7, 8, 9, 11, 14 and 18 into two individual 8.5 mL glass tubes for buffy coat collection (BD Vacutainer ACD Solution A REF364606®; BD Diagnosis, Franklin Lakes, NJ), into one 2 mL polypropylene tube for leukocyte and platelet count (BD Vacutainer K2 EDTA 3.6 mg REF367841®; BD Diagnosis, Franklin Lakes, NJ) and into two individual 10 mL glass tubes (BD Vacutainer Serum®; BD Diagnosis, Franklin Lakes,

NJ) for serum mineral concentration. Additionally, blood samples without anticoagulant were collected on days -14, 0, 7, 14, 21, 32 and 61 for serum neutralizing antibody (SNA) titers after challenge. The blood samples containing ACD Solution A were processed for buffy coat isolation as previously described (Harpin et al., 1999) and stored at -80 °C for further VI. Clotted blood samples were spun (2500 rpm × 15 min) for serum separation. Later, serum samples were stored at -80 °C for further analysis. Nasal swab samples for each calf were collected for virus isolation (VI) on study days −14, 0, 3, 5, 6, 7, 8, 9, 14, and 18. An individual cotton swab (Sterile cotton tipped applicators®; Puritan Medical Products Company LLC; Guilford, ME) was inserted in each nostril scraping the nasal mucosa, and stored in a tube containing 3 mL of PBS containing antibiotics (Penicillin 10,000 I.U.-Streptomycin 10,000µg/mL Solution 100×®; Corning; Manassas, VA) and anti-fungal (Amphotericin B Liquid 250µg/mL®; Corning; Manassas, VA). Nasal samples were transported to the laboratory in a cooler containing ice for further processing. Tubes were mixed using a vortex. Swabs were removed and the remaining solution was filtered (Millex® GP 0.22µm; Millipore Ireland Ltd.; Cork, Ireland) and stored at -80 °C for later VI.

2.5. Serum neutralizing antibody titers

Serum neutralizing antibody titers against BVDV1 and 2 were determined via a standard virus neutralization test at the University of Georgia Athens Veterinary Diagnostic Laboratory (Athens, GA). Briefly, serum samples were thawed and heat inactivated at 56°C for 30 min. Heat-inactivated serum samples in duplicate were then diluted with DMEM into a serial 2-fold dilution series, starting at 1:2 in 96-well cell

culture plates. To each well, an equal volume (25µL) of DMEM 100 $CCID_{50}$ of the appropriate cytopathic BVDV1 and 2 was added. Addition of virus took the final starting dilution of serum to 1:4. The plates were incubated in 5% CO_2 at 37°C for 1 h. Then 150µL (approximately 10^4 cells) of a MDBK cell suspension in DMEM containing 10% fetal calf serum (FCS) was added to each well. The plates were incubated in 5% CO_2 at 37°C for 4 days. An inverted microscope was used to examine the cell monolayer in each well for virus-specific cytopathic effects. The SN titer for each sample was reported as the highest dilution of serum that completely inhibited virus-induced cytopathic effects in both wells.

2.6. Virus isolation

For virus isolation (VI), 250µL of buffy coat cell suspension and nasal swab samples from each animal was added to individual 25-cm^2 tissue culture flasks containing a monolayer of MDBK cells. For cell culture, DMEM medium supplemented with 10% equine serum, L-alanyl-L-glutamine (Corning® glutagro 100× Liquid™, 200 mM; Corning Cellgro; Manassas, VA) was used. After 3 days of incubation in 5% CO_2 at 37°C, flasks were frozen at -80°C and thawed; 50µL of the cell suspension from each flask was then transferred into 3 wells of a 96-well plate seeded with MDBK cells (first plate). The inoculated 96-well plate was incubated for 3 days, and the culture medium from each well was transferred to the corresponding wells of a new 96-well plate seeded with MDBK cells (second plate). Both 96-well plates (first and second) were tested for BVDV antigen by use of an immunoperoxidase staining technique as previously described (Palomares et al., 2012).

2.7. Leukocytes and platelets count

Blood samples containing EDTA were transported in a cooler containing ice and analyzed for leukocyte and platelet count in the Pathology Department of the University of Georgia (UGA), College of Veterinary Medicine (CVM) in Athens-GA. The total leukocyte, platelet and differential leukocyte counts for each sample were determined by use of an automatic cell counter (HESKA® CBC-Diff, Vet Hematology System, Des Moines, IA).

2.8. Statistical analysis

Data were analyzed using the Statistical Analysis System (SAS® version 9.3; SAS Institute, Cary, NC, USA). Statistical assumptions of normality and constant variance were tested through Shapiro Wilk's and Levene's tests, respectively. A logarithmic base 2 transformation was applied to the antibody titers. For calculation and comparison of geometric mean (GM) antibody titers, back-transformed antibody titers were calculated for each group at days −14, 0, 7, 14, 21, 32 32 and 61after BVDV infection. The changes in mean leukocyte and platelet counts and rectal temperature over time were determined comparing the values on day 0 with the values on days 3, 5, 6, 7, 8, 9, 11, 14 and 18 using repeated-measures analysis Proc-GLIMMIX model using calf as random effect, and groups and time points as fixed effects, with the Tukey test used to adjust for multiple comparisons. Means daily sum health score, rectal temperature, body weight, average daily gain, leukocyte counts, and SNA titers were compared among the treatment groups by using repeated-measures analysis Proc-GLIMMIX model using calf as random effect, and group and time as fixed effects, with

the Tukey test used to adjust for multiple comparisons. For variables in which the groups did not have similar means at day 0, we used the first day measurement of each variable as covariate in the statistical model, to minimize the differences between groups at the beginning of the study, therefore allowing for a more accurate comparison among groups. For all analyses, values of $P \leq .05$ were considered significant, and $0.05 < P \leq .10$ was considered a tendency. All results are shown as least squared means (LSM) obtained from the statistical software SAS after analysis.

3. Results

Before BVDV challenge (d0), all groups had comparable mean rectal temperature, sum of health scores, mean body weight, total and differential leukocytes count, and platelet counts (P> .05). Mean rectal temperature significantly increased in the G3 group after BVDV challenge compared to day 0, and peaked on day 7 with values > 40°C (P= .001; Fig. 4.2). Calves in G3 had higher (P< .05) rectal temperature than G1 and G2 between days 3 and 11 after BVDV challenge (Fig. 4.2). Rectal temperature did not increase in the vaccinated calves (G1 and G2) during the experimental period, with no significant differences between groups (P= .71). Sum of health scores was higher (P = .001) in G3 compared to vaccinated calves on days 7, 8, 9, 11, 12,15 and 18 post BVDV challenge (Fig. 4.3). Calves treated with ITM had lower sum of health scores on day 5 (P= .005. Fig. 4.3), and lower nasal (P= .01) and fecal scores (P = .005) on days 5 and 8, respectively, compared to saline injected calves (data not shown). There was no significant difference regarding mean body weight among groups at any day of the study (P> .05; Fig. 4.4A). Average daily gain (ADG) during

the first seven days post challenge tended to be greater (P= .09) in G1than G3 (Fig. 4.4B). Moreover, ADG within the first 14 days after BVDV challenge was significantly greater (P= .04) in G1 compared to G3 (Fig. 4.4B). During this period, G2 had intermediate ADG values, which were not significantly different from either G1 or G3 (Fig. 4.4B). Mean total leukocytes counts significantly decreased (by approximately 40%) in the G3 calves between days 3 and 6 post challenge relative to day 0 (P< .01). These values were significantly lower than those in G1 and G2 groups (Fig. 4.5A). Vaccinated calves (G1 and G2) had similar mean leukocyte count along the study. Moreover, lymphocyte count decreased significantly in G3 calves (almost two fold) between days 3 and 7 post challenge compared to day 0 (Fig. 4.5B). Calves in G1 and G2 had a mild decrease in lymphocyte count on days 3 and 5 after BVDV challenge, with no significant difference between groups (P> .05; Fig. 4.5.B). Unvaccinated calves had a significant increase in mean granulocyte count on days 7 and 8 post challenge (P< .05; Fig. 4.5C) which was different from values observed in G1 and G2 on the same time points. A remarkable reduction in platelet counts (19–35%) was observed in G3 (between days 3 and 9) relative to the baseline levels (Figs. 4.5D). Further, there was a moderate reduction in platelet counts during the evaluation period in G1 (16–19%, on days 7, 8 and 14) and G2 (17–28% on days 6, 11, 14 and 18). Calves in G2 had significantly lower platelet counts on days 6 (P= .05) and 14 (P= .04) than calves in G1. Moreover, platelet counts also tended to be lower on days 11 (P= .10) and 18 (P= .07) in G2 calves compared to G1.Serum neutralizing-antibodies against BVDV1 were high (approximately 1:1028) on days −14 and 0 in G1 and G2 groups and did not increase dramatically after BVDV2 challenge (Fig. 4.6A). In contrast, SNA titers

against BVDV2 were moderate (approximately 1:64) on days −14 and 0 and increased significantly (almost 5-fold) by 2 weeks post BVDV2 challenge (Fig. 4.6B). There were no significant differences in SNA titers to BVDV1 and 2 between G1 and G2 throughout the experimental period (P> .05). Calves in G3 were seronegative (SNA < 1:4) for BVDV1 and 2 on days −14 and 0. In this group, antibody titers against BVDV1 and 2 progressively increased reaching seroconversion (> 4-fold increase in SNA titers) on days 32 and 61 post challenge (Figs. 4.6A and B).Bovine viral diarrhea virus was not isolated from buffy coat or nasal swab samples of any of the vaccinated calves (G1 and G2) during the experimental period. Unvaccinated calves (G3) had BVDV positive buffy coat samples between days 5 to 9 after BVDV challenge. All animals in G3 were BVDV positive on buffy coat sample at least one day during the experimental period. Moreover, BVDV was isolated from leukocytes in all five calves of G3 on day 6. Interestingly, BVDV was not detected in nasal swab samples in any of the control calves during the sampling period.

4. Discussion

The present study tested the hypothesis that administration of ITM concurrently with MLV vaccination and again 5 months later enhances the immune response and long-term protection against experimental challenge with BVDV2 in weaned dairy calves. Vaccinated calves, whether treated or not with ITM were protected from acute BVDV clinical disease, demonstrated by the absence of fever and lower clinical scores when compared to the naïve unvaccinated calves. The immunization protocol based on priming and booster vaccination administered 21 days apart demonstrated efficacy in

long-term protection (5 months after vaccination) preventing acute disease caused by BVDV challenge, reinforcing the importance of the MLV vaccines as a preventative strategy for BRDC in calves, as previously reported (Rhodes et al., 1999; Xue et al., 2010; Walz et al., 2010; Chamorro et al., 2015). Calves in the unvaccinated control group developed mild to moderate BRDC. This was characterized by showing higher daily sum health score and rectal temperature compared to vaccinated calves. Clinically remarkable differences regarding the health status after BVDV challenge were not observed between vaccinated groups. However, calves treated with ITM had lower sum of health scores on day 5, and lower nasal and fecal scores on days 5 and 8 respectively (data not shown), compared to saline-injected calves. Average daily gain was not different in calves treated with ITM from saline-treated calves. Nevertheless, only calves treated with ITM had a significantly higher ADG compared to the non-vaccinated calves within the first 2 weeks after challenge. Previous studies under field conditions without experimental challenge have shown beneficial effects of ITM on growth performance and health in highly stressed, newly received beef heifers (Richeson and Kegley, 2011). In that study, the overall ADG and total dry matter intake were greater for beef heifers receiving ITM formulations compared with control animals. Additionally, animals administered ITM had reduced BRD morbidity rates and antibiotic treatment costs compared with control heifers (Richeson and Kegley, 2011). Vaccination, whether concurrent with ITM or not protected the calves from significant BVDV-induced leukopenia and lymphopenia, a diagnostic result clearly observed in the control group. Leukopenia and lymphopenia are the main hallmarks of systemic disease occurring during BVDV infection (Ellis et al., 1998) and are believed

to result in significant immunosuppression that predisposes cattle to suffer secondary infections by commensal flora and the development of bovine respiratory disease (Walz et al., 2001a; Chase et al., 2004; Palomares et al., 2012). Unvaccinated calves had a remarkable decrease in lymphocyte counts (> 45% reduction for 7 days), while a very slight and transient reduction in lymphocyte numbers was observed after BVDV challenge in the vaccinated calves (21 and 17% reduction for G1 and G2, respectively), which agrees with previous studies (Ellis et al., 1998; Falcone et al., 2003; Chamorro et al., 2015). Interestingly, calves treated with ITM had greater platelet counts than vaccinated saline-treated calves between days 6 and 18 post BVDV challenge. A recent study to determine the effect of ITM on the onset of protection elicited by a MLV vaccine in beef calves inoculated with BVDV2 five days after vaccination, showed that the use of ITM concurrent with MLV vaccine prevented a remarkable thrombocytopenia, typical of acute infection with BVDV2 strain 890 (Bittar et al., 2016). In the current study, MLV vaccination (two doses of vaccine 21 days apart), whether in conjunction with ITM or not, prevented the viremia observed in the control calves during the week after BVDV inoculation. Viremia and viral shedding occur during the acute phase of BVDV infection, being the time when infected cattle are at higher risk of transmitting virus to herd mates (Saliki and Dubovi, 2004). Multiple vaccination challenge studies have demonstrated that most of the MLV vaccines against bovine respiratory viruses commercially available in the United States provide adequate protection from acute BVDV disease (Falcone et al., 2003; Brock, 2004; Kelling et al., 2005, 2007; Palomares et al., 2012; Chamorro et al., 2015). Further, the protective response elicited by MLV vaccination in the present study, preventing

clinical signs of disease, leukopenia and viremia upon BVDV challenge is consistent with previous reports of the efficacy of MLV-BRD vaccines on long-term protection (Goyal and Ridpath, 2005; Dean and Leyh, 1999). In the present study, the use of ITM administered at the time of vaccination and 5 months later did not enhance the level of protection from clinical disease elicited by MLV vaccination. Average daily gain was not significantly different between vaccinated groups; however, only calves treated with ITM had significantly greater ADG than non-vaccinated BVDV acutely infected calves, which could suggest an additive effect of ITM use concurrently with MLV vaccination. In a previous trial, Roberts et al. (2016) showed no effect of ITM in improving performance or morbidity when the BRD incidence was low. However, the BVDV-specific antibody response to a respiratory vaccine was greater for ITM on day 14 than for the control group, as demonstrated in our earlier study (Palomares et al., 2016). It is possible that the moderate virulence BVDV strain used for challenge, the low level of stress, and the low level of infectious pressure during this study may have favored the protection from clinical disease elicited by MLV vaccination in G1 and G2, thus masking further benefits of ITM administration on animal health and performance, that have been previously described in high-risk feedlot animals (Richeson and Kegley, 2011). In addition, in our previous study, the calves treated with ITM at the time of vaccination against BRDC pathogens had earlier and greater antibody response and mononuclear cell proliferation to BVDV than vaccinated only calves (Palomares et al., 2016). This improved antibody response has been previously correlated to enhanced protection (Richeson and Kegley, 2011). Additional studies to determine the effects of ITM on clinical protection elicited by MLV vaccines against BVDV infection in high

risk stressed calves are warranted. Shedding of BVDV in nasal secretions was not consistently detected after challenge, in contrast with previous studies (Ellis et al., 1998; Falcone et al., 2003; Fulton et al., 2005a, b; Palomares et al., 2012). It can be speculated that several factors including sampling technique, viral load, replication and shedding capacity of the BVDV strain and the laboratory techniques used for viral isolation may have affected our ability to detect BVDV in nasal secretions. The increase in serum neutralizing (SNA) antibodies specific for BVDV1 and 2 in both vaccinated groups indicated an anamnestic immune response after vaccination and subsequent experimental exposure. In the vaccinated calves, SNA titers against BVDV were high at the time of challenge, with higher SNA titers for BVDV1 than BVDV2. This is believed to have been induced by the homology between the BVDV1a vaccine strain used in this study and the BVDV isolate used in the SNA assay at the diagnostic laboratory (Goyal and Ridpath, 2005). The unvaccinated calves were seronegative at the time challenge, but the BVDV SNA titers increased significantly within 30 days after inoculation, with higher SNA titers to BVDV2 than BVDV1, which might be due to the exposure only to the homologous type 2 BVDV strain 890 during challenge. Further, the increase in SNA titers to BVDV1 in the unvaccinated calves reflected sufficient neutralizing cross-reactivity between BVDV genotypes (Dean and Leyh, 1999; Fairbanks et al., 2003). The absence or low SNA titers against BVDV in the unvaccinated control group within the first 14 days after challenge resulted in a greater susceptibility to BVDV infection and clinical disease, as previously reported in several studies (Falcone et al., 2003; Kelling et al., 2005; Chamorro et al., 2015). Several favorable features are associated with an adequate trace minerals level in calves, and

studies have shown benefits of ITM when combined with vaccination (Arthington and Havenga, 2012; Palomares et al., 2016; Bittar et al., 2016, 2018). Injectable trace minerals have been shown to enhance humoral immune response when injected concomitantly with multivalent vaccines in naïve dairy and beef calves (Arthington and Havenga, 2012; Richeson and Kegley, 2011; Palomares et al., 2016; Bittar et al., 2016, 2018). The group of animals used in this study are a subset of calves utilized in a previous study (Palomares et al., 2016) in which TM concentrations in liver and serum were demonstrated to be within normal ranges throughout the trial. Furthermore, administration of ITM was proven to result in a significant increase in hepatic Se, Cu and Mn levels as well as serum Se and Zn. To the authors' knowledge, this is the first study evaluating the effects of the use of ITM concurrently with BRDC vaccines on protection against BVDV challenge in weaned calves. While we previously showed that ITM improved some aspects of humoral and cell mediated immunity (Palomares et al., 2016; Bittar et al., 2016, 2018), in this study there were few effects attributable to ITM after BVDV2 challenge (lower sum of health scores on day 5, higher platelet counts on days 6, 11, 14 and 18 compared to saline treated calves, as well as higher ADG com-pared to non-vaccinated calves). The low correlation between these studies may have been due to the low infectious pressure, mild virulence of the BVDV2 strain used and low stress conditions to which the calves were submitted. In conclusion, the use of ITM administered at the time of vaccination against respiratory viruses and five months later did not enhance the level of protection from BVDV2 clinical disease elicited by MLV vaccination in dairy bull calves. Further studies are necessary to

effectively assess the effects of ITM on long-term clinical protection elicited by MLV vaccines against BVDV infection in high risk growing calves.

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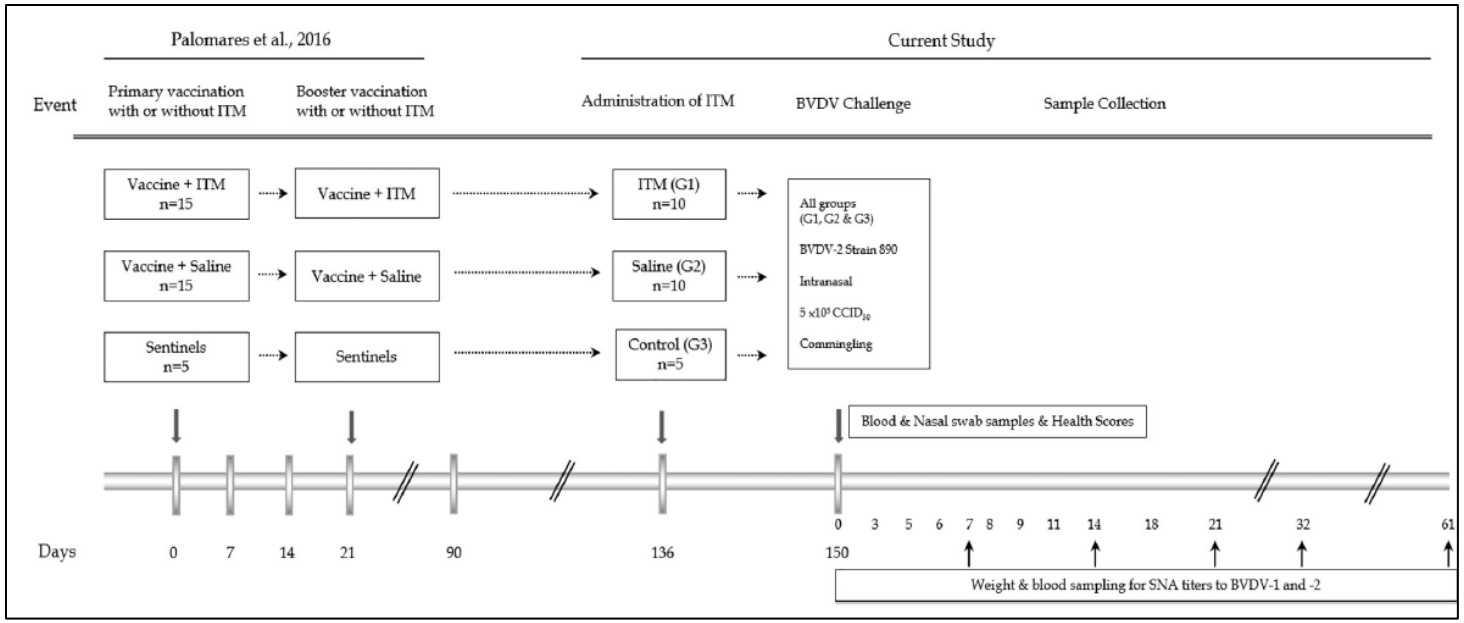


Fig. 4.1. Experimental design of the previous study (Palomares et al., 2016) administering injectable trace minerals (ITM) or saline concurrent with BRDC-MLV vaccination in dairy calves (at 3.5 months of age and 21 days later); and the current study administering another dose of ITM or saline 14 days before BVDV-2 challenge (approximately at 8 months of age).

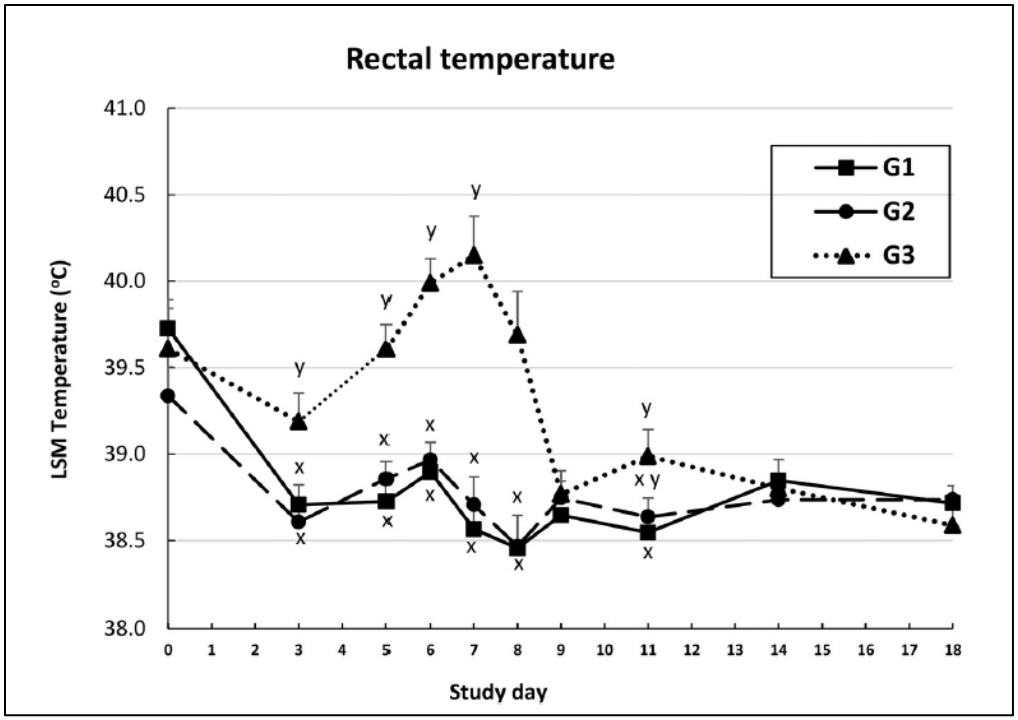


Fig. 4.2. Least square means (LSM) and standard error of the mean (SEM) of rectal temperature in dairy calves treated with ITM concurrent with BRDC-MLV vaccines and challenged with ncp BVDV-2890 strain 5 months later. [x,y] Significant difference between groups for each study day ($P < .05$).

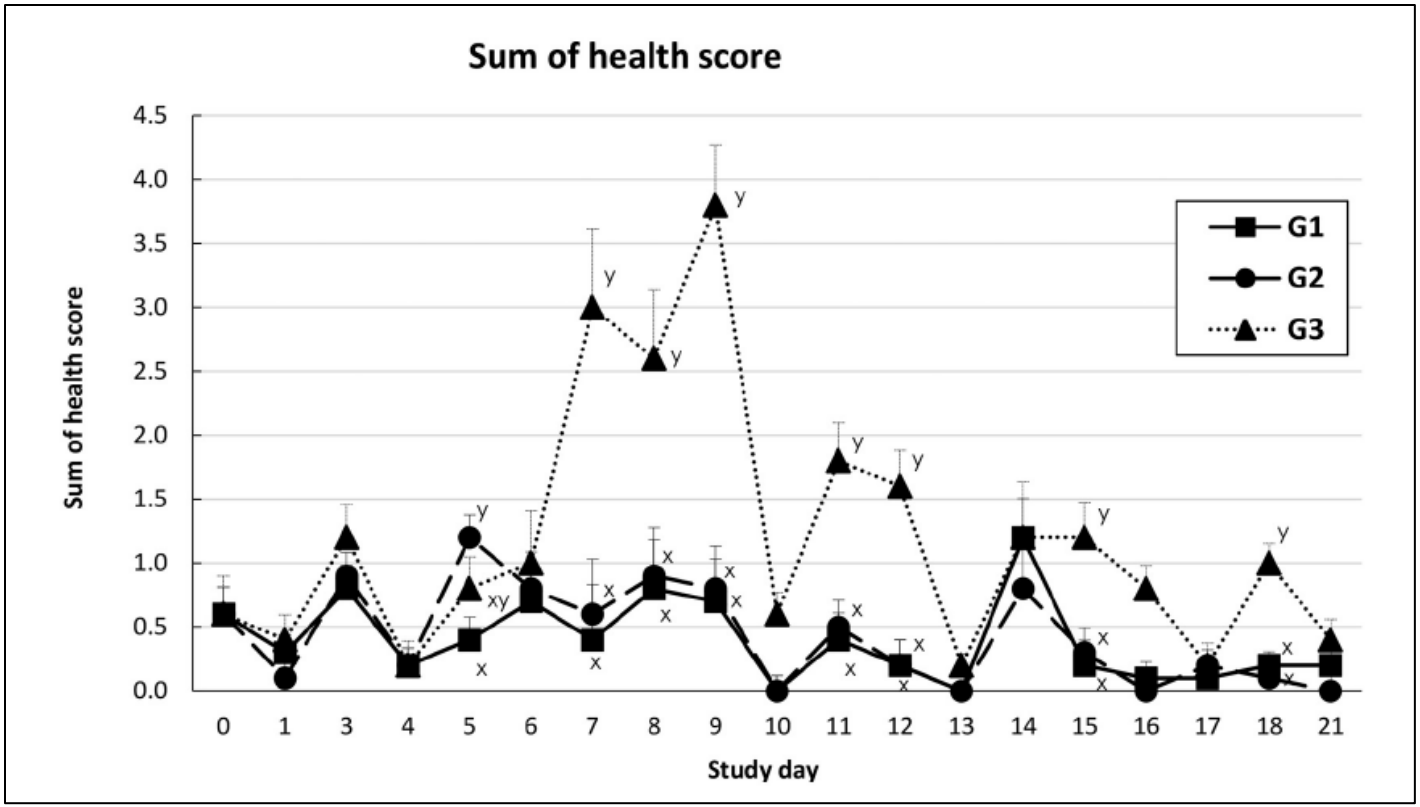
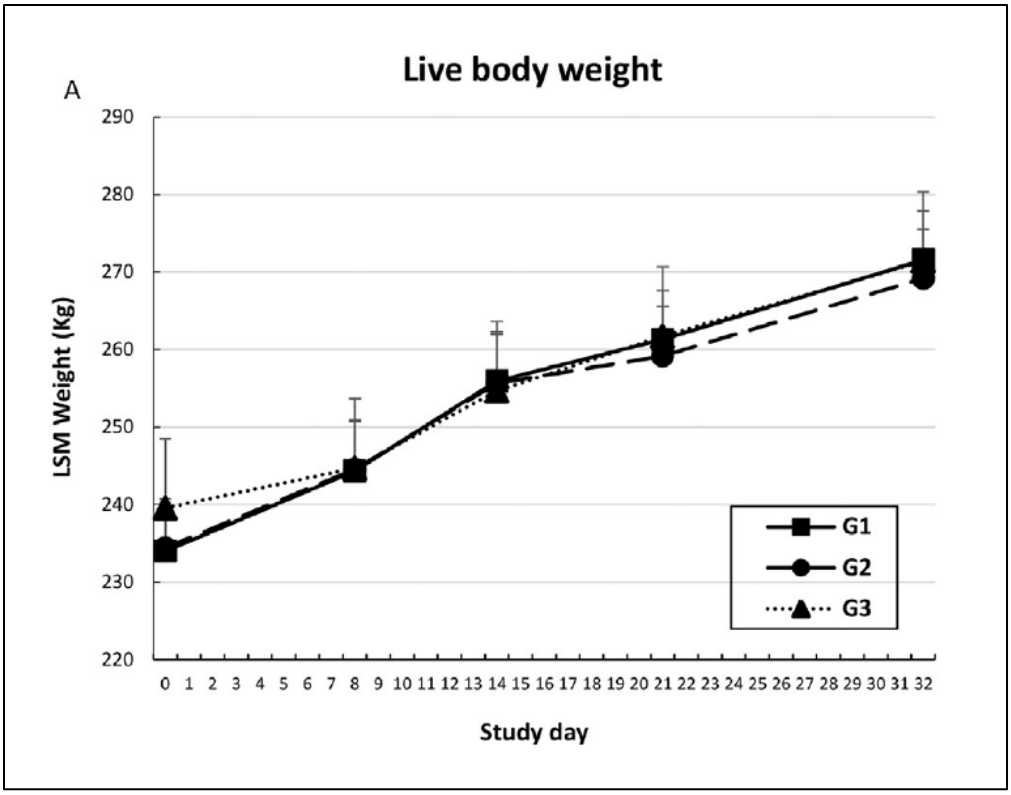


Fig. 4.3. Least square means (LSM) and standard error of the mean (SEM) of sum of health scores in dairy calves previously treated with ITM or saline concurrent with BRDC-MLV vaccines (Palomares et al., 2016) and challenged with ncp BVDV-890 strain 5 months later. ^{x,y} Significant difference between groups for each study day ($P < .05$).



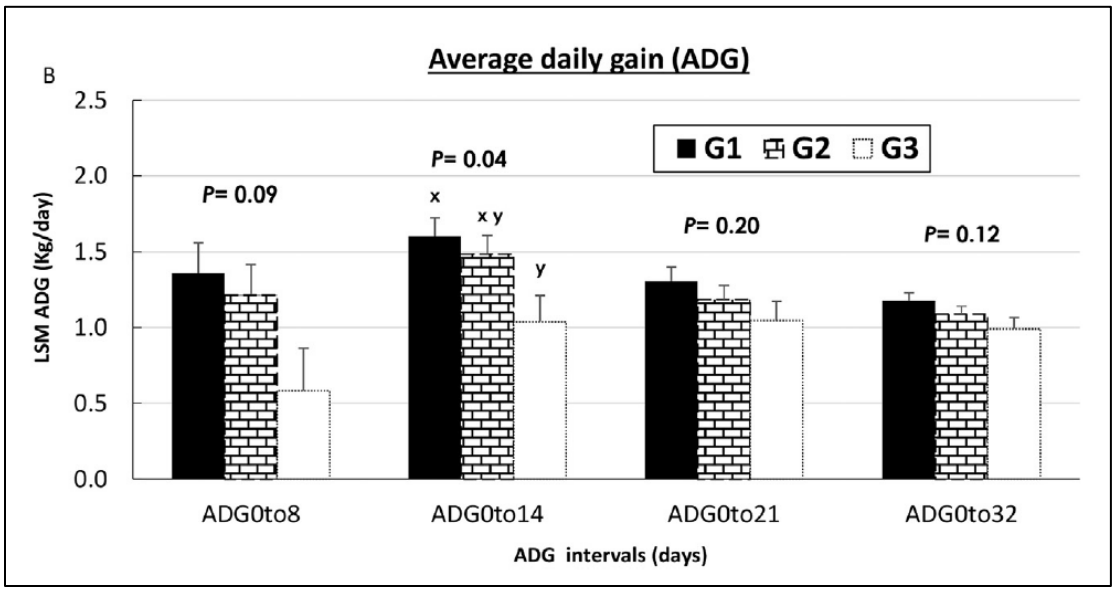


Fig. 4.4A-B. Least square means (LSM) and standard error of the mean (SEM) of live body weight (A) and average daily gain (ADG, B) in dairy calves previously treated with ITM concurrent with BRDC-MLV vaccines (Palomares et al., 2016) and challenged with ncp BVDV-2890 strain 5 months later. Average daily gain was

calculated for different study days (8, 14, 21, and 32) relative to baseline weight values on day 0. [x,y] Significant differences between groups.

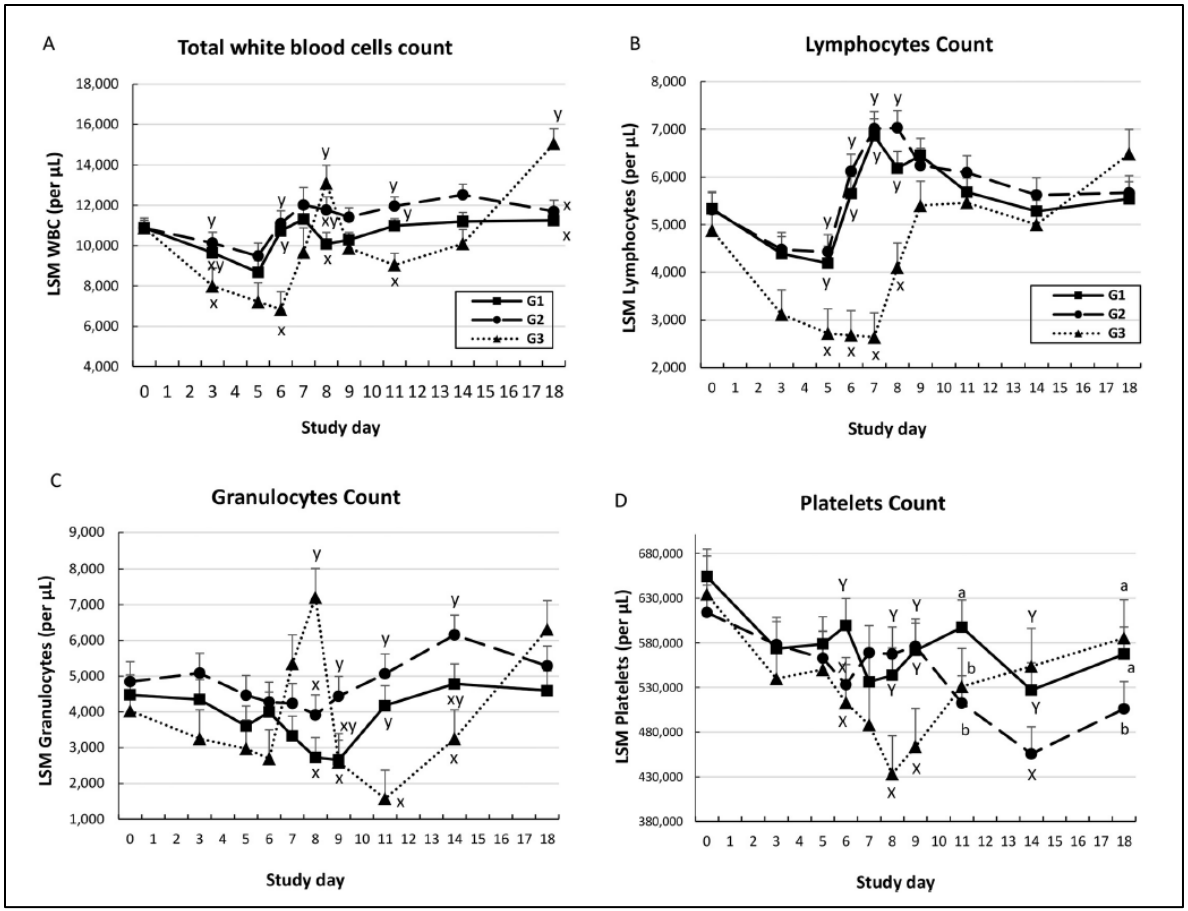


Fig. 4.5A-D. Least square means (LSM) and standard error of the mean (SEM) of total white blood cells (A), lymphocyte (B), granulocyte (C), and platelet (D) counts in dairy calves previously treated with ITM concurrent with BRDC-MLV vaccines (Palomares

et al., 2016) and challenged with ncp BVDV-2890 strain 5 months later. [x,y] Significant difference between groups by each study day ($P \leq .05$). [a,b] Values tended to be different between groups by each study day ($0.05 < P \leq .10$).

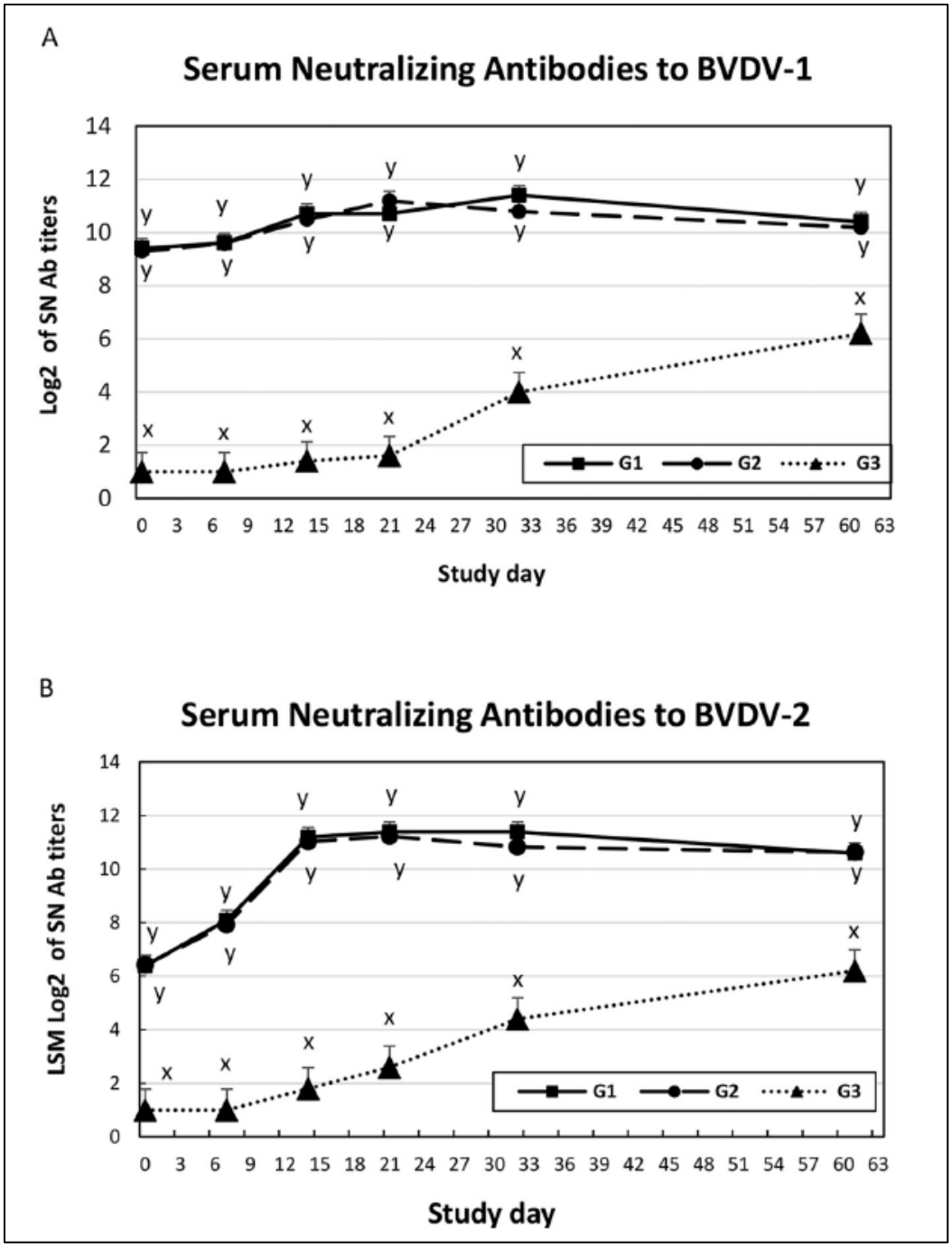


Fig. 4.6A-B. Least square means (LSM) and standard error of the mean (SEM) of serum neutralizing antibody titers to BVDV-1 (A) and to BVDV-2 (B) in dairy calves previously treated with ITM concurrent with BRDC-MLV vaccines (Palomares et al.,

2016) and challenged with ncp BVDV-2890 strain 5 months later. [x,y] Significant difference between groups for each study day ($P < .001$).

CHAPTER 5

IMMUNE RESPONSE AND ONSET OF PROTECTION FROM *BOVINE VIRAL DIARRHEA VIRUS* 2 INFECTION INDUCED BY MODIFIED-LIVE VIRUS VACCINATION CONCURRENT WITH INJECTABLE TRACE MINERALS ADMINISTRATION IN NEWLY RECEIVED BEEF CALVES[3]

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Abstract

The objective was to determine if the use of injectable trace minerals (ITM; Se, Zn, Cu, and Mn) concurrent with a modified-live virus (MLV) vaccine enhances the immune response and onset of protection against BVDV2 infection in beef calves inoculated 5 days after vaccination. Forty-five BVDV-naïve newly received calves were randomly assigned to one of three groups (15/group): VAC+ITM, received MLV vaccine and ITM (Multimin®90) subcutaneously (SC); VAC+SAL, received the same vaccine and saline SC; or UNVAC, unvaccinated. Five days after vaccination (d.0), calves were challenged intranasally with BVDV2 strain-890. Health status and rectal temperature were evaluated. Blood samples were collected for complete white blood cell counts, BVDV1 and 2 serum neutralizing antibody (SNA) titers, BVDV-PCR, percentage of $CD4^+$, $CD8^+$, $WC1^+$ and $CD25^+$ T-cells. Rectal temperature was higher (d.5 to 9) in UNVAC than the vaccinated calves. VAC+ITM had lower health scores than UNVAC (d.8 and 9). VAC+ITM had higher BVDV1 & 2-SNA titers than VAC+SAL and UNVAC on d.21 and 28. Lymphocyte counts decreased in UNVAC but not in VAC+ITM or VAC+SAL (d.3 to 11). $CD4^+$% significantly decreased in UNVAC and VAC+SAL (d.3). VAC+ITM had higher percentage of $CD4^+$% than UNVAC (d.3 and 7). Circulating activated $CD4^+$ and $CD8^+$ consistently increased in UNVAC and VAC+SAL (d.3, 7, 14, and 18) but not in VAC+ITM, which kept a plateau until day 14. VAC+ITM had lower activated $CD4^+$% and $CD8^+$% than UNVAC (d.7). Vaccinated calves had lower number of BVDV-PCR positive buffy coat and nasal swab samples than UNVAC. In summary, vaccination with or without ITM induced a rapid protection against BVDV2 infection. Administration of ITM was associated with

increased SNA response to BVDV1 & 2, enhanced health status, increased platelet counts, mitigated the decrease in $CD4^+$ T-cells, and reduced T-cell activation in calves challenged with BVDV2 five days after immunization. These results support the strategic use of ITM concurrently with vaccination, especially when a rapid protection is needed in newly received beef calves.

Key words: trace minerals, beef calves, BVDV, modified-live virus vaccine, protection

Introduction

Bovine respiratory disease (BRD) is the most economically important disease of beef cattle in North America (Taylor et al. 2010; Griffin, 1997; USDA APHIS, 2010). The infectious agents most commonly involved in BRD include *Bovine viral diarrhea virus* (BVDV), *Bovine herpes virus 1* (BHV1), *Bovine respiratory syncytial virus* (BRSV), *Parainfluenza 3 virus* (PI3V), *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis*. Acute infections with some BVDV strains cause immunosuppression, which potentiates infections by other pathogens involved in BRD (Rosenquist et al., 1970; Fulton et al., 2000). The control and prevention of BVDV is based on biosecurity measures to avoid the introduction and spread of the virus in the herd, identification and elimination of persistently infected animals, allied with vaccination (Brock et al., 2004; Walz et al., 2010).

Vaccination with inactivated or attenuated vaccines is a powerful tool to prevent and lessen the impact of BVDV infections (Dean and Leyh, 1999; Newcomer et al., 2017). Despite the commercial availability of several highly efficacious vaccines in the

USA, a significant number of producers do not vaccinate their calves against BVDV or other BRD pathogens. The efficacy of vaccination to protect against BVDV infection can be indirectly measured by the vaccine's ability to induce neutralizing antibodies and a strong cell-mediated immune response able to control virus replication, viremia and shedding (Rhodes et al. 1999; Reber et al., 2006). Another important factor in the evaluation of BRD vaccine efficacy is its rapidity in inducing an adequate immune response to protect cattle from infection and disease when they are exposed to the virus within a few days after vaccination. This trait is particularly important in stockyards and feedlots, where frequent introduction of highly stressed cattle with unknown vaccination history and infectious status increases the risk of infections that could result in severe respiratory disease with high morbidity and mortality rates and substantial economic losses for producers.

Previous studies characterized the onset of protection elicited by modified-live virus (MLV) vaccines administered 7, 5, or 3 days before BVDV inoculation with high or low virulence BVDV strains (Brock et al., 2007; Palomares et al., 2012). In both trials, calves vaccinated 5 and 7 days before BVDV inoculation were protected from clinical disease, viremia, leukopenia, and virus shedding compared with unvaccinated calves (Brock et al., 2007; Palomares et al., 2012). Those trials were performed under controlled experimental conditions with low stress level and infectious pressure ensuring appropriate animal comfort and welfare. Stressors imposed to calves, such as abrupt weaning, lack of pre-conditioning, weather extremes, irregular vaccination, long transportation, comingling and excessive location and diet changes can compromise the immune function and the response to vaccination due to oxidative stress, affecting

animal health and performance. Moreover, the current global pressure for reducing antibiotic use in food animal production engender the need for vaccination protocols that induce enhanced protection and limit disease transmission more efficiently.

Several studies have demonstrated the impact of trace minerals such as zinc (Zn), manganese (Mn), copper (Cu), and selenium (Se) on the immune function (Chirase et al., 1994; Percival, 1998; Underwood and Suttle, 1999), health status and growth in cattle (Spears and Kegley, 2002), due to their role in the structure and function of antioxidant enzymes (e.g. superoxide dismutase, glutathione peroxidase); improving neutrophil migration and phagocytic function, lymphocyte proliferation and antibody production (Pina et al., 2002; Tomlinson et al., 2008; Arthington and Havenga, 2012; Haase and Rink, 2014; Bonaventura et al., 2015). The use of trace mineral supplementation may become particularly important in highly stressed cattle, such as newly received feeder calves (Duff and Galyean, 2007). Administration of injectable trace minerals (ITM) has shown positive effects on humoral and cell mediated immune response to BRD vaccines in dairy (Palomares et al., 2016; Bittar et al., 2018a) and beef cattle (Arthington and Havenga, 2012; Roberts et al., 2016). We previously demonstrated that the use of ITM concurrently with an MLV-BRD vaccine in dairy calves resulted in earlier and more robust antibody titers to BVDV1 and *Mannheimia haemolytica*, and more rapid and stronger mononuclear cell proliferation upon stimulation with BVDV1, BRSV and *Pasteurella multocida* than the control calves (Palomares et al., 2016; Bittar et al., 2018a). After this study, there was a question as to whether the use of ITM could also favor the induction of a more rapid and improved immune response and protection shortly after vaccination in situations

where a fast protection is required, such as that of newly received calves vaccinated at arrival and shortly exposed to BRD pathogens. Therefore, in the present study we hypothesized that administration of ITM at the time of MLV-BRD vaccination would be beneficiary the immune response and protection in newly received BVDV-naïve beef calves experimentally inoculated with BVDV five days after vaccination. The objective of this study was to determine if the use of injectable trace minerals (containing Se, Zn, Cu, and Mn) supplementation concurrent with a MLV vaccine is able to enhance the immune response and onset of protection against an experimental BVDV2 infection in newly received BVDV-naïve beef calves inoculated 5 days after vaccination.

Materials and Methods

Experimental design, calves, vaccination, and treatments

The study was performed at the University of Georgia (UGA) Oconee Farm (Watkinsville-GA) from May through June 2016. The research protocol was approved by the University of Georgia, Institutional Animal Care and Use Committee (UGA-AUP# A2014 02-005-Y3 A8). This study was performed using 45 weaned Angus and Angus-crossbred calves (7 months old) purchased from a commercial ranch in Calhoun, GA. The calves were BVDV-naïve confirmed via standard virus neutralization test for serum neutralizing antibodies (SNA) against BVDV1 and 2, and ear notch biopsy for immunohistochemistry (BVDV antigen) done at the University of Georgia, Athens Veterinary Diagnostic Laboratory (Athens, GA). The calves and their

dams were not vaccinated with BRD vaccines on the farm of origin before the beginning of the study in order to maintain the calves' BVDV-naïve status. In addition, the calves and their dams were kept in an isolation pasture away from the main herd from birth to weaning to avoid contact with MLV from vaccinated cattle. At the farm of origin, the calves grazed rye grass (*Lolium hybridum*) with free access to bermuda grass hay (*Cynodon dactylon*), and water *ad libitum*.

On day -7, calves were transported for approximately 8 hours from the farm of origin in Calhoun, GA to the Oconee Farm of the College of Veterinary Medicine at University of Georgia (UGA) at Watkinsville, GA. On the experimental farm, the calves grazed fescue grass (*Festuca arundinacea*) with free access to bermuda grass hay (*Cynodon dactylon*), and water *ad libitum*. In addition, calves received daily supplementation (2.5 Kgs per calf) of a commercial ration (Cattleman's special beef; Godfreys Warehouse; Madison-GA) offered in two meals. No additional mineral supplementation was provided.

On day -5, calves were randomly assigned to one of the three groups: VAC+ITM (n= 15): calves received 2 mL of a 5-way MLV vaccine containing BHV1, BVDV1 and 2, BRSV, PI3V (Express 5®, Boehringer Ingelheim, Vetmedica, St. Joseph, MO) subcutaneously (SC) and a dose of injectable trace minerals (ITM, 1 mL/45 Kg of body weight SC; Multimin® 90, Multimin USA Inc, Fort Collins, CO); VAC+SAL (n= 15): calves received a dose of the same MLV vaccine as the previous group and an injection of sterile saline (1 mL/45 Kg SC; Vetone Sterile Saline®; Nova-Tech Inc., Grand Island, NE); or UNVAC (n=15): calves were not vaccinated or received neither ITM nor saline. Administration of ITM provided 15, 60, 10 and 5

mg/mL of Cu, Zn, Mn, and Se respectively. All injections followed guidelines of the Beef Quality Assurance Program (Beef Quality Assurance 2010®; Centennial, CO). Calves in UNVAC were isolated in a pasture separated from the vaccinated calves until the day of challenge, in order to prevent infection with vaccine virus shed by calves in VAC+ITM and VAC+SAL.

Bovine viral diarrhea virus challenge

Five days after vaccination and treatment administration, all calves were intranasally inoculated with a non-cytopathic (ncp) BVDV2 isolate (strain 890). The BVDV2 strain 890 was originally obtained from the APHIS Center for Veterinary Biologics in 1989 (Ames, IA) and has been stored frozen at -80ºC, where it was subjected to propagation in Madin-Darby bovine kidney (MDBK) cells at least every other year to maintain viable stock. The stock BVDV-2 strain 890 used in the present study was biologically cloned via successive passages by use of limiting dilutions with subsequent minimal propagation to produce an adequate amount of stock virus for characterization and animal challenge exposure studies during the six-month period prior to the challenge study. This strain has been previously proven to successfully induce lymphopenia and neutrophilia with mild to moderate clinical disease in non-vaccinated calves (Walz et al., 2001; Bittar et al., 2018b).

The inoculum consisted of an infected cell culture supernatant containing $1x10^5$ 50% cell culture infectious dose ($CCID_{50}$) per mL of ncp BVDV-2 strain 890. The BVDV isolate was propagated in monolayers of MDBK cells using Dulbecco's

Modified eagle's medium (DMEM®; Cellgro, Manassas, VA) supplemented with 10% equine serum (HyClone™ Donor Equine Serum U.S.; Fischer Scientific; Pittsburgh, PA), 1% L-alanyl-L-glutamine (Corning® glutagro 100X Liquid™, 200mM; Corning Cellgro; Manassas, VA), 0.2% anti-fungal (Amphotericin B Liquid 250µg/mL®; Corning; Manassas, VA) and 0.4% antibiotic (Penicillin 10,000 I.U.-Streptomycin 10,000 µg/mL Solution 100X®; Corning; Manassas, VA) as previously described (Bittar et al., 2018b). The inocula remained frozen at -80°C until the day of inoculation. One hour prior to inoculation, the frozen inocula were thawed. Then, 5 mLs of the BVDV2 inoculum were aliquoted into individual 12 mL sterile syringes and held in ice until use. The inoculation was performed by intranasal aerosolization of 5 mL of inoculum (2.5 mL in each nostril) with 10 cm long tip-fenestrated cannula coupled to a 12 mL syringe for each calf. After inoculation all calves were comingled in an 8-acre pasture with adequate shade during the whole experimental period. One sample of the inoculum was transported on ice to the lab to determine the $CCID_{50}$/mL after all inoculations were completed.

Clinical evaluation

Health status including rectal temperature, hydration, attitude, nasal discharge and fecal consistency of the calves was evaluated using the scoring system developed at the University of Wisconsin (http://www.vetmed.wisc.edu/dms/fapm/fapmtools/8calf/calf_health_scoring_chart.pdf). Clinical examination was performed on each calf on study days -6, -5, 0, 2, 3, 4, 5, 6,

7, 8, 9, 10, 11, 12, 13, 14, 15, 18 and 21 relative to the day of BVDV challenge. However, rectal temperature were measured only on study days -6, -5, 0, 3, 5, 6, 7, 8, 9, 11, 14 and 18. Clinical signs were assessed by evaluation of six variables individually (hydration status, nasal secretion, head-ears [attitude], feces, eyes, and cough) on a scale from 0 to 3, with 0 representing a lack of clinical signs and 3 representing severe clinical signs. The daily sum health score was the total sum of each individual clinical sign variable, and was generated by adding all individual scores of the six variables evaluated, to the extent that the minimum daily sum health score being equal to 0 (zero) and the maximum equal to 18 (i.e. if the score of all six variables that compose the daily sum health score were the maximum value of 3, then, 3 times 6 is equal 18). Three experienced veterinarians, who were not aware of calves' treatment allocation, assessed clinical signs and health scores.

Sample collection and processing

From the calves in each group, blood samples were collected via jugular venipuncture using an 18-gauge x 2.5-cm single sample needle (Vacuette®; Nipro Medical Industries Ltd., Gunma, Japan) into vacuum tubes (Vacutainer®, BD Diagnosis, Franklin Lakes, NJ) with and without anticoagulant to obtain whole blood and serum, respectively. Blood samples were collected on study days -14, -6, -5, 0, 3, 5, 6, 7, 8, 9, 11, 14, 18, 21 and 28. Blood collection was done into four individual 8.5 mL glass tubes for buffy coat collection (BD Vacutainer ACD Solution A REF364606®; BD Diagnosis, Franklin Lakes, NJ), and into one 2 mL polypropylene

tube for leukocyte and platelet count (BD Vacutainer K2 EDTA 3.6mg REF367841®; BD Diagnosis, Franklin Lakes, NJ). In addition, we collected blood into two individual 10 mL glass tubes without anticoagulant (BD Vacutainer Serum®; BD Diagnosis, Franklin Lakes, NJ) for serum mineral concentration, and serum neutralizing antibody (SNA) titers to BVDV1 and 2.

The blood samples containing ACD Solution A were processed for buffy coat isolation as previously described (Harpin et al., 1999) and freshly used for staining for flow cytometry analysis or stored at -80ºC for further real-time *RT*-PCR for BVDV nucleic acid detection. Clotted blood samples were spun (900x g for 15min) for serum separation, and serum samples were stored at -80ºC for further analysis for determination SNA titers against BVDV1 and 2.

Nasal swab samples were collected from each calf for BVDV nucleic acid detection using real-time reverse transcription polymerase chain reaction (real-time RT-PCR) on study days 0, 5 and 7. An individual cotton swab (Sterile cotton tipped applicators®; Puritan Medical Products Company LLC; Guilford, ME) was inserted in each nostril scraping the nasal mucosa, and stored in a tube containing 3 mL of PBS containing antibiotics (Penicillin 10,000 I.U.-Streptomycin 10,000 µg/mL Solution 100X®; Corning; Manassas, VA) and anti-fungal (Amphotericin B Liquid 250µg/mL®; Corning; Manassas, VA). Nasal swab samples were transported to the laboratory in a cooler containing ice for further processing. Tubes were mixed using a vortex. Swabs were removed and the remaining solution was filtered (Millex®GP 0.22µm; Millipore Ireland Ltd.; Cork, Ireland) and stored at -80ºC for later VI.

Trace mineral status of calves was assessed in liver biopsy samples (10 - 15 mg of liver tissue) collected from each calf on days -14, and 21 relative to the day of vaccination. Liver biopsies were collected using sterile biopsy needles (14 g and four inches long) with the assistance of ultrasonographic imaging (Ibex®Lite, E.I. Imaging, Loveland, CO) and local anesthesia (Lidocaine injectable. Aspen Veterinary Resources, Ltd. Liberty, MO). Mineral content in liver samples was determined at the Diagnostic Center for Population and Animal Health at Michigan State University, Lansing, MI.

Serum neutralizing antibody titers

Blood samples for serum neutralizing antibodies titers against BVDV1 and 2 were collected on days -14, -5, 0, 7, 14, 21 and 28. Serum neutralizing antibody titers against BVDV1 and 2 were determined at the University of Georgia Athens Veterinary Diagnostic Laboratory (Athens, GA) via a standard virus neutralization protocol as previously described (Bittar et al., 2018b). In summary, after heat inactivation of serum samples (56^{o}C for 30 min), serum samples were serially diluted with DMEM into a 2-fold dilution series, starting at 1:2 in 96-well cell culture plates. An equal volume of the appropriate cytopathic BVDV1 and 2 was added to each well (25 µL of DMEM with approximately 100 $CCID_{50}$ of the respective viruses) making the starting serum dilution of 1:4. After incubation (5% CO_2 at 37^{o}C for 1 h) MDBK cell suspension was added to each well (150 µL suspension with approximately 1.8 x 10^4 cells in DMEM containing 10% fetal calf serum [FCS]).

The cell monolayer was examined with an inverted microscope for signs of virus-specific cytopathic effects after plates were incubated for 4 days (5% CO_2 at 37ºC). The SN titer for each sample was reported as the reciprocal of the highest dilution of serum that completely inhibited virus-induced cytopathic effects.

Leukocyte and platelet counts

Blood samples for total white blood cells, lymphocyte, granulocytes, monocytes and platelets count were collected on days -14, -6, -5, 0, 3, 5, 6, 7, 8, 9, 11, 14 and 18. After blood collection, tubes with uncoagulated blood samples containing EDTA were kept cooled in ice and cooler, and posteriorly transported to the Department of Veterinary Pathology of the University of Georgia (UGA) in Athens, GA for complete blood count (CBC). Blood samples were then kept in room temperature on an orbital shaker to be stirred for 10 minutes before analysis. Total leukocyte, platelet and differential leukocyte counts for each sample were determined by use of an automatic cell counter (HESKA® CBC-Diff, Vet Hematology System, Des Moines, IA).

Expression of CD4, CD8, CD25 and WC1 T-cells in peripheral leukocytes by flow cytometry

Blood samples for T-cells phenotyping (CD4, CD8, and WC1) including CD25 expression were collected on days -6, 0, 3, 7, 14 and 18. For each collection sample a buffy coat was prepared. The leukocytes were assessed for cell viability (exclusion in

0.04% trypan blue) and cell number using a hemocytometer chamber. The cells were suspended to a concentration of approximately $6x10^6$ cells per mL. The samples all had 85% viability or grater. Each sample was diluted in 3 mL PBS-1X in individual pre-labeled tubes (12x75 mm polystyrene. Falcon, BD Biosciences, San José, CA). The cells were washed twice by centrifugation (300 x g for 5 minutes). The supernatant was discarded after each wash. The cells were suspended in 1.5 mL of FACS buffer (PBS with 0.5% bovine serum albumin, and 0.1% sodium azide) and mixed uniformly by vortexing. For staining, 100 µL of cells suspension (approximately $3x10^6$ cells) were added per well to a 96-wells round bottom microtiter plate. Plates were centrifuged (300 x g for 5 minutes) and snapped sharply to remove the supernatant. Each plate was vortexed with lid on to suspend the cells in the residual volume. At this point, 20 µL of each antibody tested (at the minimum saturating concentration) was added to the desired wells, and wells that were dual stained (combination of CD25 with either CD4, CD8 or WC1). The primary antibodies were mouse anti-bovine CD4 isotype IgG2a antibody at a 1:5 dilution (clone CC8, MCA1653F, BioRad, Hercules, CA), mouse anti-bovine CD8 isotype IgG2a antibody at a 1:5 dilution (clone CC63, MCA837F, BioRad, Hercules, CA), mouse anti-bovine WC1 (gamma-delta T-cell) isotype IgG2a antibody at a 1:5 dilution (clone CC15, MCA838F, BioRad, Hercules, CA), and mouse anti-bovine CD25 isotype IgG1 antibody at a 1:7 dilution (clone IL-A111, MCA2430PE). FACS buffer was added to wells to function as an unstained control. A combination of CD25 and each of the other T-cell (CD4, CD8 and WC1) antibodies were used to stain the cells. Plates were incubated (4^oC for 45 minutes). Following staining, the plates were washed three times with FACS buffer (300 x g for 5 minutes).

At the end of each wash, the plates were snapped sharply to remove the supernatant. Each plate was vortexed with lid on to suspend the cells in the residual volume. After the last wash, the supernatant was discarded and the cells were suspended in FACS fix (4% formalin in FACS buffer). Each well received 100 µL of FACS fix. The plates were incubated overnight (18-20 hours) at 4^{o}C. Well contents were transferred into a pre-labeled 1.5 mL MCT containing 300 µL of FACS buffer. After dilution of FACS buffer, samples were stored for flow cytometry analysis at 4^{o}C. All samples were assessed within one week of fixation. Flow cytometry was done using a BD Accuri C6 first generation cytometer and C-flow plus software (BD Biosciences, Franklin Lakes, NJ). Samples were gated using FALS and 90LS two parameter histograms to identify the lymphocyte, monocyte and granulocyte cells in the sample based on previous validation with highly enriched populations of each. Compensation to minimize cross talk between FL1 and FL2 signal was established using single color samples at minimum saturating concentration to eliminate green in orange and orange in green color bleed. The positive and negative windows for FL1 and FL2 single color analysis, and FL1-FL2 dual parameter histograms were established in preliminary trials using staphylococcal enterotoxin B stimulated buffy coat cells under the same staining conditions and set in a template for flow collection. Approximately 10,000 events were recorded in the lymphocyte gate for each sample. Following data collection, the lymphocyte gate was confirmed using the list mode data for each sample to assure that the same population was measured for each sample across the total experiment. The C-flow plus software was used to generate values for percent positive cells for each marker, and for CD25 positive cells within each marker. In addition, values for the

mean fluorescent intensity were generated from the analysis gates for each marker and for the CD25 population within each marker for all samples in the trial.

BVDV nucleic acid detection by RT-PCR on peripheral leukocytes and nasal swab samples

Buffy coat and nasal swab samples that had been stored at -80 C were thawed at room temperature for BVDV nucleic acid detection. Total RNA was extracted from buffy coats and nasal swab solutions using RNeasy Mini Kit (QIAGEN. Germantown, MD). Total RNA samples were submitted to the Athens Veterinary Diagnostic Laboratory where a BVDV1 and 2 multiplex real-time *RT*-PCR was performed as previously described (Letellier and Kerkhofs, 2003). Based on conserved regions of the 5' UTR of BVDV1 and 2, the primer pair F2: 5' CTCGAGATGCCATGTGGAC 3' (position 224–242 of the NADL sequence) and PESTR: 5' CTCCATGTGCCATGTACAGCA 3' (position 391–371 of the NADL sequence) were used. Two probes differing by three nucleotides were utilized: the 5' FAM CAGCCTGATAGGGTGCTGCAGAGGC TAMRA 3' probe was specific of BVDV1 whereas the 5' VIC CACAGCCTGATAGGGTGTAGCAGAGACCTG TAMRA 3' was used for the detection of BVDV2. Only results of BVDV2 detection by real-time RT-PCR are reported in the present manuscript.

Statistical analysis

Data were analyzed using the Statistical Analysis System (SAS® version 9.3; SAS Institute, Cary, NC, USA). Statistical assumptions of normality and constant variance were tested through Shapiro Wilk's and Levene's tests, respectively. A logarithmic base 2 transformation was applied to the antibody titers. For calculation and comparison of geometric mean (GM) antibody titers, back-transformed antibody titers were calculated for each group at days -14, -5, 0, 7, 14, 21, and 28 after BVDV infection. Means daily sum health score, liver trace mineral concentration, rectal temperature, leukocyte and platelet counts, SNA titers, and percentage of T-cells phenotypes were compared among treatment groups by using repeated-measures analysis Proc-GLIMMIX model using calf as random effect, time points as fixed effect, and group and time as fixed effects. Tukey test was used to adjust for multiple comparisons. For variables in which the groups did not have similar means at day -5, we used the first day measurement of each variable as covariate in the statistical model, to minimize the differences between groups at the beginning of the study, therefore allowing for a more accurate comparison among groups in this study. Results of real-time *RT*-PCR for BVDV2 detection on buffy coat samples and nasal swab specimens were analyzed by use of a frequency procedure and compared by use of a χ^2 test. For all analyses, values of $P \leq 0.05$ were considered significant, and $0.05 < P \leq 0.10$ was considered a tendency. All results are shown as least squared means (LSM) obtained from the statistical software SAS after analysis.

Results

Calves used in the study did not show any inflammatory reaction at the injection site after treatment with ITM. The hepatic trace mineral concentrations (Se, Cu, Zn and Mn) before starting the study (day -14) were comparable in all groups (Fig. 5.1A to 5.1D; $P > 0.05$) and within normal reference ranges (Herdt and Hoff 2011). Injectable trace mineral administration was associated with increased hepatic Se (Fig. 5.1B; $P < 0.001$) and Cu (Fig. 5.1A; $P < 0.01$) concentrations in the VAC+ITM group compared with VAC+SAL and UNVAC groups. Hepatic Zn concentrations increased in all three groups on day 21 compared with day -14, and it was higher in the UNVAC group compared with both vaccinated groups ($P < 0.04$. Fig. 5.1C). There were no differences in hepatic Mn concentrations on day 21 among groups or relative to baseline levels on day -14 (Fig. 5.1D).

Rectal temperatures increased in the UNVAC group after BVDV2 challenge, peaking by days 7 and 8 (Fig. 5.2). Unvaccinated calves had significantly higher rectal temperature than both vaccinated groups from days 5 to 9 after BVDV challenge ($P < 0.05$). There was no difference in rectal temperature between vaccinated groups during the experimental period. Sum daily health scores increased on days 3, 7, 8 and 9 post BVDV challenge in the UNVAC group (Fig. 5.3). Calves receiving ITM had lower health scores than UNVAC calves from day 7 to 9 ($P < 0.05$). During this period, VAC+SAL group had intermediate health scores, which were not significantly different from either VAC+ITM or UNVAC groups ($P > 0.05$). Calves in VAC+ITM group had lower head-ears (attitude) scores compared to UNVAC (day 7) and VAC+SAL group (days 8, 9 and 11; $P < 0.05$. Data not shown).

Mean total leukocytes count significantly decreased in the UNVAC group from day 0 to day 5 (approx. 66%. Fig. 5.4A). These values were significantly lower than those in the vaccinated groups ($P< 0.05$). Vaccinated groups (treated with ITM or not) had similar mean leukocytes count along the study. Mean lymphocytes, granulocyte and monocyte counts significantly decreased in the UNVAC group between days 3 and 7 after BVDV challenge ($P< 0.05$; Fig. 5.4B, 5.4C and 5.4D), which was statistically different from vaccinated groups on the same days. Calves in both vaccinated groups had a slight decrease in lymphocyte, granulocyte and monocyte counts after vaccination and again on days 5 and 6 after BVDV challenge, with no significant difference between groups (Fig. 5.4B, 5.4C and 5.4D). Platelets count significantly declined after BVDV inoculation in the UNVAC calves until days 8 and 9 (Fig. 5.4E). Differently, platelets count increased in the VAC+ITM group after day 3, while they stayed unaffected in the VAC+SAL group. Vaccinated calves that were treated with ITM had significantly higher platelet count compared to VAC+SAL (days 5, 6, and 9) and UNVAC calves (days 3, 5, 6, 7, 8, 9, and 11. $P< 0.05$. Fig. 5.4E).

Serum neutralizing-antibody (SNA) titers against BVDV1 and BVDV2 were low (on average <1:8) before inoculation (on days -14, -5, and 0) in all groups (Fig. 5.5A and 5.5B). Mean BVDV1 SNA titers dramatically increased by >4 $\log_2$ within two weeks after BVDV challenge in both vaccinated groups (Fig. 5.5A). Calves in VAC+ITM had higher BVDV1 SNA titers on day 28 compared with the VAC+SAL group ($P< 0.05$). Unvaccinated calves had lower SNA titers against BVDV1 after challenge than both vaccinated groups. There was a moderated increase by

approximately 2.5 log_2 in BVDV1 SNA titers only by day 28 post inoculation in the UNVAC group (Fig. 5.5A). Moreover, SNA titers against BVDV2 dramatically increased by 5.2 and 7.2 log_2 in VAC+ITM group within three and four weeks after BVDV challenge, respectively (Fig. 5.5B). The BVDV2 SNA titers was also increased in VAC+SAL group, but in lower levels than in VAC+ITM group (3 and 5.4 log_2 increase within three and four weeks after BVDV challenge, respectively). Calves in the VAC+ITM group had higher BVDV2 SNA titers compared to the other groups on days 21 and 28 ($P< 0.05$). Calves in the UNVAC group had also an increase in BVDV2 SNA titers by approximately 3.5 log_2 within four weeks after BVDV challenge, which were significantly lower than those in VAC+ITM and VAC+SAL groups ($P< 0.05$).

The percentage of $CD4^+$ T-cells significantly decreased in the UNVAC and VAC+SAL groups on day 3 after BVDV2 inoculation (Fig. 5.6A. $P< 0.001$). On days 3 and 7, the VAC+ITM group had higher number of $CD4^+$ T-cells than the UNVAC group. During this period, calves in the VAC+SAL group had intermediate values of $CD4^+$ T-cells compared with the other groups. Infection with BVDV2 also caused a significant reduction in the percentage of $CD8^+$ T-cells in the UNVAC calves by 3 days after BVDV challenge ($P< 0.001$. Fig. 5.6B). Vaccinated calves (treated with ITM or not) had a slight decline in the percentage of $CD8^+$ T-cells after BVDV2 challenge, which rebounded to initial values by day 7 post BVDV challenge. There were no statistical differences in the percentage of $CD8^+$ T-cells among groups during the study ($P> 0.05$). However, values of $CD8^+$ T-cells tended to be numerically greater in the VAC+ITM treated calves on day 7 post BVDV inoculation when compared with the other groups. Circulating activated T lymphocytes ($CD4^+CD25^+$ and $CD8^+CD25^+$)

consistently increased in UNVAC and VAC+SAL calves on days 3, 7 (peak), and 14 (only for $CD4^+CD25^+$) after BVDV2 challenge (Figs. 5.6C & 5.6D respectively. $P< 0.05$), but not in the VAC+ITM calves, in which activated T-cells increased on day 3, but kept a plateau until day 14 post inoculation. Calves treated with ITM had significantly lower percentage of $CD4^+CD25^+$ T-cells than the VAC+SAL group on day 7 (Fig. 5.6C. $P< 0.05$) and significantly lower percentage of $CD8^+CD25^+$ T-cells than the UNVAC group (Fig. 5.6D. $P< 0.05$). Percentage of $WC1^+$ T-cells significantly increased in the UNVAC group on days 7, 14 and 18 after BVDV2 infection (Fig. 5.6E). On day 18, calves in VAC+ITM and VAC+SAL also had a significant increase in $WC1^+$ T lymphocytes. Between days 7 and 18 after BVDV2 challenge, the VAC+ITM group had significantly lower percentage of $WC1^+$ T-cells compared to the UNVAC calves ($P< 0.05$). Activated WC1 T-cells ($WC1^+CD25^+$ T lymphocytes) significantly increased in all groups on day 3 (Fig. 5.6F. $P< 0.05$). Moreover, unvaccinated calves maintained high $WC1^+CD25^+$ T-cells lymphocytes levels until day 18 post BVDV2 challenge, while vaccinated calves had a decay in the percentage of $WC1^+CD25^+$ T-cells from day 7 to day 18 (Fig. 5.6F). On day 7, ITM-treated calves had significantly lower percentage of $WC1^+CD25^+$ T-cells compared with unvaccinated calves ($P< 0.05$).

All calves had negative PCR results for BVDV2 in nasal swabs or buffy coat samples on day 0 before BVDV2 challenge. Fourteen of 15 calves (93.3%) in the UNVAC group had at least one BVDV2 positive buffy coat sample on days 5 and 7. The percentage of BVDV2 positive buffy coat samples on days 5 and 7 was significantly greater in the UNVAC group compared with the vaccinated groups ($P <$

0.05. Table 5.1). Only one calf in VAC+ITM and one calf in VAC+SAL had a BVDV2 positive buffy coat sample on day 5 and 7 post challenge, respectively. Moreover, 46.7% (7 out of 15) of the calves in the UNVAC group had at least one BVDV positive PCR result from nasal swab samples on days 5 and 7 post inoculation, which was greater than the frequency observed in vaccinated calves. There was no difference in the proportion of BVDV positive nasal swab specimens between both vaccinated groups.

Discussion

In the current study, vaccination enabled rapid protection from experimental infection with BVDV2 in newly received beef calves. The efficacy of vaccination was demonstrated by lesser health scores (associated to better health status accordingly to the evaluation system used in this study), adequate leukocytes counts (absence of leukopenia and lymphopenia), lower proportion of BVDV2 positive nasal swab and buffy coat samples, and greater percentages of $CD4^+$ T-cells in the vaccinated groups when compared to unvaccinated control calves, which succumbed to mild clinical disease, had a significant leukopenia, thrombocytopenia, viremia and virus shedding and a notable drop in $CD4^+$ and $CD8^+$ T-cells caused by the moderately virulent BVDV2 strain 890. Moreover, administration of ITM resulted in increased hepatic concentrations of Se and Cu, and provided additional benefits of stronger immune response and protection after BVDV challenge (greater number of $CD4^+$ T-cells, higher BVDV1 and 2 SNA titers, and lesser health scores) compared with the other groups.

The experimental approach of performing vaccination and then virus inoculation a few days after arrival was used to evaluate the onset of protection by attempting to mimic a common scenario of BVDV exposure upon arrival and commingling with cattle of unknown vaccination history. A rapid onset of protection induced by primary vaccination in susceptible calves with unknown immune status would be crucial in beef cattle production, where high BRD-risk calves are subjected to management practices that involve significant stress including weaning, transportation, comingling, and abrupt changes in diets. Previous studies evaluated the rapidity of protection induced by MLV vaccination in beef calves challenged with low or high virulence BVDV at 3, 5 or 7 days after primary vaccination (Brock et al., 2007; Palomares et al., 2012). These studies demonstrated rapid protection against BVDV acute infection when vaccination was given 5 or 7 days before challenge. However, a high proportion of calves inoculated 3 days after vaccination shed BVDV after challenge. At least in the context of BVDV prevention, our results of adequate immune response and rapid protection elicited by vaccination provide evidence that may support strategic vaccination at the time of cattle arrival, reducing quarantine periods and intervals between vaccination and commingling with other cattle, which might facilitate farm management practices.

Severe BVDV-induced leukopenia and thrombocytopenia were observed in the unvaccinated calves, while vaccinated groups experienced a slight decrease in white blood cells after BVDV2 inoculation. In addition, platelets counts were significantly increased in the VAC+ITM group compared with the other groups. A known hallmark of BVDV2 infection is the reduction in leukocyte and platelet counts. The degree of

leukopenia and thrombocytopenia is correlated to virulence of some specific strains of BVDV (Walz et al., 2001). Leukopenia induced by BVDV has been associated with apoptosis and necrosis of leukocytes, as well as leukocyte migration from circulation into tissues where viral replication occurs (Walz et al., 2010). BVDV-induced leukopenia results in immunosuppression and plays a major role in the pathogenesis of BRD, as it potentiates secondary infection by commensal flora in the upper respiratory tract responsible for causing mild to fatal bronchitis and pneumonia (Brogden and Guthmiller, 2002). Reduction in platelet number is a common hematological finding in infections with some BVDV2 strains and has been associated with a hemorrhagic syndrome (Rebhun et al., 1989). Administration of ITM at the time of MLV vaccination induced a significant increase in platelet counts in calves inoculated with BVDV2. The benefits of injectable trace mineral (Se, Cu, Mn and Zn) supplementation for mitigating the negative effects of BVDV2 infection on platelet numbers is a remarkable finding of this study. Comparable effects of ITM administration on platelets counts were observed in our previous trial evaluating the long-term protection elicited by MLV vaccination and ITM supplementation against BVDV2 infection in dairy calves (Bittar et al., 2018b).

The commercial vaccine used in this study was able to reduce viremia and viral shedding after BVDV infection, as represented by the lower number of BVDV2 positive buffy coat and nasal swab samples in both vaccinated groups on days 5 and 7 after experimental BVDV2 challenge compared with the unvaccinated group, which is in agreement with previous studies using different BVDV strains (Brock et al., 2007; Palomares et al., 2012). However, supplementation with ITM showed no additional

beneficial effects reducing BVDV detection in peripheral leukocytes and nasal swab samples.

The humoral immune response post BVDV challenge was greater in both vaccinated groups compared to the unvaccinated calves. Calves treated with ITM had greater SNA titers to BVDV1 and BVDV2 compared to the other groups. Previous field studies have shown association between higher SNA titers and health performance in calves at feedlot receiving (Fulton et al., 2002; Campbell, 2004). Production of neutralizing antibodies is one of the major features of the immune system to prevent viral infections such as BVDV. Neutralizing antibodies hamper BVDV replication and transmission in the host cells, diminishing the chances of a successful viral infection and shedding within cattle populations. Similarly, positive effects of using ITM concurrent with MLV vaccination on improvement of SNA production against BRD viruses and animal health and performance have been shown in previous studies in dairy and beef cattle (Richeson and Kegley, 2011; Arthington and Havenga, 2012; Palomares et al., 2016). In contrast, our previous study evaluating the effects of ITM given at the time of vaccination on the long-term protection against BVDV infection in dairy calves showed no improvement in BVDV SNA titers or protection from clinical disease in calves treated with ITM (Bittar et al., 2018b). The absence of clinically remarkable differences between groups was attributed to the moderate virulence BVDV strain used for challenge, low level of stress and limited infectious pressure, which favored protection elicited by two doses of MLV vaccination. On the other hand, the greater BRD risk scenario of the current trial (one dose of MLV vaccine given to naïve cattle, short interval between vaccination and BVDV challenge, 8-hour

transportation before vaccination and the diet change) might explain discordance between these studies. Thus, the current experimental conditions enable to better demonstrate the effects of ITM concurrently with MLV BRD vaccine, especially when a fast immune response and protection is needed.

Infection with BVDV caused a significant reduction in number of $CD4^+$ and $CD8^+$ T lymphocytes in the unvaccinated calves, which agrees with previous reports (Howard et al., 1992; Chase et al., 2004). Calves treated with ITM had a less pronounced reduction in $CD4^+$ T-cells (with higher percentage on days 3 and 7) and a lower number of activated $CD4^+$ $CD25^+$ and $CD8^+$ $CD25^+$ T-cells compared to the other groups. Lymphocytes expressing the surface protein CD4 also called T helper cells play an important role in the humoral and cell mediated immune response after vaccination and/or infection. They contribute with the activity of other immune cells by releasing cytokines that help stimulating or regulating the immune response (Abbas et al., 2012). They are essential in B cell antibody isotype switching, activation and growth of cytotoxic T-cells ($CD8^+$), and in maximizing phagocytic and bactericidal activity by neutrophils and macrophages, among other functions. Cytotoxic T lymphocytes or $CD8^+$ T-cells have important effector functions killing virus-infected cells, through the release of the cytotoxins perforins, granzymes, and granulysin (Abbas et al., 2012). It is known that $CD4^+$ T-cell depletion during BVDV acute infection may increase the period of virus shedding, as demonstrated by the higher proportion of unvaccinated calves carrying BVDV in nasal secretions and peripheral blood leukocytes in this trial and previous studies (Howard et al., 1992). Total and activated $WC1^+$ T-cells were consistently increased in the unvaccinated group after

challenged. Treatment with ITM appeared to prevent a significant increase in WC1 T-cells. In cattle, gamma delta T-cells expressing the WC1 phenotype accounts for up to 60 % of the total circulating lymphocytes and have been described to have regulatory functions with spontaneous IL-10 secretion, (Hoek et al., 2009; Guzman et al., 2014). Therefore, increasing activation and trafficking of gamma delta T lymphocytes may be one of the mechanisms used by BVDV to down-regulate the immune system causing immune suppression, as it has been previously reported (Palomares et al., 2015).

It is possible to infer that ITM supplementation positively influenced both humoral and cell mediated immune responses elicited by MLV primary vaccination, as previously shown (Palomares et al., 2016; Bittar et al., 2018a), resulting in an enhanced protection upon BVDV exposure. In addition, ITM may have contributed to a more efficacious innate immunity (e.g. type 1 interferon antiviral state, antigen processing and presentation) after vaccination, which may have limited BVDV replication and viral load at the site of infection, reduced virus-induced T-cell depletion (resulting in higher number of $CD4^+$ T-cells) and lowered the proportion of T-cells trafficking to secondary lymphoid tissues after virus infection, reducing T-cell activation, proliferation and differentiation. This inference might explain the lower percentage of activated $CD4^+CD25^+$ and $CD8^+CD25^+$ in the peripheral blood circulation in the VAC+ITM group.

This study had the limitations that T-cell populations were not evaluated at the tissue level (respiratory tract mucosa and lymphoid tissue), which would have provided a better understanding of the T-lymphocyte phenotypic dynamics after the BVDV challenge. Nonetheless, to the authors' knowledge, this is the first study evaluating the

ex-vivo dynamics of circulating T-cells upon BVDV inoculation occurring shortly after MLV-BRD vaccination.

In summary, MLV-BRD vaccination concurrent with ITM or not, induced a rapid protection against BVDV infection. In addition, administration of ITM at the time of vaccination was associated with increased humoral immune response to BVDV1 and 2, enhanced health status, appeared to mitigate the decrease in the percentage of peripheral blood circulating $CD4^+$ and $CD8^+$ T-cells, and reduced their activation in beef calves challenged with BVDV2 five days after vaccination. Moreover, administration of ITM at the time of vaccination was associated with significantly increased platelet counts after BVDV challenge. These results support the strategic use of ITM concurrently with vaccination protocols, especially when a rapid immune response and protection is needed in newly received beef calves.

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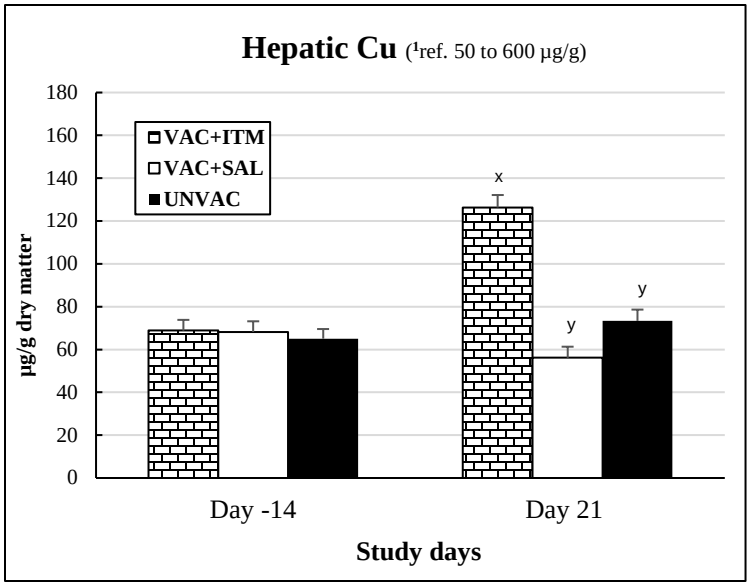
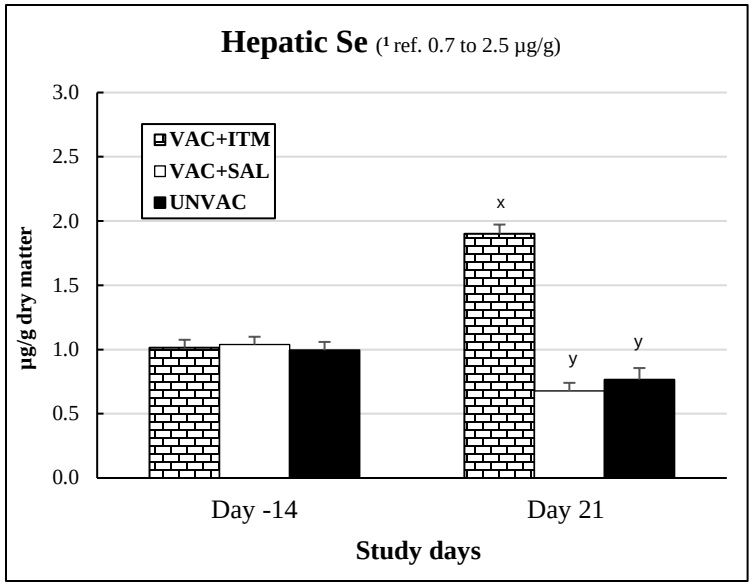
Fig. 5.1A


Hepatic Cu (¹ref. 50 to 600 µg/g)
µg/g dry matter
180
160
140
120
100
80
60
40
20
0
VAC+ITM
VAC+SAL
UNVAC
x
y
y
Day -14
Day 21
Study days

5.1B


Hepatic Se (¹ ref. 0.7 to 2.5 µg/g)
µg/g dry matter
3.0
2.5
2.0
1.5
1.0
0.5
0.0
VAC+ITM
VAC+SAL
UNVAC
x
y
y
Day -14
Day 21
Study days

5.1C **5.1D**

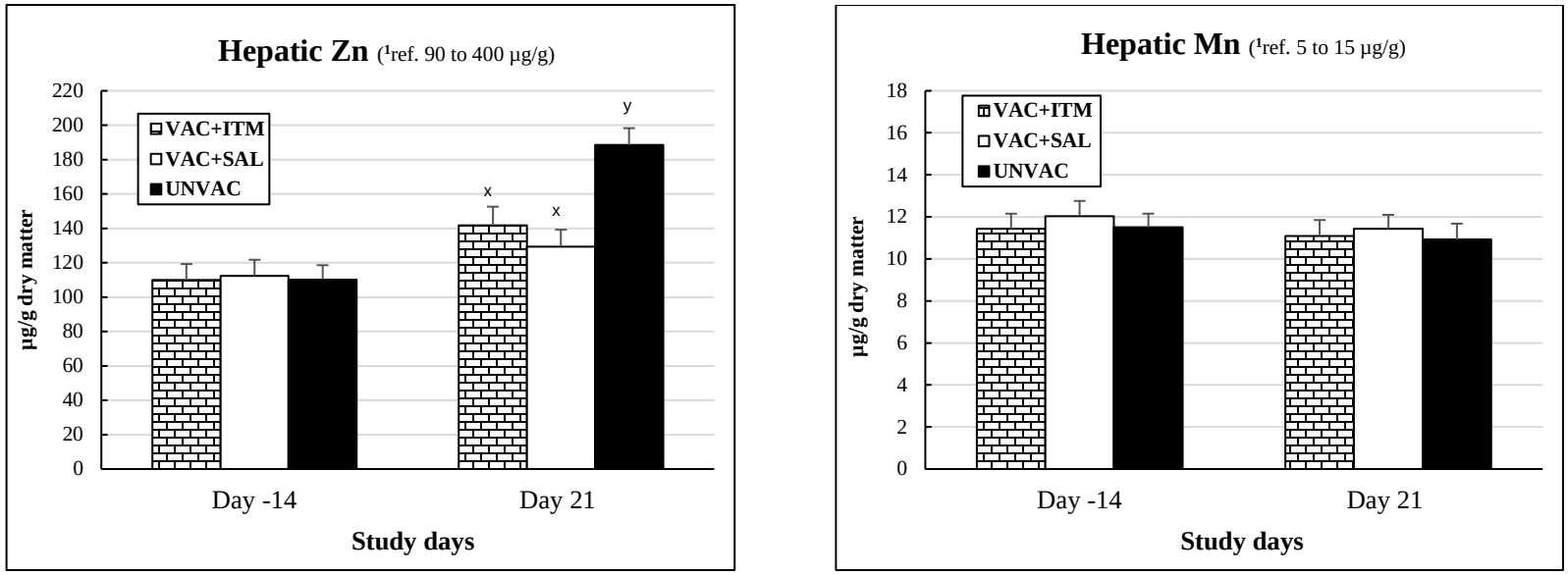


Fig. 5.1A-D. Liver trace mineral concentrations and standard error of the mean (SEM) of Se (A), Cu (B), Zn (C) and Mn (D) for beef calves treated with injectable trace minerals (VAC+ITM) or not (VAC+SAL), concurrently with MLV-BRD vaccination, or calves not treated nor vaccinated (UNVAC). Calves received treatments (ITM, saline, vaccine or nothing) at study day -5, and were individually challenged with BVDV2 (ncp strain 890) five days later (day 0). Errors bars represent the SEM. [1] Hepatic trace mineral concentrations references values based on Herdt and Hoff 2011. [x, y] Significant difference between groups ($P < 0.05$).

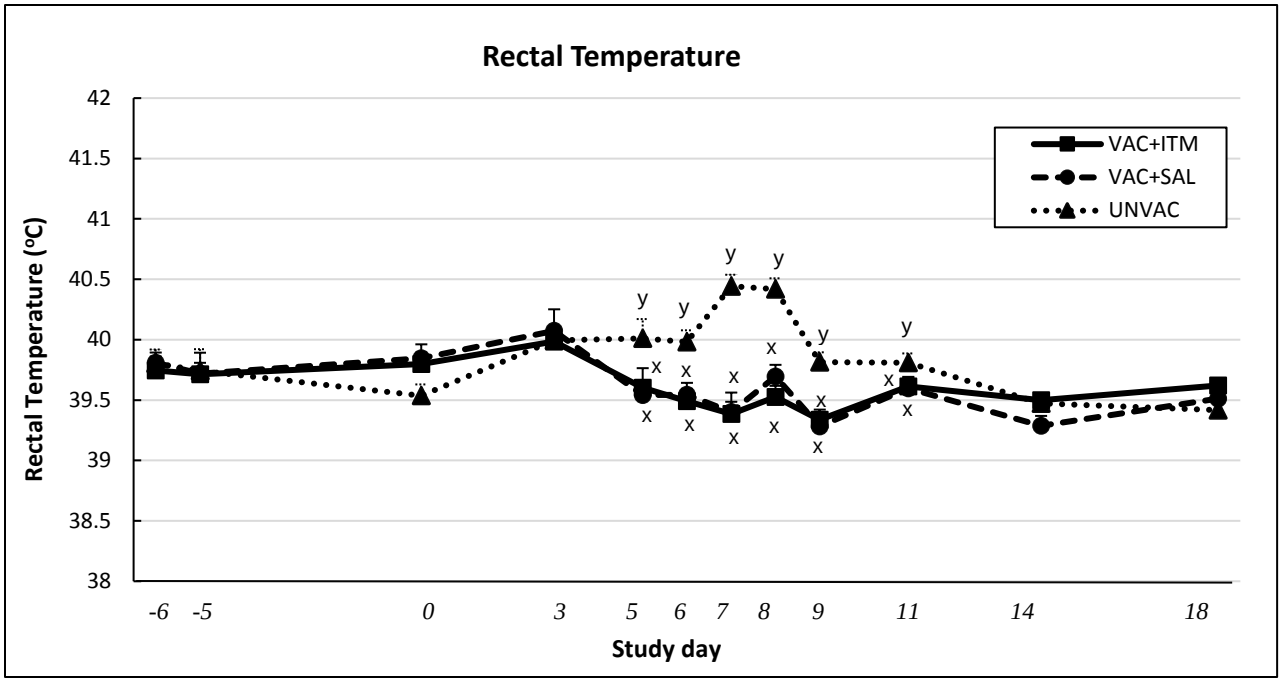


Fig. 5.2. Least square means (LSM) and standard error of the mean (SEM) of Rectal temperature for beef calves treated (VAC+ITM) or not (VAC+SAL) with injectable trace minerals (ITM) concurrently with MLV-BRD vaccination, or calves not treated nor vaccinated (UNVAC). Calves received treatments (ITM, saline, vaccine or nothing) at study day -5, and were individually challenged with BVDV2 (ncp strain 890) five days later (day 0). Errors bars represent the SEM.

[x, y] Significant difference between groups (P< 0.05).

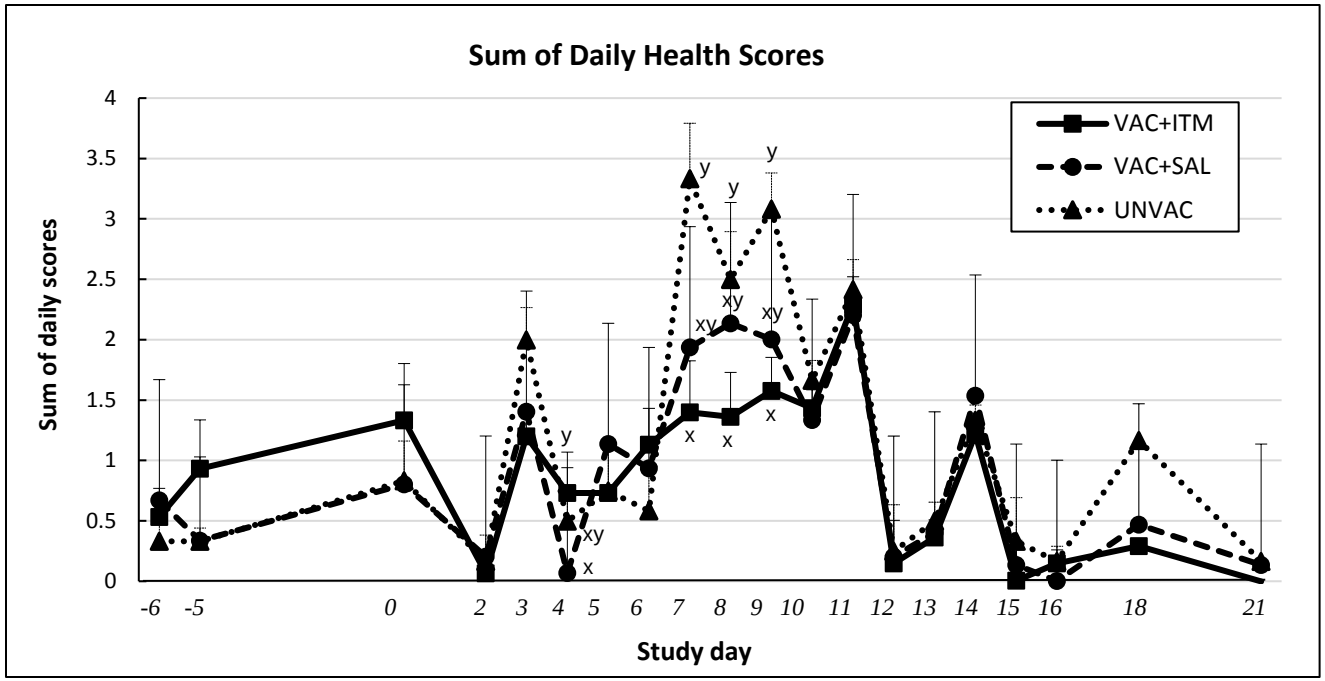
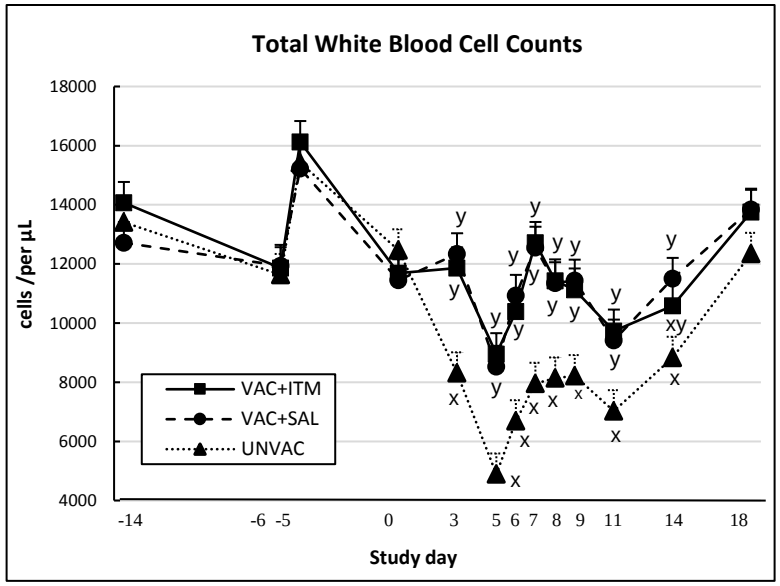


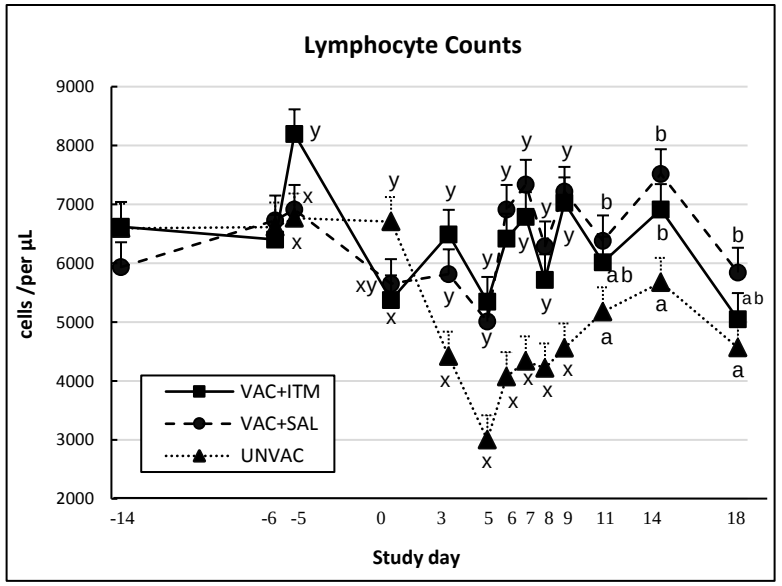
Fig. 5.3. Least square means (LSM) and standard error of the mean (SEM) of sum daily health scores for beef calves treated (VAC+ITM) or not (VAC+SAL) with injectable trace minerals (ITM) concurrently with MLV-BRD vaccination, or calves not treated nor vaccinated (UNVAC). Calves received treatments (ITM, saline or nothing, with or without vaccine) at study day -5, and were individually challenged with BVDV2 (ncp strain 890) five days later (day 0). Errors bars represent the SEM.

[x, y] Significant difference between groups ($P < 0.05$).

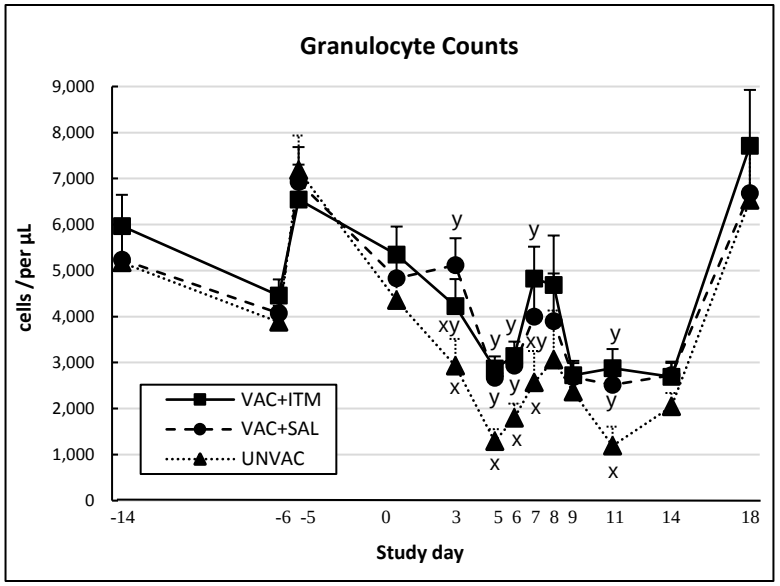
Fig. 5.4A


Total White Blood Cell Counts
cells /per µL
18000
16000
14000
12000
10000
8000
6000
4000
-14
-6
-5
0
3
5
6
7
8
9
11
14
18
Study day
VAC+ITM
VAC+SAL
UNVAC

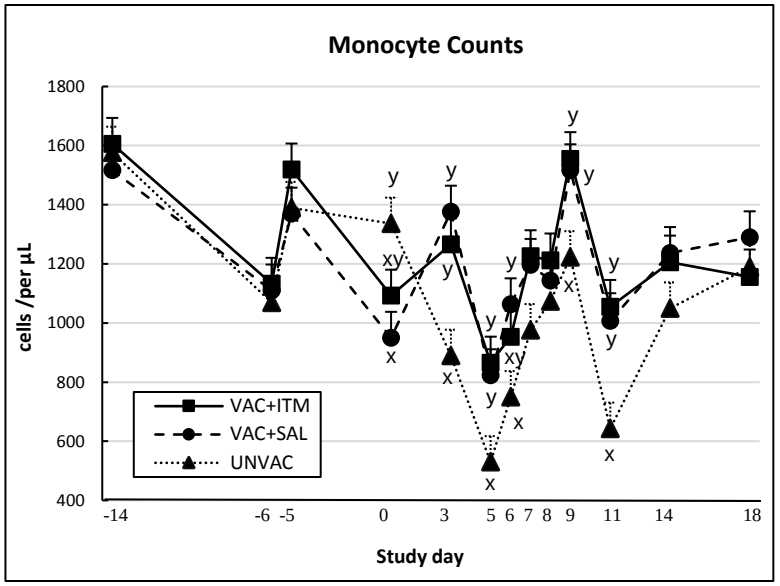
5.4B


Lymphocyte Counts
cells /per µL
9000
8000
7000
6000
5000
4000
3000
2000
-14
-6
-5
0
3
5
6
7
8
9
11
14
18
Study day
VAC+ITM
VAC+SAL
UNVAC

5.4C


Granulocyte Counts
cells /per µL
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
-14
-6 -5
0
3
5 6 7 8 9
11
14
18
Study day
VAC+ITM
VAC+SAL
UNVAC

5.4D


Monocyte Counts
cells /per µL
1800
1600
1400
1200
1000
800
600
400
-14
-6 -5
0
3
5 6 7 8 9
11
14
18
Study day
VAC+ITM
VAC+SAL
UNVAC

5.4E

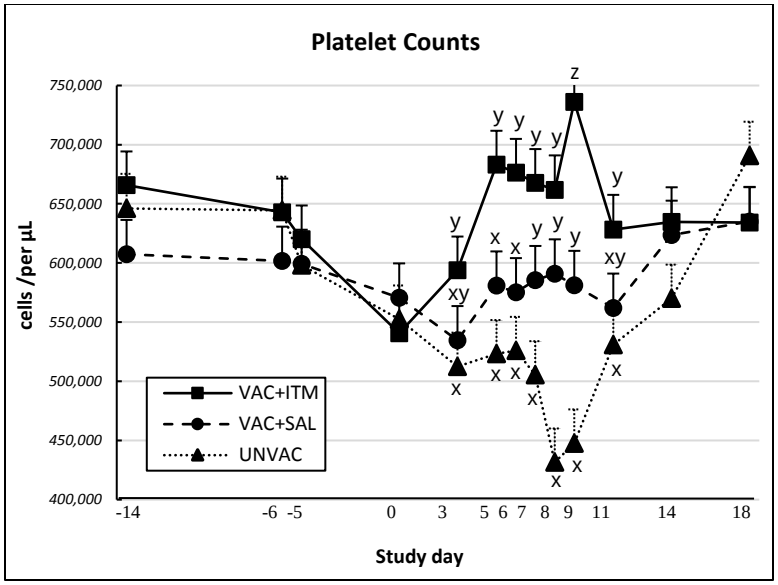
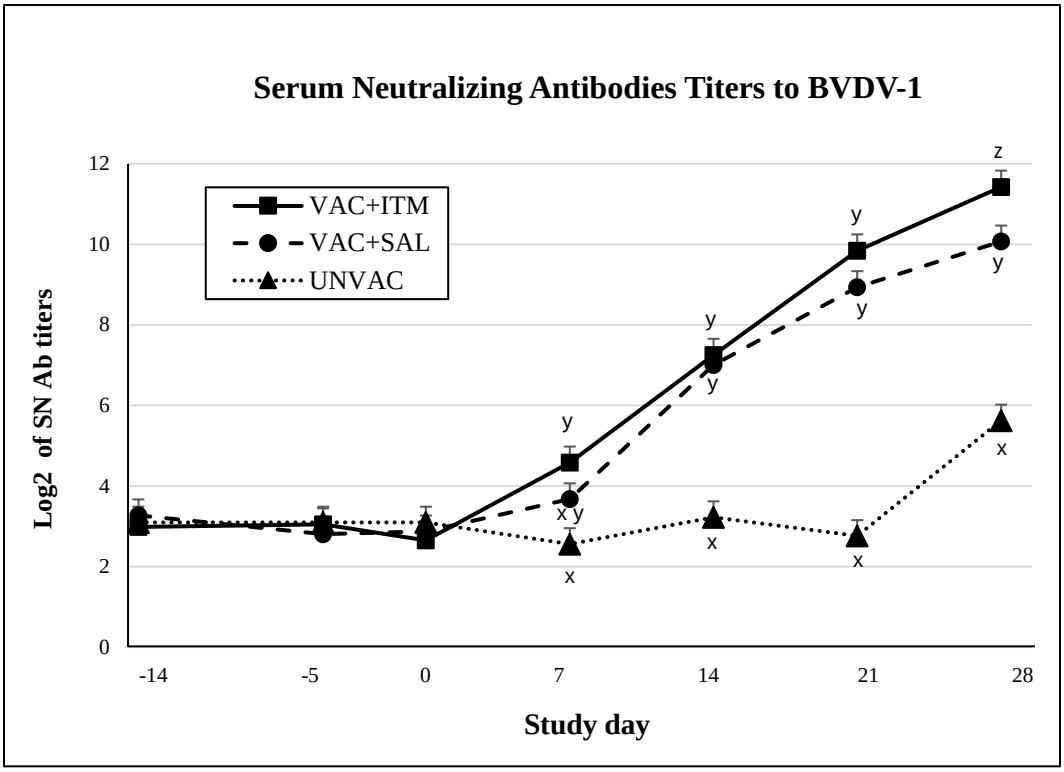


Fig. 5.4A-E. Least square means (LSM) and standard error of the mean (SEM) of total white blood cells (A), lymphocyte (B), granulocyte (C), monocyte (D) and platelet (E) counts for beef calves treated (VAC+ITM) or not (VAC+SAL) with injectable trace minerals (ITM) concurrently with MLV-BRD vaccination, or calves not treated nor vaccinated (UNVAC). Calves received treatments (ITM, saline or nothing, with or without vaccine) at study day -5, and were individually challenged with BVDV2 (ncp strain 890) five days later (day 0). Errors bars represent the SEM. [x, y, z] Significant difference between groups by each study day ($P < 0.05$).

[a, b] Values tended to be different between groups by each study day ($0.05 \leq P \leq 0.10$).

Fig. 5.5A


Serum Neutralizing Antibodies Titers to BVDV-1
Log2 of SN Ab titers
Study day
VAC+ITM
VAC+SAL
UNVAC
0
2
4
6
8
10
12
-14
-5
0
7
14
21
28
z
y
y
y
y
y
x y
x
x
x
x

5.5B

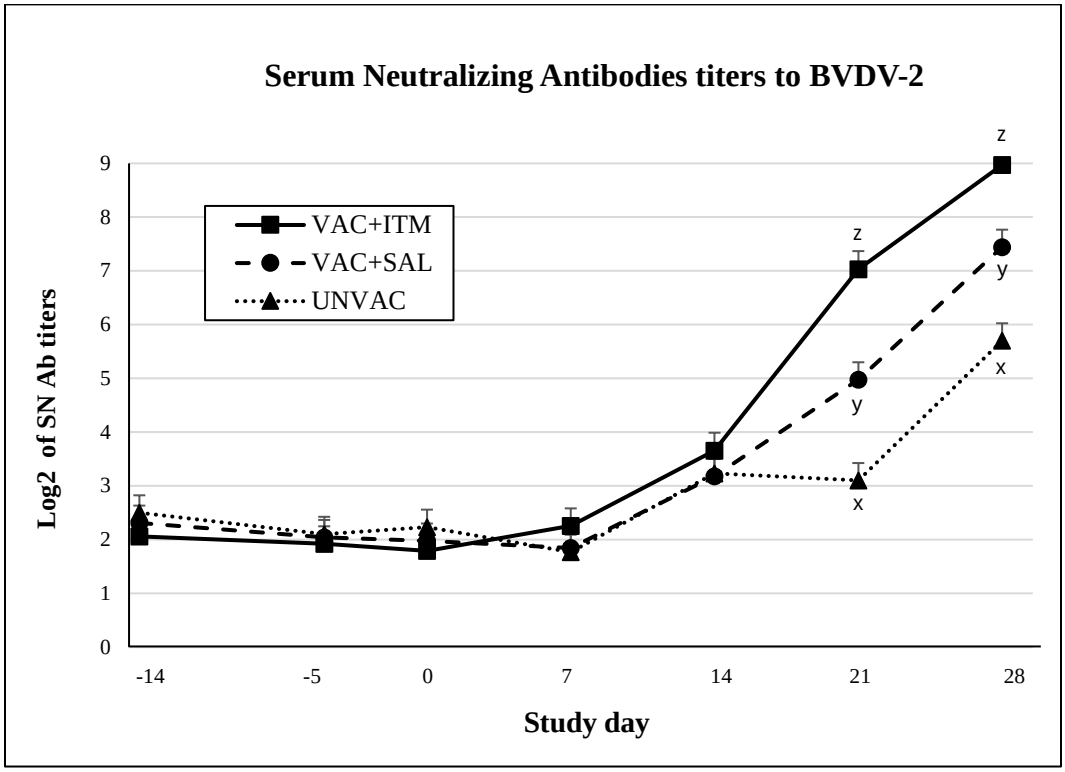
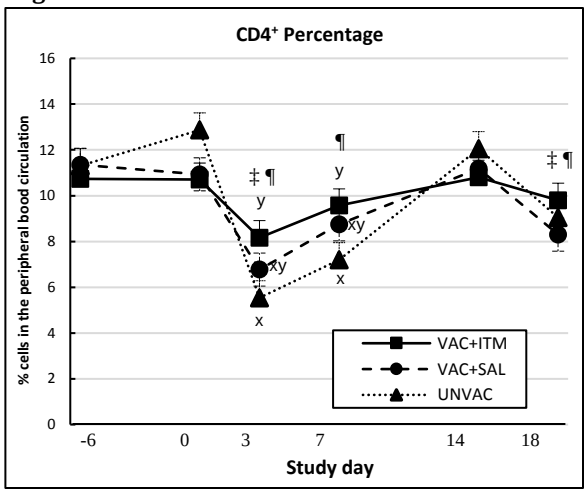

Serum Neutralizing Antibodies titers to BVDV-2
Log2 of SN Ab titers
Study day
VAC+ITM
VAC+SAL
UNVAC
0
1
2
3
4
5
6
7
8
9
-14
-5
0
7
14
21
28
z
z
y
y
x
x

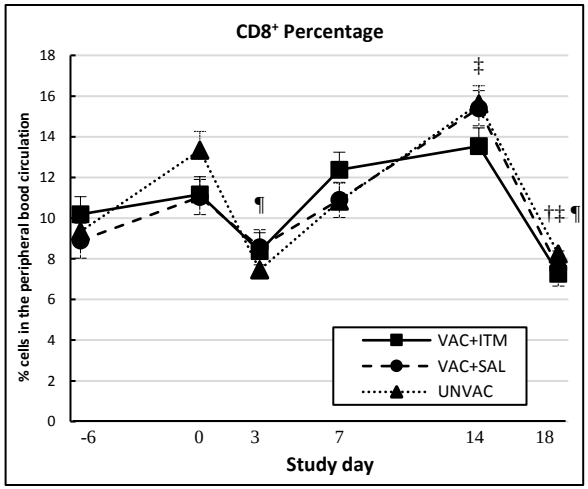
Fig. 5.5A-B. Least square means (LSM) and standard error of the mean (SEM) of serum neutralizing antibody titers to BVDV-1 (A) and to BVDV-2 (B) for beef calves treated (VAC+ITM) or not (VAC+SAL) with injectable trace minerals (ITM) concurrently with MLV-BRD vaccination, or calves not treated nor vaccinated (UNVAC). Calves received treatments (ITM, saline or nothing, with or without vaccine) at study day -5, and were individually challenged with BVDV2 (ncp strain 890) five days later (day 0). Errors bars represent the SEM.

[x, y, z] Significant difference between groups by each study day ($P < 0.05$).

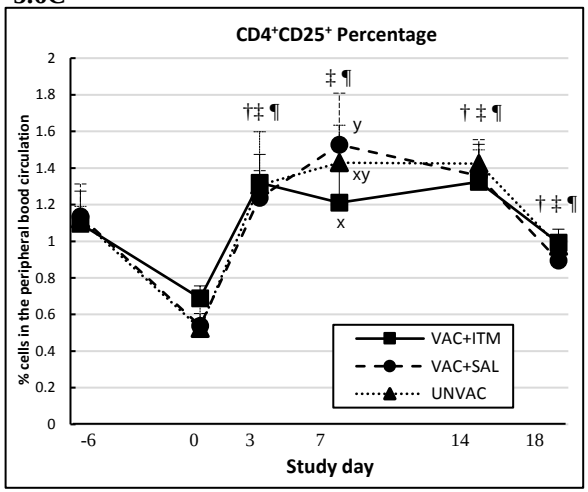
Fig. 5.6A



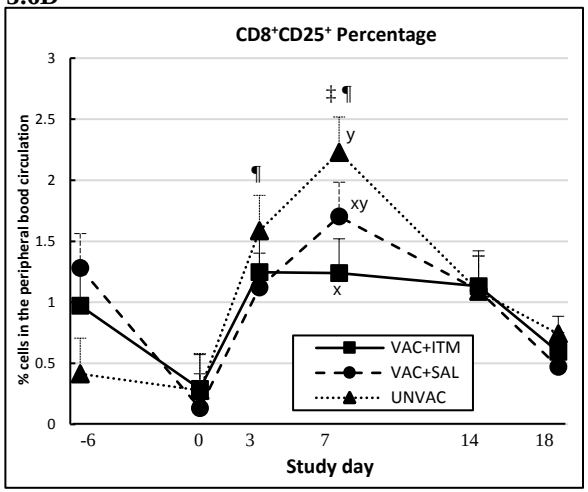
5.6B



5.6C



5.6D



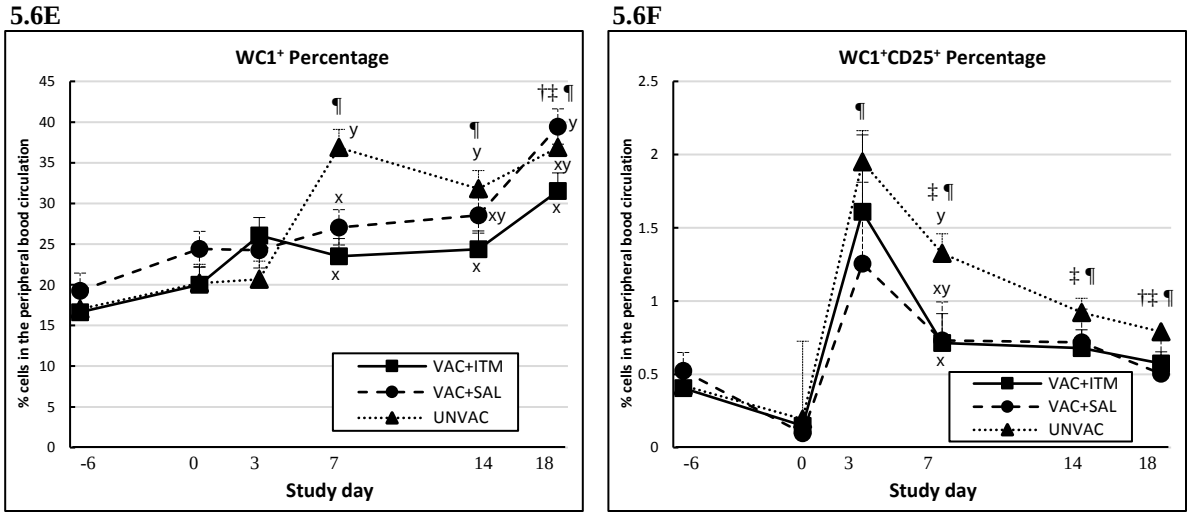


Fig. 5.6A-F. Least square means (LSM) and standard error of the mean (SEM) of percentage of CD4+ (A), $CD8^+$ (B), $CD4^+CD25^+$ (C), $CD8^+CD25^+$ (D), $WC1^+$ (E), and $WC1^+CD25^+$ (F) T-cells phenotype in the peripheral blood circulation for beef calves treated (VAC+ITM) or not (VAC+SAL) with injectable trace minerals (ITM) concurrently with MLV-BRD vaccination, or calves not treated nor vaccinated (UNVAC). Calves received treatments (ITM, saline or nothing, with or without vaccine) at study day -5, and were individually challenged with BVDV2 (ncp strain 890) five days later (day 0). Errors bars represent the SEM.

$^{x, y}$ Significant difference between groups by each study day ($P< 0.05$). † Significant difference from those on day 0 for VAC+ITM group ($P< 0.05$). ‡ Significant difference from those on day 0 for VAC+SAL group ($P< 0.05$). ¶ Significant difference from those on day 0 for UNVAC ($P< 0.05$).

Table. 5.1. Percentage (number) of positive results for BVDV 2 via real-time RT-PCR from buffy coat[1] and nasal swab[2] samples for beef calves treated (VAC+ITM; n= 15) or not (VAC+SAL; n= 15) with injectable trace minerals (ITM) concurrently with MLV-BRD vaccination, or calves not treated nor vaccinated (UNVAC; n= 15). All calves were individually challenged with BVDV2 strain 890 five days (day 0) post treatment (day -5). [3]Represents the total number of calves with at least one positive BVDV PCR result in all days out of 15 calves in each group (if a calf tested positive in more than one day, it will be counted only once).

Day	Buffy Coat[1]			Nasal Swabs[2]		
	VAC+ITM (n= 15)	**VAC+SAL** (n= 15)	**UNVAC** (n= 15)	**VAC+ITM** (n= 15)	**VAC+SAL** (n= 15)	**UNVAC** (n= 15)
0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
5	6.7% (1)	0 (0)	33.3% (5)	13.3% (2)	13.3% (2)	33.3% (5)
7	0 (0)	6.7% (1)	73.4% (11)	13.3% (2)	6.7% (1)	20% (3)
Cumulative[3]	6.7% (1)	6.7% (1)	93.3% (14)	26.7% (4)	13.3% (2)	46.7% (7)

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

The principal objective of this dissertation was to access the efficacy of ITM in promoting the health and growth of cattle. This was addressed by examining the capacity of ITM to enhance the immune response to BRD vaccines and the association of that enhanced immune capacity with the protection provided after challenge with a major component in the early phase of BRD pathogenesis, BVDV infection. The injectable trace minerals (ITM) evaluated in these studies contained Cu, Se, Zn and Mn. These minerals have been shown prior to this research to have an impact on the immune response. However, they had not previously been evaluated for their capacity to enhance the protection elicited by modified-live virus and attenuated-live bacterial vaccines in dairy and beef calves. The first study, described in Chapter Three, evaluated the immune response to a live-bacterial vaccine containing *M. haemolytica* and *P. multocida* either in concurrent treatment with ITM or without ITM in dairy calves. The bacteria contained in this vaccine represent the bacteria most commonly isolated from the respiratory tract of calves with BRD. Addition of ITM to the protocol provides a new strategy to improve the vaccine immune response against these bacteria; however, studies to evaluate the level of protection are warranted. Injectable trace minerals appear to have a positive effect on the development of useful immunity, and to reduce BRD morbidity with an associated reduction in antibiotic treatment costs. In that study,

administration of ITM concurrent with vaccination (at both the primary and booster vaccination delivery, 21 days apart) in 3-month old dairy calves resulted in an increased antibody titer against *M. haemolytica* between days 21 and 56 after primary vaccination compared with the control group (vaccine + saline). In addition, the ITM group had an enhanced PBMC proliferation following *P. multocida* antigen stimulation on day 21 after booster compared with day 0 that was not seen in the controls. Administration of ITM concomitant with vaccination appears to be a useful strategy to enhance the immune response following vaccination. Addition of ITM to the vaccine protocol has the potential to be a useful adjunct in prevention of respiratory disease in the dairy cattle. In addition, a careful review of the literature offered only a few published reports evaluating the immune response induced by bacterial vaccines in dairy calves. Thus, more research is warranted to fully evaluate and rationally develop better bacterial vaccines leading to predictable protection of calves to promote overall cattle health. In addition, studies to evaluate the level of protection (using a proven bacterial challenge model) elicited by bacterial vaccines are necessary.

The second study, presented in Chapter Four, again evaluated the effect of ITM used concurrently with MLV BRD vaccines. This study assessed the induction of long-term protection against BVDV challenge. The study utilized an intranasal moderate virulence BVDV type 2 challenge model in dairy bull calves. Long-term protection against BRD viruses is particularly important in production systems where calves cannot be frequently vaccinated. These production protocols rely on the immunity generated by a single MLV vaccine dose. This production protocol would ensure prevention of infection, and the resulting clinical disease, after exposure (e.g. BVDV-

PI calves) during the comingling process at the sale barn, backgrounder or feedlot, where nose to nose contact would commonly occur several months after vaccination. The results of this study showed that MLV vaccine administered at 3 months of age, and boostered three weeks later, was able to induce protection against moderate virulence BVDV type 2 challenge 5 months after vaccination. The protection was equivalent regardless the use of ITM in these bull dairy calves. Previous studies documented that ITM improved some aspects of humoral and cell mediated immunity (Palomares et al., 2016; Bittar et al., 2016, 2018). However, in this study, there were few effects attributable to ITM after BVDV type 2 challenge. Calves in the ITM group had lower total health scores on day 5 (lower health scores are associated with better health accordingly to the scale used in the study), higher platelet counts on days 6, 11, 14 and 18 compared to saline treated calves. In addition, they experienced higher ADG compared to non-vaccinated calves. These findings led to the conclusion that the use of ITM, administered at the time of vaccination against respiratory viruses and five months later, did not enhance the level of protection against BVDV type 2 clinical disease elicited by MLV vaccination in dairy bull calves. Further studies are necessary to effectively assess the effects of ITM on long-term clinical protection elicited by MLV vaccines against BVDV challenge, particularly in a model using high risk calves.

There is a considerable risk of newly received calves being exposed to BRD pathogens, and developing clinical disease when they arrive at the stockyard-feedlot. This risk is primarily associated with the considerable number of cattle producers who still do not vaccinate calves (USDA, 2010). An additional factor is the high level of stress calves are subjected to upon arrival, and during commingling with other cattle

from other places (that have unknown vaccination history and infectious status). Therefore, a rapid onset of protection following vaccination in naive calves would be highly beneficial to beef cattle production. The final clinical trial in this dissertation presented in Chapter Five, evaluated the development of the immune response against MLV BRD vaccine, and tested whether the onset of protection occurred by day 5 after vaccination. It also assessed if the protection elicited by the MLV used in conjunction with ITM (containing Se, Zn, Cu, and Mn) or without the ITM supplement were equivalent. The study utilized newly received, BVDV-naïve, beef calves. Five days following vaccination (with or without ITM), the calves were experimentally challenged with a moderate virulence BVDV type 2. The results of this study showed that BRD MLV vaccination, whether concurrent with ITM or not, induced rapid protection against BVDV type 2 challenge. In addition, the administration of ITM at the time of vaccination enhanced the humoral immune response to BVDV types 1 and 2, enhanced health status, appeared to mitigate the decrease of circulating peripheral blood $CD4^+$ and $CD8^+$ T-cells, and reduced T-cell activation in beef calves following challenge with BVDV type 2 five days after vaccination. Moreover, administration of ITM at the time of vaccination significantly increased platelet counts after BVDV challenge. These results support the strategic use of ITM concurrently with vaccination in novel protocols designed to enhance biological control of BRD. This is particularly true when a rapid immune response and development of protection is needed in newly received beef calves.

The overall evidence generated in this dissertation documents specific benefits of using ITM at the time of vaccination. Most of these benefits are associated with

enhancement of the immune response to vaccine following BRD vaccination in dairy and beef calves, with moderate enhanced efficacy of protection in beef calves. Some additional questions arose from the conduct of the experiments reported in this dissertation. They include; 1) Are there benefits of ITM supplementation on long-term vaccination protection under a high stress environment and in high-risk weaned calves?; 2) what would be an appropriate model that will allow for the assessment of lymphocyte subpopulations (in blood, organized lymphoid tissues and respiratory tract mucosa) after vaccination and experimental infection with BRD pathogens? This model would provide a clearer picture of the dynamics of immune response (activation, differentiation, memory, trafficking, and homing) and protection elicited by vaccination concurrent with ITM administration; and 3) What are the physiological mechanisms behind the sparing of platelets in beef calves treated with ITM as observed in the third study? Finally, the overall conclusion of this dissertation is that the use of ITM concurrent with vaccines (either MLV or live-attenuated bacteria) was able to provide benefits to the development of humoral and cell mediated immunity, and support for health and growth performance. These findings offer new insights that should advance the development of new protocols for enhancing cattle herd health in livestock production in North America. This would likely influence the capacity of producers and veterinarians to reduce the use of antibiotics without losing the ability to achieve improved health and productivity by decreasing BRD prevalence.