IMMUNE RESPONSE AND PROTECTION AGAINST BVDV-2 AND BHV-1 INFECTION ELICITED BY MODIFIED-LIVE VIRUS VACCINATION IN DAIRY CALVES. EFFECTS OF VACCINATION ROUTE AND TRACE MINERAL INJECTION

by

ALEJANDRO HOYOS JARAMILLO

(Under the Direction of Roberto A. Palomares Naveda)

ABSTRACT

Bovine respiratory disease (BRD) is a multifactorial and polymicrobial disease impacting the cattle industry, often affecting young calves. Additionally, calves undergoing pneumonia are constantly treated with antibiotics, raising concerns for future antimicrobial resistance. *Bovine viral diarrhea virus 2* (BVDV2) and *Bovine herpesvirus 1* (BHV1), play significant role in BRD pathogenesis. Prevention strategies include Modified-live virus (MLV) vaccines. These MLV have demonstrated to prevent from clinical disease. Intranasal vaccination is considered a promising tool for priming young calves. Additionally, trace minerals are important components of antioxidant enzymes and vital microelements for growth, health, immunity, and reproduction. Trace minerals have been reported to improve humoral and cell-mediated immune responses. The main objectives of this dissertation were to evaluate the effects of supplementation with trace minerals, compare the available routes for MLV vaccination, and examine the role of the humoral and cell-mediated immunity induced in protection against BRD pathogens. Moreover, we investigated the effect of trace mineral supplementation along with MLV vaccination on the induction of immunity and its correlation to the level of protection against challenge with

BVDV2 and BHV1. The first study, calves treated with ITM had significantly higher BRSV-SNA titers on day 14 ($p = 0.045$), and day 28 ($p = 0.028$) than control calves. In the second study, unvaccinated calves developed significant leukopenia than vaccinated calves. Moreover, calves treated with ITM and IN booster had a higher number of circulating $CD8^+$ T cells. In contrast, calves receiving SC booster vaccination had a higher number of circulating $CD4^+$ T cells. In the third study, calves in the unvaccinated group presented higher lesion scores by endoscopic examination, five days after BHV1 challenge than for calves receiving ITM plus vaccination (either SC or IN route; $p < 0.01$). The findings from this dissertation provided new insights about booster vaccination route in dairy calves. Further, the results indicated that ITM enhanced the immune response when delivered at priming and booster vaccination. Finally, this dissertation generated multiple new questions that need to be addressed in future research projects to define the role of vaccine immunity and improve viability of cattle rearing and management.

INDEX WORDS: Bovine respiratory disease; injectable trace minerals; cell-mediated immunity; humoral immunity; BVDV2 challenge; BHV1 challenge.

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DEDICATION

Dedicated to the ones who inspired me, trusted me, and supported me during all these years; Carlos, Vicky, Esteban, and Rose, this is all for you. To those from heaven who see us, be the glory of God to you until reuniting us all. Lucelly Salazar Lopez, Jose Duvalio Jaramillo Galvis, Carlos Eduardo Jaramillo Salazar, Dora Hoyos Hoyos. Your memories will forever pass through generations to come, as your smiles have not been forgotten by the ones still living your legacies.

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CHAPTER 1

INTRODUCTION AND OVERVIEW

A move to more intensive production by the cattle industry in response to the increased demand by consumers for milk and meat has placed a significant increase in the expectations for the capacity of livestock animals to yield higher levels of product. This has led to enhanced behavioral stress, increased exposure to environmental pathogens, due to higher stocking density, with higher incidence of disease. This is most pronounced among young animals during the developmental phase for production.

Bovine respiratory disease (BRD) is the most common cause of morbidity in the dairy and beef industry (USDA, 2013), and the leading cause of mortality in weaned dairy heifers (USDA, 2014). This polymicrobial and multifactorial disease complex is responsible for what is generally recognized as the greatest source of economic losses in the cattle industry worldwide (Griffin, 1997; McVey, 2009). Multiple factors such as environmental extremes, high stocking density, stress related to weaning, transportation, and commingling have all been identified as contributing factors to the development of BRD (Snowder et al., 2006; Schneider et al., 2009). Despite the use of prophylactic antimicrobials and programed use of vaccines, the mortality rates have remained essentially unchanged during the last decade. Under this management program, an increase of antimicrobial resistance has also been documented (Miles 2009; Gagea et al. 2006).

Viral infections, specifically Bovine viral diarrhea virus (BVDV) and Bovine herpesvirus 1 (BHV1), play significant roles in the development of clinical BRD. These viruses induced a

state of immunosuppression, disrupt the respiratory mucosa, and induce significant changes to the respiratory tract microbiota that facilitate secondary bacterial infection (Ellis et al., 2010; Walz et al., 2010; Holman et al., 2015). Therefore, the disease is characterized by coinfection and synergism between pathogens. For example, a primary infection with BHV1 is often followed by secondary infection with *Mannheimia haemolytica* that can lead to fatal bovine respiratory disease (Hodgson et al., 2005).

Vaccination programs have been shown to prevent clinical disease, but with 100% efficacy (Chamorro and Palomares, 2020). One major reason vaccines are not entirely effective is based on the ability of some viruses, even vaccine viruses, to impair the immune system. Bovine viral diarrhea virus has a specific tropism for mucosal-associated lymphoid tissue, leukocytes in lymphoid organs, and circulating lymphocytes as the major virus targets (Bruschke et al., 1998; Liebler-Tenorio et al., 2003A, Walz et al., 2010). Leukopenia induced by BVDV is associated with the apoptosis and necrosis of leukocyte populations. This is associated with migration of lymphocytes into tissues where viral replication is highly active. This often leads to depletion of both T and B cells in mucosal and systemic lymphoid tissues (Liebler et al., 1995, Kelling et al., 2002; Walz et al., 2010; Palomares et al., 2015). $CD4^+$ and $CD8^+$ T lymphocytes are the principal T cell populations affected in naïve or unvaccinated calves following challenge with BVDV2. This appears to enhance viral pathogenesis by impairing T helper and cytotoxic effector function in the infected animal (Howard et al., 1992; Brodersen and Kelling, 1999; Bittar et al., 2020).

Bovine herpesvirus type 1 remains in the body, sequestered from recognition by the immune system, following acute infection by establishing latency in the sensory neurons and leukocytes. Latency is pushed to reactivation of the virus by stress events (often cortisol),

yielding active viral replication with shedding and an increased capacity for transmission among herd mates (Jones and Chowdhury, 2010).

Another characteristic of BHV1 pathogenesis is the capacity to suppress the expression of the major histocompatibility complex class I (MHC-I) on the surface of infected cells. This inhibits recognition of infected cells by $CD8^+$ T cells and their normal immune mediated removal from the body (Nataraj et al., 1997). Further, the viral cytopathic effect on muco-epithelial cells lead to erosion of the mucosal surfaces of the upper respiratory tract. This alteration in the normal barrier function increases susceptibility to secondary infections. This generally results in enhanced and deeper bacterial colonization with subsequent development of bronchopneumonia (Jones, 2019; Panciera and Confer, 2010; Risalde et al., 2013). Moreover, similar to BVDV, BHV1 can impair the function of CD4+ and CD8+ T lymphocyte populations (Howard et al., 1992, Winkler et al., 1999).

Modified-live virus (MLV) vaccines stimulate cell-mediated and humoral immune responses by activating clones of T and B lymphocytes (Theurer et al., 2015). These immune responses in combination are able to prevent clinical disease, reduce virus replication, decrease the level of viremia, and reduce viral shedding (Rhodes et al., 1999; Kelling et al., 2007; Chamorro et al., 2016a). An essential goal of cattle health programs depends on effective vaccination and good biosecurity to prevent BRD in young calves even when they have maternal antibodies (Palomares et al., 2016). The evidence suggests that parenteral vaccination of calves in the face of maternal antibodies (IFOMA) often results in a weak or limited humoral response due to binding of the vaccine antigens by maternal neutralizing antibodies (Kimman et al., 1989; Ellis et al., 2001; Endsley et al., 2004). However, other authors have found that young animals vaccinated IFOMA can mount an immune response to vaccination that stimulate de novo cell-

mediated immunity, and more importantly, protect them from disease (Van Donkersgoed et al., 1995; Endsley et al., 2004; Rowe et al., 2004). In fact, studies in our laboratory demonstrated that dairy and beef calves vaccinated IFOMA were able to mount both enhanced humoral and cell-mediated immune responses against BVDV after vaccination, and the calves were protected when challenged with BVDV2, showing no development of clinical disease (Palomares et al., 2016; Bittar et al., 2018). These calves exhibited increased BVDV1 and 2 serum neutralizing antibody titers, had decreased evidence of viremia, and reduced levels of viral shedding in the nasal secretion after experimental BVDV2 challenge (Palomares et al., 2016; Bittar et al., 2018; Bittar et al., 2020).

The most common route of entry for BRD viruses through the nose leading to infection of the upper and lower respiratory tract (Hill et al., 2019). In a previous study, calves vaccinated intranasally with an MLV vaccine had a more rapid protective response when challenged with the combination of BHV1 and *Mannheimia haemolytica* than calves vaccinated with a parenteral MLV vaccine (Woolums et al., 2003). Moreover, intranasal (IN) MLV vaccination appears to represent the most promising route for immune priming in young calves when vaccinated IFOMA. Intranasal vaccination appears to allow a path for avoiding interference of maternal antibodies (Ellis, 2017). Further, IN vaccination has the potential to enhance the mucosal innate and IgA response to specific pathogens at mucosal surfaces. This appears to be particularly true for those entering the body by the respiratory route (Chamorro et al., 2016b, Ellis, 2017). In addition, IN vaccination has been associated with inducing a long-lasting mucosal immune memory that increased the speed of release and the level of antigen-specific secretory IgA (Hill et al., 2012; Hill et al., 2019). Intranasal vaccination also enhances the development of protective systemic immunity against viral infections (Kimman et al., 1989; Vangeel et al., 2007 and 2009;

Xue et al., 2010; Windeyer and Gamsjäger, 2019). Currently, it is common practice for cattle producers to prime vaccinate newborn dairy calves IFOMA intranasally with an MLV vaccine, with a subsequent booster vaccination at around two to three months of age (Woolums, 2007). Moreover, the booster vaccination route (parenteral vs. mucosal) is still under debate (Ellis, 2017). Despite the reported benefits of IN vaccination in young calves (Hill et al., 2012), it has been hypothesized that induction of mucosal antibody and IgA memory may limit virus replication in the nasal mucosa if a booster vaccination is given by the nasal route. This may potentially impairing the efficacy of the development of an anamnestic response following that booster vaccination (Chamorro et al., 2016b).

Trace minerals are essential nutritional elements for optimal health and performance in cattle. There are ten microminerals generally recognized to be required for proper nutrition in cattle. Of those, six have a documented practical relevance to health and growth when provided as a supplementation strategy in grazing cattle. These minerals are: Se, Cu, Zn, Mn, I, and Co (Arthington et al., 2021). Selenium is commonly deficient in cattle consuming forage-based diets. Many regions of the US have soils that is selenium deficient. Thus, supplementation with Se in these regions is clearly necessary (Stewart, 2010). The Food and Drug Administration (FDA) established limits on formulation of diets at 0.3 mg Se/kg. A deficiency in Se and Vitamin E is associated with White Muscle Disease (WMD), a syndrome representing a nutritional myo-degeneration in cattle (Van Metre et al., 2001). It is associated with an increased incidence of retention of fetal membranes (Hidiroglou et al., 1987), and other less frequent disorders. Selenium is a cofactor in several metalloenzyme systems. These include glutathione peroxidase, which is one of the most important antioxidants in mammals. Glutathione peroxidase (GPx) catalyzes the conversion of reactive oxygen species (ROS) into less reactive water and alcohols.

This prevents oxidant induced cellular damage (Sordillo and Raphael, 2013). In addition, GPx plays a significant role in neutrophil migration, bacterial phagocytosis, and microbial killing (Maddox et al., 1999; Van Metre et al., 2001; Maggini et al., 2007).

Copper is the second most commonly deficient mineral in grazing cattle (Stewart, 2010). Further, the interaction with antagonist such as sulfur has become more prevalent due to the common use of ammonium sulfate fertilizers (Arthington et al., 2002). Copper is essential for the assembly and function of antioxidant enzyme structure. Superoxidase dismutase (SOD) is a copper containing enzyme complex, and other Cu-dependent enzymes, such as ceruloplasmin, function as acute phase proteins. Copper deficiency is manifested by reduction of the antibody production, a decrease in phagocytic activity, a reduction in growth of the animal, and in a delayed onset of puberty (Phillipo et al., 1987; Arthington et al., 1995).

Zinc is an essential component of several metalloenzymes including SOD. It has an important role in nucleic acid replication and transcription. Zinc has a direct influence on rapidly dividing cells (mucosal epithelium, spermatogenesis, hoof health), and cells of the immune system (particularly T cell proliferation). There is no reliable tissue pool to serve as an indicator of Zn bioavailability in the organism. Thus, we do not fully understand the mechanisms involved in problems associated with Zn deficiency (Mortimer et al., 1999; Duff and Galyean, 2007).

Manganese is also a component of the Mn-dependent complex forming SOD. It serves as an antioxidant that is located inside the mitochondria. Mn is also a component of the glycosyl transferase complex. This complex is required for proper bone development and has an essential role in neutralizing ROS produced as byproduct of phagocytic cells (Tomlinson et al., 2008; Arthington et al., 2021).

Adequate trace mineral supplementation is required for many vital physiological processes. These included the immune response, reproduction, and animal growth (Spears and Kegley, 2002). In 2007, Duff and Galyean described the status of micronutrients as one of the core factors contributing to BRD incidence. Previous evidence showed a beneficial effect of using injectable trace minerals (ITM containing Se, Zn, Cu, Mn. Multimin-90®; Multimin –USA Inc ®) on the immune response when administered concurrently with an MLV vaccination (Arthington and Havenga, 2012; Palomares et al., 2016; Bittar et al., 2018). Our laboratory (and others) has performed studies on calves treated with ITM at the time of BRD virus vaccination that demonstrated improvement in both the humoral and cell-mediated immune responses generated. The studies documented higher specific serum neutralizing antibodies (SNA) titers, and higher levels of recall lymphocyte proliferation to vaccine antigens (Arthington et al., 2014; Palomares et al., 2016). The studies also documented an enhancement in the protection against clinical disease induced by BVDV2 experimental challenge. Further, in a study using highly stressed calves, the use of ITM induced a more rapid increase in BVDV1 specific antibody response when given on arrival at the feedlot (Roberts et al., 2016). The effects of ITM on the immune response generated and the protection elicited by IN vaccination in response to coinfection with BVDV2 and BHV1 challenge have not been previously evaluated and were subject of the research in this dissertation.

Hence, the outcomes of the research presented here may contribute significantly to the baseline knowledge of vaccine immunity to protect against the earliest stages of BRD. This will allow us to better understand the response of young dairy calves to: 1. intranasal primary vaccination, 2. booster vaccination by the IN and SC route, and 3. the role trace mineral pulse dose supplementation at the time vaccination plays in the quality and efficacy of vaccination.

The findings of this research may result in practical recommendations for calf management programs to enhance health and performance of dairy calves as they develop into replacement heifers in the herd and in developing beef calves.

This dissertation combines the results of three semi-independent randomized clinical trials examining modes of priming and booster vaccination, evaluating the immune responses generated by IN primary vaccination concurrently delivered with trace minerals supplementation (Se, Cu, Zn, and MN) in one-month old calves, both prior to the time of booster vaccination and after challenge. Further, the effects of trace mineral administration at 3.5 months of age concurrent with the booster vaccine. Finally, a trial testing experimental viral infection with both BVDV2 and BHV1 was performed seven weeks after the booster vaccinations to assess the efficacy of each booster vaccination protocol relative to trace mineral supplementation in protection of calves against development of classical clinical disease caused by these viruses. These studies were designed to compare the effects of the route of administration of the booster vaccination and the impact of trace minerals supplementation as a preventive strategies in the control BRD in young dairy calves.

Chapter two compiles a review of the literature to provide a clear context for this research with respect to the state of knowledge at the time of its design. The list of topics included here are: the epidemiology of BRD, the biology and pathology of the specific viral etiologic agents involved in the initiation of BRD (BHV1, BVDV, BRSV, BPI_3V, and BCov), calf's neonatal immunity, the factors affecting immunity of calves, mucosal immunity, an overview of the organization and function of humoral and cell-mediated immunity, a review of the diagnostic tools use in BRD assessment, and a review of current vaccination practice for BRD. Also, a summary review of the currently understood the role of trace minerals in animal health. Chapter

three represents the first trial in the project. This study was performed on a commercial dairy farm in Quitman, GA. The main objective of the studies were to determine the effect of trace minerals at the time of prime vaccination with an IN MLV vaccination on the humoral immune response and expression of pro-inflammatory & anti-inflammatory cytokines. Chapter four represents the trial in the project as it is continuation of the primary vaccination clinical trial, utilizing the same experimental animals. In this study the calves were boostered with MLV vaccines administered as IN or SC concurrent with injection of either trace minerals or saline solution. Seven weeks after booster vaccination, all calves were challenged intranasally with BVDV2. This was followed one week later with a BHV1 challenge dose. The main objectives of this trial were to evaluate the effects of the route of booster vaccination relative to the presence of trace minerals on the differential leukocyte count, dynamics of circulating T cell subpopulations, and the protection from development of classical clinical disease conferred by the vaccination after dual viral challenge. Chapter five presents the summary of the clinical outcomes observed before and after the sequential experimental challenge with BVDV2 and BHV1 to complete the reporting of the data generated by the previous studies. The main objectives were to evaluate the evidence of clinical protection induced by MLV vaccination in conjunction with trace minerals supplementation relative to BVDV2 and BHV1 challenge infection in the respiratory tissue by using endoscopy of the upper respiratory tract, an estimation of viremia, and measurement of viral shedding in nasal secretion. Finally, chapter six brings the whole program together with an overall discussion of the results focused on an integrated interpretation of the study findings. This includes extrapolation of the models examined to project potential impacts on future calf health programs.

CHAPTER 2

REVIEW OF THE LITERATURE

Bovine respiratory disease (BRD). Epidemiology in pre- and post-weaned dairy calves

Bovine respiratory disease (BRD) is a multifactorial illness that affect cattle industry resulting in substantial economic losses due to higher morbidity and mortality and the associated treatments cost. The National Health Monitoring System (NAHMS) reported in 2012 that 18.1% of preweaned dairy heifers were diagnosed with BRD in the US (USDA, 2012). A more recent study of California dairies reported that 22.8% of preweaning calves were diagnosed with and treated for BRD, and that 19% of all deaths in that age range could be attributed to BRD (Dubrovsky et al., 2019). Respiratory infections, leading to clinical pneumonia in affected calves, have a significant impact on their growth performance compared to calves with no obvious signs of pneumonia (Wittum et al., 1996). In US dairy operations, BRD is the principal cause of morbidity in preweaning calves, and the most common cause of mortality in weaned heifers (USDA, 2012 & 2014). Despite advances in treatments and preventive measures over the past several years, the prevalence of BRD in dairy calves has remained similar over the last 15 years (Chigerwe et al., 2015). The economic losses associated with BRD in dairy operations include: the cost of treatments for clinical disease, the losses associated with culling before first calving, an increase in heifers with dystocia, heifers showing reduced growth, reduced milk yields during first lactation, and other lesser costs (Windeyer et al., 2014; Schaffer et al., 2016).

The general occurrence of BRD in the US has been reported to have a prevalence of 18.1% in preweaned heifers. The prevalence of BRD in weaned dairy heifers was 11.2%, but only 1.2% in pregnant heifers. The general rate due of mortality due to BRD in dairy operations was 2.3% overall. Weaned heifer calves and pregnant heifers has a mortality rate of 1.3% and 0.2%, respectively (Dargatz et al., 2014). The factors associated with BRD morbidity and mortality include substandard housing, poor ventilation, and poor hygiene conditions that contribute remarkably to the risk of BRD in dairy operations in the US. In some regions of the US, that have harsh winter conditions, there is a need for indoor housing. This increases the risk for BRD om these herds. In contrast, regions of the US where the cold is not less extreme, outdoor housing is the most common practice and it reduces the BRD risk (Lago et al., 2006; Losinger et al., 1996; Svensson et al., 2003).

Multiple methods have been used in an attempt to define the prevalence of BRD in California dairies and on calf ranches (McGuirk, 2008; Love et al., 2014). One method included the use of a respiratory disease scoring system. This model reported an estimate prevalence of BRD in 100 dairies that were included of 6.9% (Karle et al., 2019). Thoracic ultrasonography has been recently developed as a diagnostic adjunct. This is used to detect lung consolidation patterns associated with clinical and subclinical pneumonia (Love et al., 2016; Ollivett et al., 2016). The study performed by Love and collaborators (2016) reported a BRD prevalence of 31.7% in the herds assessed. The difference among estimates may be related to the differential sensitivity of the methods used to identify the BRD clinical cases. Another study, in the same geographical region, reported that 22% of dairy calves were diagnosed and treated for BRD before weaning (Dubrovsky et al., 2019). The understanding of the high prevalence of BRD, and the role of the host immune response to the severity of respiratory infection, should help close

the gap in our knowledge of the parameters that can be modulated to reduce BRD prevalence in heifer stock. Thus, it is important that future studies on BRD be conducted.

Viral agents involved in BRD

Bovine respiratory disease is a disease that involves coinfections and interactions among several pathogens. The pathogenesis involves several viruses and multiple bacteria, including several that are common upper respiratory flora (Panciera and Confer, 2010). In addition, multiple host factors, the quality of farm management practices (including animal flow and isolation of young animals from the standing milking herd), the effects of extreme environmental conditions, and nutritional and feed management stressors are all involved in the development of BRD and the level of its severity (Edwards et al., 2010; Taylor et al., 2010). It is generally believed that several viruses can provide the initiation of the pathogenesis of BRD by disrupting the epithelial barrier of the respiratory tract, altering inflammatory homeostasis, and causing immunosuppression. These provide a pathway to predispose the calves to secondary infections caused by a change in bacterial growth and migration in the respiratory tract (Panciera and Confer, 2010). The viruses most commonly involved in early BRD pathogenesis are *Bovine herpes virus 1* (BHV1), *Bovine viral diarrhea virus* (BVDV), *Bovine respiratory syncytial virus* (BRSV), Bovine parainfluenza-3 virus (BPI_3V), and *Bovine coronavirus* (BCoV). The biology, pathogenesis, and clinical relevance of these viruses within BRD will be described in the rest of this section.

Bovine herpesvirus 1 (BHV1)

Bovine herpesvirus 1 is a double-stranded DNA virus composed of an icosahedral nucleocapsid and an envelope connected by a tegument. It belongs to the family *Herpesviridae*, subfamily Alphah*erpesvirinae* (Turin et al., 1995). There are three defined subtypes, including BoHV1.1, BoHV1.2a, and BoHV1.2b (Metzler et al., 1985). Subtype 1 target respiratory and reproductive tract, most commonly seen as infectious rhinotracheitis (IBR) and abortions. This subtype of BHV-1 is more prevalent in Europe, and in North and South America. Subtype 2a is most commonly associated with respiratory and genital infections. The most frequently diagnosed are IBR, infectious pustular vulvovaginitis (IPV), balanopostitis (IPB), and abortions. This subtype is commonly isolated in Brazil. Subtype 2b is more prevalent in Australia and Europe, and associated with respiratory disease, vulvovaginitis, balanopostitis, but not abortions (Oirschot et al., 1995; D'Arce et al., 2002).

The most common modes of transmission include nose to nose contact, airborne via droplets, and venereal transmission by either artificial insemination or natural mating (Oirschot et al., 1995; Jones and Chowdhury, 2010). Incubation period is around 2 to 6 days. Typical clinical signs of infection are known as "Red nose" and generally include common respiratory disease symptoms (fever, anorexia, coughing, nasal discharge, and dyspnea), conjunctivitis, swollen nostrils, and obstruction of upper respiratory tract with an excess of purulent secretions (Yates, 1982). It is also common that visible foci of petechial and ulcerations in the upper respiratory tract mucosa, around nasopharynx and trachea are observed (Gershwin et al., 2014); and some necrotic foci in the septal mucosa may be observed (Jones and Chowdhury, 2010). Genital infections are characterized by frequent urination and mild vaginal infection (Oirschot et al., 1995, Yates, 1982). Secondary bacterial infections in the reproductive tract and transient

infertility in female cattle are often observed. In bulls, it is common to see pustular lesions around prepuce and penis (Yates, 1982; Bowland and Shewen, 2000; Jones and Chowdhury, 2010). Regardless of the viral sub-type, BHV1 establishes lifelong latency in the trigeminal and sacral ganglia after clinical infection (Tiko et al., 1995; Jones and Chowdhury, 2010).

There are a sequential set of proteins expressed during BHV-1 infection that are encoded by a set of temporally regulated genes (Figure 1.1). These genes are called immediately early (IE), early (E), and late (L) genes. The IE genes are stimulated by the action of the tegument protein, VP16, upon viral entry (Jones, 1998 & 2003). When the IE transcription process is activated, two proteins are generated within the IE transcription unit 1, IEtu1 and IEtu2. At the same time, IEtu1 is spliced and translated into two transcriptional regulatory proteins BICP0 and BICP4 (Wirth et al., 1989 & 1991). The BICP0 protein is post-transcriptionally translated into EmRNA (E2.6). The protein BICP4 plays an important role activating the E promoter of E2.6 and regulating the transcription of IEtu1. The BICP0 protein has a "RING finger" protease function that participates in productive infection (Inman et al., 2001; Saira et al., 2008). Generally, proteins transcribed from E genes do not have structural roles. Their expression requires *de novo* DNA synthesis, including the family of DNA polymerase enzymes, thymidine kinase, ribonucleotide reductase, dUTPase, and origin binding protein. The family of L genes encode the structural proteins of the virus. They direct the virion assembly and release. Further, the family of L genes are divided in two classes, Gamma-1, and Gamma-2. Both classes play a role in transcribing *de novo* BICP0 and BICP4 (Jones, 2019). The viral replication cycle occurs inside the host cell, taking over the nuclear replication system of the cell. This is a very stable replication process generating minimal genetic changes to the viral genome. Host error correction systems involved in DNA proof reading function during BHV1 viral replication. Thus,

mutations are rarely seen (Kaashoek et al., 1996). Acute infection begins with replication in mucosal tissues. This results in high levels of epithelial apoptosis (Jones, 1998 & 2003).

The latency process is initiated when the virus replicates in the oral, nasal, or ocular cavity. From these tissues, the virus enters to the peripheral nervous system by cell-to-cell spread. The virus replicates in nerve cells until it reaches its primary target, the trigeminal ganglia. Once the virus reaches the trigeminal ganglia expression of lytic genes is shut down. To establish latency, the latency genes are switched on (Schang et al., 1997; Inman et al., 2002). The viral particles can be found in the nervous system for two to six days after acute infection (Schang et al., 1997). Certain peripheral lymphoid tissues, primarily the tonsils, are believed to be additional sites for latency of BHV1 (Muylkens et al., 2007). Latency related genes (LR) are expressed in neural tissues during the latent state. The products of some additional genes, not generally associated with acute infection, can also found during latency (Jones, 1998 & 2003; Jones et al., 2006). The LR gene family modulates the expression of the B1CP0 gene (Bratanich et al., 1992; Hossain et al., 1995). This keeps the virus in a state of latency and hidden from the host. The LR genes contain two open frames ORF1 and ORF2, that lack an important amino acids (AA) sequence required to initiate the infection. The ATG sequence is required for acute infection (Bratanich et al., 1992; Hossain et al., 1995). ORF2 has an anti-apoptotic mechanism that regulates the latency-reactivation cycle (Ciacci-Zanella et al., 1999; Shen et al., 2008; Jaber et al., 2010). Stress stimuli or its analog, dexamethasone treatment, promote the expression of IEtu1 that drives the upregulation of the viral proteins BICP0 and BICP4. This activates the lytic cycle in nerve tissue. This is the first step in re-activating the infection from its latent state (Winkler et al., 2000; Frizzo et al., 2013; kook et al., 2015). Stress-mediated re-activation of BHV-1 stimulates the family of E promoters. The encoded viral transactivators reactivate the

virus from the latent stage and support viral spread to new tissue sites (Barnes et al., 1998; Schonevild et al., 2004).

A portion of the immune response against BHV1 can be evaluated by measuring SNA titers in circulation. These antibodies recognize and attach to envelope glycoproteins: gB, gC, gD, and gH, of BHV-1 (Marshall et al., 1988; Van Drunen Littel-van den Hurk et al., 1986). Cell-mediated immunity is very important clearing acute BHV-1 infection through the action of $CD8^+$ T cells (Denis et al., 1993; Huang et al., 2005; Van Drunen Littel-van den Hurk et al., 2007). However, there are viral mechanisms that impair the immune response at several levels, including T cell recognition of infected cells (Hariharan et al., 1993; Hinkley et al., 1998; Nataraj et al., 1997), induced apoptosis of CD 4^+ T cells (Eskra et al., 1997; Winkler et al., 1999), inhibition of IFN production (Saira et al., 2007 & 2009; Jones, 2009), and impairing CD8+ T cell recognition of BHV-1 infected targets by down regulation of surface MHC I expression (Griebel et al., 1987 & 1990; Carter et al., 1989). The BICP0 protein contains transcriptional activation domains that stimulate a productive infection and disrupt antiviral IFN β promoter activity (Jones, 2009). The inhibition of IFN β occurs when BICP0 protein reduces the expression and transcription of IRF3 protein regulators in host cells (Saira et al., 2009). Another mechanism that interferes with the innate immune response involves viral BICP27 protein expressed by an early viral promoter. This immunosuppressive mechanism interferes with the stimulator of interferon genes (STING). This is a complex of genes upregulating primarily the type 1 IFN responses (Johnson et al., 2008; Sandri-Goldin, 2011). In addition, down regulation of MHC class I expression and its transport to the cell surface occur due to the action of viral Glycoprotein N (gN) of BHV1. This protein disrupts TAP-mediated transport of cytosolic components of cell surface complexes into the endoplasmic reticulum (ER) for assembly and transport. Once

trapped in the ER the peptides are degraded and recycled by the cell. Thus, in this manner BHV1 blocks the role of MHC class I in antigen presentation and recognition of infected cells by armed CD 8^+ T cells (Ambagala et al., 2004). The most abundant tegument protein present in the virion is VP8. This protein is a potent IFN antagonist. It functions by preventing STAT1 from entering to the nucleus, thus inhibiting the IFN γ production core transcriptional receptors, Jak 1 and Jak 2. All these immune disrupting mechanisms make BHV1 a successful pathogen. They also represent a significant challenge to disease control once the virus is established in the herd (Jones, 2019).

There are many commercially available vaccines constructed in different ways on the market. The most commonly licensed vaccines for BHV-1 are either modified-live virus (MLV) or inactivated vaccines (Van Drunen Littel-van den Hurk et al., 2005). Modified-live virus vaccines are usually beneficial to herd health and reduce economic losses due to BHV-1. Modified-live virus vaccines induces functional cell-mediated (CMI) and humoral immune responses, and lasting memory. Nevertheless, rarely MLV vaccines may revert to virulence in a host causing vaccine associated abortion or respiratory disease. This is most often documented when the immune system is compromised (Jones and Chowdhury, 2010). In contrast with MLV, inactivated vaccines have a greater safety profile, never reverting to virulence. They have a higher cost as they require two doses and an adjuvant to achieve the desired level of effector immune response. Killed BHV-1 vaccines also tend to induce less CMI and often do not have strong memory development. Some commercially available MLV vaccines have been shown to induce a CMI responses by activating CD 4^+ and CD 8^+ T cells when adjuvanted properly (Endsley et al., 2002). Experimental genetically engineered vaccines have been produced and tested under controlled conditions (Chowdhury et al., 2014). Some studies, including a vaccine

containing gC, gG, or TK-deleted viruses, have showed significant reactivation of latent virus following initial vaccination with some degree of virulence in vaccinated cattle. Thus, these vaccines are less efficacious and have not been licensed (Kaashoek et al., 1996a & b). Another vaccine candidate, containing an LR mutant virus, has showed promising results. It has reduced post-vaccine virulence than some whole virus MLV and will not reactivate from latency. The results suggest that this will be a good vaccine candidate for the future (Inman, et al., 2002).

Bovine viral diarrhea virus (BVDV)

Bovine viral diarrhea virus (BVDV) is an enveloped, single-stranded positive sense RNA virus. BVDV belongs to the family *Flaviviridae*, genus *Pestivirus* (Darbyshire, 1969; Schweizer et al., 2014). The viruses in the genus *Pestivirus* produce a large polyprotein that is divided after transcription into several structural and nonstructural proteins. Four structural proteins (SPs) have been identified. They are a basic core protein (C), and three envelope glycoproteins (E^{rns}, E1, E2). The envelope glycoproteins are inserted into the membrane of the envelope (Lindenbach et al., 2013). Based on nucleotide sequencing the *Pestivirus* genus has been divided into four species: classical swine fever virus, BVDV type 1 and 2 viruses, and the border disease virus of small ruminants (Simmonds et al., 2012). In addition, a few atypical pestiviruses, that have been named HoBi-like viruses, have been isolated from other animal species. The original was the Giraffe-1 pestivirus isolated during an outbreak of disease in giraffes in Kenya (Harasawa et al., 2000). The first report of HoBi-like pestiviruses in cattle came from Brazil and was associated with Brazilian fetal bovine serum (Schirrmeier et al., 2004).

There is a special feature of *pestivirus* that further divides them into two biotypes. In cultured cells some are cytopathic (cp), leading to death and lysis of the infected cells, and some

are noncytopathic (ncp), replicating in the host cells without causing obvious damaging the integrity of the cells or significantly halting cell growth (Charleston et al., 2001).

The presence of three surface proteins, E^{rns}, E1, and E2 that are co-expressed, is an unusual feature of the pestivirus family (Figure 1.2; Lindenbach et al., 2013). The N^{pro} protein is an autoprotease. N^{pro} protein cleaves its own carboxy-terminus, generating an amino-terminal end for the C protein. The C protein signals the translocation of E^{rns} into the endoplasmic reticulum. The resulting product is a C protein with a transmembrane (TM) sequence. This allows the release of the C protein from the membrane (Heimann et al., 2006). The next processing step in viral assembly is at the NS2- NS3 site. This processing plays a key role in regulation of the RNA replication (Elbers et al., 1996;). N^{pro} is the first protein encoding on the pestiviral genome. This unusual protein is important in regulating the interaction between host and virus. It induces degradation of IRF 3 in the proteasome and blocks the IFN response (Behrens et al., 1998; Ettema et al., 2003; Gil et al., 2006; Mayer et al., 2004; Bauhofer et al., 2007). The C protein has a variable length. This makes it susceptible to natural mutations and insertion of host genes (Riedel et al., 2010). E^{rns} protein is a unique protein with no homologue present in the host or other families of virus. It is translated immediately after the C protein during early infection. The importance of the E^{rns} protein as an envelope glycoprotein lays in its roll in the attachment to epithelial cells. (Iqbal et al., 2000; Lindenbach et al., 2013; Stapleton et al., 2011).

Transmission of BVDV can occur by direct nose-to-nose contact, or indirect exposure (e.g., saliva, nasal discharge, urine, feces, semen, and milk, in the air or on surfaces -including fomites). Pestiviruses are actively shed from all the mucous membranes. This increases the risk of infection for susceptible animals in contact with or near those infected. Further, vertical

transmission can occur when the virus crosses the placenta and infects the fetus. This can result in six specific outcomes: abortions, stillbirths, congenital defects, immunotolerance leading persistent infections, or production of a calf which has a normal immune system and some IgM antibody. The last happens if the infection occurs after the full immune system is developed in the fetus (Choi et al., 2002; Meyling et al., 1990; Thiel et al., 1996; Grooms, 2004).

Fetal infection occurs commonly in herds with acute infections occurring during pregnancy BVDV. Consequently, there are several possible outcomes of fetal infections. These are determined by which BVDV biotype of virus causes the maternal infection, and the stage of gestation play an important role (Kelling et al., 2013). Fetal death generally results from BVDV infection before day 40 of gestation, when the virus replicates in the conceptus causing embryonic death. The second consequence happens at early fetal stages (45 – 140 days) causing fetal death and abortion. If the fetus is infected with a low virulence ncp virus before the immune system is fully developed, the fetus develops an ongoing systemic BVDV infection and become immunotolerant to that virus, resulting in a life-long persistent infection of the animal (PI; Scherer et al., 2001; Grooms, 2004; Kelling et al., 2013). BVDV viruses are able to cross the allantoic and amniotic membranes in infected macrophages leading to congenital infections. This highlights the importance of vaccination as a tool to prevent infection of cows during early pregnancy (Swasdipan et al., 2002). As a consequence of congenital infection during the first trimester (40 to 125 days) of gestation, PI calves are permanently immunotolerant to the infecting and closely related BVDV viruses. This will result in weak antibody production or failure to produce antibody in response to viral exposure. These animals shed virus over their entire life-time. Some shed continuously, others intermittently, and represent the major source of BVDV exposure in cattle production, both between and within herds (McClurkin et al., 1984;

Swasdipan et al., 2002). Only low virulence ncp BVDV are able to yield PI calves. The BVDV PI develops in a particular time window of infection (40-150 days of gestation). At this time, the immune system does not yet discriminate self and foreign antigens (Peterhans et al., 2013). Thus, BVDV is recognized as a "self-antigen". This leaves these calves with a "hole" in their specific immune response network due to clonal deletion of responsive lymphocytes. On the other hand, an infection in utero once the immune system is developed (after 150 days of gestation) will lead to one of the following: abortion, congenital defects, or virus clearance. In this last scenario, calves are born IgM seropositive to BVDV (Dereget et al., 1995; Baker, 1987; Moennig et al., 1992).

The clinical signs resulting from BVDV infection range from subclinical to the development of mild to severe disease. BVDV induces disease depends on the state of the host immune response, and the virulence of the strain involved. A transient leukopenia often accompanies the typical sings of respiratory disease (fever, nasal discharge, coughing, dyspnea), or more rarely diarrhea, during BVDV infection. Transient leukopenia increases the risk for secondary infection by other viruses or bacteria (Potgieter, 1995; Brackenbury et al., 2003; Chase, 2013). Another virulence mechanism involved in BVDV disease is the fast dissemination of virus throughout the tissues of the host. The virus will target both primary and secondary lymphoid tissues leading to lymphoid depletion during acute infection (Liebler-Tenorio et al., 2000, 2002, & 2004). Mucosal disease (MD) is most commonly seen in BVDV PI cattle. The clinical characteristics of MD are bloody diarrhea, fever, ulcerative lesions of the mucosal tissue in the respiratory and digestive tract that are associated with anorexia, and ataxia. Death occurs rapidly after onset of these clinical signs with mortality rate of 100%. This fatal syndrome occurs when PI cattle are co-infected with a closely related cp biotype strain. The strains either arise

from mutation of the ncp virus carried by the PI animal or when the animal is vaccinated with an MLV vaccine that contains a homologous BVDV cp strain. It has been reported that the close antigenic relationship between the viruses involved generates a series of mutations, or rearrangements, that yield a cp biotype from the ncp virus that is already resident in the animal and the ncp virus has been present since fetal stage (Baker, 1987; Meyers et al., 1991; Greiser-Wilke et al., 1993; Liebler et al., 1991; Bolin, 1995; Jenckel et al., 2014).

It has been reported that BVDV interferes with the immune response through the action of the N^{pro} protein. This protein blocks the type 1 IFN response when studied *in vitro* (Tamura et al., 2014). In line with this, N^{pro} and E^{rns} might be inhibiting the regulation and response of the innate immune system at two different levels. N^{pro} inhibiting IRF3 and E^{rns} acting at a more systemic level inactivating TLR 7 function (Zurcher et al., 2014; Python et al., 2013). In contrast, there are studies reporting systemic INF production during the acute phase of infection in calves with a ncp BVDV (Smirnova et al., 2008). This observation suggests that a partial adaptive immune activation, in which BVDV does not completely evade the immune response, is characterized by an increase in the production of NF γ in calves and immunologically fetuses (Smirnova et al., 2014, Hansen et al., 2015). In PI calves, the chronic upregulation of IFN γ, and genes induced by IFN γ exposure, in the face of chronic persistent viral production are the drivers of impaired growth and their chronic predisposition to secondary infections (Munoz-Zanzi et al., 2003; Shoemaker et al., 2009).

Strategies to prevent BVDV induced losses have relied on controlling reservoirs of virus in the herd and limiting transmission of virus to susceptible animals. These involve implementing vaccination programs and practicing the culling of identified PI calves (Walz et al., 2010). Although, vaccination is the major mode used to prevent acute infections and to block the

development of PI infections or abortions, it remains important to separate pregnant cows from known or suspected PI calves to break the transmission cycle more completely. There are several commercially available vaccines, including primarily inactivated and MLV vaccines available for control of acute infection and a few for blocking the development of PI calves (Bowland and Shewen, 2000). For many years, BVDV vaccines included only Singer or NADL strains (cytopathic BVDV type 1a). These were used to prevent acute infection, abortion, and to try (mostly unsuccessfully) prevent the development of PI calves. However, the occurrence of abortion and severe disease among vaccinated cows, and the detection of BVDV type 2 viruses in many herds, has led to the addition of several cp BVDV type 2 viruses to the formulation of newer vaccines (Van Campen et al., 2000 & 2010). Generally, MLV vaccines will stimulate both humoral and cell-mediated immune responses in cattle. Further, they are more economical as fewer doses are required for long-term clinical protection. Nevertheless, the main concern with MLV vaccines is the possibility of reversion to virulence. This remains a concern in pregnant cows (Becher et al., 2001; Stevens et al., 2011; Endsley et al., 2014). On the contrary, inactivated BVDV vaccines are safer to use (as they contain no live virus). However, they are considered more costly due to the higher quantity of antigen required per dose and are less efficient because a minimum of two doses are needed to achieve an optimized immune response. Even though the label calls for it and it has been demonstrated that a single does is not fully protective, some producers do not administer the second dose (Walz et al., 2010; Liang et al., 2006). A consensus statement presented in 2020 by Walz and collaborators indicated that MLV vaccines conferred better clinical protection against fetal infection, abortion, and the development of PI calves (particularly those so labeled). Current eradication programs in some parts of Europe currently relay on the use of marker vaccines (lacking specific gene products following vaccination) that

allows the differentiation of infection with field virus and vaccinated animals (DIVA). Although, the major debate point concerning DIVA vaccines is the development of PI calves. PI animals do not produced antibodies against related strains, so infection is not detected by the serological screening methods currently employed (Dong et al., 2007). Genetically modified vaccines appear to be the rising stars. One GMV has already been certified for use in parts of Europe (Bovela®, Boehringer Ingelheim). This vaccine has been designed to generate non-functional, but immunologically recognized E^{rns} and N^{pro} genes for both BVDV type 1 and 2 in the vaccine. Studies in seronegative, pregnant heifers have shown that the vaccine virus was did not cross the placenta (Meyers et al., 2007). This indicated it was safe for use in pregnant cows. Further, it was demonstrated that this vaccine induced memory CD 8^+ T cell responses (Platt et al., 2017).

Bovine respiratory syncytial virus (BRSV)

Bovine respiratory syncytial virus (BRSV) is a non-segmented enveloped negative stranded RNA virus, member of the order *Mononegavirales*, family *Paramyxoviridae*, within the subfamily *Pneumovirinae*, genus *Pneumovirus* (Stott et al., 1985; Bunt et al., 2005). It is closely related to *Human respiratory syncytial virus* (HRSV), therefore a good model to understand the infection in humans (Van der Poel et al., 1994). The virion lipidic envelop contains three transmembrane surface glycoproteins including large glycoprotein (G), fusion protein (F), and small hydrophobic protein (SH; Collins et al., 2001). The envelope contains inside a nucleoprotein (N), a phosphoprotein (P), the viral RNA-dependent polymerase protein (L), and a genomic RNA (Figure 1.3; Collins et al., 2001). The matrix protein (M) is part of the inner layer contained inside of the envelope. The RNA genome encodes for transcriptional anti-termination factor (M2-1), RNA regulatory protein (M2-2), and two non-structural proteins (NS1 and NS2;

Collins et al., 2001). The NS proteins are one of the major differences between *Pneumovirus* genetic subtypes (Pastey et al., 1995).

Furthermore, both NS proteins have an important role regulating the response of the IFN α and β (Goodbourn et al., 2000). The short integral membrane protein SH might play a role in virus-mediated cell fusion by interacting with the F protein (Feldman et al., 2001; Karger et al., 2001). The glycoprotein G is a glycosylated non-globular protein and the major attachment protein, similar to HN and H proteins in other paramyxoviruses (Wertz et al., 1985; Langedijk et al., 1997). Moreover, the main immune reaction of the host is targeted to this protein (Levine et al., 1987). Around 80% of the G protein is produced 24 hours after infection (Hendricks et al., 1988). The G protein has a predilection for heparin-binding domains with glycosaminoglycans on the cells' membranes (Teng et al., 2001). In addition, G protein can also be secreted acting as a decoy by binding to SNA (Hendricks et al., 1988). The fusion protein (F) is responsible for fusion with the host cell membrane, virus penetration, and delivery of the nucleocapsid, producing syncytia or multinucleated giant cells, main visual characteristic of BRSV. Also, the F protein induces SNA, conferring resistance to infection. The nucleocapsid is integrated by the N, P and L proteins (Samal et al., 1991). Protective immunity against BRSV is induced by N protein as CD 8^+ T cells are able to recognize N protein (Taylor et al., 1997). N protein is an important regulation factor for viral transcription, while L protein is responsible for viral transcription and replication (Yunus et al., 1998). There are three M proteins located in the inner layer of the envelope, M2 mRNA encodes M2-1 and M2-2 proteins (Collins et al., 1990). M2-1 protein promotes transcriptional chain elongation and M2-2 regulates the switch between transcription and replication (Bermingham et al., 1999). Based in the G protein, the genetic subtypes are

divided into three groups (A, B, AB; Furze et al., 1994). Five different genotype groups have been established based on F and N protein (Valarcher et al., 2000).

The main mode of transmission of BRSV is via nasal secretions or aerosols inhalation over short distance. Once transmitted, the virus starts replicating in ciliated, and non-ciliated epithelial cells in the respiratory tract (Castleman et al., 1985). Cellular entry is initiated when G protein contact heparin-binding domain, followed by cleavage of the F protein into F1 and F2. Subunit F2 favor the entry viral penetration into host cytoplasm (Schlender et al., 2003). Inside of the cytoplasm the replication and translation of the RNA begins. N and M protein migrate to the cellular membrane, in which the F and G protein are embedded. Viral particles start the budding process using the apical membrane, leaving the cell before it bursts (Valarcher et al., 2007). In vitro studies indicated that infected cells die because of apoptotic mechanisms, which leads to progressive loss of cells in the respiratory tract (Michel et al., 2008). The loss of the respiratory epithelium (pneumocytes) caused by BRSV along with the disruption of the mucociliary function, result in establishment of bacterial infections in the lower respiratory tract, leading to severe bronchopneumonia (Ellis, 2009).

The disease resulting from BRSV infection also varies from subclinical to severe pneumonia. The clinical signs are typical of viral respiratory disease (coughing, pyrexia, nasal discharge, anorexia, and respiratory distress). Main mechanism of disease is characterized by activation of the NF-κB which leads to the induction of inflammatory chemokines and cytokines (IL12, IFN γ, TNF α, IL6, IL18, IFN α and β, CXCL8, CCL5, CCL2, CCL3), and influx of neutrophils (Sudaryatma et al., 1997; Valarcher et al., 2007). Similarly, the establishment and maintenance of the infection is based on failure of the anti-viral state by inhibition of the IFN response, then inducing immunomodulation of the cell-mediated response towards Th2 polarized

immune response (Valarcher et al., 2007; Gershwin, 2007). The interaction between dendritic cells (DC) and BRSV in the respiratory tract determines the immune response towards Th1 or Th2 paths (Gershwin, 2012). The F protein interacts with TLR 4, activating NF-κB and initiates a proinflammatory cytokine storm (Lizundia et al., 2008). These cytokines recruit additional inflammatory cells (neutrophils), leading to necrotizing bronchiolitis, which is typically seen in BRSV infection (Gershwin, 2012). The Th2 polarized response is initiated by DC and poor IL 12 stimulation to produce a Th1 response (Lizundia et al., 2008). The main mechanism to clear virus is mediated by CD 8^+ T cells. Cellular mediated CD 8^+ cytotoxic response is mediated when DC cells secrete IL 12, leading to a polarized Th1 response and production of IFN γ (Gaddum et al., 1996; Woolums et al., 2004). However, CD 8^+ T cell response is blocked by Th2 cytokine production of IL 10 and IL 4 (Bueno et al., 2008).

Multiple vaccine preparations are commercially available worldwide, most of them are multivalent formulations. The main concerns about BRSV vaccination include exacerbation response to vaccines, and virulence reversion by the modified-live virus then leading to outbreaks (Kimman et al., 1987; Gershwin, 2012). The live attenuated BRSV vaccine was demonstrated to prime CD 8+ T cells (Antonis et al., 2006). On the other hand, inactivated vaccines, and other formalin inactivated BRSV vaccines are considered risky to use due to the potential Th2 response and cytokine storm activation (Gershwin, 2012). The use of adjuvated vaccines with CpG oligonucleotides in inactivated preparations has shown positive results in mice, which may justify investigation about their use in cattle (Oumouna et al., 2005). DNA vaccines encoding for either N or F protein have demonstrated significantly lower clinical scores and higher T cell response to viral antigen in vaccinated animals compared to the control group (Lin et al., 2003).

Bovine Parainfluenza-3 virus (BPI$_3$V)

Bovine Parainfluenza-3 virus is a single-stranded, non-segmented RNA negative-sense virus with a spherical to pleomorphic capsid. It belongs to the order *Mononegavirales,* family *Paramyxoviridae,* subfamily *Paramyxovirinae,* genus *Respirovirus* (Karron et al., 2007). It is antigenically related to *Human parainfluenza virus type 1*, and genetically related to *Human parainfluenza type 3* (Karron et al., 2007). This RNA virus comprises six genes (N-P-M-F-HN-L), encoding for nine proteins (Figure 1.4). The main conserved protein, the nucleoprotein (N), along with the phosphoprotein (P) and the large proteins (L), formed the core of the virus, named the ribonucleoprotein (RNP or nucleocapsid). The P and L proteins with or without N protein, are essential for the transcription of viral mRNA and replication within the cytoplasm. The most abundant conserved protein inside of the infected cells is the nonglycosolated matrix (M). This protein is the key for virus assembly, budding, and release (Karron et al., 2007; Takimoto et al., 2004). The main proteins having a role in the attachment and penetration to the host cells are the homotetrameric hemagglutinin-neuraminidase (HN), and the homotrimeric fusion (F) glycoproteins, respectively. These two proteins (HN and F) are responsible for antibody responses when exposed in the extracellular milieu. Other nonstructural proteins (V, C, and D) are believed to inhibit the IFN α and β antiviral functions (Karron et al., 2007). Recent studies the conserved M protein have found two distinct genotypes, BPI$_3$Va and BPI$_3$Vb (Harwood et al., 2008).

The main mechanism of transmission is through the respiratory tract via droplets. Once in the respiratory tract, the HN glycoprotein will bind to the N-acetylneuraminic acid (N-salic acid) of the mucus layer. It has been hypothesized that once attached, degradation of the mucus layer

will allow for the virus to get closer to subjacent cells, to then target the epithelium (Morein et al., 1972). It is also believed that other proteins may play a role by deactivating mucins, cilia and cellular glycocalyx to get closer to the epithelial cells, similar as neuraminidase activity in *Human influenza virus* (Matrosovich et al., 2004).

At the cellular level, the pathogenesis of BPI_3V seems to exploit the same mechanisms used by other respiratory viruses, attaching to the sialic acid residues, and disrupting them through the hemagglutination activity of the HN protein (Levin et al., 1999). The cells that are documented to be targeted by the HN protein include tracheal cells, pneumocytes (type I and II), ciliated bronchial cells, and bronchiolar cells (Tsai et al., 1975; Bryson et al., 1983). Successful attachment to the host cell produces an interaction between HN and F proteins, changing the conformational structure of the virus, and resulting in a protein fusion between viral envelope and host cell membrane (Lamb et al., 2006). Once inside of the cell, M protein starts the replication process. Transcription onto viral mRNA serves as template for replication of the viral genome and translation of viral proteins (Levin et al., 1999; Tsai et al., 1975; Karron et al., 2007). As previously stated, M protein plays an important role including assembly, budding and release of virus particles to infect other cells (Karron et al., 2007). Intracytoplasmic inclusion bodies are visible under the microscope, which is possibly associated with the cytoplasmic viral replication (Tsai et al., 1975; Bryson et al., 1983). The molecular mechanisms of cell injury are not well determined yet. Infected tracheal cultures from neonatal calves showed loss of the epithelial layer height, cilia loss, swelling and desquamation, followed by syncytium formation (Campbell et al., 1969; Craighead et al., 1968). The epithelial cytopathic effect has been attributed to necrosis, beyond other mechanisms like apoptosis (Bryson et al., 1983). Finally, there are clinical reports in infants suggesting that the immune system can play a significant role

magnifying the pathogenesis of parainfluenza. This was supported by the evidence of specific IgE histamine release against parainfluenza in infants with parainfluenza viral–associated upper respiratory disease (Welliver et al., 1982). Nevertheless, there are no reports that support this hypothesis in bovine, so that studies of the role of the immune system in the BPI_3V pathogenesis are warranted.

The nonstructural proteins V, C, and D are capable of inhibiting IFN α and β, thus impairing the antiviral intracellular signaling. This immunosuppressive mechanism along with the cytopathic effects, contribute to enhance viral infection and help to establish secondary bacterial infections (Durbin et al., 1999; Komatsu et al., 2004 & 2007).

Clinical signs are highly variable from subclinical disease to severe pneumonia (Betts et al., 1964, Bryson et al., 1999). General signs of viral infection may include fever two days after exposure ranging from 40.1° – 41.4° C, lasting from seven to ten days (Frank et al., 1973), cough, mucopurulent nasal discharge, inappetence, and high respiratory rate. Seronegative calves may have severe clinical signs and are more susceptible to BPI_3V infection (Allen et al., 1978; Campbell et al., 1969). Although, clinical signs by itself are very general, it is hard to diagnose BPI_3V on clinical signs alone. It is also common to find multiple agents, *Mannheimia haemolytica* and *Mycoplasma spp* as concomitant infections (Betts et al., 1694; Allen et al., 1978; Gagea et al., 2006).

Gross pathology findings consist of atelectasis and consolidation of ventral lung lobes, especially cranial lobe. The affected areas might appear swollen with mucopurulent exudate (Bryson et al., 1999). The histopathologic findings are characterized by infiltration of a combination of inflammatory cells. Lung lesions are typical of bronchitis/bronchiolitis and alveolitis, characterized by death of epithelial cells, beginning as early as 24 hours after

infection. Sloughing of the mucosal lining of airways is a hallmark of BPI_3V-associated lung lesions (Bryson et al., 1983). The infiltration of inflammatory cells may occlude respiratory airways and increase inflammation mediated by IgE, eosinophils, and histamine (Bryson et al., 1983). About 14 days after infection, the repairing phase is characterized by airway and alveolar epithelium hyperplasia (Bryson et al., 1999 & 1983).

The prevalence of BPI_3V in cattle populations is a high, thus most of the dams transfer maternal antibodies to the raising calves (Woods, 1968). Passive transfer might be a good strategy to overcome infection because of antiviral effect of IgA in nasal secretion (McKercher, 1972). An immune response has been detected as early as 6 days after infection, and characterized by humoral responses, mostly local IgA and systemic IgG and IgM (Morein et al., 1970; Marshall et al., 1971). The immunity has been reported to last 3 to 5 months in systemic circulation, and 8 weeks in nasal secretion (Marshall et al., 1971). There was an anamnestic response after BPI_3V re-exposure and nasal antibodies lasted for 5 months after contact (Marshall et al., 1971). Cell-mediated immune response studies are scarce, but there is evidence of lymphocyte proliferation (Moren-Lopez et al., 1977; Johnson et al., 1977) and NK cytotoxic activity against BPI_3V in infected cells (Campos et al., 1982). In mice models, CD 8^+ T cells coincide with mice *Parainfluenza 1 virus* clearance and depletion of CD 4^+ T cells, suggesting an important role of CD 8^+ T cells in clinical recovery from viral infection (Hou et al., 1992; West et al., 1999).

The current strategies to control and prevent BPI_3V are based on parenteral or mucosal vaccines (inactivated or modified-live virus; Gale, 1968). Antigens that are included in the intranasal vaccines are temperature-sensitive mutants, restricting active replication in the lower respiratory tract (Zygraich et al., 1979; Vangeel et al., 2009). Maternal antibodies titers in calves

decline at 28 days after colostrum absorption from 1:8 to 1:4. Thus, vaccination is recommended when the BPI3V SNA titers are around 1:4 (Fulton et al., 2004).

Bovine coronavirus (BCovs)

Bovine coronavirus is a single-stranded positive sense RNA virus belonging to the order of *Nidovirales*, family *Coronaviridae*, subfamily *Coronaviridae,* genus *Betacoronavirus* group A (Cavanagh, et al., 1997; Brownlie, 2017). It is composed by 10 open reading frames (ORFs). The first ORF (ORF1a and ORF1b) compromises the two-thirds of the genome, encoding polyproteins that are cleaved into nonstructural proteins (Figure 1.5). The last third of the genome contained the other ORF encoding for structural protein genes: (HE)-S-E-M-N. The ORF3 encodes hemagglutinin-esterase protein (HE); the ORF4 encodes the spike glycoprotein (S), the ORF 8 encodes small membrane protein (E), the ORF9 encodes membrane protein (M), and the ORF10 encodes nucleocapsid protein (N; Hasoksuz et al., 1999; Kourtesis et al., 2001). The S glycoprotein is cleaved by an intracellular protease into two subunits S1 (N terminus) and S2 (C terminus; Cavanagh et al., 1986; Abraham et al., 1990). The S1 subunit is a variable protein that interacts with specific receptors within the immune system, inducing a specific SNA response, and hemagglutination activity (Yoo et al., 2001). The S2 subunit is a highly conserved protein and has a key role in the attachment and fusion of viral membrane with host cellular membranes (Lou et al., 1998). The HE protein is also involved in the attachment to cell receptors and hemagglutination of erythrocytes (Deregt et al., 1989). The HE and S proteins elicit specific SNA that can block viral attachment and infectivity (Saif, 2010). Variation in tropism and host range is attributed to change in the spike protein (S). Research focused on the hypervariable

region S1B has found conformational amino acid changes discriminating between respiratory or enteric strains (Yoo et al., 2001).

This ubiquitous virus is associated with mild (coughing, rhinitis) to severe respiratory disease (pneumonia) in two to six months old calves. The virus can be isolated in nasal secretions as well as in feces (Saif et al., 1990; Heckert et al., 1990). Nasal and fecal samples analyzed by RT-PCR found a high detection rates of BCoV in both specimens (Hasoksuz et al., 2002). A necropsy report from Texas found that BCoV and *Pasteurella spp* were responsible for necrotizing pneumonia (Storz et al., 2000). Nasal and fecal shedding are commonly found from 0 to 4 days after arrival (Thomas et al., 2006). Captive wild ruminants, including sambar deer (*Cervus unicolor*), white-tailed deer (*Odocoileus virginianus*), waterbuck (*Kobus ellipsiprymnus*), and elk (*Cervus elephus*) have been identified as wildlife reservoirs for BCoV (Tsunemitsu et al., 1995; Majhdi et al., 1997).

The pathogenesis of BCoV is characterized by three different syndromes, severe enteric infections in calves (neonatal calf diarrhea, NCD), winter dysentery (WD) with hemorrhagic diarrhea in adults, and respiratory infections in cattle of all ages (Saif, 2010). Respiratory disease in young calves (2-6 months) ranges from rhinitis to pneumonia (McNulty et al., 1984; Heckert et al., 1990). Challenge studies confirmed effective shedding in fecal and nasal samples (McNulty et al., 1984; Reynolds et al., 1985). Clinical signs caused by BCoV infection are likely to be exacerbated by stress and coinfections with other viruses or bacteria (Saif, 2010). BCoV seronegative cows that were exposed to calves infected with a winter dysentery strain, developed mild signs of respiratory disease (mucopurulent discharge, fever, mild cough; Traven et al., 2001). The theory behind the spreading BCoV from the nasal to the enteric system, lays on the extensive replication in the nasal mucosa and possible swallowing of high amounts of virion

particles to the gastro-enteric system, resulting in infection, replication, and colonization (Saif, 2010).

The resulting evidence of acute respiratory disease produced by viruses is hard to obtain because by the time the calf succumbs to disease, bacterial infections have already colonized the lower tract (Ellis, 2019). For this reason, histological lesions are hard to attribute to viral infections. In the last decade, field outbreak investigations of acute respiratory disease have shown evident gross and histopathological lesions including, tracheal petechiae, ulcers in the upper respiratory tract, along with typical respiratory signs of coughing, and nasal mucopurulent discharge (Hick et al., 2012). On the other hand, histopathological finding included bronchointerstitial pneumonia with intra-bronchiolar syncytial cells in the ciliated airway, likely compatible with BCoV infection. Furthermore, BCoV viral RNA was detected by qRT-PCR in 2 out of 15 lungs tested (Hick et al., 2012).

Despite the circumstantial evidence that BCoV might be strongly associated with BRD, the role of BCoV has been debated due to unsuccessful attempts to recreate the clinical disease in the field (Saif et al., 1986; Cho et al., 2001; Park et al., 2007). Furthermore, there are not vaccines developed to prevent BCoV-associated pneumonia in young calves (Saif, 2010). Limited studies have suggested that SNA against BCoV and BCoV antibody specific isotypes (IgA, IgG1, and IgG2) might be a marker of protection of naturally infected calves (Lin et al., 2000; Cho et al., 2001; Thomas et al., 2006). However, there is no correlation between the level of protection and the type of clinical disease (enteric or respiratory; Saif, 2010). In a study performed by Plummer et al. (2004), a BCoV MLV vaccine for neonatal diarrhea was administered intranasally, inducing cross protection against field strains. This confirmed a possible cross protection among BCoV strains. Further research is needed to develop vaccines

that are able to protect against the different field strains and distinct clinical syndromes (Saif, 2010).

Neonatal immunity in the calf

The immune development of the calf starts in the uterus. The synepitheliochorial placenta represents a sterile environment that filters out all macromolecules and cells, thus, prevent transfer of cellular or humoral immunity from the dam to the fetus (Haeger et al., 2016, Cortese, 2010). This characteristic results in the newborn calf being born agammaglobulinemic (Barrington, 2001; Cortese, 2010). However, there is a progressive development of the cellular innate and adaptive immune components during the development of the fetus. Calves are born with an immature immune system. The immune elements are not yet organized to respond to external threats effectively. The calf depends on the rapid delivery of colostrum and time for maturation of the network to reach a fully functional immune response (Smith et al., 2020). Immune cells develop slowly within the fetus. They take over 130 days to be released into the systemic circulation. Thus, there is minimal immune protection of the fetus before the fifth month of pregnancy (Barrington, 2001). In late gestation, the innate immune response is primarily mediated by neutrophils that are capable of producing a low level of phagocytic activity. Before parturition the innate response is impaired by high levels of fetal cortisol (Tizard, 2000). At the same time, T and B lymphocytes are developing in the fetus. They differentiate from pluripotent precursor in primary immune development sites. These cells to later move from the bone marrow to the liver, spleen, and thymus for further development (Barrington, 2001). The primordial thymus is seen at 30 days of gestation. It reaches full size about mid gestation. The thymus is most active in the very young calf. By one year of age thymic involution is

almost complete (Anderson, 1922; Jordan, 1976). In contrast, B cells differentiate in fetal bone marrow (Senogles et al., 1979). During the third trimester of pregnancy, fetal T and B lymphocytes traffic from the primary lymphoid organs begin to populate organized secondary and mucosal lymphoid tissues. This reduces the number of circulating fetal cells by the time of birth (Casaro et al., 1971; Kampen et al., 2006; Senogles et al., 1979). Cytokines and interferon can be produced by the fetus beginning at 60 days of gestation (Charleston et al., 2001). The induction of an immune reaction in a fetus is dependent on the age of the fetus when it is exposed to a pathogen. At 120 days of development, a fetus can produce IgM antibodies against PI3V; by 190 days IgM against BVDV (Banks et al., 1989).

The ingestion of colostrum during the few first hours of life is very important for the immunologic protection of the calf for at least the first four to eight weeks after birth (Chase et al., 2008). Calves should ingest about 4 L of high-quality colostrum within three hours of birth and an additional 2 L at about 12 hours of age (Godden, 2008). Colostral absorption is mediated by a transient receptor in the intestine, FcRn, and by micro-pinocytosis by the digestive tract epithelium. The capacity to absorb these macromolecules present in the colostrum declines beginning at 6 hours after birth and ends about 36-48 hours after birth (Rischen, 1981). Once the ingestion stimulates the filling reflex, the population of gut epithelial cells start to turn over and mature so that they do not allow absorption of maternal antibody or large proteins (Rischen, 1981). Neonatal corticosteroids play a crucial role increasing the absorption of colostrum (USDA, 2002). The main components of colostrum are antibodies, cytokines, and maternal cells, including leukocytes (Riedel-Caspari et al., 1991). The major antibody in colostrum is IgG1. IgG1 comprises about 80-90% of total antibody transferred. In bovine colostrum, IgA, IgG2, IgE, and IgM are present but in lower quantities than IgG1 (Godden et al., 2019). Endogenous

production of IgM does start until day 4 when it soon is seen in circulation. In general, the adult level of antibody in circulation takes about four months to achieve (Husband et al., 1975). The cells found in fresh colostrum include macrophages/mammary epithelial cells (40-50%), lymphocytes (22-25%), and neutrophils (25-37%). Transfer of maternal cells from the gut to the circulation reaches its peak levels in the calf 24 hours after birth. More T lymphocytes than B lymphocytes are transferred to the calf from colostrum (Reber et al., 2005 & 2006; Liebler-Tenorio et al., 2002). The two major enhancements in the calf receiving colostrum are the improvement in the phagocytic activity of neutrophils, and more rapid development of antigen-presenting cells (Reber et al., 2005). The origin of the cytokines in colostrum is still undefined. It is postulated that they are secreted by cells in the mammary gland during colostrogenesis and/or produced by the leukocytes that are transferred to the calf (Hagiwara et al., 2000). Proinflammatory cytokines present in colostrum (IL1β, IL 6, TNF β, INF γ) might be required for the recruitment of neonatal innate cells and lymphocytes into mucosal tissues to seed normal development and to improve the phagocytic function of resident macrophages and recruited neutrophils (Menge et al., 1998). There are also larger quantities of anti-inflammatory cytokines (IL 4, TGF β, IL 10) present in colostrum. These likely playing a role dampening the inflammation induced by rapid exposure to microbes and facilitating commensal colonization (Wheeler et al., 2007). In conclusion, calves that do not passive transfer of immunity due to poor colostrum management or malabsorption, will still mount immune responses with time. However, they risk developing neonatal disease that is more severe and life threatening than calves receiving an adequate level of good quality colostrum (Godden et al., 2019).

The immune system in calves matures with time and continues to develop until puberty (Reber et al., 2006). Maternal cells that were absorbed by the calf home to lymph nodes and

mucosal lymphoid tissues where they appear to remain for a period of about 5-14 days (Donovan et al., 2007). Further, cells present in colostrum appear to be primed to respond to bacterial and viral agents, circulating in the calf until 48 hours after ingestion (Donovan et al., 2007; Meganck et al., 2014). The cytokine balance between pro-inflammatory and anti-inflammatory activities, in conjunction with hormones produced by the placenta (progesterone, estrogen, and prostaglandin E2), and fetal cortisol produced before parturition combine to promote a bias toward the Th2 response. This suppresses cell mediated responses and limits B cell antibody isotype switch following birth. Under this environment, the early antibody production is of endogenous IgM (Morein et al., 2002; Mao et al., 1994). Therefore, by the first week of life the immunoglobulin-producing cells are highly active increasing the number of circulating IgM positive cells until five weeks of life (Husband et al., 1972). It is generally accepted that calves with failure of passive transfer are at higher risk of disease and have reduce growth rates (Raboisson et al., 2016). Further, maternal antibodies titers in post-suckling calves are highly variable. This may reflect either the quality of colostrum or the level of absorption of macromolecules by the calves. Loading of maternal antibody depends on many factors, including farm practices, dystocia management and calf stress, c-section, time of the first colostrum feeding, and other less common problems (Windeyer and Gamsjäger, 2019).

The function of innate immunity in young calves is dependent of the complement system. The levels of complement components are low at birth in calves. However, they increase to 50% of the adult level by one moth of age, and to reach normal levels at approximately six months of age (Cortese, 2009; Firth et al., 2005). At birth, the secondary lymphoid organs are already populated with cells, but they function poorly until they developed under antigenic stimulation. Resident macrophages located in these tissues direct tissue-level immune homeostasis. These

macrophages recruit other cells (monocytes and neutrophils) to address an immediate innate response (Smith, 2020). By one week of age, neutrophils are able to mount a typical innate immune response. Neutrophil function gradually improves in speed and robustness until the calf reaches five months of age (Hauser et al., 1986). Dendritic cells at birth are functional, but present at very low numbers in the tissues. Their antigen-presenting capacity and the ability to create a link between the innate and adaptive responses is functional. Dendritic cell function is greater in calves that receive adequate passive immunity (Morein et al., 2002).

The development of the acquired immune response in calves is enhanced by the transfer of humoral and cellular component from the dam. The adaptive immune response depends on the efficiency of antigen presentation based on MHC I and II expression. This process is enhanced by the ingestion of colostrum, and enhanced capacity is detectable at 7 days after an adequate colostrum absorption (Reber et al., 2006). Further, B cells gradually increase over time to 20% of total lymphocytes by eight weeks of life (Kampen et al., 2006). Circulating IgA, IgG1 and IgG2 are usually detectable in circulation at about 32 days of age (Husband et al., 1975). T cells represent a 28-34% of total lymphocyte population, with total $CD4^+$ approximately 20% and $CD8^+$ 10% of the T cell population in calves of about 1-3 months old (Kampen et al., 2006). Gamma delta T cell, the largest T lymphocyte population in the adult bovine (60% of circulating T cells; Davis et al., 1996), account for 25% of the total lymphocytes at the first week of life, decreasing and reaching 16% by the third week of life (Kampen et al., 2006).

Neonatal immunity is an important topic for investigation and discussion. It provides the baseline to establish vaccination programs and has a significant influence on the health of young calves.

Factors affecting immunity in calves

Welfare in dairy and beef calves is improved through management strategies focused on minimizing stress, improving biosecurity, and optimizing nutrition to prevent disease occurrence. In addition to the cost associated with treating sick calves, the reduction in growth and increased mortality are factors to consider when planning for strategies to improve health in calves (Windeyer et al., 2014). Difficult parturition and birth trauma decreases the newborn vitality (Murray et al., 2013). In addition, calves experiencing difficult births are more likely to have failure in passive transfer (Hulbert et al., 2016), which leads to higher risk of neonatal diarrhea and mortality in calves less than one month of age (Lora et al., 2018). On the other hand, 20% of the calves affected by FTP presented with bovine respiratory disease (Windeyer et al., 2014). Neonatal diseases could be prevented by improving colostrum management. Therefore, a normal birth accompanied by adequate consumption of high-quality colostrum access, with normal intestinal absorption decreases the risk of morbidity and mortality in newborns (McGuirk et al., 2004). As a normal physiological process, the levels of glucocorticoids in calves increase before partition. At the same time, the increase level of glucocorticoids along with colostrum ingestion is a very important mechanism to improve liver function, and immune homeostasis (Liggins, 1994; Bayne, 2003). The short immunosuppressed stage at birth prevents excessive inflammatory responses to commensal bacteria colonizing the mucosal surfaces (Stilling et al., 2014; Bayne, 2003). The development of the mucosal microbiome and associated barrier is key factor to prevent enteric and respiratory disease (Chase et al., 2019).

The principal farm management practices are full of potential stressors. These include: parturition and birth, transportation, disbudding, castration, weaning, and commingling (Hulbert

et al., 2016). The resilience and rapid development of immunocompetence in young calves is an important factor to allow calves to cope with their susceptibility to disease (Morel et al., 2015).

Transportation imposes high stress on both beef and dairy calves. In a report from NAMHS (2007), 1 of 10 heifers are transported during the period of 0 – 2 days of age to specialized calf raising facilities. This stressor causes the release of glucocorticoids, cytokines, acute phase proteins, disrupts immune homeostasis and disturbs the regulation of the innate immune responses (Arthington et al., 2003; Earley et al., 2010). Older calves are better suited to respond to this stressor as they are better able to thermoregulate and control dehydration (Tao et al., 2013; Arieli et al., 1995; Gebresenbet et al., 2012). Methods to improve transportation are proposed and call for by the NRC (2006). These include management of stocking density, control of high or low ambient temperature, and being aware of the distance of transport. Other factors such as the duration of transport, the age at transport, and the addition of rest stops with water and feed, have also been proposed (Knowles et al., 1997; De la Fuente et al., 2012).

The metabolic demands of calves increase during periods of high stress. A diet that has too few calories, too little roughage, too little protein and too few essential minerals combined with stress can lead to an increase of oxidative stress (Sordillo and Aitken, 2009). The innate immune response under oxidative stress leads to an increase in peroxidation of inflammatory cells. This results in tissue damage caused by the actions of reactive oxygen species (ROS, Spears at el., 2008). Adequate nutrition, including trace mineral supplementation, is a very important factor for growth and development of young calves (Nagaraja et al., 1998).

Vaccination is a common strategy that influences the immune system. The effectiveness of vaccination programs depends on an interaction of several factors. These include: the type of antigen involved, the type of vaccine used (modified-live or inactivated vaccine), the age at

vaccination, the presence of maternal antibodies, the level of disease occurrence and amount of infectious agent pressure, the level and type of stress, and if the at pathogen exposure is a permissive window (Cortese, 2010). Mucosal vaccines have recently taken on a greater role. This is because of their ability to circumvent the typical effects of circulating maternal antibodies (Windeyer et al., 2019; Chamorro et al., 2016). The age of the calf at primary vaccination has become a topic generating controversy. In general, the intent of vaccinating younger calves is to enhance maternal antibody prevent infections immediately, as well as to develop the capacity of the immune system to respond before the typical age of contact with specific pathogens by the calves (Woolums, 2007). Finally, an ideal vaccination program is one that protect calves from disease in the field by including the development of preformed or memory immune responses that last across the production cycle of the animal.

Mucosal immunity

Mucosal epithelia function as biological barriers that provide a context for the host immune system to interact with the commensal bacteria in the microbiota. Most interactions are designed to promote a symbiotic balance between the immune response capacity of the host, to manage interaction of useful organisms, and to avoid colonization and invasion of external pathogenic organisms (Osman et al., 2018). Organized mucosal epithelial barriers are present in the gastroenteric, respiratory, and reproductive tracts (Chase et al., 2019). The respiratory tract is a major point of entry for many pathogens. The tight junctions between epithelial cells of the pseudostratified columnar mucosal epithelium establish a physical barrier against pathogens and limit the entry of toxic substances (Sato et al., 2012). The Goblet cells within the respiratory epithelium are responsible for producing mucus. Mucus is a defense system tool. Mucus forms a

thick layer over the surface that traps foreign substances and microorganisms (Evans et al., 2009). Mucus is composed by a complex mixture of polysaccharides containing mucins that provide a protective barrier. Several polysaccharides have been characterized in human mucus. The full composition of mucus has not yet investigated in calves (Thornton et al., 2008).

The innate immune response on the mucosal tissues is triggered principally by pattern recognition receptors (PRR; including- Toll-like receptors, NOD-like receptors, and retinoic acid-inducible gene-I). These are activated by components of microbes that represent "pathogen associated molecular patterns" (PAMPs) or by materials released from damaged cells that are collectively known as "danger-associated molecular patterns" (DAMPs). The PRRs play an important role identifying the activity of pathogens and in initiating and modulating the response to vaccines. This occurs by linking the innate to the adaptive immune responses in the tissue, then systemically (Ackermann et al., 2010). Another component of mucosal immunity is the family of antimicrobial peptides. There is evidence of binding, and inactivation, of *Mannheimia haemolytica* by antimicrobial peptides in cattle (Heidari et al., 2002). Furthermore, beta-defensins were the first antimicrobial peptide found in ruminants. They have been shown to have activity against Gram-positive and -negative bacteria (Schonwetter et al., 1995). Several types of effector cells are also present as actors in the mucosal innate immune response. Tissue macrophages and dendritic cells are present in newborns at birth and enhanced by 21 days of age. Natural killer (NK) cells and neutrophils show increasing activity in local tissues during the first three months of life (Kampen et al., 2006; Fries et al., 2011). Activation of the innate immune response is the first and principal line of defense in calves during the period over which the immune system becomes fully competent (Chase et al., 2008; Smith et al., 2020).

Adaptive immunity within the structure of the mucosal epithelium is characterized by development of organized lymphoid foci that function like the larger lymph nodes in the central tissues. These foci are called “mucosal-associated lymphoid tissue” (MALT) centers. They consist of both inductive sites for antigen processing and presentation, and effector centers where B and T lymphocyte clonal expansion occurs after antigen presentation in multiple compartments (Pabst, 2015). The primary function of these centers is to generate an antigen-specific response characterized by production of IgA secretory cells and their memory, foci of activated T cells (including gamma-delta cells) that provide local effector and helper function and their memory (Osman et al., 2018). The structure is organized as a series of isolated lymphoid follicles, within which a zone rich in T cells, known as interfollicular zone (IF) is the manager of the overall local response. The organized lymphoid foci contain multifold (M) cells and dendritic cells that function in sampling antigen, processing antigen, presenting antigen, provided the draining function to feed antigen to the central immune tissues, and communicating with local lymph nodes by sending activated cells, DC, and cytokines/chemokines to them for further immune expansion (Kraal, 2004; Stanley et al., 2001). In the upper respiratory tract, the MALT tissue is most commonly referred to as “nasal-associated lymphoid tissue” (NALT). Specialized MALT in the gastroenteric system features regionally differentiated “gut-associated lymphoid tissues” (GALT) that provide strong local focus to the response (Liebler-Tenorio et al., 2006).

The immune system continues to mature over time. Local lymphoid tissues of the upper respiratory tract, including: the pharyngeal tonsils, palatine tonsils, lingual tonsils, and soft palate tonsils play differential roles in the response and have a somewhat different distribution of adaptive cells representing the environment (Liebler-Tenorio et al., 2006). The pharyngeal and palatine tonsils are present by 95 days of gestation. The tonsils are populated by cells but do not

fully develop prior to birth. Over the period of two weeks to two months of age, a significant lymphocyte infiltration into the tonsils occurs. During that time the germinal centers begin to form. This lymphocyte infiltration includes T cells, B cells, monocytes-macrophages, and dendritic cells (Schuh et al., 1992; Palmer et al., 2011). The germinal centers keep growing in size and function until the adulthood (Velinova et al., 2001). The cellular migration to the local lymphoid tissues is directed by chemokines, primarily CXCL13, CCL19, and CCL21 (Fukuyama et al., 2006).

Neonatal calves are susceptible to respiratory diseases largely because the immune system is not fully functional at birth. Further, at an early age the state of mucosal immunity has a Th2 response bias. Neonatal mucosal tissue has a reduced density and functional capacity of mucosal dendritic cells. This leaves the calf susceptible to intracellular pathogens including most viruses (Marshall-Clarke et al., 2000). Recent studies have shown that mucosal immunity in young calves can be primed to overcome the Th2 bias in their response. Priming increases production of INF gamma, development of memory response, and the production of local antibody production recognizing the priming pathogens (Vangeel et al., 2007 & 2009; Hill et al., 2012 & 2019).

The primary humoral response at mucosal surfaces is the secretory IgA (sIgA). The main role of sIgA is neutralizing pathogens and toxins by binding them to block their attachment and entry. IgA can play a role in maintaining the balance between the organisms that make up the commensal microbiota and those that are primarily pathogens (Sato et al., 2012). Antigens are processed and presented by dendritic cells (DCs) to T cells that are located in the germinal centers, then intact antigen and helper products of the T-cells activate naïve B cells to expand and differentiate into cells capable of producing antibody. This process activates an IgA class

switching in the expanding B cell population, increases the production of specific IgA antibodies for secretion. (Mora et al., 2006).

The immune response in mucosal tissues may be independent of the systemic system in cattle. This may be why local immunization has proven to be an efficacious tool in arming some tissues against local challenge (Pabst, 2015). Intranasal vaccines are a common strategy to circumvent maternal antibodies interference (Hill et al., 2012). Furthermore, IN vaccination with modified-live virus vaccines stimulate production of mucosal IgA in young calves (Hill et al., 2012 & 2019; Palomares et al., 2021). In general, IN vaccination with MLV also provides some level of systemic antibody and CMI activation. In a recent study, IN booster vaccination given 5 weeks after IN prime vaccination, induced an increase in the IgA titers. This confirmed the enhanced response of young calves when primed and boostered with an IN MLV vaccine (Hill et al., 2012). There is also evidence of clinical protection afforded by IN vaccination. In a study performed by Ellis et al. (2013), significant protection was observed at 7 and 9 weeks after vaccination with an IN MLV BRSV vaccine. Additionally, there was an increase of specific IgA against BRSV in vaccinated calves vs controls in that study.

Mucosal immunity studies have allowed us to understand the potential of priming MALT in the upper respiratory tract. Further vaccine–challenge studies have shown the efficacy of IN vaccination in young calves as an important strategy in modulating respiratory disease (Ellis et al., 2013; Vangeel et al., 2007 & 2009). In addition, it is also important for us to understand the role of the microbiome and how it is impacted by management practices including mineral supplementation, metaphylaxis, and vaccination. The microbiome plays an important role in connecting the immune system with the recognition of pathogen in the respiratory tract (Holman

et al., 2015). Understanding this interaction and how it influences the immune response during mucosal vaccination should contribute to enhance vaccine efficacy.

Cell-mediated immunity and humoral immunity

The adaptive immune response consists of multiple steps for its development to full function. These progress from recognition of the antigen by antigen presenting cells (APC), binding of lymphocytes to antigen and the "APC co-receptors" that allow activation of lymphocytes, clonal expansion of responsive clones, differentiation of adaptive cells to the functional state, trafficking of armed functional cells in the body, binding and attacking the pathogen leading to its destruction. Once the last step is concluded, the response changes focus, calling for cells from the population of clones that are differentiated to drive the restoration of homeostasis. Another function of the clonal pool is the generation of antigen-specific memory cells to provide an enhance pool of cells for future encounters with the same antigens. The two major pathway for adaptive immunity are the humoral response (mediated by B lymphocytes and producing circulating antibody), and the cell-mediated response (mediated by armed and activated T lymphocytes (Abbas et al., 2018). In mammals, the T lymphocytes originate in the thymus. The B cells originate in the bone marrow. Each arises in these primary lymphoid tissues from lymphoid precursor cells from the pluripotent pool. The T cells are produced as a larger fraction of the cells that circulate in the blood than do the B cells. T cells generally represent about 60-80% of circulating lymphocytes and B cells only 10-40%. Both populations traffic to organized lymphoid tissues representing secondary immune system anatomy to populate primary, secondary, and tissue associated lymph nodes. B cells are more prevalent in the cortex of the lymph nodes, follicles in Peyer's patches (PP), and marginal zone of the white pulp in the spleen.

While T cells are located in the paracortex of lymph nodes, the periarteriolar lymphoid sheaths of the spleen, and the interfollicular areas of the Peyer's patches. T cells are divided into two families of antigen receptors pairs, αβ and γδ. With the γδ being more primitive and arising earlier in both evolution and development. The T cell antigen receptor (TCR) is associated with the signaling adaptor, CD3, that is shared by all T cells. The αβ T cell receptor complex involves two context setting surface proteins, CD4 and CD8. CD4 is found on T cells that primarily have helper function and require the presence of MHC class II antigen on the APC for activation. While the CD8 requires specific binding to the MHC class I protein complex on APC (Tizard, 2009; Abbas et al., 2018). Bovine γδ T cells generally expressed WC1 on their surface. WC1 is a transmembrane glycoprotein closely related to CD163 (Holm et al., 2009). Gamma delta T cells are a nonclassical T cell population with activation independent of either main MHC complex. They generally require whole antigen and are often stabilized by the members of the CD1 complex. They are found in high numbers in epithelial sites (skin and mucus membranes), Hemal-lymph nodes, and in the thymus (Guzman et al., 2014; Tizard 2009). A significant number of gamma-delta T cells circulate in cattle. The highest fraction of circulating gamma-delta T cells is found in calves with declining numbers in older animals.

Protective immunity against viral infection involves both humoral and cell mediated immune responses. T cell responses involving both $CD4^+$ and $CD8^+$ cells have been shown to be important against viral infections including influenza, BVDV, and BHV1 (Platt et al., 2011; Endsley et al., 2002a and 2002b). The major roles of T helper cells ($CD4^+$ lymphocytes) are the release cytokines (primarily IFN-gamma) to stimulate the action of cells of the innate immune response. These include activating neutrophils and arming macrophages. Moreover, $CD4^+$ T cells play a role directing the actions of $CD8^+$ T lymphocytes and the maturation and

differentiation of B lymphocytes during the development of a specific adaptive immune response (Boehm et al., 1997; Doherty et al., 1997). $CD8^+$ T cells have a primary role as cytotoxic cells. They generate and release potent antiviral compounds. These may serve as inflammatory mediators and provide effector function for cellular antiviral action (Howard et al., 2004). γδ T cells have a wide range of functions. These include: providing transition from innate to classical adaptive responses, immune regulation in cattle, cytokine production (including IFN-gamma), a role in immunosurveillance of the mucosal epithelium, and several others that are not as well characterized (Chen et al., 2013; Price et al., 2007; Guzman et al., 2012). The humoral immune response is mediated by antibody produced by B cells. Stimulated and expanded clones of B cells produce antibodies after vaccination or antigen exposure (Endsley et al., 2002a). Differentiated B cell foci also produce mucosal IgA after class switch. This helps to block microbial attachment to epithelial cells, prevents viral entry of epithelial cells and viral or bacterial translocation across epithelial cells to the basolateral surface (Hill et al., 2012).

Three important population of T helper cells have been identified, T helper 1 (Th1), T helper 2 (Th2), and T helper 17 (Th17). The function of each is driven by signature cytokines, each type having distinct functions. Th 1 cells are matured by IL-12 release from antigen presenting cells - APC (dendritic cells, macrophages, B cells) and epithelial cells, then the Th1 cells produce IFN-gamma and other cytokines that feed back to the APC populations and epithelial cells in target tissue. This upregulates CMI directed immunity targeted against intracellular pathogens (e.g., Mycobacteria, and virus). Th 2 cells responds to IL1 alpha produced by resident macrophages and epithelial cells. Th2 cells release of IL4 and IL-10. This suppresses IFN-gamma production and function. Th2 cells stimulate B cell clonal expansion, then differentiation to antibody production, primarily IgM. Later, Th2 cells stimulate antibody

class switch from IgM to IgG, IgA, or IgE depending on the context of the danger, damage, and antigen signals. Class switch to IgE will generate immunity against parasites and reduce the response to intracellular organism. Further, IL 4 alternatively drives macrophages to the M2 phenotype. This includes a change in function to an anti-inflammatory release of IL-10 and TGF-beta for wound repair and fibrosis. Th17 cells effect their function using a combination of cytokines, primarily IL 17, IL 22, IL 23, and IL 21 with some IL 6, TGF-beta also produced. The critical cytokine in the TH17 complex is IL 23. Its production will enhance IL 17 production and keep Th 17 cells viable and functional. Th17 cells principal functions are development and regulation of inflammation, recruiting of granulocytes to damaged and infect tissues, and targeting extracellular bacteria and fungi for destruction (Abbas et al., 1996; Abbas et al., 2018; Tizard, 2009).

CD 25 is expressed on the surface of lymphocytes that are activated and on monocytes after exposure to IFN gamma. CD25 represents the beta chain of the common family of receptors for many cytokines, including IL-2, IL-4, IL-13, and IL-15. When CD25 is concomitantly expressed with other T cell markers (CD4, CD8, WC1) indicates the cells have been activated recently by specific antigens (Quade et al., 1999; Minami et al., 1993). However, this activation is not clearly indicative of the function of cells with this phenotype. CD25 expression is induced readily by viral infection, proinflammatory (IFN γ) or anti-inflammatory (IL 10) cytokine release (Endsley et al., 2004). Furthermore, proinflammatory cytokine expression patterns (TNF α, IL-1 β and IL-6) are rapidly expressed by monocytes or macrophages, often yielding a CD25+ phenotype (Abbas et al., 2018). The immune response can sometimes be overwhelmed. Thus, to balance the multiple responses initiated, it is necessary to achieve an immunologic balance. The T regulatory cell populations play this important role controlling overzealous inflammatory

responses by releasing regulatory cytokines (IL 10, TGF-beta, and IL-35) which dampen the response and limit inflammatory injury (Lippolis, 2008). Further, T-regulatory cells inhibit the expansion and arming of adaptive cells (particularly T-cells) by blocking the cytokine effects in the secondary tissue and at the site of arming of effector cells.

The cell-mediated immune response is an important component of the immune response overcoming intracellular acute infections with BVDV (Howard et al., 1992). Bovine viral diarrhea virus (BVDV) plays an important role in the bovine respiratory disease complex by inducing an immunosuppressive effect over T cells, impairing the immune system, which makes the animal more susceptible to suffer secondary infections (Kelling, 2007). The consequences of the mechanism of this transient immunosuppression induced by BVDV relay on downregulating the immune function, and causing death of leukocytes including, granulocytes, monocytes, macrophages, dendritic cells, $CD4^+$, $CD8^+$, γδ T cells, and B cells (Sopp et al., 1994; Chase et al., 2004; Walz et al., 2001). The level of viremia produced by BVDV has an inverse relationship with leucocyte counts (Walz et al., 2001). Molina and collaborators (2012) reported a marked decrease in lymphocytes and other white blood cells (monocytes, neutrophils) 3 and 9 days after intranasal inoculation of ncp BVDV in dairy calves (8-9 months old). In the same study, a sharp decline in $CD4^+$, $CD8^+$T cells and B cells was observed. However, there was an increase in the level of the leukocytes, reaching baseline values at 12 days after inoculation.

The decrease of $CD8^+$ T cell seems to have no effect in the magnitude of viremia. Nevertheless, $CD4^+$ T cells critically impair immune response against BVDV (Howard et al., 1992; Chase et al., 2004). It has been hypothesized that γδ T cells may not play an important role against BVDV infection (Howard et al., 1992), although there might be a production of inflammatory cytokines at early stages of infection, including IL 12, TNF α, and IFN γ by these

cells (Sopp et al., 2001; Baldwin et al., 2021). Even though, some reports have shown decay of γδ T cells and then increase around 12 days after infection, there might be an immunomodulation effect favoring viral infection (Molina et al., 2012; Bittar et al., 2020) controlling the inflammation (Pollock and Welsh, 2002).

Vaccination against BRD

Preventive strategies to decrease BRD prevalence include colostrum management, metaphylactic use of antimicrobial, and the use of vaccination programs. This last item is the most flexible tool in the disease prevention arsenal for cattle. Vaccination represents a challenge to the producer because there are not 100% effective. Vaccines can be delivered at any production stage with some expectation of efficacy. It can be given in a form that is compatible with the state of the animal at the point in time when delivering the vaccine seems best in the management program. Not all commercial vaccines are equal in their capacity to induce immunity or protection for a specific purpose. Thus, vaccination must be planned and fitted to the goals and limits of the producer.

A study vaccinating calves at 2 or 5 weeks of age with a modified-live virus (MLV) vaccine showed no impact on BRD incidence at 3 months of age (Windeyer et al., 2012). Selecting the best protocol to vaccinate a population of animals depend on many factors including the prevalence of the disease (pathogen pressure), type of vaccine to be used (modified-live vs. inactivated virus), number of antigens or genotypes included (monovalent vs. multivalent), possible deleterious effects and safety concerns, level of efficacy required and expected, age of the animals to be vaccinated, health status of the animals to be vaccinated and

production system specific limitations (nutrition, stress and housing, planned shipping, planned comingling).

The main reason for using inactivated virus vaccines is safety. There are concerns when using MLV vaccines in pregnant or immune stressed animals as they contain live agents and may possibly revert to a virulent state. Inactivated, or killed-virus (KV) vaccines do not generate revertant agents with pathogen potential and are generally not immunosuppressive (Kelling, 2004), except for high concentration gram negative killed whole organism vaccines. Killed-virus vaccines (KV) are primarily used in pregnant animals in an attempt to avoid vaccine-related abortions. Killed antigens have no replication capacity. Thus, they require adjuvants to produce an immune response by emulating cell damage pathways. For this same reason, KV require more antigen in the formulation than MLV vaccines. KV must deliver all the antigen needed for the response, but MLV produce most of the antigen in the tissues of the host. KV generally require more than one dose to achieve a sufficient level of protection. In addition, the onset of immunity is often delayed with KV relative to MLV (Newcomer et al., 2017). On the other hand, MLV vaccines induce a faster onset of immunity, demonstrating both cell-mediated and humoral response three to five days after vaccination (Brock et al., 2007). It has also been postulated that MLV vaccines produce a superior cell-mediated response than most KV vaccines (Woolums et al., 2013). The core concern with MLV is the that in pregnant heifers or stressed (immunosuppressed) animals live agent may have unwanted infectious consequences (Newcomer et al., 2017).

Vaccination of calves in face of maternal antibodies (IFOMA) has been topic of research recently (Chamorro et al., 2016b, Endsley et al., 2003). There are several different interpretations of how much IFOMA vaccination is impacted depending on the outcome of each trial. It has

been normal to evaluate vaccination success based on seroconversion. The most common definition used is a 4-fold increase of antibody titers post-vaccination (Kirkpatrick et al., 2008). While this is a measure of B cell response, it has been recognized that CMI is often an important part of the immune response against many viral pathogens. When maternal antibody decay reaches about 80%, there is a window of susceptibility to disease in calves. To protect calves at this time, vaccination is needed for induction of calf produced immune responses to close the susceptibility gap (Chase et al., 2008).

Considering vaccination against BVDV, the results of several studies have documented that MLV vaccination reduces clinical disease associated with post-vaccine viral challenge (Platt et al., 2009; Zimmerman et al., 2006 & 2009; Stevens et al., 2011; Palomares et al., 2012; Bittar et al., 2020). In addition to clinical signs, another important factor for protection of seronegative calves is the reduction of viremia post-challenge with the associated reduction in virus shedding. These have been mediated by MLV vaccination (Kelling et al., 2007; Palomares et al., 2012). Calves vaccinated IFOMA are capable of mounting an active immune response without obvious seroconversion. The post-vaccine immune response was characterized by a reduced rate of decay of maternal serum neutralizing titers, an apparent anamnestic response upon re-exposure to the agent, and demonstrated development of specific T cell response against BVDV after MLV vaccination. The outcome generally includes clinical protection after BVDV challenge (Endsley et al., 2003; Fulton et al., 2004; Chamorro et al., 2016a; Platt et al., 2009). Conversely, when the levels of maternal antibodies are high, standard parenteral vaccination do not appear to induce significant levels of specific humoral immune response to the vaccine antigens (Zimmerman et al., 2006). Another similar report found that calves vaccinated at 2 to 5 weeks of age, that had a high concentration of maternally derived BVDV serum neutralizing antibody (SNA) titers

responded poorly after challenge with a homologous virus four months after vaccination (Ellis et al., 2001). In both cases, it was suggested that a high level of maternally derived antibodies interfered with development of a BVDV-protective immune response (Ellis et al., 2001; Zimmerman et al., 2006). The efficacy of parenteral vaccines in seronegative, preweaned calves has been reported. This population of calves remains at high risk of disease (Ellis et al., 2001). Summing up, the lack of consensus on this topic warrants further research to explore appropriate strategies for priming vaccination in preweaned calves.

Intranasal (IN) vaccination has become more attractive as a strategy to bypass the interference of maternal antibodies. Intranasal priming of the mucosal surfaces improves the humoral response against the vaccine viral agents. Moreover, IN vaccination IFOMA with MLV vaccines have proved effective (Xue et al., 2010; Chamorro et al., 2016b). While the immune system is not fully mature in young calves, the nasal-associated lymphoid tissue (NALT) is capable of producing an immune response during the first week of life (Osman et al., 2018). In a study by Kimman (1989), calves IN vaccinated IFOMA generated better protection than that were calves vaccinated parenterally (IM). This clinical protection was characterized by a strong mucosal IgA response in lung washes, higher serum antibody titers against BRSV following BRSV challenge, and a lack of nasal shedding. Similar protection was conferred in calves IN vaccinated IFOMA and then challenge with BVDV. They produced antigen-specific T cell response that could be measured *in vitro* (Ridpath et al., 2003; Endsley et al., 2004).

Intranasal vaccination with MLV vaccines has been proven to increase IgA levels in mucosal secretions and develop long-term persistence of memory B and T cells (Hill et al., 2019 & 2012, Takahashi et al., 2007; Demberg et al., 2014). Additionally, calves that were booster vaccinated 35 days after priming, had a clear anamnestic response (Hill et al., 2012). The

protective response induced by IN vaccines reduced clinical signs and BHV1 shedding following challenge (Hill et al., 2019). Furthermore, IN vaccination enabled immunity against BHV1 and BRSV mediated by increases specific IgA nasal titers against both viruses (Mahan et al., 2016, Palomares et al., 2021). This indicates the importance of secretory IgA in protection of mucosal surfaces. Mucosal surfaces are the major route of exposure to viral pathogens and the transmission of viral pathogens (Chase et al., 2019). Moreover, calves vaccinated IN at 3 weeks old, then challenged with BRSV, had reduced nasal shedding of virus and reduced clinical symptoms (Vangeel et al., 2006). Another study from the same lab with treated calves under the same conditions, but challenged against PI_3V, had reduced nasal shedding of virus. However, the clinical symptoms were similar for both the vaccinated and control groups (Vangeel et al., 2009). In conclusion, IN MLV vaccination has proven efficacy in calves challenged with the virus contained in the vaccine.

Bovine respiratory diagnostic tools

Diagnosis of BRD is frequently performed based on clinical signs (clinical diagnosis) and determining the presence of specific pathogens often verifying the lesions that confirm the disease (infectious or etiologic diagnosis). Moreover, the presence of antibodies indicates exposure to the agent, not prior disease (Fulton and Confer, 2012). Diagnosis of a disease is often used as first step in a plan to prevent disease. Examination of individuals or populations starts with a careful evaluation of the clinical history. This information will lead to knowledge about the possible organs involved (distribution of disease), possible etiologic agent, and possible metabolic alterations involved (Fulton and Confer, 2012).

Clinical diagnosis

Clinical signs and behavioral changes have been evaluated in young animals as a proxy to identifying disease. Clinical and nonspecific signs, including fever, anorexia, diarrhea, and depression, have been systematically scored for different clinical evaluation scoring methods to try to understand the best time to treat calves (McGuirk, 2008; Love et al., 2014; Maier et al., 2019). These scoring systems were designed to help assist the veterinarian diagnose disease and provide tools for farm personnel (with proper training) to identify most common signs of disease. This push for a standardized case definition for bovine respiratory disease is an important step forward (McGuirk, 2008). The main disadvantages of clinical score systems are the broad largely non-specific definition of the clinical score elements (Buczinski et al., 2016). For instance, using something as commonly associated with many diseases as high temperature in a scoring system does little to define a specific clinical problem, but more indicates general proinflammatory cytokine release or the effect of heat stress. Another disadvantage is the underdiagnoses of subclinical respiratory disease. Subclinical disease will often produce low scores masking the true diagnosis and facilitating the establishment of infection in a herd (Pardon et al., 2020). In the recent years, other respiratory disease monitoring systems have been developed, these are generally based on statistical methods which avoid much of the subjectivity in the outcomes (Love et al., 2014). The typical body temperature cut point was recently change to 39.2°C. This variable was introduced as a dichotomous predictor rather than an ordinal predictor. Further, an additional dichotomous predictor was introduced attempting to evaluate the respiratory effort as a co-variable in the model. Finally, the definition of a BRD case was commonly confirmed by finding a PCR positive for one of the associated respiratory viruses, or a combination of positive culture of an aerobic respiratory bacteria plus a clinical score of > 4, or culture of *Mycoplasma*

bovis plus a clinical score >4 (Love et al., 2014). Respiratory scores are not intended as a gold standard of diagnosis (Ollivett et al., 2016). Rather, they stand as a proxy for standard diagnosis to evaluate the severity of respiratory disease in calves in the field.

Thoracic ultrasound (TUS) is a novel application of the ultrasound technique. It is readily available on the farms as most have access to an ultrasound machine to validate pregnancy (Ollivett et al., 2016). Clear images from TUS have been recently correlated with the typical clinical manifestations and show strong agreement in results by multiple evaluators (Buczinski et al., 2013 and 2018). Ultrasonographic finding recognize;, lung consolidation, patchy pneumonia, and the severity of pneumonia affecting more than one lobe (Ollivett et al., 2016). TUS has a scoring system providing a US score from 0 – 5. This is based on a series of observations ranging from normal aerated lung with no consolidation, scored 0; Diffuse signs of comet-tail artifact with no consolidation, scored 1; Signs of lobular or patchy pneumonia, scored 2; Lobular pneumonia that affects only one lobe, scored 3; or Pneumonia affecting two lobes, scored 4 (with the cranial and caudal division of the cranial lobe scored individually). Lobular pneumonia affecting three or more lobes is scored 5 (Ollivett et al., 2016). TUS scores 0 to 1 are considered normal, while scores equal to or greater than 3 are considered bacterial bronchopneumonia. Small lobular lesions are associated with viral infections and may not warrant treatment (Ollivett et al., 2013 and 2015).

Endoscopy is a minimally invasive diagnostic tool that allows for direct observation of the upper respiratory tract (URT) mucosa. The first report using endoscopy on the upper respiratory tract was performed on jersey cows by Anderson et al. (1994). This tool might be useful for identifying inflammatory changes of the mucosa in subclinical calves before epithelial lesions develop. Thus, early identification of sick animals by endoscopy may have a significant

impact on disease control. This diagnostic method has been widely used in horses for evaluation of several respiratory disorders (Holcombe et al., 2001, Slovis, 2004, Sheta E., 2017). Generally, it is useful for identifying lymphoid hyperplasia, tracheitis, or neoplasms in the URT (Holcombe et al., 2001, Slovis, 2004). No studies had been performed concerning endoscopic evaluation of the upper respiratory tract of calves infected with respiratory viruses before our studies in this dissertation.

Etiologic diagnosis

The main prevention strategy in ongoing outbreaks is an accurate diagnosis allowing optimally design the intervention. However, the accuracy of BRD diagnosis is impaired by multiple factors and the large number of interactive pathogens involved. The prompt use of diagnostic tools to address each case can be definitive. The information available at the time of designing interventions to halt the infectious cycle is critical.

Herd surveillance, focused on the youngest calves, helps in detecting BVDV PI calves in the herd (main source of infection within the herd). This is a common practice across US (Newcomer et al., 2016). The most common surveillance practices in both beef and dairy cattle are bulk tank milk testing for BVDV antigen or antibody, looking for serum neutralizing antibodies (SNA) in heifers before the first pregnancy. BVDV testing in pooled ear notches (pre and post weaned) and placing sentinels within herds. These have proven to be useful in detecting PI calves (Newcomer et al., 2016)

However, BHV-1 surveillance is less common. This is in the face of a high prevalence of BHV-1 in many regions. Almost universal exposure of cattle to BHV-1 and latency tend to make less useful the diagnosis of the presence of this virus.

Bulk tank milk PCR testing allows to test the general cow population for several different pathogens in one sample. This is a common practice allowing cheap, fast diagnosis, and less labor than individual testing (Hill et al., 2010). Molecular techniques for monitoring the bulk tank have been used to increase sensitivity of pathogen detection (Renshaw et al., 2000). Furthermore, detection of antibodies before and at the time of colostrum consumption is a cost-effective tool. Finding serological evidence of a pathogen in colostrum provides a pathway that might be missed in pooled samples from the bulk tank milk, from pooled serum, or pooled ear notches. This is not currently a common practice (Schefers et al., 2008).

On the other hand, the gold standard diagnostic tool for BVDV and BHV-1 is virus isolation from swabs, secretions, or tissues of infected animals (Saliki and Dubovi, 2004; Van Donkersgoed and Babiuk, (1991). Molecular techniques have also been used to detect viral infection; but the presence of viral nucleic acids does not confirm infection and can represent vaccine antigen (Givens et al., 2013). Other commonly used diagnostic techniques are based on antigen detection often using Enzyme-linked immunoassay (ELISA) methods, or immunohistochemistry (IHC) with monoclonal antibodies (Edmondson et al., 2007; Gripshover et al., 2007).

Advances in viral detection have evolved during the last few decades. The used of nasal swab sampling has enhanced the range of pathogens detected to include bovine coronavirus and influenza D virus as recognized parts of the Bovine respiratory disease repertoire (Fulton, 2020; Hause et al., 2014). Molecular detection is currently one of the most widely used techniques in diagnostic labs. Furthermore, primer and probe sequences for most of the viruses of interest are openly available for research and diagnostic use without restriction, offering a common standard for detection (Fulton, 2020).

Serology is commonly used to test for infectious pathogens. Other methods still employed are: microbial culture, Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS), Polymerase Chain Reaction (PCR), and Next-Generation sequencing. Serology is the most commonly used technique to evaluate vaccination programs and in general screening for pathogens circulating in the herd. Serology is cheap and low tech. The main disadvantages of serology are: the time required for processing, the duration of time before seroconversion occurs in the herd, a high variation in sensitivity and specificity between tests due to the use of "lab specific antigens or strains", and problems to identify vaccinated from infected herds. Delays in serology interpretation can reduce the effectiveness in clinical therapeutics when they are delayed (Pardon et al., 2020). Microbial culture is most commonly used for aerobic and frequently encountered bacterial pathogen identification. Time lab for culture and reporting are also a matter of concern related with difficult to culture organisms or those that overgrow under standard lab conditions (Van Driessche et al., 2017). In addition, uniform tests for antimicrobial sensitivity and specificity have not been established universally for most of bacteria involved in BRD (Bokma et al., 2020). Molecular techniques, including PCR, are commonly used to identify bacteria and viruses involved in BRD. Molecular biology diagnostic tools, represented by PCR, are usually more expensive tools. However, the capacity to pool five animals for testing without affecting sensitivity and specificity help make them more useful (O'Neil et al., 2014). The main disadvantage in molecular diagnosis is that pathogens can be identified that are not actively causing infection. This may be because they are "residents" that may be opportunistic pathogens, but are not always involved, or that may currently not participate in the active infection (Pardon et al., 2020). Next-Generation sequencing is now becoming available in many labs. This is because of greater availability of the platforms

and commercial access to the required sequences. This technique is the based on the body of microbiome studies. The method has a great advantage of identifying whole genomes of the agent (virus and bacteria), and at the same time identifying all probable pathogen present in the sample (Conceicao-Neto et al., 2015). The main disadvantage of Next-Gen is its high cost. In addition, the computational tools, and the knowledge to understand the output requires additional training and experience to be useful.

Trace minerals and health

Trace minerals play an important physiological role in growth, immune function, reproductive function, catalytic activity in metabolism, organization, and assembly of structural components of cells,, and multiple biological activities that are not yet fully described (Suttle, 2010, Spears and Kegley, 2002; Rabiee et al., 2010). These elements are needed in small quantities, yet their bioavailability is vital for essential life processes (NRC, 2001). As structural components, trace minerals function as cofactors for several enzymes (Glutathione peroxidase, Superoxide dismutase, Ceruloplasmin), have a role in stabilizing the functional structure of important bio-molecules, and in regulation of gene replication, transduction, and transcription (McDowell, 2003; Suttle, 2010; Smart et al., 1981). The required concentration of trace minerals in soils is highly variable across the USA. Several regions of the US have soils with deficient levels of the trace minerals Se, Zn, Co, and Cu. This is directly reflected in the forages produced in those places. Thus, trace mineral supplementation is recommended in many regions of the US (McDowell, 2003; Suttle, 2010; Swecker, 2014). Subclinical deficiency of minerals is not usually evident on gross examination. Thus, animals with marginal levels of mineral often see a decrease over time. The levels become critically low affecting immunity and enzyme functions

first, followed by a reduction in growth efficiency, normal muscle and nerve development, and late onset or failure of fertility. Gross deficiency is evident as chronic subfertility and the development of a family of clinical issues (Fraker, 1983; Wikse, 1992).

Copper (Cu) has important roles in metabolism, growth, and immunity. Cu is component of several macromolecules and enzyme complexes. Cu is an integral part of the cytochrome C oxidase system, a mitochondrial enzyme complex necessary for cellular energy production yielding ATP. Copper is a component of Ceruloplasmin, a plasma protein responsible for transport of Fe. Serum concentrations of this enzyme are often used as an estimate of Cu status. Ceruloplasmin is elevated during acute inflammation. It has been documented to have anti-inflammatory function (Frieden and Hsieh, 1976). Further, Cu is a vital component of the Cu-Zn Superoxide Dismutase complex. This is an antioxidant enzyme complex that catalyzes radicals, particularly reactive oxygen species (ROS, Graham, 1991; Rucker et al., 2008; Suttle, 2010). Copper deficiency affects growth performance, immunity, general health, and reproductive efficiency (Wikse et al., 1992). Neutrophil phagocytic activity is dependent on Cu-containing enzyme complexes and leads to leukocytes membrane damage caused by the excessive ROS action due to the lack of antioxidant capacity (Graham, 1991; Suttle, 2010). Copper negatively interacts with Mo and S in diets. This results in failure to absorbable these mineral components (Gould et al., 2011).

Manganese (Mn) has important roles in immunity, growth, and reproduction. It is a crucial structural component of many metalloenzymes. Mn has an important role in the carbohydrate metabolism as it regulates the activity of the Mn-Metalloenzyme, pyruvate carboxylase and an Mn-activated enzyme, phosphoenolpyruvate carboxykinase, and helps control the glycogen levels in liver (Baly et al., 1986). Mn is also an important constituent of

Mn-Superoxide Dismutase (SOD2) found in mitochondria. This enzyme has an important role in controlling oxidative stress caused by ROS (Rucker et al., 2008; Suttle, 2010). Mn deficiency can be observed as defects in bone development, impaired metabolism of lipids and carbohydrates, impaired growth, and immunosuppression (Rucker et al., 2008).

Selenium (Se) is frequently deficient in soil and thus, in forage. Selenium is an important structural element of the antioxidant enzyme Glutathione Peroxidase (GPx). This antioxidant enzyme has a main function in cellular membrane protection against radicals produced during oxidative stress and as the result of lipid peroxidation (Beckett et al., 2005). Selenium has a direct impact in innate immune function. It has a role in neutrophil migration following inflammation (Maddox et al., 1999). Selenium is involved in T lymphocyte blastogenesis (Spears et al., 2000). Moreover, Se supplementation was shown to reduce the prevalence, severity, and duration of mastitis with a reduction in the somatic cells count observed as well (Weiss and Hogan, 2005). Although, the main role of Se is in antioxidant defense, deficient levels of Se can also affect growth, reproduction, and the other functions of the innate and adaptive immunity responses. Deficient levels of Se have been associated with retention of placenta, increased embryonic mortality, and reduction in sperm mobility (Smart et al., 1981; Suttle, 2010).

Zinc (Zn) is an essential trace mineral component of greater than 2500 known enzymes. These enzymes are important in immunity, metabolism, reproductive and growth. It is a component of the Cu-Zn Superoxide Dismutase (SOD1) complex. Its role is reducing the effects of radicals produced during oxidative stress and limiting cellular damage induced by ROS. Zinc is important for differentiation and maturation of T cells. Zinc is an essential component of several transcription factors, impacting chromatin structure, and in regulating the

cell cycle. Enzymes containing Zn have roles in nucleic acid replication and transcription, and enzymes regulating apoptosis of cells (Dardenne et al., 1994; Falchuk, 1998; Spears, 2000; Spears and Kegley, 2002). Deficiency in Zn affects mRNA expression and the building of protein. This deficiency leads to the downregulation of some important genes (Falchuk, 1998). Other consequences of Zn deficiency are impaired T cell-mediated responses, impaired reproduction, and poor growth and development of young stock (Dardenne et al., 1994; McDowell, 2003; Suttle, 2010).

There are many strategies to supplement cattle with trace minerals. Often formulations are designed around organic or inorganic sources for delivery of the minerals desired. Organic sources are generally more readily absorbed by the animal. This is often beneficial during periods of high stress or low feed intake (Greene, 1995; Rabiee et al., 2010). Methods for the administration of trace minerals ranges from oral supplementation based on powder formulations to be placed in drinking water, salt blocks, rumen boluses (often with delayed delivery), to injectable presentations for immediate uptake. All presentations have different targets with respect to when and how bioavailability is to be achieved. For instance, the fastest way to increase the levels of trace minerals in liver tissue is by parenteral injection. However, the boluses with delay delivery are sustained over time showing better controlled concentrations of Se in liver tissue 120 days after placing trace minerals in the animal (Jackson et al., 2020). Injection of trace minerals bypasses the metabolic effect of the rumen where the microbiome and other mineral sinks will reduce absorption (Jackson et al., 2020).

There is an active role of trace minerals in the control of oxidative stress. Innate immune defense is modulated by soluble cytokines producing either an anti- or pro-inflammatory environment. For instance, TNF α and IL1 β are expressed rapidly after infection is initiated.

They produce a potent inflammatory effect. In contrast, IL 4 and IL10 produce a strong anti-inflammatory effect (Bannerman, 2009). Macrophages, dendritic cells, and neutrophils respond to these cytokines and migrate to the site of infection. There they begin the phagocytic process to remove the invading pathogen. In the process of phagocytosis, there is release of ROS and a family of hydrolytic enzymes (Babior, 1999; Sordillo, 2016). Although, ROS have a beneficial effect being capable of destroying the invading pathogens, an excessive accumulation of ROS leads to host cell damage, principally by peroxidation of cell membrane lipids leading to cell death if conditions persist (Valko et al., 2007). Antioxidant defense is characterized by a combination of radical scavengers and antioxidant enzymes systems that achieve and maintain ROS homeostasis (Sordillo and Raphael, 2016). Trace minerals are important components of the antioxidant enzymes families (glutathione peroxidase -Se; superoxide dismutase – Cu, Zn, Mn).

Previous studies have shown beneficial effects of injectable trace minerals (ITM; Se, Zn, Cu, Mn; Multimin-90®; Multimin –USA Inc ®) on the immune response and health of dairy cattle (Machado et al., 2013; Teixeira et al., 2014; Palomares et al., 2016; Bittar et al., 2018b). A study evaluating the effect of injectable trace minerals on lactating Holstein cows found a positive effect of trace mineral supplementation on udder health characterized by lower somatic cell count in multiparous cows, lower incidence of subclinical mastitis, reduced incidence of clinical mastitis on multiparous cows, and decrease incidence of stillbirths and endometritis. This was explained as a probable effect of the Se and Cu supplementation improving udder health and improving the innate and adaptive immune response (Machado et al., 2013). Further studies in dairy calves, early in postnatal life, demonstrated a beneficial enhancement of the immune response and reduction in oxidative stress status. In this study, calves receiving trace minerals had an improve glutathione peroxidase activity, lower incidence of disease at preweaning

(diarrhea, pneumonia, and otitis), and improve neutrophil function (Texeira et al., 2014). The supplementation of trace minerals in both studies improved the oxidative stress status along with the particular effects of each mineral contributing to an improve immune response.

Several studies have reported the effects of trace minerals when used concomitantly with MLV vaccination in beef cattle at times of high stress (i.e., before transportation, upon arrival, and at the time of vaccination). In a study including steers (10-12 months old), that were vaccinated with a multivalent MLV vaccine, the animals receiving trace minerals had enhanced humoral response to BHV-1 following vaccination. This contributed to an improve health status in the herd (Arthington and Havenga, 2012). In another study, highly stressed crossbred beef heifers received an MLV vaccination and were supplemented with two different preparations of commercial injectable trace minerals at initial processing. They found that an improved overall gain and feed efficiency was achieved during the receiving period in calves receiving the trace mineral. In addition, the antibiotic treatment cost was lower for all heifers receiving trace mineral. A similar response was observed when comparing either trace mineral product (Richeson and Kegley, 2011). In a study conducted in 2014, a combination of highly stress calves and steers were parenterally vaccinated (MLV multivalent vaccine) and given trace minerals injection or no trace minerals treatment upon arrival to the feedlot. The study concluded that animals receiving trace minerals had a higher BVDV antibody response than the control animals. Thus, trace minerals enhanced the humoral immune response to the vaccine, but no effect was observed on growth performance (Roberts et al., 2016). A study in beef cattle demonstrated the efficacy of trace minerals at time of high stress and higher metabolic demand. Periods of high stress are generally followed enhanced concentrations of glucocorticoids in plasma, higher oxidative stress, and dampened immune responses (Anderson et al., 1999).

Strategies to mitigate oxidative stress in this herd included vitamin supplementation (Vitamin E and C), trace mineral supplementation, especially trace minerals involved in metalloenzymes (Se, Zn, Cu, Mn), and synthetic antioxidants (Deters and Hansen, 2020).

There are several studies reporting the effect of trace minerals in dairy calves at the time of MLV vaccination. A study preformed in 2016 by Palomares and collaborators, 35 dairy calves were enrolled in a randomized control trial. Calves were vaccinated with an MLV vaccine and randomly assigned to trace mineral or saline treatment groups. The study concluded that calves receiving trace minerals had an increased BVDV1 SNA titer in serum, and higher peripheral blood mononuclear cell proliferation to BVDV1 and BRSV. Another report that was part of that study, assessed the response to an *M. haemolytica* and *P. multocida* killed vaccine. They found that calves receiving trace minerals had an increased titer to *M. haemolytica* at 21 and 56 days after vaccination (Bittar et al., 2018a). Hence, both studies demonstrated an enhanced immune response in the presence of trace minerals given concomitantly with the MLV or killed vaccines. A series of experimental challenges with intranasal inoculation of BVDV2 after MLV vaccination in the presence or absence of trace minerals were reported by the same lab. One of these studies was performed in weaned dairy bull calves given an MLV booster at 4.5 months old then challenged with BVDV2 two weeks later the study reported evidence of clinical protection against challenge following the MLV vaccine. There was no enhancement of the immune response in calves receiving the trace minerals (Bittar et al., 2018b). On the other hand, in a study performed on recently transported beef calves, an MLV booster given at 7 months of age followed by a challenge with BVDV2 five days after the booster, reported an increase in the humoral response against BVDV1 & 2, increased platelet absolute number relative to the challenge, and reduced decline in CD 4^+ and CD 8^+ T cells in response to challenge in the calves

receiving the trace minerals (Bittar et al., 2020). In conclusion, trace minerals, given at the time of vaccination with an MLV have been shown to enhance the humoral and cell-mediated responses against the BRD pathogens that were in the vaccine and monitored in these studies. These results support the strategic use of trace mineral supplementation at the time of vaccination in young dairy calves and in newly received beef calves at the stocker or feedlot.

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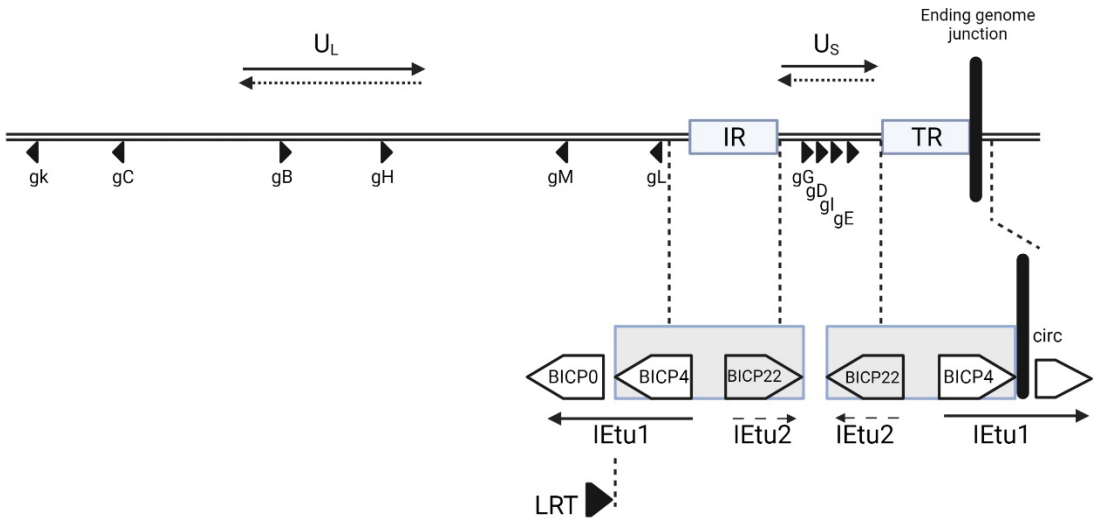

Figure 1.1: Organizational scheme of the BHV1genome. There are two sequences, one is the long sequence, referencing the L gene (U_L), and a short sequence (U_S). The short sequence is flanked by an internal repeat (IR), and by a terminal repeat (TR). The location of the 10 genes encoding the glycoproteins is represented by black arrows along the main line. The promoters of the two immediately early proteins (IEtu1 and IEtu1) are located in the IR and TR sequences. The activation IEtu1 located in IR leads to the transcription of BICP4 and BICP0 genes, whereas the activation IEtu1 located in TR leads to the transcription of BICP4 and circ genes. The transcription of BICP22 is mediated by IEtu2 promoter. Latency Related Transcript (LRT) region is represented by black arrow at the bottom of the graphic. This region is actively expressed during latency period. Created with BioRender.com. Adapted from B. Muylkens et al., 2007.

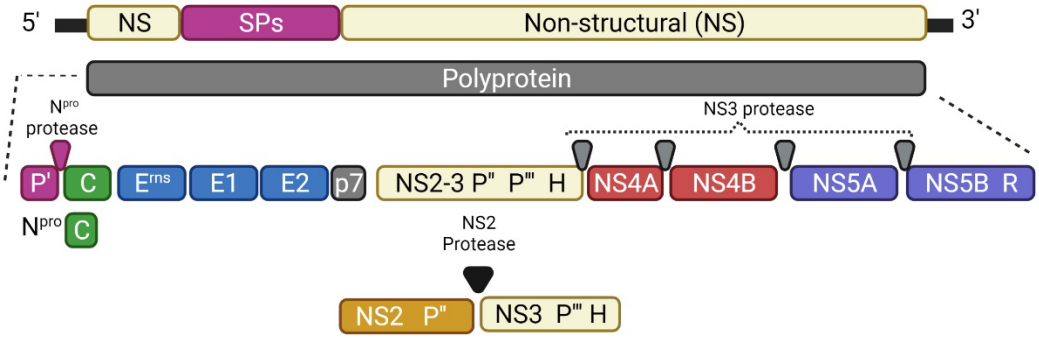

Figure 1.2: BVDV genome organization diagram depicting the main proteins. Each section represents an individual mRNA. The nonstructural proteins are depicted as NS. In noncytopathic BVDV, NS2-3 cleavage is detectable for a short time. Meanwhile, in the cytopathic BVDV, NS3 is continuously produced along with NS2-3. Created with BioRender.com. Adapted from Fenner's Virology, 5th edition.

Figure 1.3: BRSV genome organization diagram depicting the main proteins (3' – 5'). Each section represents an individual mRNA. Created with BioRender.com. Adapted from Fenner's Virology, 5th edition.

Figure 1.4: BPI$_3$V genome diagram organization depicting the main proteins (3' – 5'). Each section represents an individual mRNA. Created with BioRender.com. Adapted from Fenner's Virology, 5th edition.

Figure 1.5: Bovine Coronavirus genome organization diagram depicting the main proteins (3' – 5'). Each section represents an individual mRNA. The downstream of ORF1b *Betacoronavirus* can represent diversity of different species and subspecies.

Created with BioRender.com. From King, A.M., Adams, M.J., Carstens, E.B., Lefkowitz, E.J. (Eds.), Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses, p. 808. Copyright ® Elsevier (2012).

CHAPTER 3

EFFECT OF ADMINISTRATION OF TRACE MINERALS (SE, ZN, CU, AND MN) ON SYSTEMIC AND MUCOSAL IMMUNE RESPONSES ELICITED BY PRIMARY INTRANASAL MODIFIED-LIVE VIRUS VACCINATION IN DAIRY CALVES[1]

[1] Hoyos-Jaramillo A, Palomares RA, Bittar JHJ, Hurley DJ, Saliki J, Stanley, S, Gutiérrez A, Urdaneta J, Hamrick B, Rodríguez A, Graham J. To be submitted to Vaccine, December 2021.

Abstract

Intranasal (IN) vaccination with modified-live virus (MLV) vaccines is a common part of the management designed to prevent bovine respiratory disease (BRD). Injectable trace mineral (ITM) supplementation given in conjunction with MLV vaccines has been proven to enhance both the immune response and health status of dairy calves. The objective of this study was to document the effects of ITM administered at the time of primary IN MLV vaccination of young dairy calves on the level of serum neutralizing antibody (SNA) titers to BHV1, BRSV, and BPI_3V, the level of cytokine expression in circulating cells, and the level of BHV1-specific IgA in nasal secretions following the vaccination. A total of 60 calves (1 month old) were administered an IN MLV vaccine containing BHV1, BRSV, BPI_3V (Inforce 3®), and randomly assigned to one of two experimental groups: ITM (n=30; Multimin®90 containing Se, Cu, Zn and Mn) or SAL (n=30; sterile saline). There was a consistent decay in virus-specific SNA titers in both groups. Calves treated with ITM had significantly higher BRSV-SNA titers on day 14 ($p = 0.045$), and day 28 ($p = 0.028$) than the control calves. No significant differences were observed in the NA titers for BHV-1 or BPI_3V between groups. There was a significant increase in BHV-1-specifc IgA in nasal secretion in both groups, but no significant difference between groups. In conclusion, primary IN vaccination of young dairy calves with high levels of maternally derived SNA did not produce a significant increase in SNA titers to the vaccine viruses but did stimulate a significant IgA response to BHV-1 in nasal secretion. Injectable trace mineral supplementation was associated with a delayed decrease of BRSV SNA titers. These were significantly higher on days 14 ($p = 0.045$) and 28 ($p = 0.028$) after primary IN vaccination with ITM compared to control group. Administration of ITM was associated with lower clinical scores and BRD morbidity and mortality compared to the control group. Treatment with ITM did

not affect the serum titers for BHV1 and BPI3V, or the BHV-1-specific IgA level in nasal secretion.

Keywords: Intranasal vaccination, Modified-live virus vaccines, Trace minerals, Dairy calves.

Introduction

Bovine respiratory disease (BRD) is the most common and costly cause of morbidity in the dairy and beef industry (USDA, 2013). BRD is the leading cause of mortality in weaned dairy heifers (USDA, 2014), and poses a constant welfare concern for dairy farms (Maier, 2019). BRD is a polymicrobial and multifactorial disease that represents a heavy economic burden to the cattle industry worldwide (Griffin, 1997; McVey, 2009). Several risk factors, including extremes in climates, high stocking density, weaning, transportation-related stress, introduction of new calves from outside sources, and poor nutrition have been identified as contributing to the development of BRD (Snowder et al., 2006, Woolums 2013). Viral infections play an essential role in BRD pathogenesis. Infection with *Bovine herpesvirus 1* (BHV1), *Bovine respiratory syncytial virus* (BRSV), and *Bovine Parainfluenza type 3 virus* (BPI_3V) cause a disruption of the respiratory mucosa leading to secondary bacterial infections and lead to consequential bronchopneumonia (Cusack 2003, Ellis 2010, Walz 2010). Moreover, *Bovine viral diarrhea virus* (BVDV) and BHV1 cause immune suppression. This leaves the animals more susceptible to infection by resident commensal bacteria of the upper respiratory tract that become pathogens under these conditions (Panciera and Confer, 2010).

An essential goal of cattle health programs is the application of effective vaccination and adequate biosecurity measures to reduce the impact of BRD on young calves that still have relatively high levels of circulating maternal antibodies (Palomares et al., 2016). Published studies provide evidence that suggests that parenteral vaccination of calves in the face of maternal antibodies (IFOMA) often results in a limited immune response to vaccine antigens. This appears to be due to inhibition of the recognition, processing and response to vaccine antigens mediated by circulating maternal antibodies (Kimman et al., 1989; Ellis et al., 2001; Endsley et al., 2004). At present, it is a common management practice to prime vaccinate young dairy calves IFOMA with an intranasal (IN) MLV vaccine against the family of viruses commonly associated with BRD viral pathogenesis (Woolums, 2007). Circulating maternal antibodies do not appear to interfere with the processing and response to IN MLV vaccine antigens (Hill et al., 2012; Ellis 2017). In addition, IN vaccination will enhance the mucosal immune response against the vaccine antigens. This is particularly useful for those infecting the animal by the respiratory route (Chamorro et al., 2016b, Ellis, 2017). Intranasal vaccination has been associated with inducing a long-lasting mucosal immune memory. This results in increased levels of antigen-specific secretory IgA at the time of natural antigen exposure (Hill et al., 2012; Hill et al., 2019). IN MLV vaccines also induce systemic protective immunity against viral infections (Kimman et al., 1989; Vangeel et al., 2007 and 2009; Xue et al., 2010; Windeyer and Gamsjäger, 2019).

Adequate trace minerals are required for the optimal function of many physiological processes. Trace minerals impact the immune response, general health vigor, and promote growth (Enjalbert 2006, Spears and Kegley, 2002). Previous studies have shown beneficial effects of using injectable trace minerals (ITM; Se, Zn, Cu, Mn; Multimin-90®; Multimin –USA

Inc ®) to enhance the immune response and improve protection against BRD pathogens when combined with MLV vaccination, (Arthington and Havenga, 2012; Roberts et al., 2015; Palomares et al., 2016; Bittar et al., 2018a, Richeson and Kegley, 2011). Studies on calves treated with ITM at the time of vaccination demonstrated improvement in both humoral and cell-mediated immune responses against respiratory pathogens. These were reflected in increased specific circulating antibody against BVDV and *M. haemolytica,* and enhanced leukocyte proliferation in response to BVDV, BRSV and *P. multocida* in culture (Arthington et al., 2014; Palomares et al., 2016, Bittar 2018b). Further, in a study utilizing highly stressed calves, injection of trace minerals induced a quicker rise in BVDV1 specific antibody titers upon arrival at the feedlot (Roberts et al., 2016). Studies in our laboratory have also demonstrated that dairy and beef calves vaccinated IFOMA were capable of mounting specific humoral and cell-mediated immune responses and providing clinical protection against BVDV2 challenge after MVL vaccination concurrent with ITM delivery (Bittar et al., 2018a; Bittar et al., 2020). However, the effects of ITM on the systemic and mucosal immune response after IN vaccination of young dairy calves had not been fully evaluated. Thus, it is the main focus of this study.

We hypothesized that administration of ITM concurrently with IN MLV vaccination would enhance circulating SNA titers against BHV1, BPI_3V, BRSV, and enhance mucosal BHV1-specific IgA in nasal secretions of young dairy calves. We also hypothesized that calves receiving ITM concurrent with IN MLV vaccines would have enhanced mRNA expression in circulating cells following vaccine response for both pro-inflammatory and anti-inflammatory cytokines. Our objectives were to determine the effects of ITM administered at the time of primary IN vaccination on the development of NA against BHV1, BRSV, and BPI_3V, and production of mucosal BHV1-specific IgA in nasal secretions in young dairy calves; and to

measure the expression of pro-inflammatory and anti-inflammatory cytokines in calves relative to the administration ITM.

Methods

Study location and animal husbandry

This study was performed at a commercial dairy farm located at Quitman, Georgia under commercial management conditions. The experimental protocol was reviewed and approved by clinical research committee of the College of Veterinary Medicine, University of Georgia. This farm had approximately 7,000 milking cows at the time this study was done. Eighty dairy bull calves born during May 5th through the 11th of 2017 were all initially reviewed for use this study. Calves were fed 4 L of pasteurized pooled colostrum from primiparous and multiparous cows using a bottle. If the calves were not able to suckle, the colostrum was delivered using an esophageal tube feeder (Jorvet; Jorgensen Laboratories, Loveland, CO). The calves were managed in individual outdoor hutches (1.5 m wide by 2 m long) in an area separate from the rest of herd. The individual pens were bedded with sand, which was changed every week. Within the first week, the calves were fed a total of 8.5 L of milk divided equally three times per day (06:00, 12:00, and 17:00 h). Water and calf starter diets were offered *ad libitum* starting at day 3 of life to all calves. Calves were weaned at 2 months of age. Nutritional values of the feed starter and milk replacer met the nutritional requirements for growing dairy calves by the NRC (2001) (Table 3.1).

Inclusion criteria and data collection

The calves were evaluated every day after birth by trained farm personnel and once a week by two experienced veterinarians. To be included in the study, a baseline immunological and clinical evaluation was conducted, the calves were shown to be free of apparent clinical disease [e.g., fever (rectal temperature ≥101 °F), respiratory signs (cough, dyspnea, nasal, or eyes secretion) and showed no signs of diarrhea (loose to watery stools)] prior to inclusion in the study. They were shown to have an adequate passive transfer (total IgG ≥ 1000 mg/dl)]. All calves utilized were negative for BVDV by antigen capture ELISA from skin biopsies. Once included, they were vaccinated, and the study initiated.

Experimental design, animals, and treatments

On study day 0, a total of 60 healthy calves (about 1 month of age) were selected and administered 2-mL (1 mL in each nostril) of an intranasal (IN) modified-live virus (MLV) vaccine containing BHV1, BRSV, BPI3V (Inforce 3®Zoetis Animal Health NJ, USA.), and randomly assigned to one of two experimental groups:

1. ITM (n=30): calves were administered injectable trace minerals (ITM, Multimin®90, 1 mL/100 lb of body weight) containing Se, Cu, Zn & Mn subcutaneously (SC). Each ml of the trace mineral supplement contains 60 mg of Zn, 10 mg of Mn, 5 mg of Se, and 15 mg of Cu (Multimin ® 90, North America Inc., Fort Collins, CO).
2. SAL (n=30): Calves received an injection of sterile saline solution (1 mL/100 lb of body weight) SC.

Randomization was done using a random number generator (www.random.org) with stratification based on total circulating IgG and average daily gain prior to the start of the study.

Treatments (ITM and SAL) were administered in the neck region on the right side following the Beef Quality Assurance Program guidelines (Beef Quality Assurance 2010®; Centennial, CO). No adverse local inflammatory reactions were observed.

Sample collection and sample processing

Blood samples were obtained by jugular venipuncture into vacuum tubes (Vacutainer® 10 ml, BD Diagnosis, Franklin Lakes, NJ). These contained anticoagulant (acid citrate dextrose - ACD) for obtaining whole blood or nothing for serum.

For obtaining serum, blood samples with no anticoagulant were incubated at 37 °C for 30 minutes following collection to facilitate the retraction of the clot. All samples were centrifuged at 671 x g for 15 minutes and the serum was collected in 1 mL volumes in micro-centrifuge tubes. The whole blood samples with ACD were processed within five hours after collection to isolate the buffy coat cells following the procedure as previously described (Harpin et al., 1999).

Nasal secretion samples were collected from each individual calf using two foam sponges cut to the size of each nostril and attached to a red string to facilitate its visualization for removal. These sponges were introduced one at a time into each nostril and kept in place for 30 to 60 seconds. The sponge was removed from the nostril by pulling the string and was introduced into a 12 mL syringe without the plunger. The sponge was squeezed using a barrel, and the nasal secretions collected in sterile microcentrifuge tubes.

Blood and nasal samples were collected on days -21, 0 (vaccination), 7, 14, 21, 28, 42 and 60 relative to the day of priming vaccination plus ITM or SAL treatment. Serum, nasal secretion, and buffy coat cells were all stored at -80 °C until processing for assessment. Serum was assessed for SNA titers against BHV1, BRSV, and BPI3V using serum. Buffy coat cell

samples were analyzed to determine the level of mRNA expression for both pro- and anti-inflammatory cytokines. Finally, the nasal secretions were tested for BHV1-specific IgA level using an ELISA measurement. Serum samples were also used to determine the trace mineral status at each time point by the Michigan State University Veterinary Diagnostic laboratory.

Total Ig G concentration in serum

Serum samples collected at one week after colostrum feeding were submitted to the University of Georgia Tifton Veterinary Diagnostic laboratory (Tifton GA) to assess the passive transfer status as represented by the total IgG concentration in serum. The IgG quantity was measured using the method described in Chelack et al. (1993). Briefly, serum samples were diluted 1:4 then added to wells of a single radial immunodiffusion kit. Quantity was measured using antiserum against bovine IgG (heavy and light chains; Jackson Laboratories, West Grove, Pennsylvania, USA) antibody. The radial diffusion plates were commercially prepared from 2% agarose and quality assured by the company. The test was run by adding antiserum in phosphate-buffered saline at 2.5% with a pH of 7.25. All samples were tested in triplicate and incubated at 25°C for 18 to 24 h in a humid chamber before measurement of the precipitate. The standard curve mean for the assay (range 1.06 to 8.5 g/L) were obtained using duplicates of bovine IgG serum calibrators (Midland BioProducts Corporation, Boone, Iowa, USA). The validity of the test was verified using a reference serum from the Center for Veterinary Biologics, Animal and Plant Health Inspection Service, USDA, Ames, Iowa. The values for the ring diameters were digitally measured with a computer-assisted plate reader (The Binding Site Group, Birmingham, England) and the quantities of antibody in serum calculated using a linear analysis regression program.

Morbidity and health status evaluation

Morbidity was assessed everyday by farm personnel for the initial group of 80 calves. General signs of illness - anorexia and depression, respiratory symptoms (cough, nasal, or eye secretions, and respiratory effort) or diarrhea was scored and recorded by the farm personnel in charge of management and care of the animals. In addition, the general health status was evaluated once a week by two veterinarians using the health scoring system developed at the University of Wisconsin by McGuirk (2018). Clinical signs of illness and the average daily weight gain (ADG) were evaluated on days 0, 7, 14, 21, 28, 42, and 60 of the trial by these experienced veterinarians. Clinical signs recorded by two veterinarians that were blinded to each treatment group and calves IDs. Clinical signs included: rectal temperature, evidence of coughing, nasal and eyes secretion, position of head-ears (attitude), depression (poor attitude), consistency of the feces, and hydration status (skin pinch). Each sign was scored on a scale 0 to 3, with zero representing absence of clinical problems, and 3 indicating a severe clinical problem indicated. The total maximum possible score was fifteen. Finally, the total score was calculated by adding all the individual scores for these variables (Bittar et al., 2018b). Calves reporting moderate or severe respiratory disease or having indications of diarrhea were isolated in a quarantine area and treated with antibiotics. These calves were removed from the immunological evaluation protocol. For the purpose of this study, the health scores of the sick calves were included only until the day the moderate to severe clinical signs of disease were identified. As these calves were isolated in quarantine, no further samples were collected, and no further clinical scores were not recorded on subsequent study days. The exclusion was performed to avoid bias during the evaluation of the immune response after vaccination relative to trace mineral injection.

Serum neutralizing antibody (SNA) titers against BHV1, BRSV and BPI$_3$V

Serum neutralizing antibody (SNA) titers against BHV1, BRSV and BPI$_3$V were measured by the Athens Veterinary Diagnostic Laboratory at the University of Georgia (Athens, GA). They used a standard clinical virus neutralization test on Madin-Darby bovine kidney cells (MDBK). Briefly, serum samples were thawed at room temperature and heat inactivated at 56°C for 30 min in a water bath. The samples were diluted in duplicate as a serial 2-fold dilution with Dulbecco minimum essential medium (DMEM). The starting point for the assay was 1:2. The assay was conducted in flat bottom 96-well cell culture plates. Each virus was diluted in DMEM to a concentration of 100 $TCID_{50}$ per 25 µL and 25 µL of each virus was added in each test well. This final addition of virus made the final starting dilution of serum 1:4. The plates were incubated in 5% CO_2 at 37°C for 1 h. At this point 150 µL (10^4 cells) of a MDBK cells suspension in DMEM containing 10% fetal bovine serum (FBS) was added to each well. The plates were incubated in 5% CO_2 at 37°C for 4 days. An inverted compound microscope was used to evaluate the cell monolayer for virus-specific cytopathic effects relative to each virus. The SN titer for each sample was reported as the highest dilution of serum that completely inhibited virus induced cytopathic effects in both wells.

BHV1-specific IgA ELISA in nasal secretions

Total IgA concentration in nasal secretions was determined using an ELISA developed in house for quantification of bovine IgA (Bovine IgA ELISA Quantitation Set, Bethyl Laboratories Inc, Montgomery, Tex.). To measure IgA recognizing BHV-1 specifically, each well of a 96-well plate flat bottom ELISA plate (MaxiSorp™, Millipore Sigma, Merck, Germany) was coated with 100 µL of a 1:400 dilution of binary ethyleneimine inactivated BHV1 Cooper strain in pH 9.7

carbonate buffer (coating buffer). This dilution was determined to yield maximal BHV1-specific signal from a positive BHV-1 nasal secretion from vaccinated calves and minimal background signal from fetal bovine serum. The plat was incubated overnight at 4°C. The next morning, the plate was washed three times with PBS containing 0.05% Tween 20 (wash buffer). The plates could be prepared before the assay day and stored dry at -20°C until they were needed. On the day of the assay, the plates were hydrated with wash buffer. The plates were blocked by adding 200 µL of PBS solution containing 0.5% bovine serum albumin in each well and incubated at room temperature for 1 hour. The plates were washed three times with wash buffer. While plates were being blocked, the nasal secretion samples, thawed at 4ºC the day before were brought to room temperature and diluted in wash buffer starting to 1:3.125. This resulted in a final sample dilution of 1:50. Each sample was vigorously mixed to homogenize it using a vortex before testing and plated in duplicates to be diluted across the plate. Eight samples per plate were tested. The first two duplicates had 100 µL of the 1:3.125 diluted sample, the next four wells contained 200 µL of wash buffer. The plates were incubated at room temperature for 1 hour. The plates were washed five times with wash buffer. To each well, a 1:500 dilution of horseradish peroxidase conjugated rabbit anti-bovine Ig A (Bethyl Laboratories, Montgomery, TX) was added. These plates were incubated at room temperature for 30 minutes. The plates were washed five times with wash buffer. Finally, 100 µL of 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS, Sigma) with 0.3% H_2O_2 was added to each well. The plates were incubated in a dark chamber at ambient temperature for 20 minutes or until the required color intensity was observed for the positive control samples. Plates were read at 405 nm with a microplate reader (Epoch, BioTech, Whiting, VT). Titers were reported as the inverse of the last dilution yielding greater than or equal to twice the mean optical density of the negative control (low IgG FBS,

Gibco, ThermoFisher®, Waltham, MA) at a 1:100 dilution. Samples with an endpoint surpassing the range of detection were assessed at higher dilutions and the samples that were negative at all dilutions were retested at a lower dilution.

Cytokine mRNA expression analysis

RNA extraction and cDNA synthesis for mRNA expression analysis

Total RNA extraction was performed for samples collected on days 0 (prime vaccination), 7, 14, 21 and 28. Frozen buffy coat cell samples were thawed at room temperature and total RNA was extracted using an RNAeasy[2] mini kit according to manufacturer instructions. Total RNA concentration was assessed by spectrophotometry using a Nanodrop[3]. If samples did not meet the spectrophotometry quality standards (having 260:280 ratios between 2.0 and 2.2) were discarded and re-assessed. cDNA templates were synthesized by using the high-capacity cDNA[4] Archive Kit with 100ng of RNA as a template.

Optimizing primer concentration PCR efficiency

Primer concentrations and *q*RT-PCR conditions were optimized to increase the PCR efficiency producing maximum amplicon with the expected optimal melting curve for each specific target gene. During this procedure, different primer concentrations were tested (50, 300, 900 nM) to generate optimal amplification signal of the following target genes IFN-γ, IL-12, IL-4, IL-10, TGF-β, FOXP3, and 18S ribosomal subunit as housekeeping gene (Table 3.2). PCR

[2] RNeasy®, QUIAGEN, Inc, Germantown, MD.
[3] ND-1000 UV-Vis Spectrophotometer, Nanodrop Technologies, Wilmington, DE.
[4] High-capacity cDNA Reverse Transcription Kit Applied Biosystems, Inc, Foster city, CA.

efficiency was evaluated for each target gene by establishing a standard curve with half log dilutions (1-100 pg/ml) of a sample with higher cDNA yield.

Quantitative real-time PCR

Quantitative real-time PCR was performed using SYBR green nucleic acid stain[4]. In a 96-well plate, the PCR mix was prepared using 15 µL SYBR green Master Mix, 1 µL each forward and reverse of primers at the specific concentration, 1 µL of dd H_2O, and finally 2 µL of cDNA template (100 ng/ µL) were added to each well. The PCR was set up for pre-incubation at 95 °C, followed by 45 cycles of denaturation at 95 °C for 15 seconds, annealing at 60 to 71 °C (primer dependent) for 10 seconds, and extension at 72 °C for 10 seconds. Melting curves were performed for 60 seconds at each step with temperature increasing gradually by 0.1 °C/second from 65 °C to 95°C. All samples were processed in triplicates and an average of the Ct values was used to calculate the relative amount of mRNA expressed for each cytokine target gene. One sample with the highest yield cDNA was used as a variation control in each plate, and dd H_2O was used as a negative control.

Normalization and relative quantification of target genes

The Ct value is indirectly proportional to the amount of mRNA express in the sample. The greater the mRNA is expressed in the sample the faster threshold is met reaching a lower Ct value. Normalization process was done using the comparative $^{\Delta\Delta}$Ct method (Livak and Schmittgen, 2001). Gene expression analysis was performed by relative quantification of the

mRNA level or fold regulation of target genes normalized to the housekeeping gene 18S (unaffected by the studied conditions) and compared with baseline values on day zero.

Statistical analysis

Data were analyzed using Statistical Analysis System version 9.3 (SAS® Institute, Cary, NC, USA). Serum neutralizing antibody titers against BHV1, BRSV, BPI3V, and specific IgA titers against BHV1 in nasal secretions were $\log_2$ base transformed to normalize the data and then back transformed into geometric means. Shapiro Wilk's test was performed to assess the statistical assumption of normality, and Levene's test was performed to assess the assumption of constant variance. Means for SNA titers, specific BHV1 IgA, $^{\Delta\Delta}$Ct RNA cytokine expression, serum mineral concentration, and weekly health score were compared between groups by repeated-measurements analysis Proc-GLIMMIX model from SAS®, with calf variable as a random effect and the interaction between group and time as a fixed effect. Data were adjusted for multiple comparisons by using Tukey-Kramer's test. For SNA, BHV1-specific IgA in nasal secretions, serum mineral concentration, and weekly health scores, values on day -21 were considered baseline. For cytokine expression, day 0 was considered baseline ($^{\Delta\Delta}$Ct fold change). The baseline days were used as covariates to decrease the variation among the samples at the initial sampling point. For all the results, $p \leq 0.05$ was considered significant, and $0.05 < p \leq 0.10$ was considered a tendency.

Results

All calves had total serum IgG concentrations higher than 1,000 mg/dl, indicating adequate maternal passive transfer of maternal antibody. There was no significant difference in the total serum IgG concentration between the experimental groups (Fig. 3.1).

There was an effect of time after treatment on the serum concentrations of Cu, Se, Mn, and Zn ($p < 0.0001$). Serum trace mineral levels were within the normal ranges for dairy calves and were significantly greater (Se on day 7, $p = 0.015$) or tended to be greater (Cu on day 7, $p = 0.062$; and Zn on day 60, $p = 0.075$) in the ITM calves compared to the SAL calves (Figs. 3.2. A-D). Although, no significant differences were observed for serum concentrations of these trace minerals during other times during the study; overall, the ITM group had a higher level of the supplemented trace minerals.

There was a significant effect of time after treatment on body weight (BW; $p < 0.0001$). As expected, calves began to gain weight after the first week of age with a significant increase within each group after day 14 ($p < 0.05$; Fig 3.3A). There were no significant effects of treatment or the interaction treatment x time on BW and ADG (Figs. 3.3A and B).

Weekly clinical scoring generated only low values during the study on days recorded for both groups (Fig. 3.4). There was a significant effect of time on the clinical scores ($p < 0.0001$). Values for the clinical scores significantly increased in both groups on days 42 ($p < 0.0001$) and 60 ($p < 0.0001$). On days 42, and 60, calves treated with ITM had lower clinical scores than SAL calves ($p = 0.0017$ and $p = 0.0315$, respectively). During the development of this study (June-July 2017) seven of 30 calves (23.3%) in the SAL group had moderate to severe clinical signs of respiratory disease and were treated with antibiotics. Four of these calves (13.3%) died or were

euthanized. In contrast, only four calves in the ITM group had evidence of BRD, and only one (3.3%) died.

There was a significant effect of time on the SNA titers against all viruses (BRSV, BHV1, and BPI_3V; $p < 0.0001$). A consistent decay in virus-specific SNA titers was observed during the duration of the study in both groups. Treatment had a significant effect on SNA titers to BRSV ($p < 0.05$), with greater titers for the ITM group on days 14 ($p = 0.045$) and 28 ($p = 0.028$) than for the SAL calves (Fig. 3.5A). In addition, ITM calves appeared to have numerically greater SNA titers to BRSV on day 60 than SAL calves, but no statistical difference was observed ($p = 0.132$). No significant effects of treatment or the interaction treatment x time were observed in SNA titers against BHV1 (Fig. 3.5B) or BPI_3V (Fig. 3.5C).

There was a significant effect of time on the BHV1-specifc IgA titers in nasal secretions ($p < 0.0001$). Both treatment groups had a significant increase in BHV1-specifc IgA titers in nasal secretions starting on day 14 after vaccination relative to baseline values on day -21 (Fig. 3.6). Treatment with ITM did not have an effect on the BHV1-IgA titers in nasals secretions at any point in the study.

Transcription of mRNA was measured on days 7, 14, 21, and 28, using the values observed before primary vaccination as the gene expression reference (Fig. 3.7 A-F). There was a significant effect of time on the expression of the target genes ($p = 0.01$). In addition, treatment significantly affected the expression of the pro-inflammatory cytokine IFN γ (Fig. 3.7 A). On day 28, calves treated with ITM had lower IFN γ mRNA expression ($p = 0.034$) and a tendency of lower IL12 transcription ($p = 0.077$) than the SAL calves (Fig. 3.7 A and B). On the other hand, the IL4 mRNA levels were up-regulated on days 14 and 21 in both treatment groups, and enhanced expression was seen on day 28 in the SAL group as well. This was significantly

different from values observed for the ITM calves ($p = 0.001$; Fig. 3.7 C). Upregulation of IL10 (on days 14 and 21) and TGF-β（on days 14 and 28) was observed in both treatment groups. There was no significant difference between groups in these measurements (Fig 3.7 D and E). Expression of the regulatory transcriptional factor FOXP3 was increased after vaccination in both treatment groups. We observed significantly higher values for FOXP3 on day 28 in the SAL calves than the ITM treated calves ($p = 0.03$; Fig. 3.7 F).

Discussion

Primary IN vaccination of young dairy calves that have high levels of maternal antibodies under commercial management conditions did not induce a significant increase in neutralizing antibody titers against BHV1, BRSV and BPI_3V. This agrees with previous reports in dairy and beef calves (Vangeel et al., 2007; Hill et al., 2012; Palomares et al., 2021). However, IN vaccination was able to induce a functionally interesting increase in BHV1-specific IgA titers in nasal secretions and evidence of local antigenic stimulation of IgA production, as previously reported (Kimman et al., 1989; Cortese et al., 2017; Hill et al., 2019; Palomares et al 2021). Treatment with ITM slowed the decay of neutralizing antibody against BRSV. This resulted in a significantly higher BRSV-SNA titer in calves on days 14 and 28 post-vaccination. This was not observed in the vaccinated calves concurrent saline-treatment. Further, ITM supplementation appeared to contribute to more robust general health in the calves. This was demonstrated by significantly lower clinical scores on day 42 and 60, with lower BRD morbidity and mortality in these calves. Nevertheless, ITM supplementation did not impact the production or duration of neutralizing antibody against BHV1 and BPI_3V or the production of BHV1-specif IgA found in nasal secretions.

Previous studies demonstrated that supplementation with ITM during early postnatal life offers significant benefits with respect to the anti-oxidant status, immunity (e.g., neutrophil function) and general health (lower incidence of diarrhea, otitis, and respiratory disease) of dairy calves under commercial rearing conditions (Teixeira et al., 2014). This supports the results of the present study.

This trial was performed on a commercial dairy under standard production management protocols and during a period of high temperature –humidity in the environment (average of 85 °F, 75 % humidity during June and July). On this farm, BRD is enzootic and the main cause of morbidity and mortality of their weaned calves. Under such stress conditions, there may be an imbalance between generation and neutralization of pro-oxidants (Duff and Galyean, 2007). This will result in accumulation of reactive oxygen species (ROS). Oxidative stress conditions may cause significant damage to immune cells, resulting in immunosuppression and increase susceptibility to pathogens. Trace minerals, such as Se, Zn, Cu, and Mn, play an important role in the immune system since they act as structural and functional components of the antioxidant enzyme systems responsible for the reduction of the excessive levels of ROS (Sordillo and Aitken, 2009). When these systems are fully functional they decrease the odds of leukocyte damage. In addition, these trace minerals have specific roles in cell migration, phagocytic function, and the killing ability of neutrophils. Trace minerals participate in the antibody production cascade and are also involved in aspects of cell proliferation during adaptive immune capacity clonal expansion (Spears, 2000; Tomlinson et al., 2008, Machado et al., 2013).

Previous studies have demonstrated solid evidence that ITM supplementation benefits the function of the immune response and enhances protection elicited by vaccination (Arthington and Havenga 2012, Palomares et al., 2016, Bittar et al., 2018a and b, Bittar et al., 2020). In those

studies, calves treated with ITM at the time of parenteral MLV vaccination had a stronger response and generated a faster humoral and cell-mediated immune response against BVDV, BRSV, and BHV-1 documented by an increased level of neutralizing antibody and stronger in vitro leukocyte proliferation when stimulated with each vaccine pathogen (Arthington et al., 2014; Palomares et al., 2016). Moreover, the effects of ITM supplementation on vaccine-induced protection have been demonstrated in terms of the ability of ITM- treatment to mitigate the expected reduction in circulating $CD4^+$ and $CD8^+$ T cells following BVDV2 experimental infection (Bittar et al., 2020).

In the present study the trace mineral requirements of the calves were met. After treatment the serum trace mineral concentration in serum was above the lower limit for each mineral in both the treatment and control groups. Under adequate nutritional management including trace mineral supplementation of pregnant cows, calves are born with higher serum and hepatic levels of trace minerals (Van Saun et al., 1989; Stanton et al., 2000; Ahola et al., 2004). It has been demonstrated that a positive correlation between serum Se concentration in young calves and their dams is mediated by an efficient transport of Se through the placenta during late pregnancy (Campbell et al., 1990). In addition, transfer of Se with colostrum may also affect the serum Se concentration of the calf (McConnell and Roth, 1964; Campbell et al., 1990). Since Se is a vital component of the glutathione peroxidase enzyme complex, calves with a greater Se reservoir can cope better with oxidative stress (Sordillo and Aitken, 2009). The enhanced health status and greater titers of BRSV neutralizing antibody observed in ITM-treated calves here suggest that additional strategic trace mineral supplementation may be beneficial to young calves. Their immune system is exposed to many new pathogens (and vaccines) during their first months of life.

The calves studied here had adequate transfer of passive immunity with high titers of neutralizing antibody recognizing BRSV, BHV1 and BPI_3V before vaccination. These antibodies decayed overtime as expected. We observed no significant increase in these antibody titers after vaccination with or without ITM given concurrently with the vaccine. Maternal antibodies are the primary source of protection against new bacterial, fungal, and viral infections in neonatal calves (Chamorro et al., 2016). However, the great level of variability in the elements of passive immune activity to dairy calves represents a challenge for achieving predictable and effective protective immunity in response to vaccination. This is a larger problem when calves face high infectious pressure. Although, high concentration of maternally derived antibodies may interfere with *denovo* endogenous antibody production following vaccination, the rate of antibodies decay in vaccinated calves might slow down as a result of adequate antigenic stimulation (Endsley et al., 2004; Windeyer et al., 2014). Vaccinating young calves with an IN MLV vaccine is a common practice in dairy operations in the USA. This protocol has become popular based on the belief that IN vaccines will avoid antigenic interference from maternal antibodies that circulate after birth. High levels of maternal antibody in serum have been observed to interfere with responses when using parenteral vaccines (Windeyer, 2019; Hill et al., 2012). Ollivett et al. (2018) evaluated the capacity of IN trivalent vaccination in young calves within the first week of life to impact the natural occurrence of BRD. These researchers observed a reduction in lung lesions associated with BRD. However, IN vaccination did not reduce the risk of respiratory disease as demonstrated by those calves having high respiratory scores after vaccination (Ollivett et al., 2018).

In this study, we provide evidence of increased of BHV1-specific IgA in the nasal secretion of both treatment groups after the priming vaccination. However, there was no

significant differences in IgA level between the treatment groups. This antibody response in a mucosal tissue lasted until the booster vaccine was delivered two months later (at 3.5 months of age; data not shown herein). Immunoglobulin A, as secretory IgA is the primary humoral immune response on the mucosal barriers in the respiratory tract. IgA plays an important role blocking binding and entry of pathogens (Osman & Griebel 2018). Neonatal intranasal immunization primes the nasal-associated lymphoid tissues, inducing by inducing a local IgA response against the specific viral antigens, to help dampen future encounters with the natural pathogen. This makes the nasal epithelium an optimal target for mucosal vaccines in young cattle (Brandtzaeg et al., 2011; Osman et al., 2018). Similar to the results observed in this study, a study performed by Hill et al (2012) indicated that calves immunized with an IN vaccine had increased IgA production after vaccination that did not impact the production of serum neutralizing antibody.

Trace mineral supplementation at the time of IN vaccination appears to be associated with a more balanced expression of pro-inflammatory and regulatory cytokines. The observed gene expression profiles in the SAL calves could be the result of inflammation induced by the intranasal vaccine. In addition to the upregulation of IFNγ and IL12, this group had significantly higher expression of IL4 and FOXP3 than the ITM-treated calves. Modified-live vaccines generally induced a cell-mediated immune response driven by virus replication in the epithelial cells and for some viruses, leukocytes. These virus-infected cells initiate antigen presentation in the context of both MHC class I & MHC class II, activate chemokine and cytokines production in the infected tissues that leads to either Th1 or Th2 immune responses as required for pathogen management (Tizard, 2009). We believe that the combined higher expression levels of inflammatory and regulatory molecules in the SAL calves might be a consequence of the level of

vaccine virus replication yielding more robust cellular injury and requiring activation of a stronger repair environment to modulate the inflammation caused by the viruses in the MLV intranasal vaccine. This has been reported for BHV1 found in MLV causing local inflammation due to high levels of local replication of the attenuated virus (Jones and Chowdhury, 2010). We speculate that ITM may either dampen the expression of the stimulatory and regulatory proteins through their control of ROS levels, eliminating subsequent cell damage and ongoing inflammation (Sordillo and Aitken, 2009; Sartori et al., 2016). Enhancing the focus of the immune mechanisms that prevented further inflammation in response to vaccine virus replication to nearer optimal levels for initiation of a functional immune response. Further research is warranted to better understand the Th1/Th2 bias of the response after priming IN vaccination and how ITM effects this when delivered concomitantly.

In conclusion, primary IN vaccination of young dairy calves that have high levels of maternal antibodies circulating was not able to significantly increase the production of new neutralizing antibody that circulated in the calf. IN priming vaccination did stimulate a significant IgA response against BHV1 in nasal secretions. Trace mineral supplementation appeared to slow down the decay in BRSV neutralizing antibodies and was associated with higher BRSV titers on days 14 and 28 after primary IN vaccination. ITM was associated with significantly lower clinical scores and less BRD morbidity and mortality than seen in control calves. Treatment with ITM did not affect *denovo* production of neutralizing antibody recognizing BHV1 or BPI_3V, or the level of BHV1-specific IgA in nasal secretions. Further, treatment with ITM was associated with what appeared to be a more balanced expression of the pro-inflammatory and regulatory cytokines measured in this study. The use of injectable trace

minerals may be an important and practical tool to enhance the health of preweaned calves and make them ready for the next stages of production.

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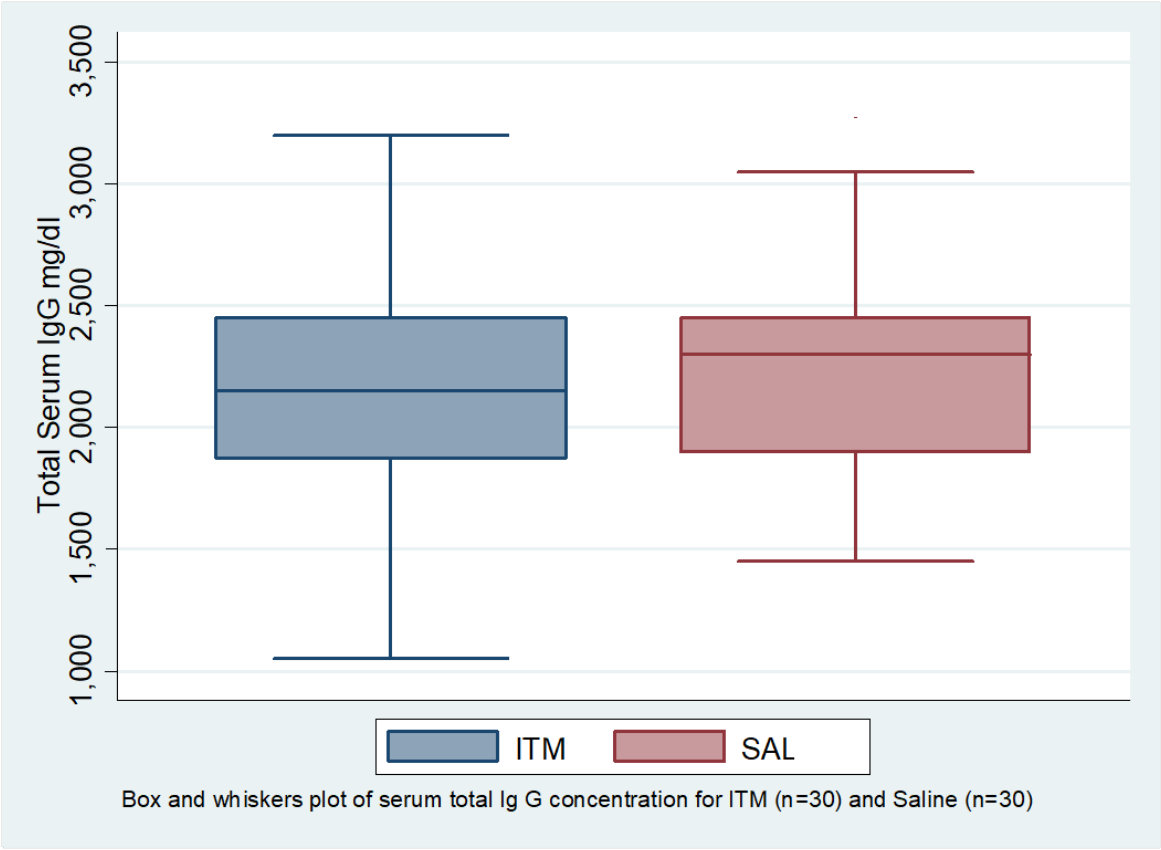
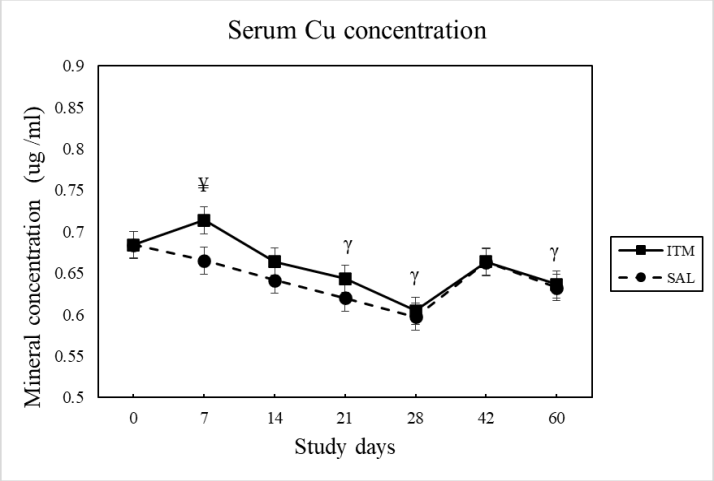
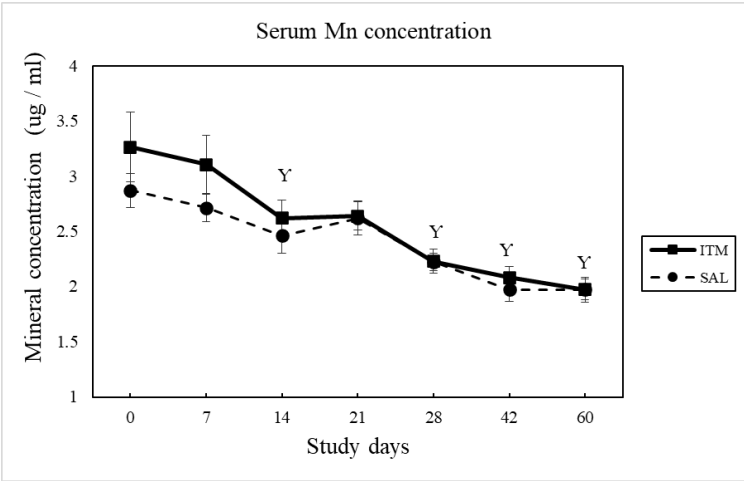


Figure 3.1 – Total serum IgG. Error bars represent the standard error of the means (SEM). As inclusion criteria calves should have had an adequate level of passive transfer measured by total IgG in serum. Calves that had total serum IgG ≤ 1000 were not included in the study. There were no significant differences between groups.

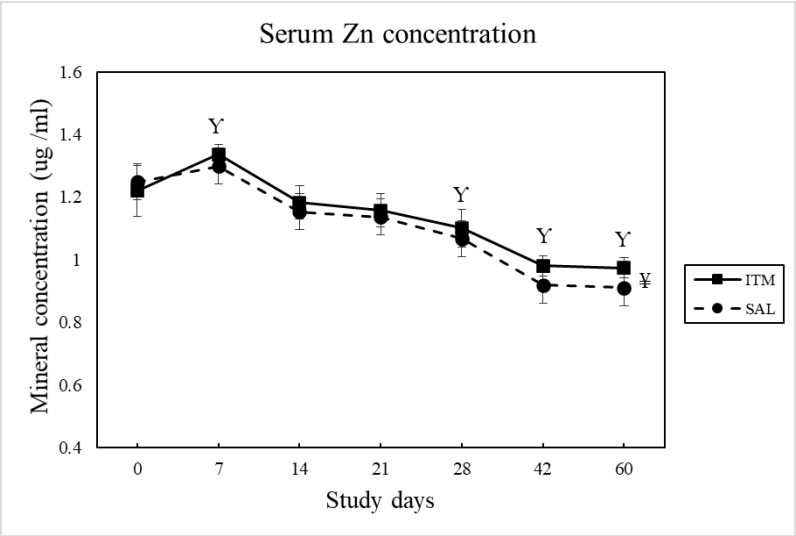
A


Serum Cu concentration
Mineral concentration (ug /ml)
0.9
0.85
0.8
0.75
0.7
0.65
0.6
0.55
0.5
¥
γ
γ
γ
γ
0
7
14
21
28
42
60
Study days
ITM
SAL

B


Serum Mn concentration
Mineral concentration (ug / ml)
4
3.5
3
2.5
2
1.5
1
Y
Y
Y
Y
Y
0
7
14
21
28
42
60
Study days
ITM
SAL

C


Serum Zn concentration
Mineral concentration (ug /ml)
1.6
1.4
1.2
1
0.8
0.6
0.4
Y
Y
Y
Y
¥
0
7
14
21
28
42
60
Study days
ITM
SAL

D

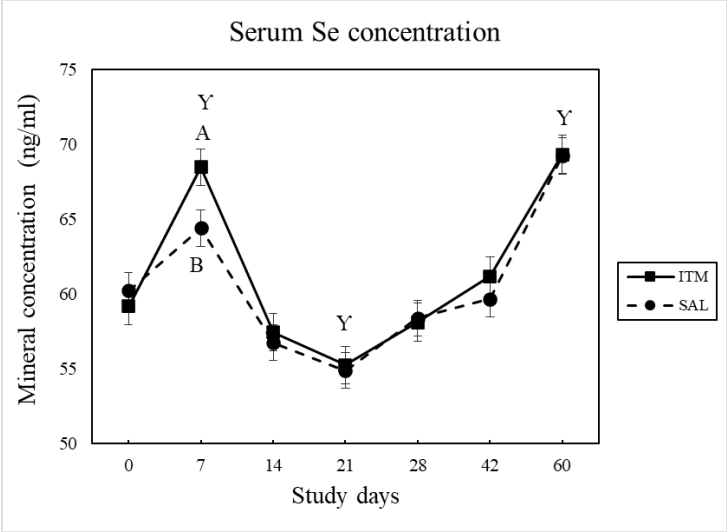
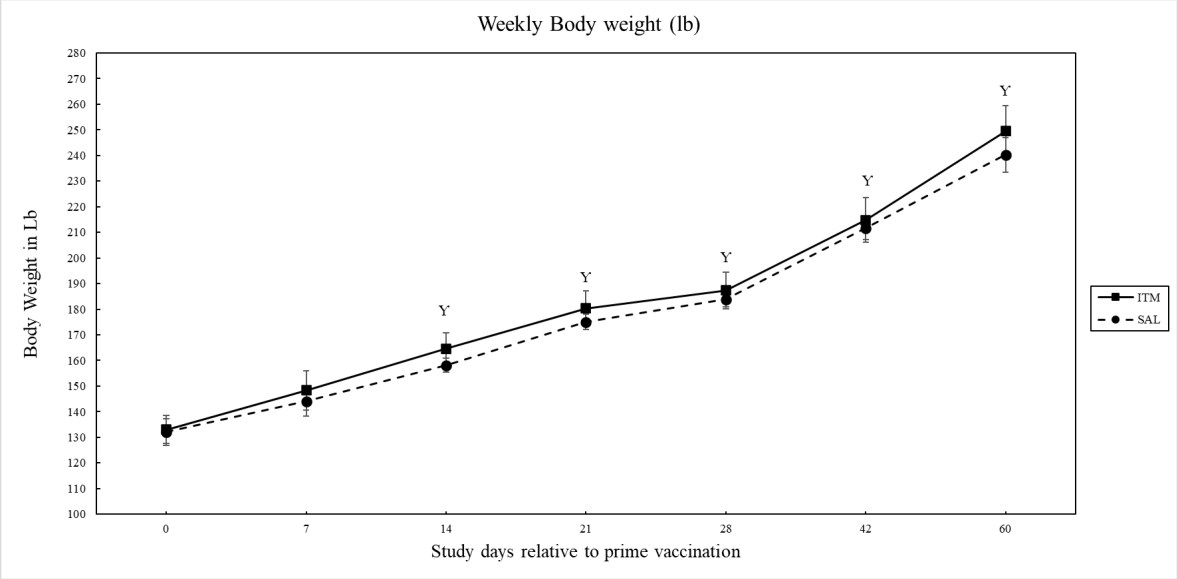

Serum Se concentration
Mineral concentration (ng/ml)
75
70
65
60
55
50
Y
A
B
Y
Y
0
7
14
21
28
42
60
Study days
ITM
SAL

Figure 3.2 – Serum trace minerals concentrations for Copper (A), Manganese (B), Zinc (C), and Selenium (D). Calves received injectable trace minerals (ITM) or saline along with primary intranasal (IN) MLV vaccine (day 0). Errors bars indicate standard error of the means (SEM). Values on day 0 were considered baseline and covariate. Significant difference was found between groups in selenium (Se) serum concentration (day 7; $p = 0.015$), and there were tendencies for greater copper and zinc serum concentrations at days 7 ($p = 0.062$), and 60 ($p = 0.075$), respectively. There was an effect of time for all serum trace minerals (Cu, Mn, Zn, Se; $p = < 0.0001$). ^{A,B} Significant difference between groups ($p < 0.05$). $^{¥}$Tendency between groups ($0.05 < p \leq 0.10$). ^{Y} Significant difference ($p < 0.05$) within the ITM group or SAL group when compared with day 0.

A


Weekly Body weight (lb)
Body Weight in Lb
280
270
260
250
240
230
220
210
200
190
180
170
160
150
140
130
120
110
100
Y
Y
Y
Y
Y
ITM
SAL
0
7
14
21
28
42
60
Study days relative to prime vaccination

B

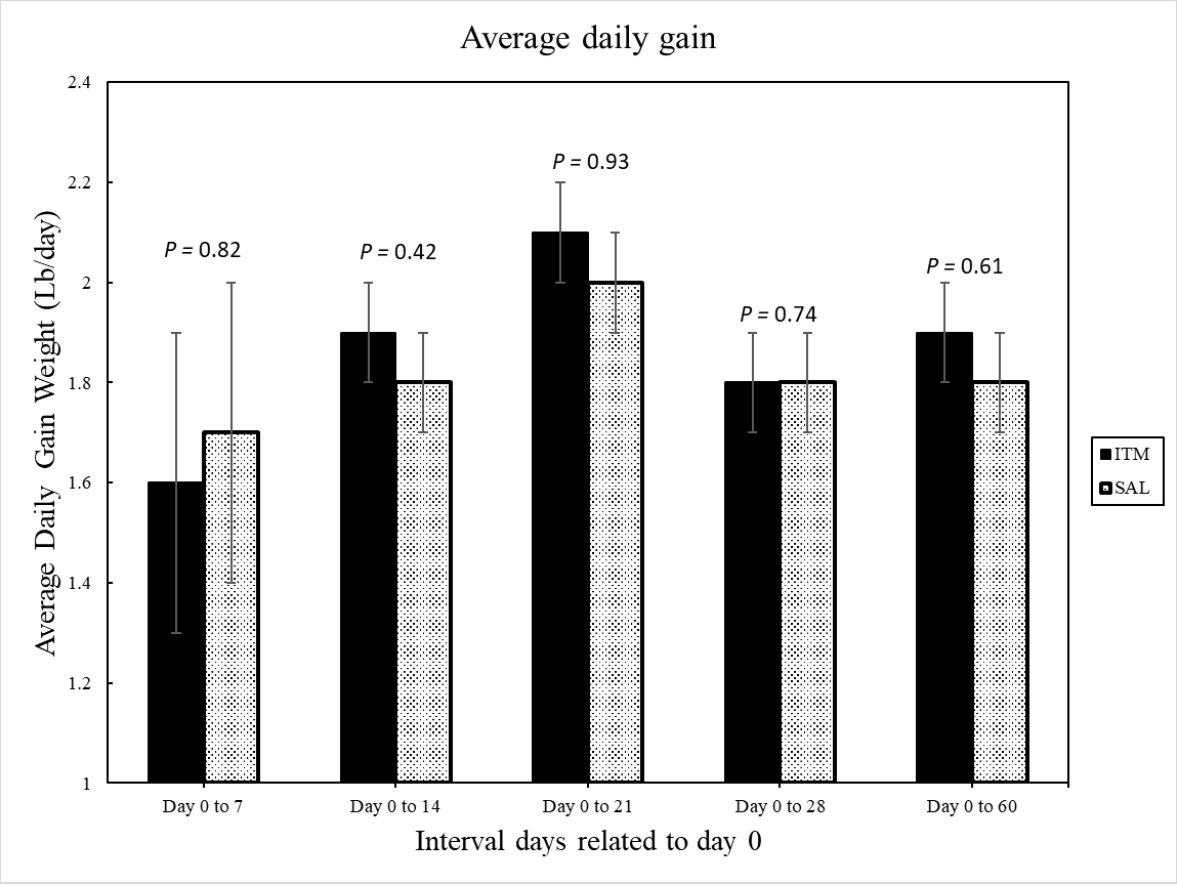

Average daily gain
Average Daily Gain Weight (Lb/day)
2.4
2.2
2
1.8
1.6
1.4
1.2
1
P = 0.82
P = 0.42
P = 0.93
P = 0.74
P = 0.61
ITM
SAL
Day 0 to 7
Day 0 to 14
Day 0 to 21
Day 0 to 28
Day 0 to 60
Interval days related to day 0

Figure 3.3 – Weekly body weight (A) and average daily gain (B) in dairy calves prime vaccinated with an IN MLV vaccine along with either trace minerals (ITM) or saline solution (SAL). Error bars represent the standard error of the means (SEM). Calves increased weight weekly with significant difference ($p < 0.05$) within groups after day 14 (A). There was not significant difference between groups on ADG (B). ^{Y} Significant difference ($p < 0.05$) within ITM and SAL groups when compared with day 0.

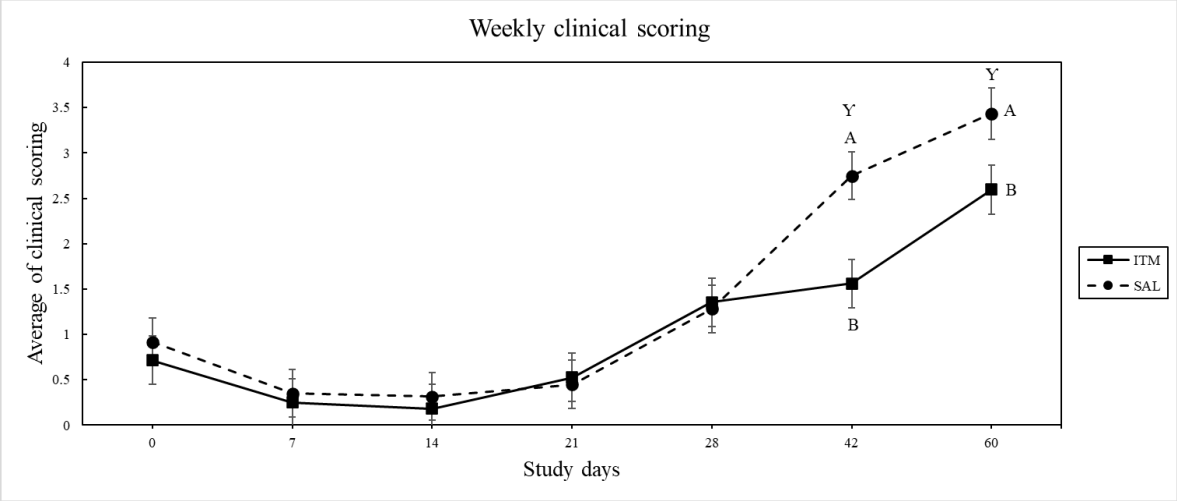
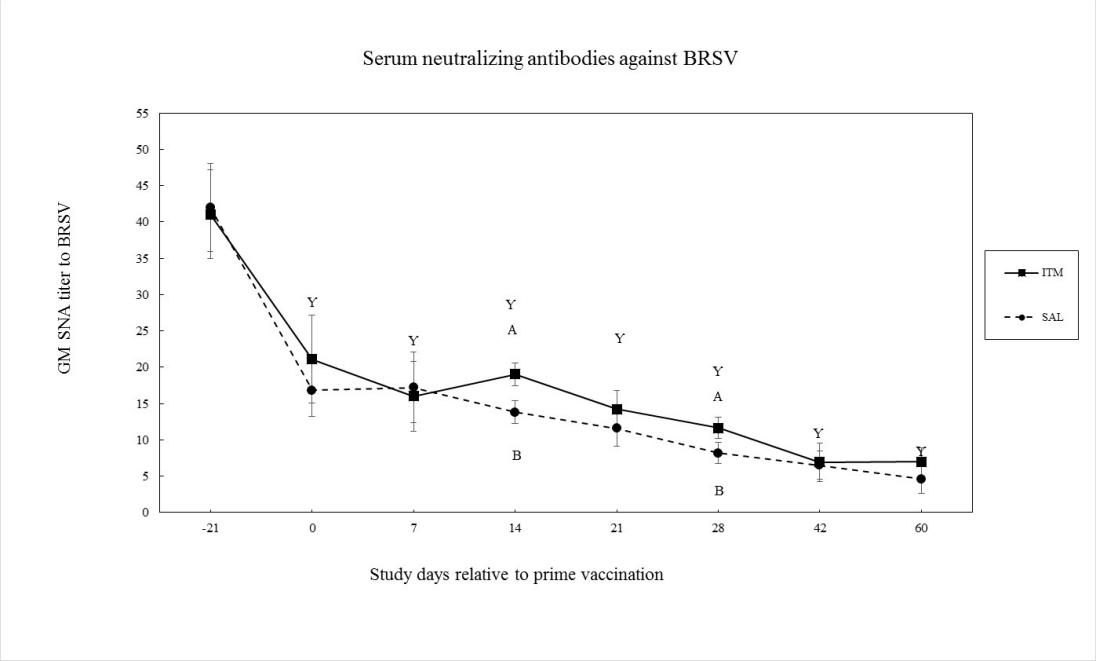
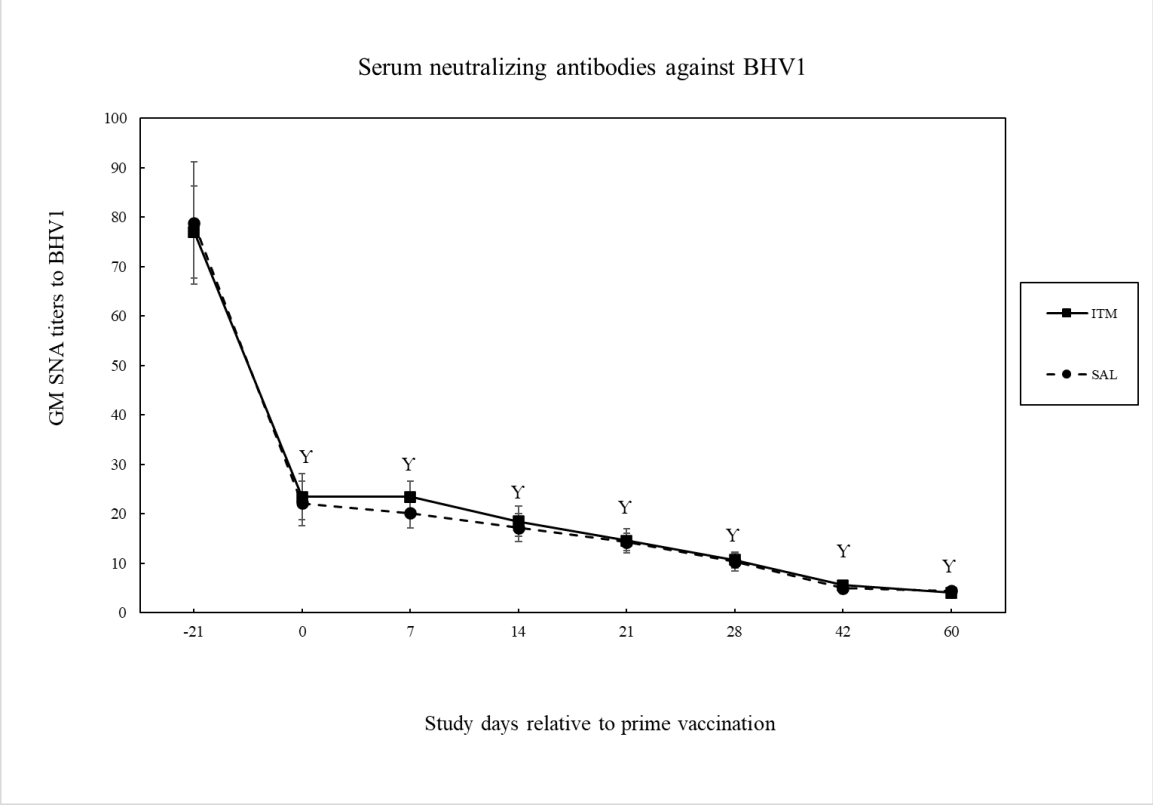


Figure 3.4 – Clinical scoring was evaluated once a week by trained personnel. Error bars represent the standard error of the means (SEM). *On day 42 and 60 SALINE group have a significant greater clinical score compared with ITM ($p = 0.0017$ and $p = 0.0315$, respectively). There was a significant effect of time with clinical scores increasing after day 28 ($p < 0.0001$). A,B Significant difference between groups ($p < 0.05$). Y Significant difference ($p < 0.05$) within ITM or SAL groups when compared with baseline on day -21.

A


Serum neutralizing antibodies against BRSV
GM SNA titer to BRSV
Study days relative to prime vaccination
ITM
SAL
55
50
45
40
35
30
25
20
15
10
5
0
-21
0
7
14
21
28
42
60
Y
Y
Y
A
Y
Y
A
Y
Y
B
B

B


Serum neutralizing antibodies against BHV1
GM SNA titers to BHV1
Study days relative to prime vaccination
ITM
SAL
100
90
80
70
60
50
40
30
20
10
0
-21
0
7
14
21
28
42
60
Y
Y
Y
Y
Y
Y
Y

C

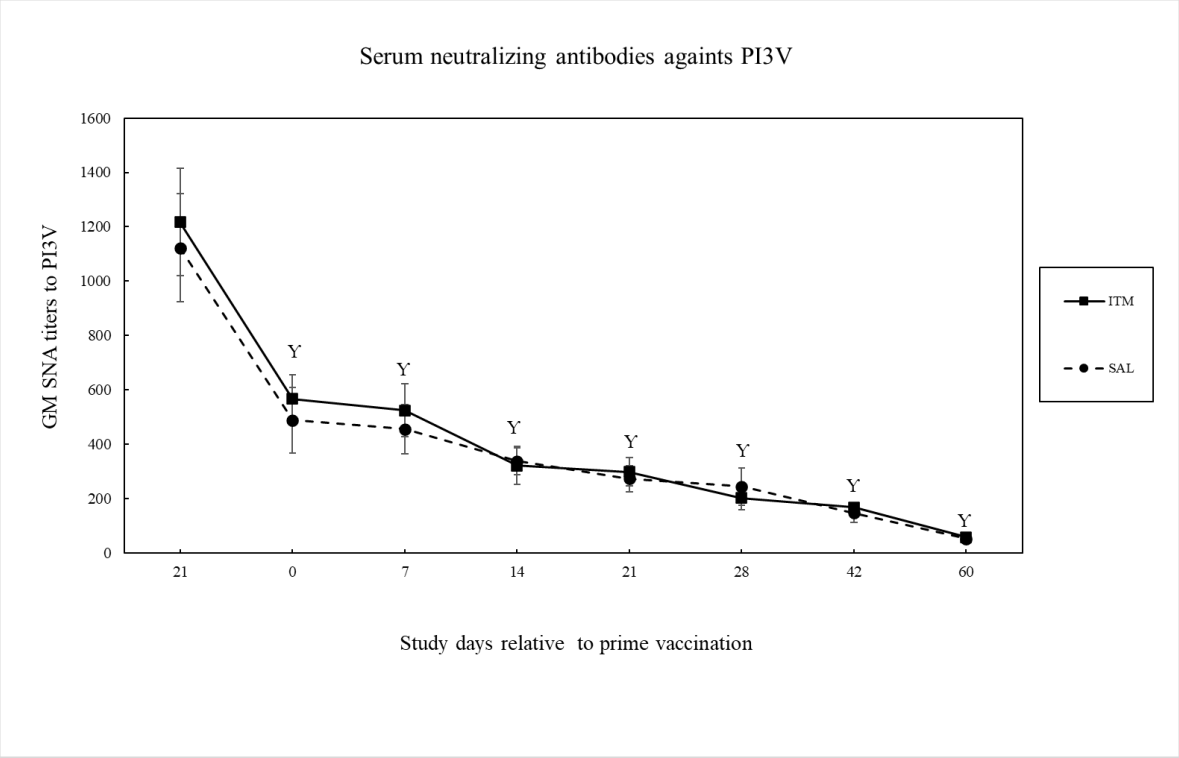


Figure 3.5 – Geometric means serum neutralizing antibody (SNA) titers against BRSV (A), BHV-1 (B), and PI3V (C) in dairy bull calves prime vaccinated with an IN MLV vaccine along with trace minerals (ITM) or saline solution (SAL). There was an effect of time for all SNA ($p <$ 0.0001), and effect of group on SNA BRSV ($p = 0.05$). ^{Y} Significant difference ($p < 0.05$) within ITM and SAL groups when compared with baseline on day -21. ITM group had significant greater SNA titers to BRSV on day 14 (A, $p = 0.045$), and day 28 (A, $p = 0.028$). Significant differences were not observed for SNA against BHV1 and BPI3V between treatment groups.

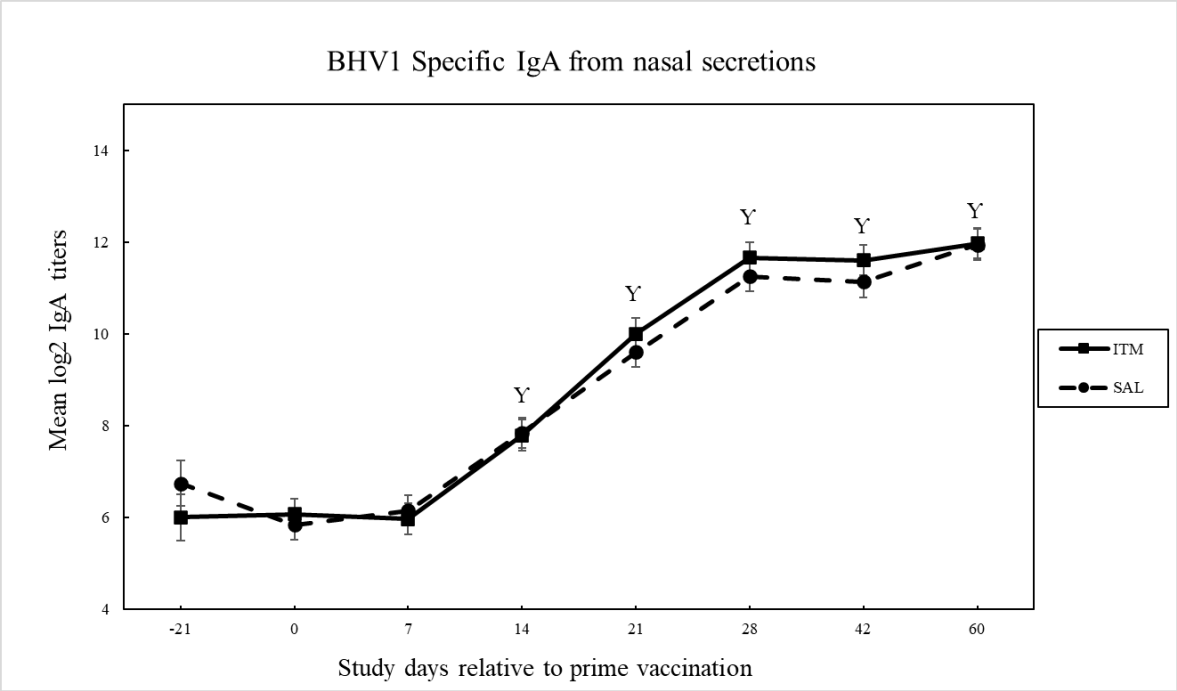
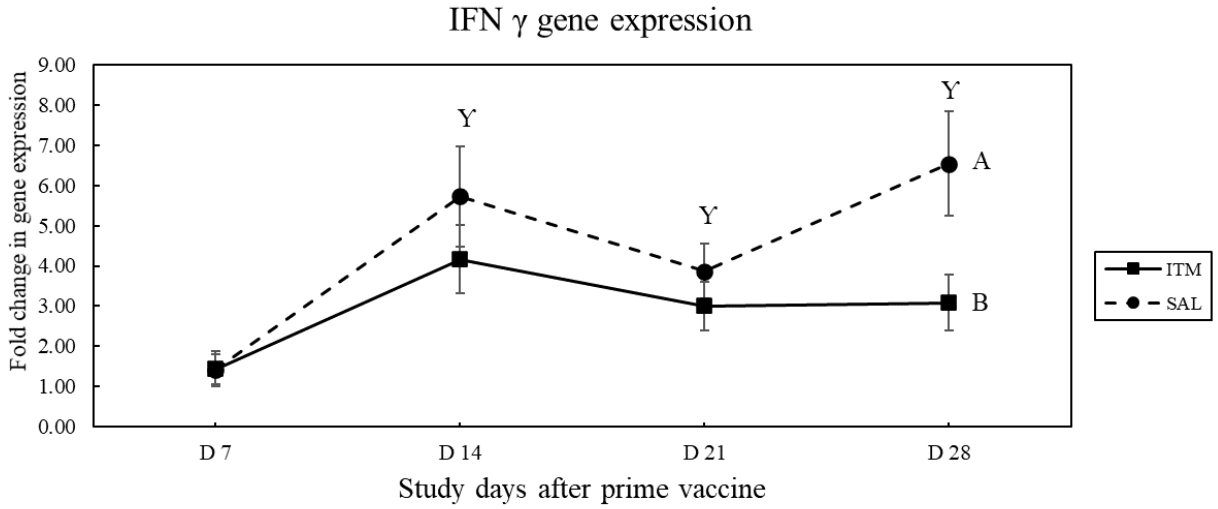
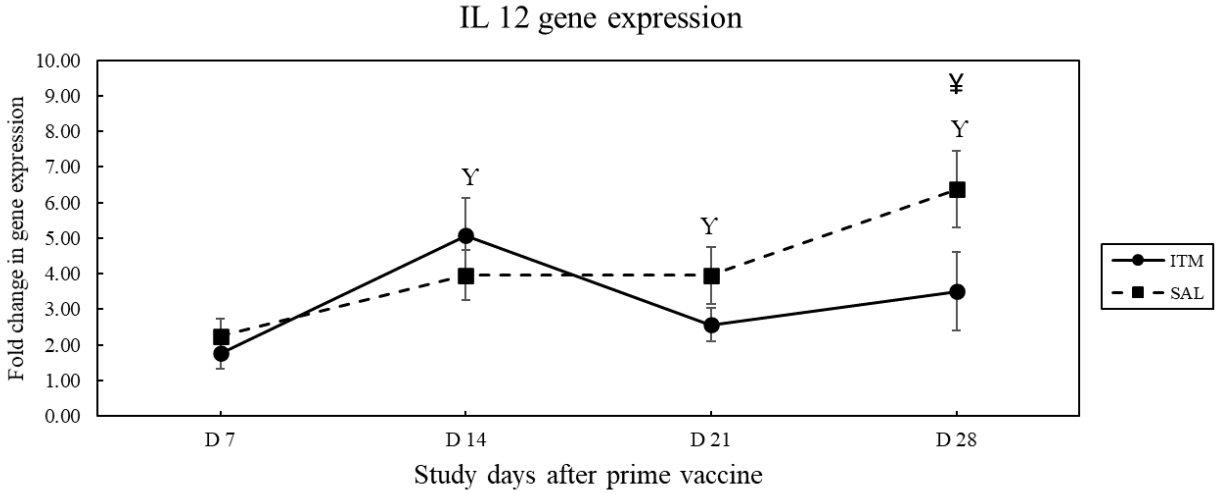


Figure 3.6 – Mean BHV1-specific IgA titer in nasal secretions in calves prime vaccinated with an intranasal (IN) MLV vaccine and treated with injectable trace minerals (ITM) or saline (SAL). There was an effect of time with a significant increase in titers in both groups after day 14 when compared with day -21 ($p < 0.0001$). ^{Y} Significant difference ($p < 0.05$) within ITM and SAL groups when compared with day -21.

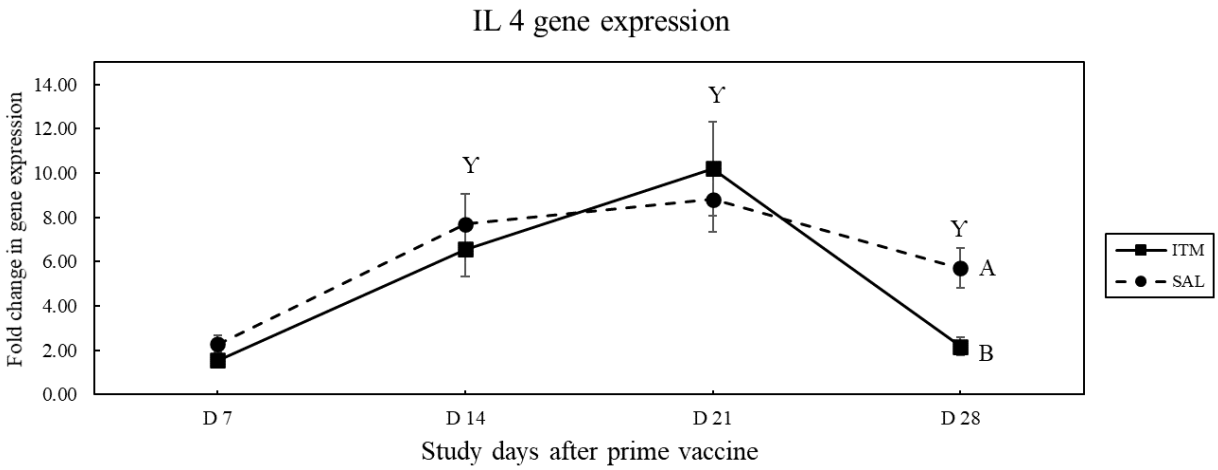
A


IFN γ gene expression
Fold change in gene expression
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Y
Y
Y
A
B
D 7
D 14
D 21
D 28
Study days after prime vaccine
ITM
SAL

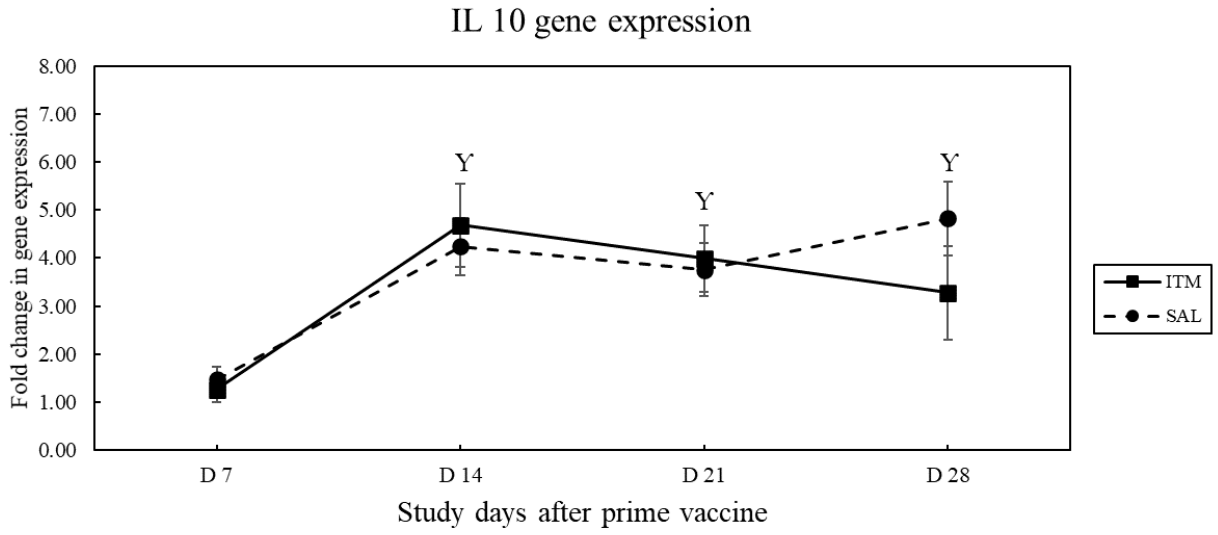
B


IL 12 gene expression
Fold change in gene expression
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
¥
Y
Y
Y
D 7
D 14
D 21
D 28
Study days after prime vaccine
ITM
SAL

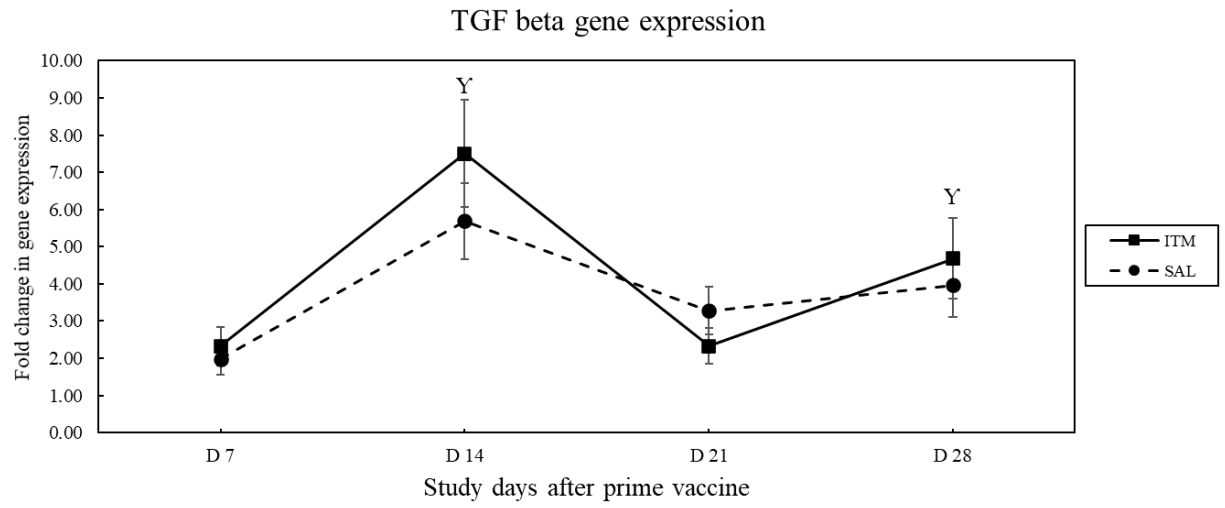
C


IL 4 gene expression
Fold change in gene expression
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Y
Y
Y
A
B
D 7
D 14
D 21
D 28
Study days after prime vaccine
ITM
SAL

D


IL 10 gene expression
Fold change in gene expression
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Y
Y
Y
D 7
D 14
D 21
D 28
Study days after prime vaccine
ITM
SAL

E



F

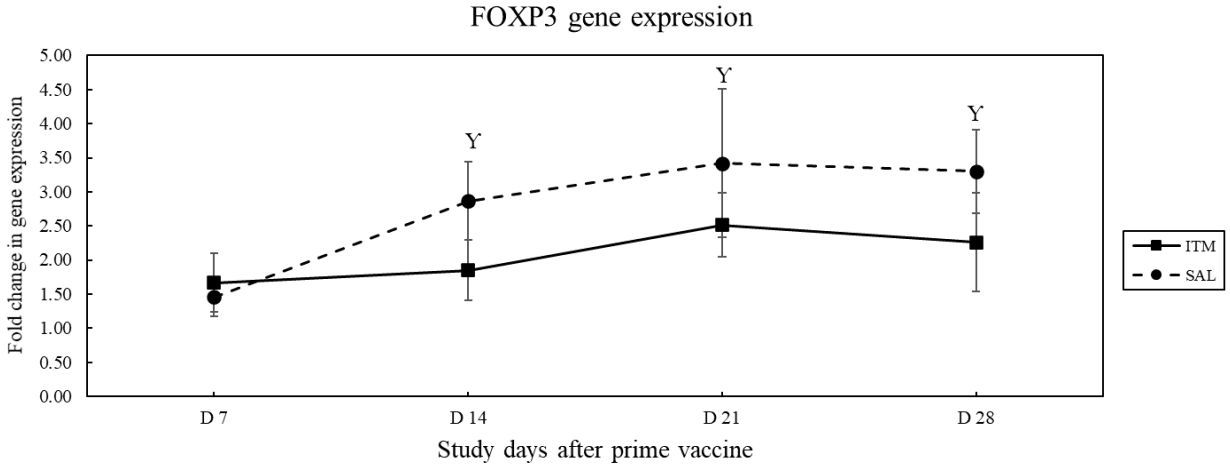


Figure 3.7 – Changes in mRNA expression of pro-inflammatory (A,B), anti-inflammatory cytokines(C,D,E), and regulatory transcriptional factor (F) of calves prime vaccinated concomitant with either trace minerals (ITM) or saline solution (SAL). Data is presented as fold

change of mRNA expression normalized with the housekeeping gene (18S), using day 0 as a baseline and covariance. There is an effect of time for all variables ($p = 0.01$). [A,B] Significant difference when compared between groups ($p < 0.05$). [¥] Tendency when compared between groups ($0.05 < p \leq 0.10$). [Y] Significant difference ($p < 0.05$) within ITM and SAL groups when compared with day 7.

Table 3.1 – Nutrient composition of milk replacer (dry) and grain starter served to the calves. Calf starter grain was offered *ad libitum* starting at 3 days of life.

Item	Milk replacer (dry) [a]	Grain starter [b]
--- % (DM basis) ---		
CP	29.6	23.8
CF	28.7	14.1
TDN	122	83
ADF	-	7.8
NDF	-	14.1
Ash	5.41	7.5
--- mg/kg (DM basis) ---		
Ca	6827	7377
P	5943	4131
Mg	1064	2002
K	10839	10119
Na	4375	2218
S	3966	2583
Fe	112	89
Zn	89.0	66.1
Cu	15.9	16.7
Mn	54.8	58.7
Se	9.3	9.1
Mo	2.1	2.0
Co	0.95	1.41

[a] Milk replacer is composed of dry whey, and bovine plasma as CP (26%), maltodextrin (2.5%), crude fat (0.50%), crude fiber (0.15%), vitamin A, D, and E (0.20%), total viable bacteria 4.8 million CFU per oz.

[b] Grain starter composed of soybean (45.03%), corn gluten (50.04%), molasses (2.25%), calcium (1.5%), and salt (1.0%), trace mineral (0.1%) vitamin A, D, and E (0.05%) offered *ad libitum*.

Table 3.2 – Primers used for quantitative RT-PCR mRNA expression of cytokines in calves that were prime vaccinated with MLV intranasal vaccine along with trace minerals (ITM) or saline solution (SAL).

Genes	Gene ID	Description	Primer sequences	Reference
IFN - γ	EU276066	Bovine Interferon gamma	Fwd: 5'GTAGCCCTGTGCCTGATTTC3' Rev: 5'CACATTGTCCCTTCCCAGAG3'	Baldwin, 2008
IL - 12	U11815	Bos taurus interleukin 12	Fwd: 5'CATCAGGGACATCATCAAACCA3' Rev: 5'CCTCCACCTGCCGAGAATT'3'	Seo et al., 2007
IL – 4	M77120	Bovine interleukin 4 (Bos taurus)	Fwd: 5'AGGAGCCACACGTGCTTGA 3' Rev: 5'TTGCCAAGCTGTTGAGATTCC3'	Seo et al., 2007
IL – 10	U00799	Charolais interleukin-10 (Bos taurus)	Fwd: 5'TTCTGCCCTGCGAAAACAA3' Rev: 5'TCTCTTGGAGCTCACTGAAGACTCT3'	Seo et al., 2007
TGF-β	M36271	Bovine transforming growth factor Beta-1	Fwd: 5'CATCTGGAGCCTGGATACACAGT3' Rev: 5'GAAGCGCCCGGGTTGT3'	Seo et al., 2007
FoxP3	506053	Forehead-box p3	Fwd: 5'AAGAGCCCAGGGACAACTTTC3' Rev: 5'GGGTTCAAGGAGGAAGAGGAA3'	Seo et al., 2007
18S	LOC493779	18S ribosomal RNA	Fwd: 5' GTAACCCGTTGAACCCCATT3' Rev: 5 CCATCCAATCGGTAGTAGCG'3'	Piper et al., 2008

CHAPTER 4

CIRCULATING T CELL SUBPOPULATIONS IN DAIRY CALVES INFECTED WITH *BOVINE VIRAL DIARRHEA VIRUS 2* AND *BOVINE HERPES VIRUS 1* AFTER MODIFIED-LIVE VIRUS BOOSTER VACCINATION. EFFECTS OF THE ADMINISTRATION ROUTE AND TRACE MINERAL SUPPLEMENTATION[2]

[2] A. Hoyos-Jaramillo, R.A. Palomares, J.H.J Bittar, D.J. Hurley, A. Rodríguez, E.A. González-Altamiranda, S. Kirks, A. Gutierrez, S. Wall, K. Miller, J. Urdaneta, K. Skrada, D. Lopez, M. Fenley. To be submitted to PLOS ONE Veterinary Medicine, December 2021.

Abstract

The objective of this study was to document the effects of the route of administration of modified live vaccine (MLV) and the impact of the concurrent use of injectable trace minerals (ITM) at booster vaccination on the dynamics of leukocytes and changes in T cell subpopulations in dairy calves following sequential challenge with *Bovine viral diarrhea virus 2* and *Bovine herpes virus 1*. Sixty male Holstein calves were used in this study. Forty-eight calves were administered an MLV intranasal (IN) vaccine that contained BHV1, BRSV, PI3V (Inforce 3®), then randomly assigned to subcutaneous (SC) administration of injectable trace minerals (ITM, n=24) or saline (SAL, n=24). Ten weeks later, the calves received a booster vaccination by either the SC or IN route, and a second dose of ITM, or saline, as previously administered to yield: ITM-SC (n=12), ITM-IN (n= 12), SAL-SC (n=12), and SAL-IN (n=12). An additional 12 calves did not receive any vaccine or treatment (UNVAC, n=12) to serve as basal controls. Forty-nine days after booster, all calves were challenged with BVDV2 then seven days later with BHV1. Blood samples were collected on days -7, 0, 3, 6, 7, 10, 12 and 14 relative to initial challenge to allow determination of the number of circulating leukocytes and the distribution of T cell subpopulations ($CD4^+$, $CD8^+$, $WC1^+$ and $CD25^+$). Unvaccinated calves showed a significant leukopenia relative to the vaccinated calves. There was a significant decrease in both $CD4^+$ and $CD8^+$ T cells over time following BVDV2 challenge, that was most pronounced in the UNVAC calves. Calves receiving SC vaccination appeared to have a greater fraction of circulating $CD4^+$ T cells than the UNVAC calves. Calves treated with ITM had a greater fraction of circulating $CD8^+$ T cells than the other groups. Calves in the ITM-IN group had a significantly greater fraction of circulating $CD8^+$ T cells on days 6 and 7 ($p < 0.01$) than the other groups. All vaccinated groups had steady enhancement in the fraction of circulating $CD4^+CD25^+$ T cells and

a slight enhancement in circulating $CD8^+CD25^+$ T cells following booster vaccination. In contrast, UNVAC calves showed a significant enhancement in the fractions of circulating $CD4^+CD25^+$, $CD8^+CD25^+$ and $WC1^+CD25^+$ T cells on day 14 after challenge. In conclusion, the route of administration of the booster vaccine, and the concurrent use of trace minerals with vaccination impacted the fraction of both circulating $CD4^+$ and $CD8^+$ T cells following challenge with BVDV2+BHV1 infection in vaccinated and unvaccinated calves The changes in T cell subpopulations circulating was impacted by both the route of booster vaccine and the administration of ITM, but not significantly in most cases. Trace minerals supplementation concurrent with MLV vaccination might generate improved protective cellular immunity against viral agents involved in respiratory disease based on the dynamics of circulating cells measured here.

Keywords: trace minerals, T lymphocyte subpopulations, vaccination route, BVDV, BHV1, BRD.

Introduction

Bovine respiratory disease is a polymicrobial, multifactorial disease associated with substantial economic losses for the cattle industry worldwide (McVey, 2009). Multiple factors – extreme environments, stocking density, stress of weaning, stress from transportation, stress associated with receiving and commingling have all been identified as contributors to the development of BRD (Snowder et al., 2006; Schneider et al., 2009). Several viral infections, most commonly *Bovine viral diarrhea virus* (BVDV) and *Bovine herpesvirus 1* (BHV-1), are well recognized as playing a significant role in the development of clinical BRD. These viruses

have the ability to induce immunosuppression, disrupt the respiratory mucosa, and alter the respiratory tract microbiota that provide enhanced conditions for the development of secondary bacterial infection (Ellis et al., 2010; Walz et al., 2010; Holman et al., 2015). Bovine viral diarrhea virus has a strong tropism for cells in mucosal lymphoid tissues, lymphoid organs, and is capable of infecting circulating lymphocytes (Bruschke et al., 1998; Liebler-Tenorio et al., 2003A, Walz et al., 2010). This results in a depletion of circulating and organized areas of T cell and B cells. This can be seen as a pattern of apoptosis in mucosal and systemic lymphoid tissues (Liebler et al., 1995, Kelling et al., 2002, Palomares et al., 2015). Leukopenia produced by BVDV is associated with apoptosis and necrosis of leukocyte populations and strong migration of leukocytes into tissues where the viral replication is active (Walz et al., 2010, Palomares et al., 2015). Previous studies have reported that $CD4^+$ and $CD8^+$ were the primary lymphocyte populations affected by certain BVDV2 infections in either unvaccinated or immunologically naïve beef calves (Howard et al., 1992; Brodersen and Kelling, 1999; Bittar et al., 2020). Bovine herpesvirus type 1 infection is associated with impairment of the effector function of lymphocytes by suppressing the expression of major histocompatibility class 1 (MHC-1) complex antigen on target cells surfaces that block killing by $CD8^+$ T cells. Further, BHV-1 induces $CD4^+$ T cell apoptosis resulting in the depletion of T cells from the lymphoid tissues (Howard et al., 1992, Winkler et al., 1999; Nataraj et al., 1997, Jones and Chowdhury, 2010).

Vaccination of young dairy calves with modified-live virus vaccines has been documented to confer protection from disease induced by experimental challenge with respiratory viruses including BVDV1 and 2, BHV1, BRSV and BPI_3V (Adair et al., 2000; Fairbanks et al., 2003; Kelling et al., 2005; Ellis et al., 2009 & 2010). However, there is not enough evidence of a universal capacity of MLV vaccination to prevent naturally occurring

disease in dairy calves (Chamorro and Palomares 2020). A common vaccination protocol utilized in many dairy operations consists of prime vaccinating the calves during the first weeks of life with an intranasal MLV vaccine, followed by a booster with a parenteral vaccine at the time of weaning (Hill et al., 2012; Palomares et al., 2021). The use of IN vaccines for priming vaccination of young calves has been suggested to reduce the interference of maternal antibodies and provide earlier protection. It is believed that IN priming pushes forward the development of strong and consistent memory immune responses that are enhanced by the parenteral booster vaccine (Xue et al., 2010; Chamorro et al., 2016). Moreover, some trials have indicated that the among the benefits of using an IN booster vaccinate is a strong induction of secretory IgA leading to a reduction in viral shedding after BHV1 challenge (Hill et al., 2012 & 2019; Palomares et al., 2021). The route of booster vaccination of diary calves is still an open question. Therefore, studies to compare the protective immunity generated against respiratory virus in dairy calves vaccinated IN or SQ are warranted.

Adequate trace minerals are required for optimization of many physiological processes. The immune response, predictable reproduction, and the expected level of growth are all dependent on the adequate delivery of minerals either in feed or by supplementation (Spears and Kegley, 2002). Moreover, the levels of several micronutrients is one of the main factors contributing to BRD incidence (Duff and Galyean, 2007). Previous evidence indicates that there are beneficial effects of giving injectable trace minerals (ITM) containing Se, Zn, Cu, Mn (Multimin-90®; Multimin –USA Inc ®) to the immune response. This is clearest when used concomitantly with MLV vaccination (Arthington and Havenga, 2012; Palomares et al., 2016; Bittar et al., 2018). Studies on calves treated with ITM at the time of vaccination demonstrated improvement in the humoral and cell-mediated immune response against BVDV, BRSV, and

BHV-1. Increased neutralizing antibody in the serum and in *in vitro* leukocyte proliferation in response to each vaccine virus were observed (Arthington et al., 2014; Palomares et al., 2016). In the trial present here, we evaluated the effects of the route of booster vaccination as augmented by the administration ITM at the time of vaccination. We measured the efficacy of protective cell-mediated immunity generated in response to BVDV2 and BHV1 challenge. Our hypothesis was that the route of booster vaccination combined with the use of ITM would have an effect on the robustness of the cell mediated immune responses following BVDV2 and BHV1 challenge in dairy calves. We also hypothesized that concurrent administration of ITM with MLV booster vaccination would impact the typical decrease in the fraction of both $CD4^+$ and $CD8^+$ circulating T cells normally observed after BVDV2 & BHV1 challenge of dairy calves. Thus, our objective was to determine the effects of the route of administration (IN vs. SC) and the use of concurrent ITM with that vaccination on the dynamics of leukocytes and the fractions of circulating T cell subpopulations in circulation in dairy calves following challenged with BVDV2 and BHV1.

Methods

Study location and animal management

This study was conducted using 60 dairy calves derived from a commercial dairy farm located in Quitman Georgia. The calves were transported to the University of Georgia River Bend Farm located in Athens, GA. The calves were cared for in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010), and the study was reviewed by the University of Georgia institutional Care and Use Committee and approved (IACUC, AUP# A2016 11-012-Y3-A3). Further, all protocols were reviewed and approved by the Institutional Biosafety Committee (IBC, license # 2017-0006).

At the farm of origin, calves were managed in individual outdoor pens containing hutches. The study calves were housed in an area separated from the rest of the calves and from the adult herd. The calves were fed 4L of moderate to high quality pooled colostrum (≥22.0% Brix refractometer) within 1 hour after birth using a bottle with a nipple. At twelve hours after birth, calves were given an additional 2 L of colostrum. During the first week, the calves were fed a total of 8.5L of milk daily, divided three portions per day. The volume of milk was increased the through week 6 (11.5L) based on average calf weight. Each week. Water and calf starter diet were offered *ad libitum* starting at day 3 of life to all calves. Calves were weaned at 2 months of age. Four weeks later, the calves were transported to the University of Georgia farm. On the day of transportation, all the calves were administered a dose of Tilmicosin (10 mg/kg of body weight, Micotil®300, Elanco Animal Health, Indianapolis IN) subcutaneously as a metaphylactic treatment to prevent the occurrence of respiratory disease associated with shipping. After arrival at the farm, the calves were allowed to acclimatize for two weeks on the same feeding program before booster vaccination.

The calves were housed on an 8-acre pastures with a mixture of Bermuda grass (*Cynodon dactylon*) and fescue grass (*Festuca arundinacea*) and shade was provided. The calves had no access to mineral supplements during this period. All calves had access to hay (Bermuda grass and fescue grass) and water ad libitum for the duration of the study. All calves received 2.7 kg/head/day of concentrated feed supplement (Bulk Cattleman's Special; Godfrey's Warehouse; Madison-GA) divided into two meals. Nutritional values in feed concentrate and pasture met the requirement established by the NRC (2001, Table 4.1).

Study design, vaccination, and treatment

The experimental design was a fully randomize clinical trial. At the farm of origin, 48 of the 60 calves received a priming dose of an intranasal (IN) modified-live virus (MLV) vaccine that contained BHV1, BRSV, BPI3V (Inforce 3® Zoetis Animal Health), and either trace minerals (ITM, n=24; Multimin®90) or saline solution (SAL, n=24) based on random assignment. At 3.5 months of age, at the University of Georgia farm, the vaccinated calves were randomly divided (www.random.org) into two groups. One receiving an MLV booster vaccination by the subcutaneous (SC) route and the other by the intranasal (IN) route. Concurrent with the vaccination, the groups received either a second dose of ITM or saline solution, subcutaneously, according to their previous assignment. The trace minerals provided 15, 60, 10 and 5 mg/mL of Cu, Zn, Mn, and Se, respectively. Four experimental groups were as follows:

1. ITM-IN (n=12): Calves received a 2mL dose of the same IN MLV vaccine (Inforce 3®) at 3.5 months of age, along with a second dose of ITM (Multimin®90; 1ml/100 lb SC).
2. ITM-SC (n=12): Calves received a 2mL dose of a SC MLV vaccine containing BHV-1, BRSV, PI3 and BVDV1 & 2 (Bovi-shield Gold® 5 Zoetis Animal Health®) along with a second dose of ITM (Multimin®90; 1ml/100 lb SC).
3. SAL-IN (n=12): Calves received a 2mL dose of Inforce 3® IN and a second injection of saline solution (1ml/100 lb SC).
4. SAL-SC (n=12): Calves received a 2mL dose of a SC MLV vaccine (Bovi-shield Gold® 5) and a second injection of saline solution (1ml/100 lb SC).

The IN boostered calves also received an SC dose of a MLV vaccine containing BVDV1 & 2 (Bovi-shield Gold® BVD, Zoetis Animal Health®). It was delivered to match the spectrum

of antigens that SC boostered calves received so that only the route of booster vaccination for BHV1, BRSV, and BPI3V differed. Additionally, calves receiving the same vaccination route were commingled in the same pasture and separated from the other avoiding nose-to-nose contact.

In addition, 12 unvaccinated calves did not receive any MLV vaccine or other treatment so they could serve as a double negative control group (5. UNVAC, n=12). The unvaccinated calves were isolated on a separate pasture until the day of BVDV2 challenge. This avoided possible cross infection from nasal shedding or nose-to-nose contact from live vaccinated calves.

BVDV2 and BHV1 virus challenge

Forty-nine days after of booster vaccination (defined as day 0), all calves (including UNVAC) were inoculated intranasally with BVDV2 (890 strain). This was followed seven days later by intranasal challenge with BHV1 (Colorado-1 strain). The BVDV2 isolate was obtained from APHIS Center for Veterinary Biologics in 1989 (Ames, IA). The virus was propagated in Madin-Darby Bovine Kidney (MDBK) cell culture. Once the strain arrived at our lab, the virus was propagated multiple times one MBDK cells as previously described (Bittar et al., 2018). The inoculum contained 1×10^5 50 % tissue culture infectious dose ($TCID_{50}$) per ml. The challenge was performed by nasal aerosolization of the inoculum performed with 10-cm long fenestrated cannula that was attached to 12-ml syringes. Each calf received a total dose of 5 ml of challenge virus. It was divided into 2.5 ml volumes for each nostril. Following BVDV2 challenge, all calves were commingled on the same pasture throughout the rest of the study.

Seven days after the BVDV2 challenge (day 7), all calves were also challenged with BHV1 (Colorado-1 strain). This strain was obtained from American Type Culture Collection

(ATCC; Manassas, VA USA). The virus was propagated in MDBK cells for generation of the challenge inoculum. The inoculum consisted of a lysed culture suspension containing 8×10^6 $TCID_{50}$ per ml. The inoculation process was comparable to that described for BVDV2 challenge, also with a 5 mL total dose delivered.

Health assessment and clinical scoring

Health assessment was conducted while calves were at the chute on days -7, 0, 3, 5, 7, 10, 12, and 14 following BVDV2 challenge. The assessment was based on the Wisconsin health scoring system as published by McGuirk (2008). The evaluation of clinical signs was done and recorded by the team. The following were monitored: rectal temperature, the position of the head-ears (attitude), evidence of hydration, cough, ocular or nasal secretions, and fecal consistency. The clinical signs were each evaluated on a scale from 0 to 3 (where 0 indicated a lack of clinical signs, while 3 severe clinical signs), with a possible maximum score of fifteen. The sum of the daily health score was calculated by adding all individual score values for all the recorded clinical variables by two veterinarians blinded for treatment and calf ID, as previously reported (Bittar et al., 2018).

Sample collection and processing

Whole blood samples were collected on days -7, 0, 3, 5, 7, 10, 12, and 14 relative to BVDV2 challenge by jugular venipuncture using 20 gauge+1'' single needle (Vacuette®; Nipro Medical Industries Ltd., Gunma, Japan) into vacuum tubes containing anticoagulant (either EDTA, and ACD solution; Vacutainer®, BD Diagnosis, Franklin Lakes, NJ) or no additives. Blood samples containing EDTA anticoagulant (5 ml) were used for whole blood circulating

differential cell count assessment. Blood samples were collected for buffy coat separation (8.5 ml with ACD) on the same days. Blood samples collected for serum with no additives were done on days 0 and 14. All serum samples were stored at -80 °C until further serum neutralizing antibody (SNA) titers were evaluated. All whole blood samples were kept on ice following collection until arrival to the laboratory (within two hours).

Hepatic trace mineral concentration

Trace mineral levels in each calf were determined using a liver biopsy samples on days -5, and 21 relative to the day of booster vaccination and trace mineral injection. The tissue samples were shipped to the Diagnostic Center for Population and Animal Health at Michigan State University, Lansing, MI. Liver biopsies were performed by trained veterinarians. They collected approximately 10 mg of liver tissue from each calf. Samples were taken by locating the liver from the right caudo-dorsal thoracic area using an ultrasound with a probe of 5-8 MHz (Evo Ibex®Lite, E.I. Imaging). Local anesthesia (2 mL of Lidocaine injectable, Aspen Veterinary Resources, Ltd.) was used to manage pain of the procedure. The liver tissue samples were collected using sterile surgical semi-automatic biopsy needles (16 g and four inches long; BIOPSYBELL S.R.L.; Mirandola). Meloxicam (1mg/kg; 7.5mg/ tablet) was administered orally (16 tablets) 12-18 hours before liver biopsy and a second dose immediately before the procedure for overall pain management.

White blood cell counts

Blood samples were transported to the laboratory in a cooler with ice within two hours after collection for determination of total white blood cell, lymphocyte, granulocyte, monocyte,

and platelet counts using an automatic cell counter (HESKA5® CBC-Diff, Vet Hematology System). Blood samples were kept stirring for 10 min at room temperature on an orbital shaker before analysis.

Serum neutralizing antibodies titers

Blood samples for determination of serum neutralizing antibody (SNA) titers for BHV1 and BVDV2 were collected on days 0 and 14 after the initial viral challenge. Serum neutralizing antibody titers were determined at the University of Georgia Veterinary Diagnostic Laboratory (Athens, GA) using the standard diagnostic laboratory protocol as previously described (Bittar et al., 2018). Briefly, serum samples were heat inactivated in water bath at 60 °C for 30 minutes. After heat inactivation, samples were diluted two-fold in duplicate starting at 1:2. A volume of 1:2 serum was placed in wells of a flat bottom 96-well cell culture plate (Falcon ®, Corning™, Corning, NY). Further dilution was done in the plate to test for the endpoint. An equal amount of medium containing the virus of interest was added to the each well increasing the dilution 2-fold for the final analysis. 25 µl of DMEM containing a target of 100 $TCID_{50}$ of virus was used. After one hour of incubation, 150 µl of MDBK cell suspension (target 1.8×10^4 cells) in DMEM containing 10% bovine fetal serum, was added to each well. The plates were incubated for four days. At this point, the cell monolayer was evaluated for virus-specific cytopathic effect. The end point titer was reported for each sample as the reciprocal of the highest dilution that completely inhibited virus-specific cytopathic effect.

T cell phenotyping in peripheral leukocytes by flow cytometry

Blood samples for T cell phenotyping were collected in 8 mL tubes with ACD anticoagulant on days -7, 0, 3, 5, 7, 10, 12, and 14 following initial challenge. Buffy coat cells were isolated as previously described (Harpin et al., 1999) and immediately stained for T cell phenotype by flow cytometry. Single ($CD4^+$, $CD8^+$, $WC1^+$, and $CD\ 25^+$) and dual ($CD4^+CD25^+$, $CD8^+CD25^+$, and $WC1^+CD25^+$) T cell phenotypes were measured by flow cytometry. Cell staining started immediately with an assessment of cell viability using Trypan blue (0.04%) and a hemocytometer. Each sample was used if it had ≥ 85% of cell viability. The T cell phenotyping protocol was previously described (Bittar et al., 2020). Briefly, the cells were suspended in PBS to a total volume of 3 ml that contained a target of 6 x 10^6 cells/ml. The cells were transferred to prelabeled tubes (12 × 75mm polystyrene, Falcon, BD Biosciences, San José, CA). The cells were washed by centrifugation (300 x g for 5 min at room temperature) twice. 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 uniformly mixed by vortexing. The staining procedure was initiated by adding 100 µl of each cell suspension per well to wells of a 96-well rounded bottom plate (Falcon ® 96-well, Corning™, Corning, NY). The plate was previously labeled (with sample ID and antibodies to be added). After loading the cells, the plates were centrifuge (300 x g for 5 min). The supernatant was removed by snapping the plate but retaining the cells. Each plate was vortexed in the residual volume to suspend the cells fully. The primary antibodies were added to each pre-labeled well (25 µl/primary antibody) at concentrations based on the minimum saturating concentration previously determine in preliminary studies (Bittar et al., 2020). The primary antibodies used were: mouse anti-bovine CD4 antibody, IgG2a isotype at 1:5 dilution (clone CC8, MCA1653 F, BioRad, Hercules, CA),

mouse anti-bovine CD8 antibody, IgG2a isotype at 1:5 dilution (clone CC63, MCA837 F, BioRad, Hercules, CA), mouse anti-bovine WC1 antibody, IgG2a isotype at 1:5 dilution (clone CC15, MCA838 F, BioRad, Hercules, CA), and mouse anti-bovine CD25 antibody, isotype IgG1 at 1:7 dilution (clone IL-A111, MCA2430PE). Each individual phenotype ($CD4^+$, $CD8^+$, $WC1^+$, and CD 25^+) was evaluated and combination phenotypes of $CD25^+$ with the other surface markers ($CD4^+CD25^+$, $CD8^+CD25^+$, and $WC1^+CD25^+$) were assessed. FACS buffer alone served as a non-staining, negative control. Once all the single and double staining combinations were added to the wells, the plate was incubated at 4 °C for 45 minutes. After incubation, the plates were washed three times with FACS buffer. After the last wash, 100 µl of FACS fix suspension (4% formalin in FACS buffer) was added to each well. This was incubated overnight at 4 °C. The next day all samples were transferred to pre-labeled 1.5 ml microcentrifuge tubes containing 300 µl of FACS buffer for analysis. These samples were stored at 4 °C until reading in the flow cytometer, but not longer than 3 days. Flow cytometry was performed using a BD Accuri C6 first generation cytometer (BD Franklin Lakes, NJ) and the data obtained was evaluated using the C-flow plus software (BD Biosciences, Franklin Lakes, NJ). A lymphocyte population was defined in a polygonal gate from among the total of events based on size and granularity (P2). All samples were gated using FALS and 90LS two parameter histograms to map the cell populations including lymphocytes, monocytes, and granulocytes cells based on previous studies. To minimize cross talk between FL1 and FL2 singles, compensation of the signal was established using single color samples that allowed eliminating green from orange and orange from green. The positive and negative windows for FL1 and FL2 were established using single color analysis. The FL1-FL2 dual parameter histograms were established in preliminary trials.

Approximately 10,000 events were collected for the lymphocyte gate for each sample. The gates were standardized for all samples to assure the collection of the same population throughout the experiment. The C-flow plus software was used to determine the percent of positive cells for each marker, and to map the CD25 positive cells within each marker. In addition, values for the mean fluorescent intensity (MFI) were recorded for each analysis gate for all samples.

Statistical analysis

All data were analyzed using the Statistical Analysis System (SAS version 9.3; Cary, NC, USA). All data were tested for normality and constant variance by Shapiro Wilk's and Levene's tests, respectively. A logarithmic base 2 transformation was applied to the SNA titers. Flow cytometric analysis were reported as an Expression Index, which is a ratio of the T cells responsiveness from each animal versus the unresponsive T cell specific subset. The sum of health score, hepatic trace mineral concentrations, SNA titers, circulating differential leukocyte numbers, and distribution of T cell subpopulations were compared among the four treatment groups using a repeated measures analysis model (Proc-GLIMMIX of SAS®). Calf ID was used as a random effect, and the interaction among treatment group and time as fixed effects. The Tukey test was applied for multiple comparisons. Baseline from day -7 was considered as baseline for the variables clinical score, differential leukocyte number, T cell subpopulations percent and MFI index. The results were considered significant if $P \leq 0.05$, A statistical tendency was declared when $0.05 < P \leq 0.10$.

Results

All calves in the study had adequate hepatic concentrations of Se, Cu, Zn and Mn before and after booster vaccination. There was a significant effect of time on the hepatic levels of Se, Zn and Mn ($p = 0.0001$; Fig. 4.1 A - D). Copper and Se concentrations decreased overtime after vaccination in the vaccinated, but saline-treated calves and in the unvaccinated and unsupplemented calves (Se: $p < 0.001$ in SAL-IN, SAL-SC and UNVAC groups and Cu: $p < 0.01$ only in the SAL-IN group, Figure 4.1A & B). Hepatic Cu and Se concentrations were increased after booster vaccination in the ITM-treated calves relative to baseline values before vaccination and treatment (with a significant increase of Cu in the ITM–SC group, $p = 0.01$ and Se in the ITM–IN group, $p = 0.035$). Hepatic Se levels were significantly greater ($p < 0.0001$) in the ITM-IN group than those observed in the vaccinated, but saline-treated calves (SAL-IN and SAL-SC), and in the UNVAC group. All groups had a similar variation in hepatic levels of Zinc (reduction) and manganese (increment) after baseline collection on booster vaccination ($p < 0.05$; Fig 4.1C & D). No significant differences were observed on Cu, Zn and Mn levels among any of the groups (Fig. 4.1A).

There was a significant effect of time on the clinical scores observed ($p < 0.0001$; Fig 4.2). All groups had a comparable increase in clinical scores between days 7 and 14 after BVDV2 challenge ($p < 0.01$). On day 10, unvaccinated calves presented a higher score compared to the vaccinated groups ($p < 0.05$). The sum of health scores was compatible with moderate BRD from days 10 to 12 after BVDV2 challenge. Yet, no significant differences were observed in the sum of health scores among vaccinated calves after BVDV2 and the following BHV1 challenges.

There was an effect of time ($p < 0.0001$), group ($p < 0.0001$), and the interaction of group*time ($p < 0.0001$) on the differential white blood cell numbers in circulation. A significant leukopenia (on days 3 to 10; Fig. 4.3A), accompanied by reduction of total circulating lymphocytes (on days 3 to 7, $p < 0.001$; Fig. 4.3B), circulating monocytes (on days 3 to 7, $p < 0.01$; Fig. 4.3D), circulating neutrophils (on day 6, $p < 0.01$; Fig 4.3C), and circulating eosinophils (on days 3 to 7, 12 and 14, $p < 0.05$; Fig. 4.3E) count were observed in the UNVAC group following BVDV2 challenge (Fig. 4.3). Moreover, the number of circulating platelets was significantly lower from day 6 to 14 for UNVAC than any vaccinated group ($p < 0.05$, Fig. 4.3G). On day 6 and 7 ITM-SC and SAL-IN circulating platelet numbers were significantly higher than those of the UNVAC ($p < 0.05$). On day 10 only SAL-IN was found to be significantly different from UNVAC ($p < 0.05$). On day 12 ITM-SC, SAL-IN, and SAL-SC were all significantly different than the UNVAC calves for circulating platelet numbers ($p < 0.05$). On day 14, only ITM-SC was significantly different in the number of circulating platelets than the UNVAC calves ($p < 0.001$).

On day 3, the ITM-SC group had significantly greater number of circulating total WBC number ($p = 0.047$) and circulating lymphocytes ($p = 0.004$) than the ITM-IN group. On day 6, the ITM-IN calves had a significantly greater circulating total WBC number than the SAL-IN group ($p < 0.0001$). Calves receiving SC vaccination had higher circulating lymphocyte numbers on day 14 than calves receiving IN vaccination or the UNVAC calves ($p < 0.01$). On day 10, the UNVAC calves had a significant increase in circulating monocytes and basophils (likely associated with inflammation), than the other experimental groups ($p < 0.0001$; Fig. 4.3D and 4.3F, respectively). On the same day, ITM-treated calves (ITM-IN and ITM-SC) had significantly higher numbers of ($p < 0.05$; Fig. 4.3C) circulating neutrophil than the UNVAC

calves. ITM-IN calves had a higher number of circulating neutrophils on day 6 than either SAL-IN and UNVAC calves ($p < 0.001$).

There was an effect of time ($p < 0.001$), and the interaction group*time ($p = 0.01$) on the fraction of $CD4^+$ T lymphocytes in circulation. There was a significant decrease in the fraction of circulating $CD4^+$ T cells relative to the time after BVDV2 challenge relative to the baseline values ($p < 0.0001$; Fig 4.4B). This reduction was more pronounced and prolonged in the unvaccinated calves most clearly seen between days 3 and 12 following BVDV2 challenge. On day 3 following BVDV2 challenge, calves receiving SC booster had a larger fraction of circulating $CD4^+$ T cells than the UNVAC calves. Only the values for the SAL-SC group were statistically significant ($p = 0.003$). There was an effect of time ($p < 0.001$), group ($p < 0.001$), and the interaction group*time ($p = 0.01$) on the fraction of circulating $CD8^+$ T lymphocytes within the groups (Fig. 4.4C). Challenge with BVDV2 resulted in a reduction in the circulating fraction of $CD8^+$ T cells 3 days after challenge in all groups. These values remained low until day 12 after initial challenge in the unvaccinated calves. There was a rebound in the circulating fraction of $CD8^+$ T cells in all the vaccinated groups on day 6. This was significantly greater than in the UNVAC group ($p < 0.001$). At this time point, calves treated with ITM concurrent with either booster vaccination showed a numerically increase in the circulating fraction of $CD8^+$ T cells relative to the other groups ($p = 0.01$). Calves in the ITM-IN group had the circulating fraction of $CD8^+$ T cells on days 6 and 7. These were significantly higher than those in the SAL-SC, SAL-IN and UNVAC groups ($p < 0.01$). There was an effect of time ($p < 0.001$), and the interaction group*time ($p = 0.01$) on the circulating fraction of activated T cells in the $CD4^+CD25^+$ and $CD8^+CD25^+$ T cell subpopulations on days 10, 12 and 14 (Fig. 4.4E and 4.4F, respectively). All the vaccinated groups had a comparable and consistent response in the

circulating fraction of $CD4^+CD25^+$ T cells with a small increase in the circulating fraction of $CD8^+CD25^+$ T cells after the delivery of both the BVDV2 and BHV1 challenges. In contrast, UNVAC calves had a significant increase in the circulating fraction of both $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells on day 14 after BVDV2 challenge n (7 days after BHV1 challenge). This was significantly different than the values observed for the vaccinated calves under all vaccine treatments ($p < 0.05$ and $p < 0.01$, respectively).

There was an effect of time ($p < 0.001$) on the circulating fractions of $WC1^+$, and $WC1^+CD25^+$ T cells. There was a significant decrease in the circulating fraction of $WC1^+$ T cells on day 6 compared to baseline values prior to BVDV2 challenge ($p < 0.0001$; Fig. 4.4D and 4.4G). Thereafter, the circulating fraction of WC1+ T cells increased over time, with a significant increase seen on days 7 and 10 ($p < 0.0001$) and a stable circulating fraction on days 12 and 14. No significant differences were observed between treatment groups.

The double positive circulating fraction of $WC1^+CD25^+$ decreased on day 3 following BVDV2 challenge ($p < 0.05$; Fig. 4.4G). The circulating fraction of these cells steadily increased over time until day 14 ($p < 0.05$). On day 14 post initial challenge, the UNVAC group had a significantly increased circulating fraction of WC1+CD25+ cells than any of the vaccinated groups ($p < 0.05$).

The SNA titers for BHV1 and BVDV2 were measured on days 0, and 14 relative to the initial challenge (Fig. 4.5 A & B). There was a significant effect of time ($p < 0.0001$), group ($p < 0.0001$), and the interaction groups * time ($p < 0.0001$) on SNA to both BHV1 and BVDV2. Antibody titers against BHV1 were significantly higher on day 14 for ITM-SC and SAL-IN compared to the UNVAC calves ($p < 0.0001$; Fig. 4.5A). However, the SAL-SC and ITM-IN calves had an intermediate response in terms of neutralizing antibody ($p = 0.184$). Calves

receiving ITM concurrent with a multivalent SC MLV vaccine had significantly greater SNA titers against BVDV2 compared with the other treatment groups ($p < 0.05$; Fig. 4.5B).

Discussion

This study investigated the effects of the route of booster vaccination and the effect of administration of ITM concurrent with MLV vaccination on the dynamics of the fraction of circulating T cell subpopulations following serial challenge with BVDV2 then BHV1 in dairy calves as an indication of the protective cell-mediated immunity induced against BVDV2 and BHV1 infection. This was analyzed relative to the four vaccination protocols tested. Vaccination regime including priming at one month of age with a booster two months later. This was designed to mitigate the expected drop in total leukocytes, specific leukocyte populations and platelets and the fraction of several T lymphocyte subpopulations in circulation following BVDV2 challenge. This was achieved in this trial with only a few remarkable differences observed among vaccinated groups. The circulating fraction of the major T cell subsets were significantly enhanced in calves receiving the multivalent SC vaccination ($CD4^+$ T cells on day 3), and further when combined with trace mineral supplementation ($CD8^+$ T cells on days 6 and 7).

In this study, MLV vaccination protected animals from serial BVDV2 and BHV1 challenge induced clinical disease. There were no clinically relevant differences among vaccinated groups. This agrees with the results reported in previous trials on weaned dairy or beef calves (Xue et al., 2010; West et al., 2000; Vangeel et al., 2007; Bittar et al., 2020). The clinical protection observed in all vaccinated groups was very similar. It may be associated with indications of comparable activation of CMI that we observed among all groups.

Experimental challenge with BVDV2 resulted in a significant leukopenia in the unvaccinated calves. This was characterized as a reduction of all major types of white blood cell in circulation. Challenge with BHV1 seven days later did not cause further reduction in the circulating differential leukocyte numbers in circulation. These returned to their baseline numbers rapidly after BHV1 challenge. All the fractions of circulating lymphocyte subpopulations ($CD4^+$, $CD8^+$ and $WC1^+$) we studied were significantly reduced following BVDV2 challenge. This was more pronounced in the unvaccinated group. Leukopenia and lymphopenia, believed to be caused by apoptosis, necrosis, and trafficking of many cells to viral replication zones are considered a major hallmark of BVDV pathogenesis (Walz et al., 2010). The magnitude of the effects of BVDV infection on the total population of WBC in circulation, and particularly on circulating T-lymphocytes, is highly dependent on the strain virulence (Chase 2013). Infection with isolates of BVDV having different virulence properties has resulted in more or less pronounced decreased T-lymphocyte subpopulations. Most commonly, the $CD8^+$ T cells have the greatest decline during BVDV infection, with the $CD4^+$ T lymphocytes showing a lesser effect (Endsley et al., 2004; Brodersen and Kelling 1999; Chase et al., 2004). A similar reduction in the number of circulating $CD4^+$ and $CD8^+$ T cells was observed in a previous study by our team in newly received beef calves challenged with BVDV2 (Bittar et al., 2020).

Overall, MLV vaccination, regardless the route of administration or if accompanied by trace mineral supplementation prevented the expected effects of BVDV2 challenge on the reduction in peripheral WBC numbers seen in unvaccinated animals. Platelet counts in unvaccinated calves were significantly lower on days 6 to 14 compared to those in vaccinated calves under any of the vaccine protocols used. A similar response was observed in a clinical trial evaluating the immune response of MLV vaccines with concurrent treatment with trace minerals

(Bittar et al., 2020). The reduction in circulating platelet numbers has been associated with the hemorrhagic syndrome produced by some BVDV2 strains (Rebhun et al., 1989). Thus, there might be an effect of trace minerals that protects against the thrombocytopenia mediated by BVDV. In addition, vaccination appeared to have a protective effect on the elements of cell-mediated immunity by mitigating the decay of the circulating $CD4^+$ and $CD8^+$ T cells on days 3 to 7 relative to what was observed in unvaccinated calves. In previous studies, MLV vaccination also resulted in preservation of the circulating fraction of $CD4^+$, $CD8^+$ and $WC1^+$ T cell memory responses in young calves (Platt et al., 2006 & 2009; Bittar et al., 2020).

A decrease in the circulating fraction of $CD4^+$ T cells was observed three days after BVDV2 challenge in all vaccinated groups. This dropped rapidly rebounded, as seen on day 6. Calves receiving multivalent SC booster vaccination (Bovishield® Gold 5) 60 days after IN priming vaccination had a higher circulating fraction of $CD4^+$ T cells after BVDV2 challenge. A larger fraction of $CD4^+$ T cells might confer benefits to the early immune response after viral infection, as T helper cells control expansion of both humoral and cellular components of the adaptive immune response. T-helper cells produce factors that differentiate and arm effector cells of CMI and provide the major regulation of the proliferation, differentiation, and antibody class expression of B lymphocytes (Tizard, 2009; Chase, 2013). In addition, calves receiving trace mineral supplementation concurrent with SC booster vaccination had a higher amplification of SNA response against BHV1 at 21 days after booster and 14 days following BHV1 challenge compared to the other treatment groups. This may be associated with the enhanced circulating fraction of $CD4^+$ T cells.

The protocol referred as “IN prime-SC boosting” vaccination has been documented to be effective in both dairy and beef cattle operations. Further, prime-boost vaccination with different

vaccine formulations using different delivery routes can elicit improved immunogenicity and protection (Lu, 2009). A previous study in our lab concluded that beef calves receiving IN prime-SC boost vaccination resulted in greater SNA titers for BRSV and a stronger production of mucosal IgA specific for BRSV and BHV1 (Palomares et al., 2021). Moreover, a similar report concluded that SC booster vaccination administered to beef calves five months after the IN priming vaccination resulted in increased antibody response to BRSV (Ellis et al., 2013). There are a few available reports comparing parenteral with. IN vaccination in beef cattle (Hill et al., 2019; Erickson et al., 2020). However, there is limited evidence examining the efficacy of different routes of administration routes at booster vaccination in dairy calves on prevention of BRD (Chamorro and Palomares, 2020).

Trace minerals supplementation at the time of booster vaccination that generate a larger circulating fraction of $CD8^+$ T cell after BVDV2 challenge. Calves receiving ITM concurrent with IN booster vaccination had a significantly larger circulating fraction CD8+ T cells on days 6 and 7 following BVDV2 challenge. This finding may have implications. It appears to indicate that protective CMI elicited by these vaccination protocols may be improved. As $CD8^+$ T cells, that often function as cytotoxic T lymphocytes, are a critical player in the acquired immune response to intracellular infectious agents, including viruses.

Supplementation with the trace mineral solution significantly increased the levels of Se and Cu in the ITM-IN and ITM-SC groups, respectively. This may have an effect on the observed enhancement in $CD8^+$ T cell circulation following combined BVDV2 and BHV1 challenge in these calves. Trace minerals represent vital elements for the function of cattle immunity and in general health. Trace minerals, specifically Se, Cu, Zn and Mn, are structural components of antioxidant enzymes that help manage and reduce the excessive ROS in tissues.

This excessive ROS has been associated with significant damage to cells, including white blood cells, and may lead to dampening or delayed activation of the immune response (Sordillo, 2013, Teixeira et al., 2014; Sordillo and Aitken, 2009). In addition, zinc has a significant role in the function of the cell cycle involved in enzymes critical to nucleic acid replication and transcription. These enzymes are crucial to the process of lymphocyte clonal expansion. In cattle, the administration of ITM has been documented to enhance humoral and cell-mediated immune responses and enhance protection after parenteral vaccination against virus and bacteria that cause BRD (Arthington and Havenga, 2012, Palomares et al., 2016, Bittar et al., 2018a, 2018b, & 2020).

There was a decay in circulating γδ T cells in the circulation of all groups after BVDV2 challenge. This is likely to have resulted from trafficking of these cells to the sites of viral replication, particularly in mucosal tissues (Palomares et al., 2015). Gamma delta ($WC1^{+}$) T cells are known to have important roles in managing infection in mucosal tissues in mammals. In cattle, they have been demonstrated to play a role in most intracellular infections, particularly fairly early in the infection (McGill et al., 2016). Thus, migration from the circulation is expected. The fraction of gamma delta was measured as the larges fraction of T cells in young calves. It has been previously documented be a large fraction of lymphocytes in circulation and most tissues in very young animals and to decline in circulation with age (Tizard, 2009). These cells have multiple roles in the immune response. They are non-classical effector cells that do not need MHC class I or II presentation of antigen to be activated. They also appear to the major population of T regulatory lymphocytes in cattle (Guzman et al., 2012). It has been suggested that γδ T cells may not play a significant role in direct management of BVDV infection (Howard et al., 1992). Some reports indicate a decrease in circulating γδ T cells early then an increase

around 12 days after infection. This is taken as an indication of their immunomodulating role that may favor the viral infection (Molina et al., 2012; Bittar et al., 2020). Others, suggest that they have a role in controlling the damage induced by inflammation that follows the acute phase of infection (Pollock and Welsh, 2002; Baldwin et al., 2021).

Unvaccinated calves had significantly greater number of activated T cells subpopulations ($CD4^+CD25^+$, $CD8^+$ $CD25^+$, and $WC1^+$ $CD25^+$) in circulation following BVDV2 and BHV1 challenges than did the vaccinated calves. Only activated $CD8^+$ T cells ($CD8^+$ $CD25^+$) were increased in circulation over baseline values, and these were seen on days 10, 12 and 14 in the vaccinated calves. However, that the cells carried activation marker does not provide clear information about their function. The phenotype expressed is a consequence of cellular activation in the presence of viral infection that elicits either proinflammatory (IFN-gamma) or anti-inflammatory (IL-10) cytokine release (Endsley et al., 2004). It does not assure that the cells observed in circulation are specific for the infecting agent (many bystander cells are active in this environment) or that they are fully differentiate and armed to enter the tissues and remove virally infected cells. Moreover, attenuated viruses contained in MLV vaccines have the ability to replicate in the host cells, stimulate T cell activation (to control viral replication) and induce regulatory cells and products that modulate the immune response (Platt et al., 2006, 2009, and 2017). The increase in circulating activated T cells subpopulations may reflect an immune response supporting enhanced inflammation, could reflect activation in a common danger and damage context of memory cells, or could represent a true response of infection. Further research is warranted to understand the role of the activated T cell subpopulations in circulation as they relate to viral pathogenesis.

In conclusion, MLV vaccination protected calves from the effects of experimental serial challenge with BVDV2 and BHV1. Vaccination prevented leukopenia, thrombocytopenia, and reduced the reduction in the circulating fraction of the T cell subsets we measured. Furthermore, there was an effect of the route of administration and if trace minerals were concurrently administered on the circulating fraction of $CD4^+$ T cells (greater in calves receiving SC vaccination) and CD8+ T cells (greater in calves receiving ITM), respectively. Trace minerals are essential for growing calves. Supplementation is often necessary to achieve this. The use of ITM concurrent with MLV vaccination appears to generate improved protection from disease and may enhance the elements of immunity that are in circulation designed to clear viral infections involved in respiratory disease.

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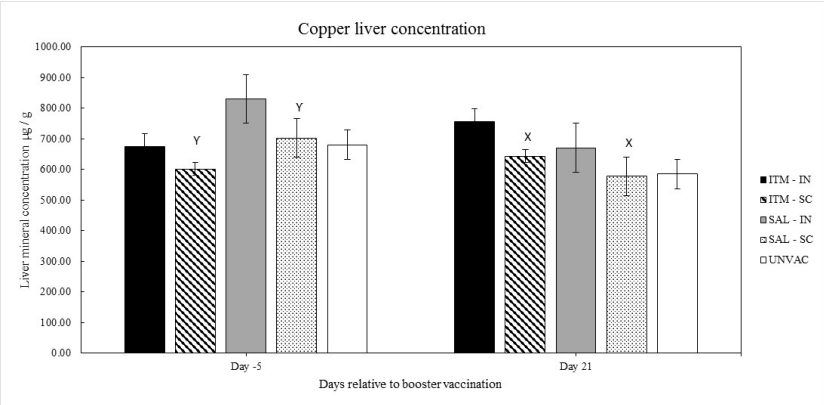
A



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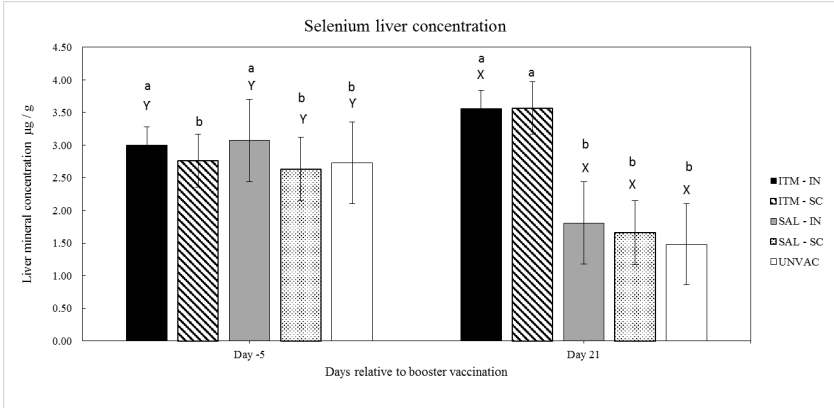

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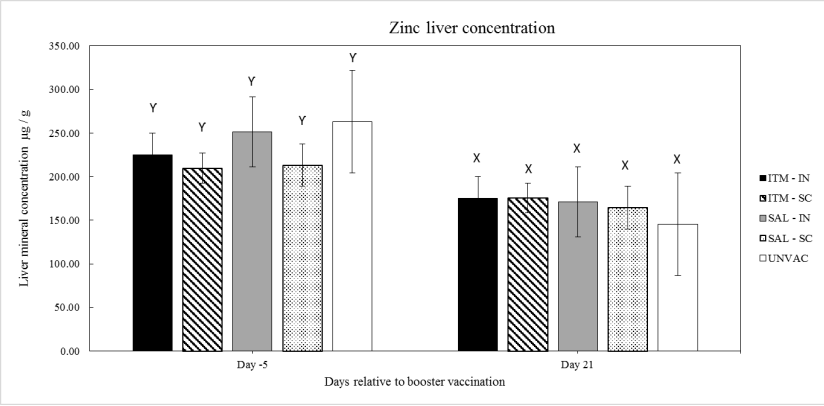

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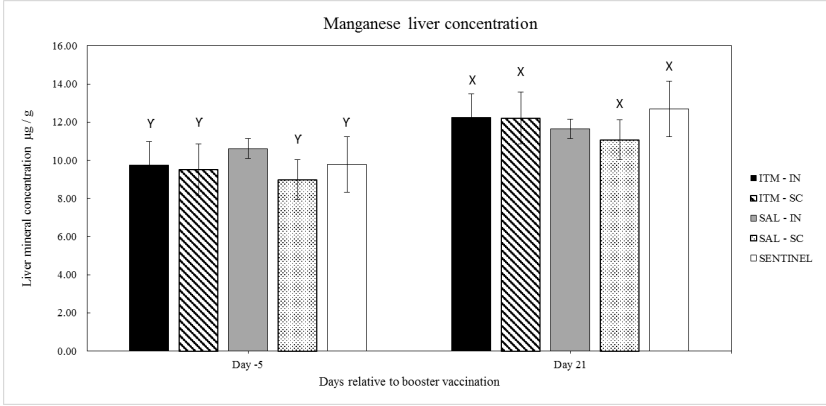


Figure 4.1 – Liver tissue trace minerals concentrations on day -5 and 21 relative to booster vaccination for Copper (A; reference range = 500-600 µg/g), Selenium (B; reference range = 0,7–2,5 µg/g), Zinc (C; reference range = 90-400 µg/g), and Manganese (D;

reference range = 5-15 μg/g). Error bars represent the standard error of the means (SEM). All calves received trace minerals or saline along with intranasal booster MLV vaccination (study day 0). Liver biopsy samples were done five days before vaccination. Errors bars indicate standard error of the means (SEM). There was an effect of time for liver concentrations of Se, Zn, and Mn ($p < 0.0001$). ^{A,B} Significant difference between groups on the respective day. $^{\gamma x}$ Significant difference among groups when compared day -5 vs day 21 ($p < 0.05$).

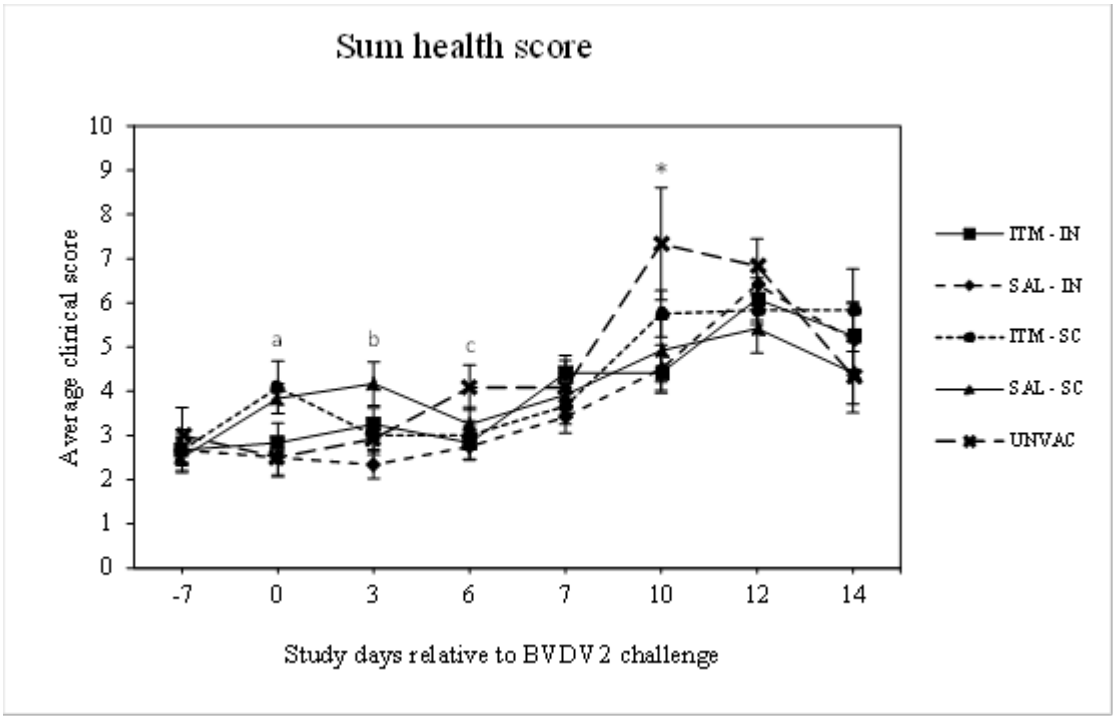
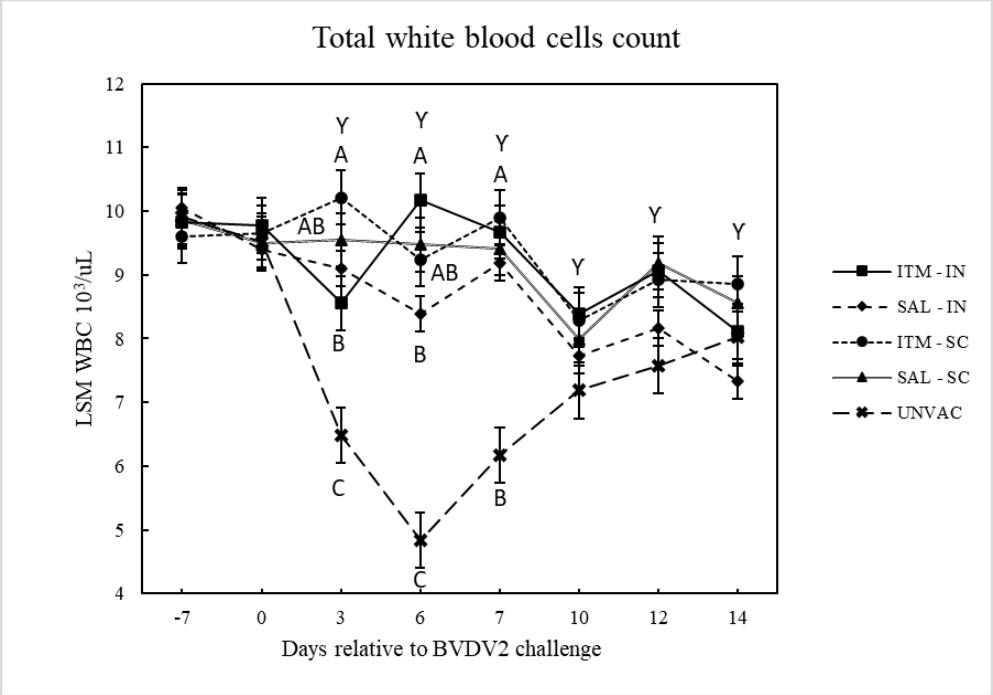
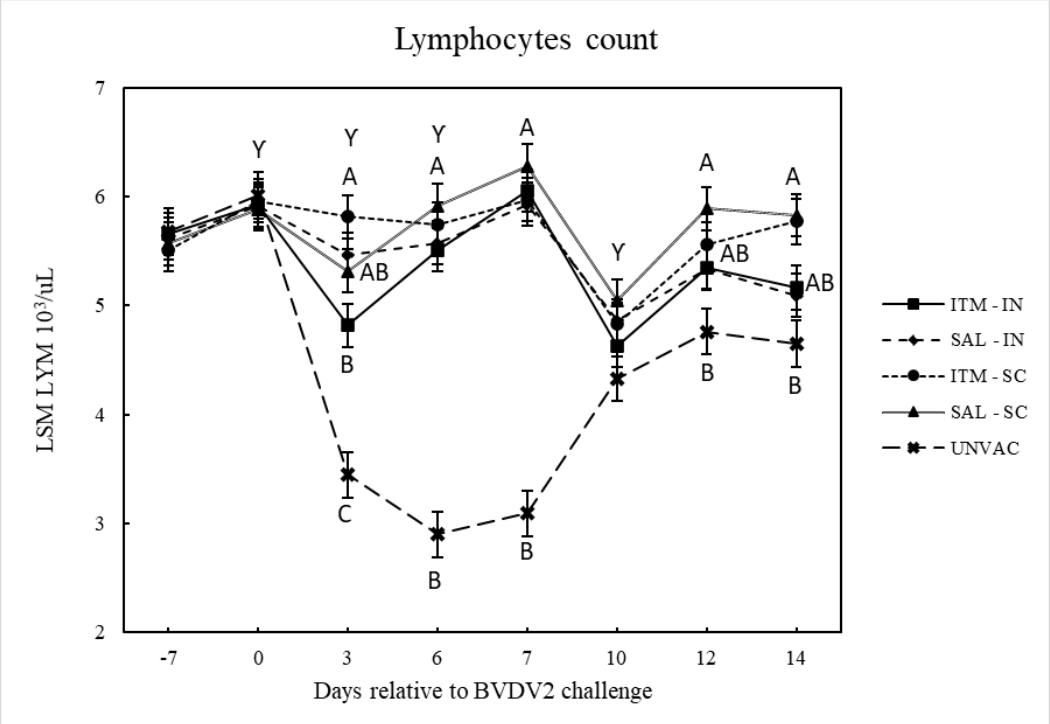


Figure 4.2 – The Sum clinical scoring. The clinical scoring baseline was day -7, while day 0 was considered the BVDV2 challenge. Error bars represent the standard error of the means (SEM). [a] Values in ITM-SC and SAL-SC groups were significantly greater ($p < 0.05$) than those in UNVAC calves on day 0. [b] Sum health score in SAL-SC group was significantly greater ($p < 0.05$) than that in the UNVAC group on day 3. [c] The sum of the health scores was greater in the unvaccinated calves on day 6 after BVDV2 challenge compared to SAL-IN ($p = 0.03$), and tended to be greater than ITM-IN ($p = 0.06$), and ITM-SC ($p = 0.08$). [*] The sum of the health scores was significantly greater ($p < 0.05$) on day 10 in the UNVAC group compared with all vaccinated groups.

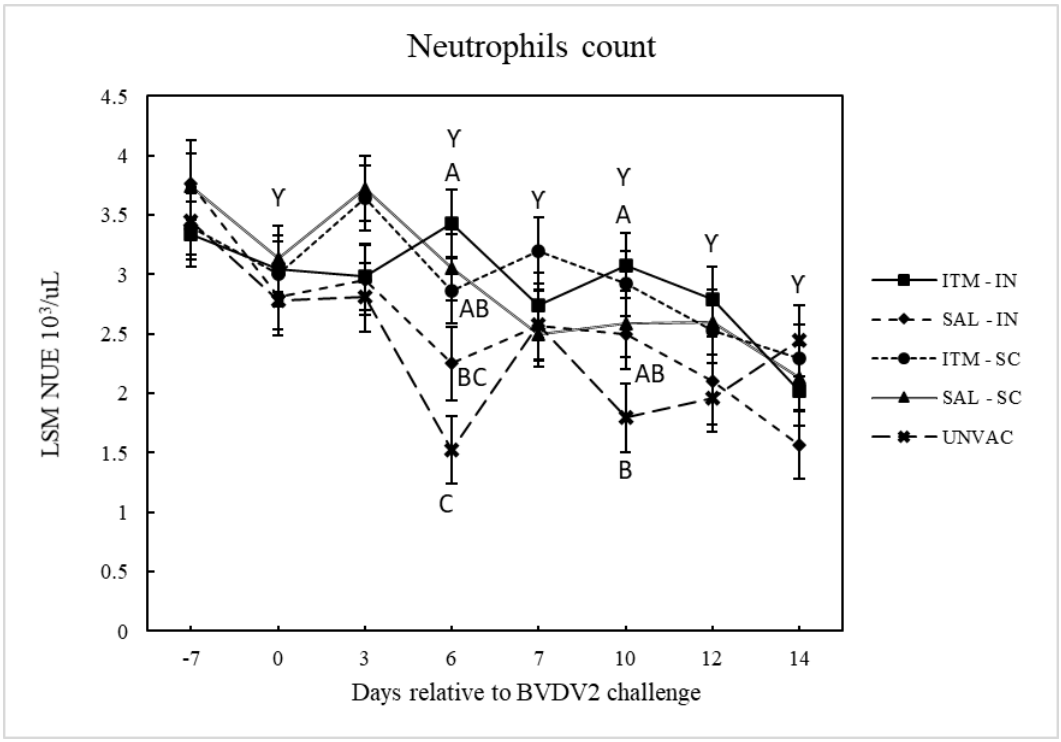
A


Total white blood cells count
LSM WBC 10³/uL
Days relative to BVDV2 challenge
ITM - IN
SAL - IN
ITM - SC
SAL - SC
UNVAC

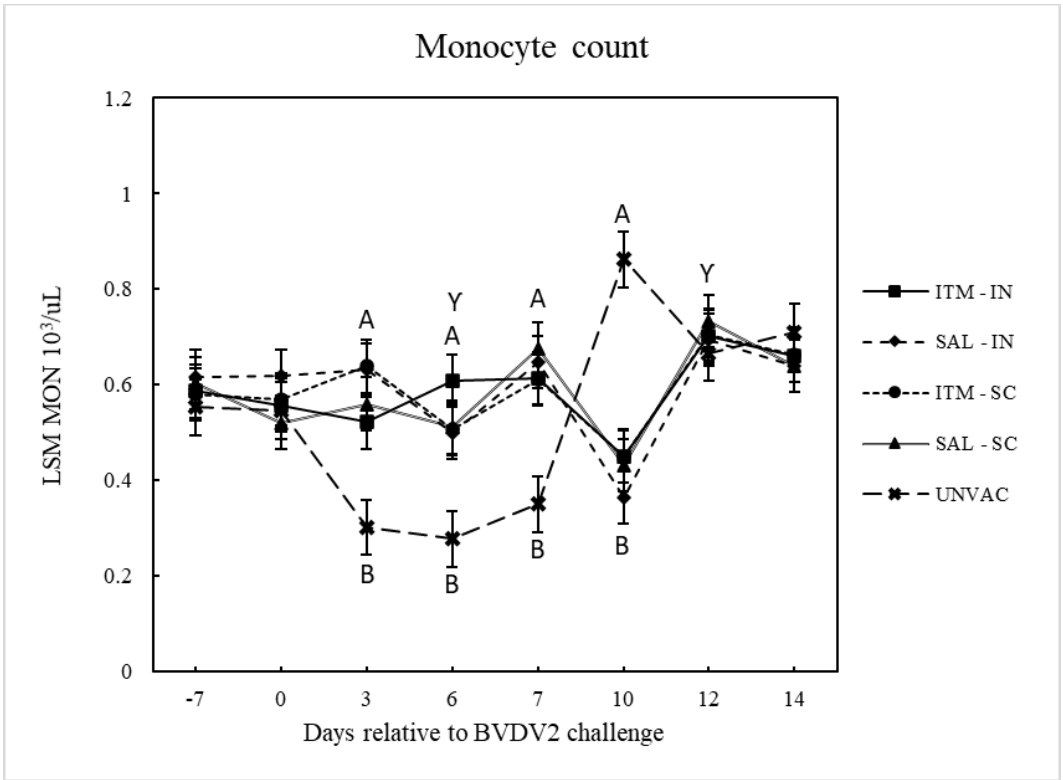
B


Lymphocytes count
LSM LYM 10³/uL
Days relative to BVDV2 challenge
ITM - IN
SAL - IN
ITM - SC
SAL - SC
UNVAC

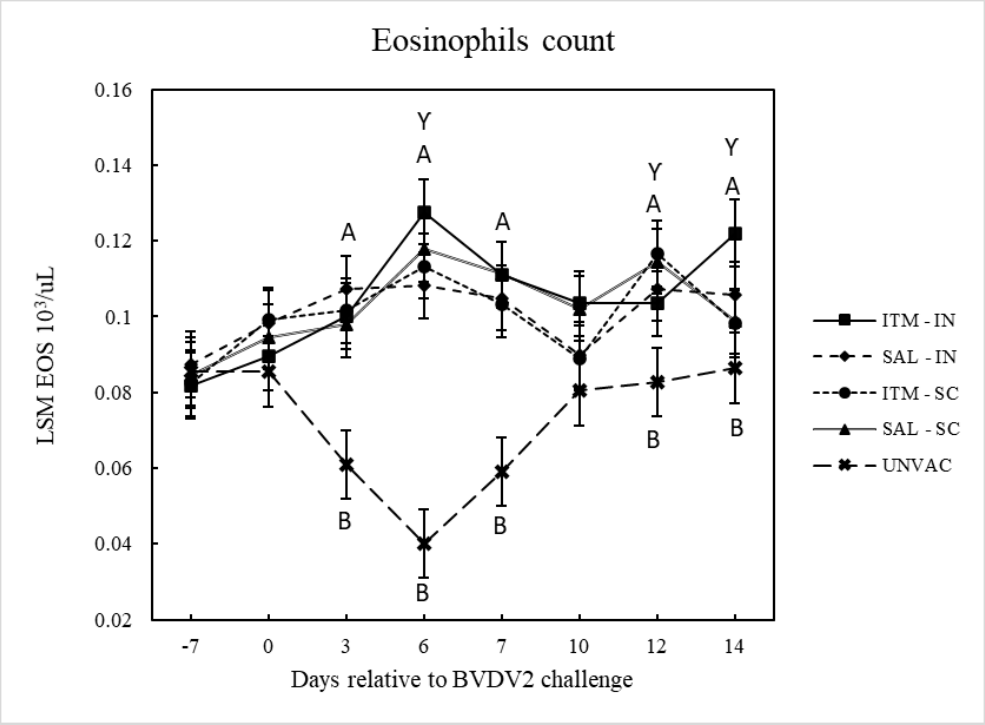
C


Neutrophils count
LSM NUE 10³/uL
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Y
A
Y
Y
A
Y
Y
AB
BC
AB
B
C
-7
0
3
6
7
10
12
14
Days relative to BVDV2 challenge
ITM - IN
SAL - IN
ITM - SC
SAL - SC
UNVAC

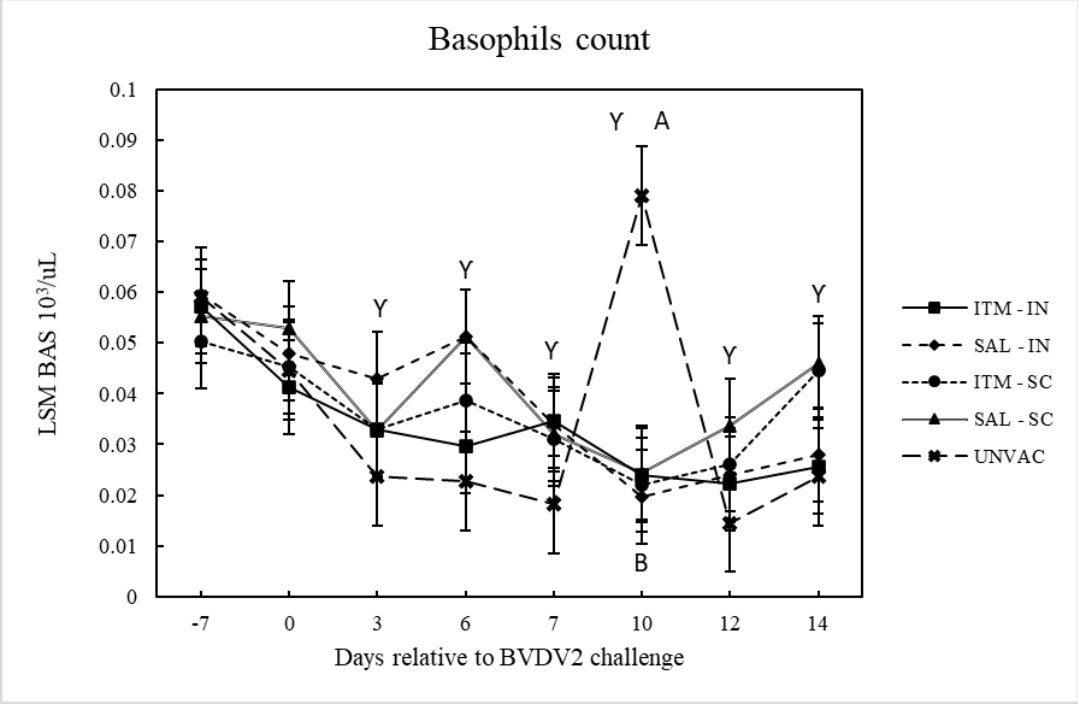
D


Monocyte count
LSM MON 10³/uL
1.2
1
0.8
0.6
0.4
0.2
0
A
Y
A
A
Y
B
B
B
B
-7
0
3
6
7
10
12
14
Days relative to BVDV2 challenge
ITM - IN
SAL - IN
ITM - SC
SAL - SC
UNVAC

E


Eosinophils count
LSM EOS 10³/uL
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
-7
0
3
6
7
10
12
14
Days relative to BVDV2 challenge
ITM - IN
SAL - IN
ITM - SC
SAL - SC
UNVAC

F


Basophils count
LSM BAS 10³/uL
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
-7
0
3
6
7
10
12
14
Days relative to BVDV2 challenge
ITM - IN
SAL - IN
ITM - SC
SAL - SC
UNVAC

G

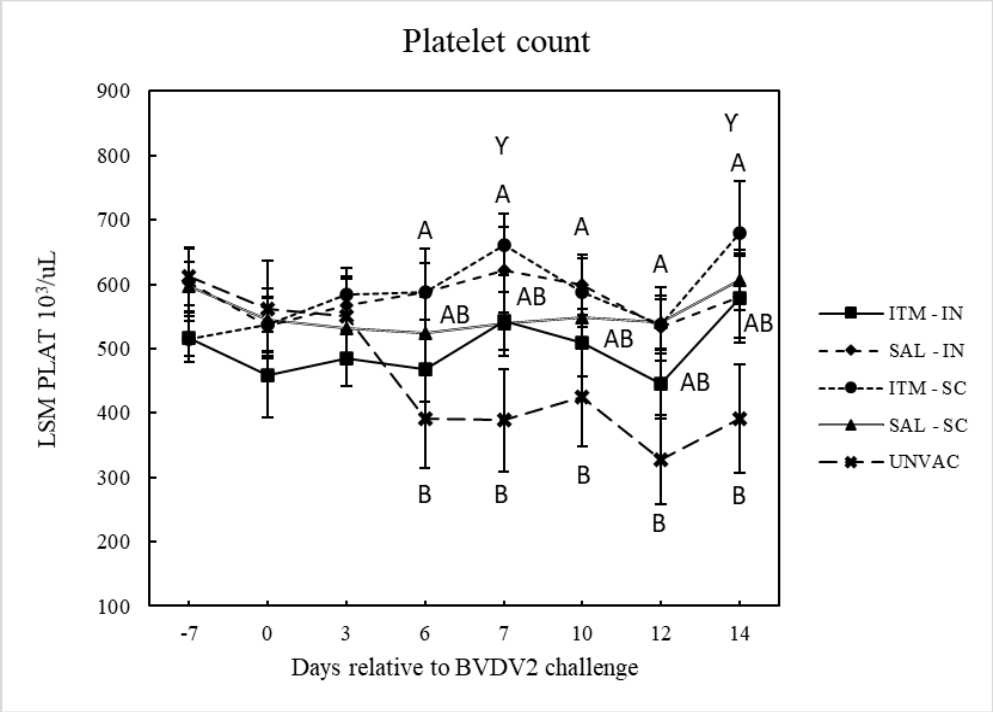
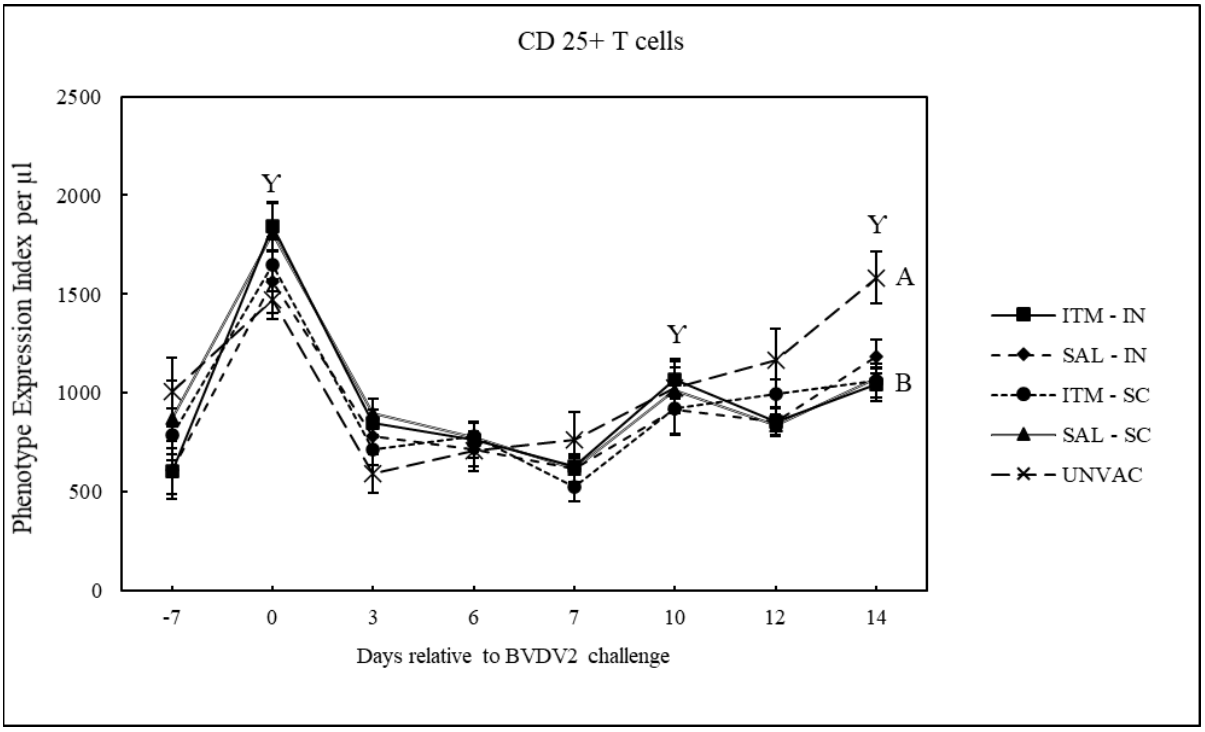
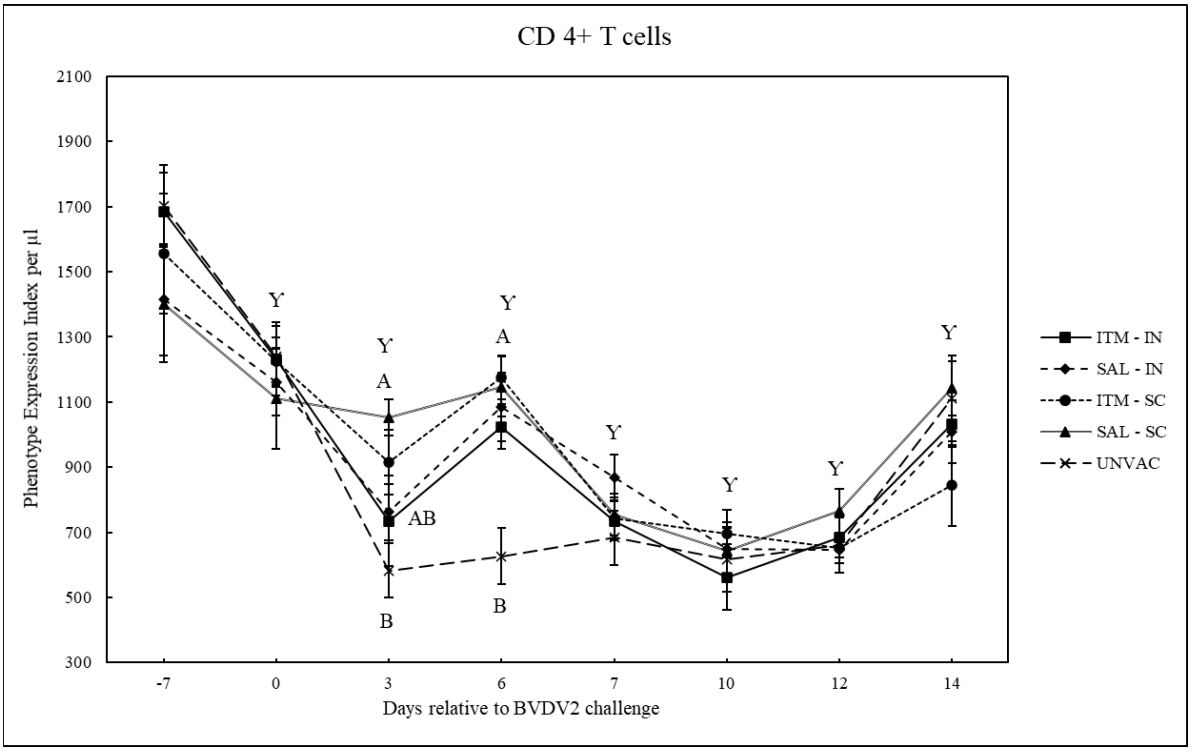


Figure 4.3 – Least squeare means (LSM) for White blood cell differential count and differential leukocyte numbers. Total white blood cell absolut number (Number/µl) - WBC (A), Lymphocytes absolute number - LYM (B), Neutrophils absolute number – NUE (C), Monocyte absolute number – MON (D), Eosinophils absolute number – EOS (E), Basophils absolute number - BAS (F), and Platelets absolute number - PLAT (G). There is a general leukopenia (A-E) in the UNVAC group after day 0 (BVDV2 challenge). ^{A,B,C} Significant difference between groups on the respective day ($p < 0.05$). $^{\gamma}$ Difference among groups when compared with baseline ($p < 0.05$).

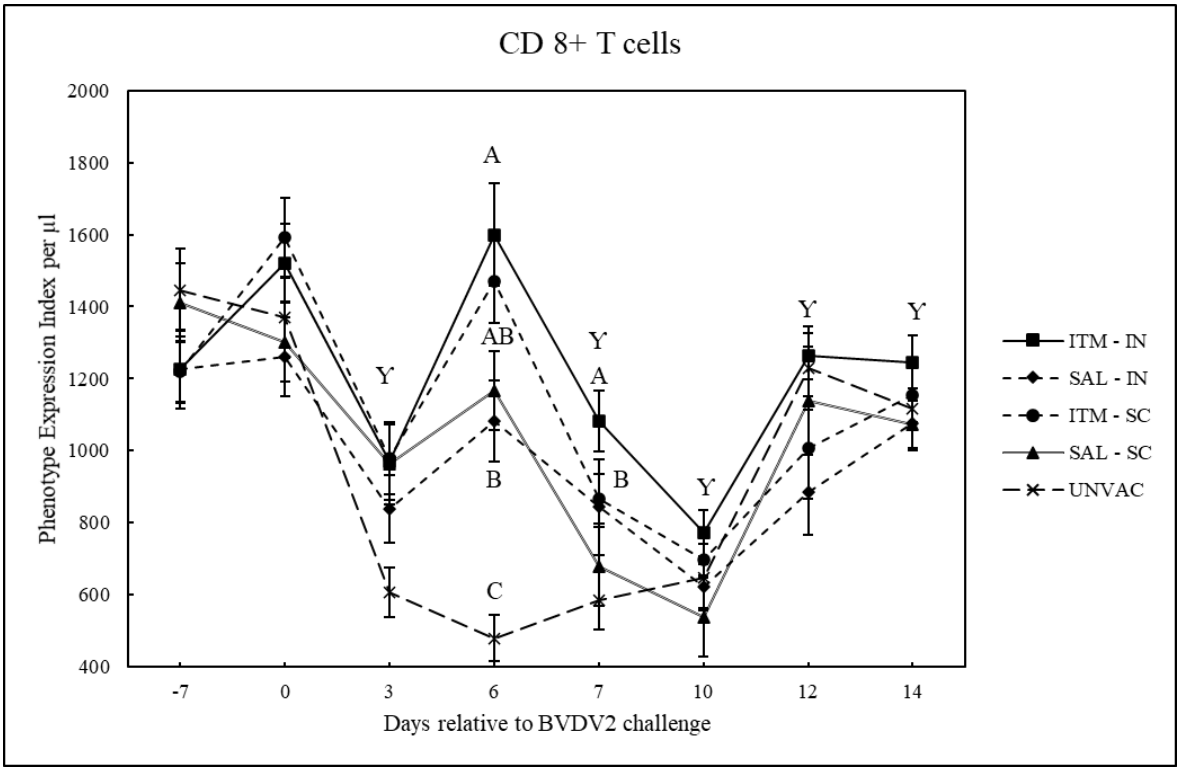
A


CD 25+ T cells
Phenotype Expression Index per µl
2500
2000
1500
1000
500
0
-7
0
3
6
7
10
12
14
Days relative to BVDV2 challenge
Y
Y
Y
A
B
ITM - IN
SAL - IN
ITM - SC
SAL - SC
UNVAC

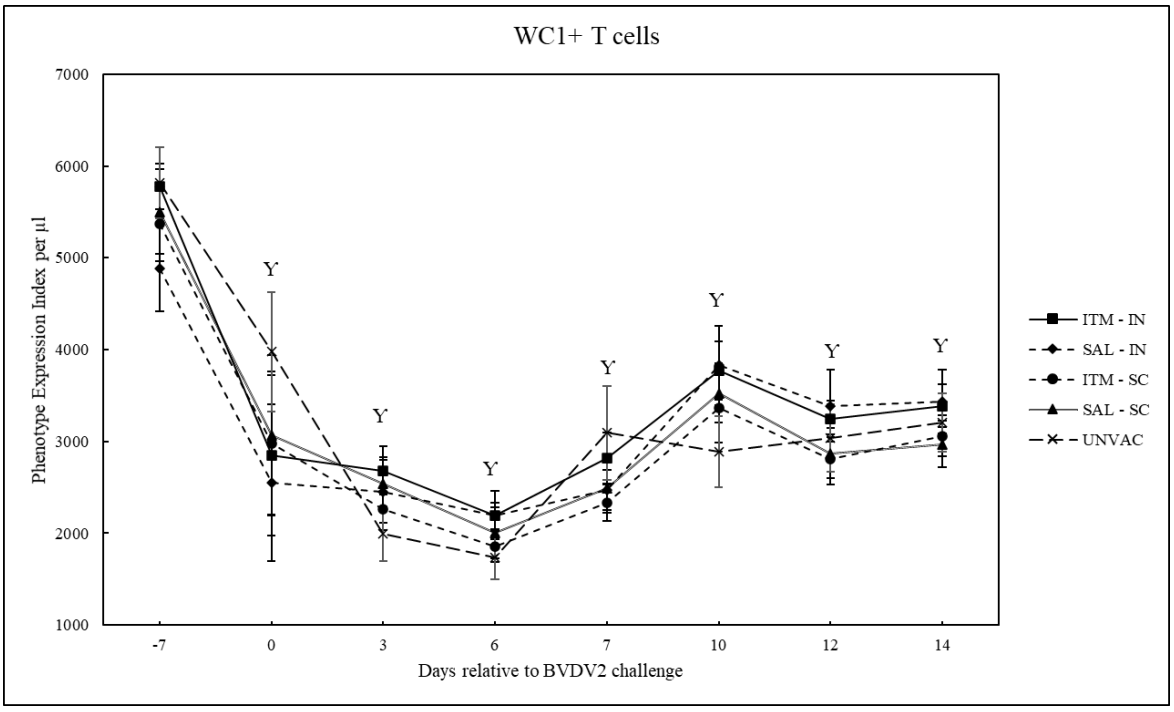
B


CD 4+ T cells
Phenotype Expression Index per µl
2100
1900
1700
1500
1300
1100
900
700
500
300
-7
0
3
6
7
10
12
14
Days relative to BVDV2 challenge
Y
Y
Y
A
Y
A
AB
B
B
Y
Y
Y
ITM - IN
SAL - IN
ITM - SC
SAL - SC
UNVAC

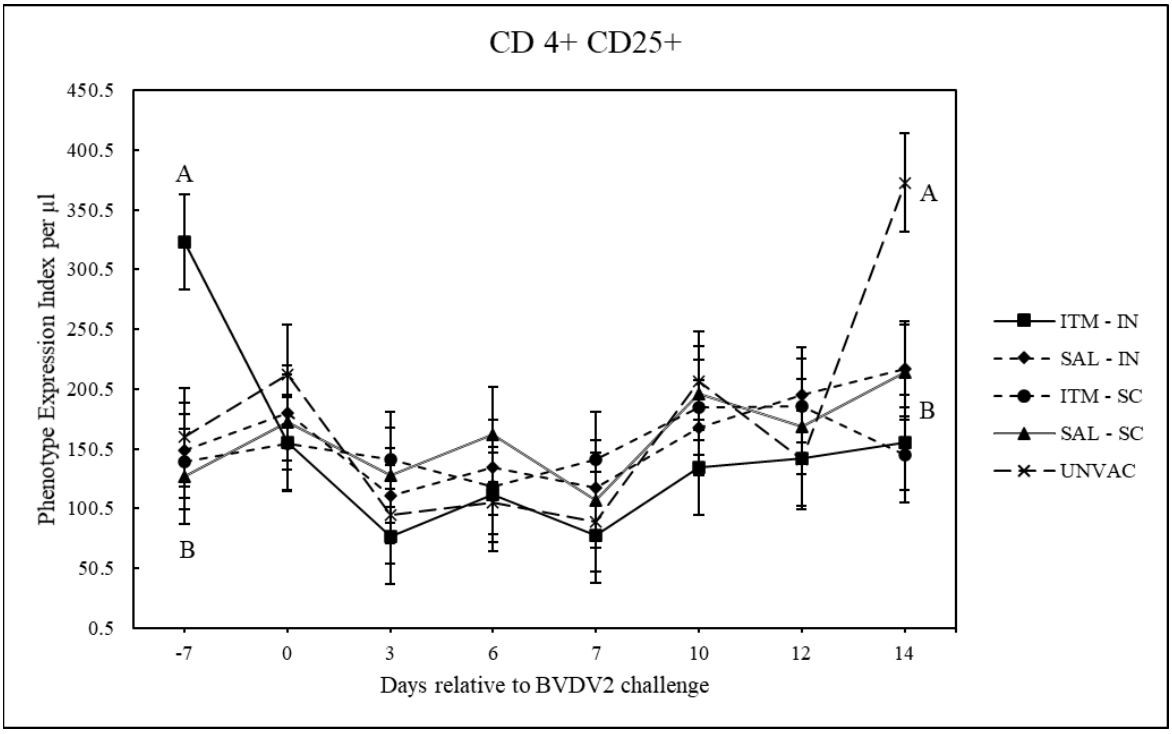
C


CD 8+ T cells
Phenotype Expression Index per µl
2000
1800
1600
1400
1200
1000
800
600
400
-7
0
3
6
7
10
12
14
Days relative to BVDV2 challenge
A
AB
B
C
Y
ITM - IN
SAL - IN
ITM - SC
SAL - SC
UNVAC

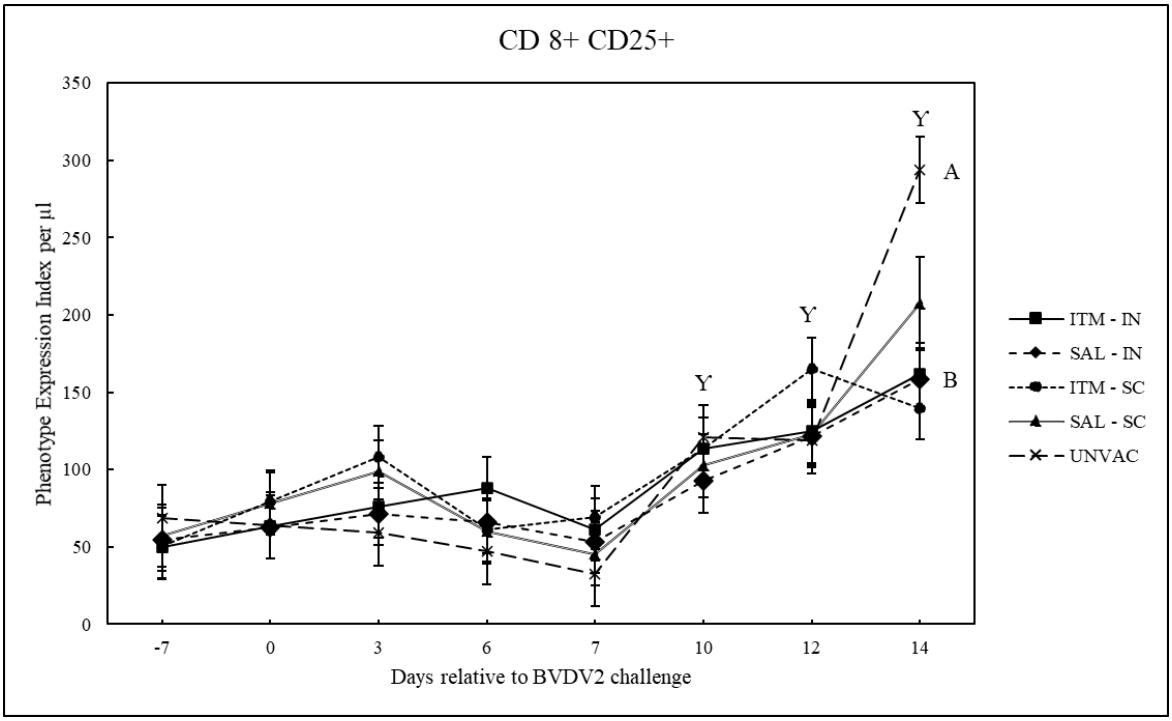
D


WC1+ T cells
Phenotype Expression Index per µl
7000
6000
5000
4000
3000
2000
1000
-7
0
3
6
7
10
12
14
Days relative to BVDV2 challenge
Y
ITM - IN
SAL - IN
ITM - SC
SAL - SC
UNVAC

E


CD 4+ CD25+
Phenotype Expression Index per µl
450.5
400.5
350.5
300.5
250.5
200.5
150.5
100.5
50.5
0.5
A
B
A
B
-7
0
3
6
7
10
12
14
Days relative to BVDV2 challenge
ITM - IN
SAL - IN
ITM - SC
SAL - SC
UNVAC

F


CD 8+ CD25+
Phenotype Expression Index per µl
350
300
250
200
150
100
50
0
Y
A
Y
Y
B
-7
0
3
6
7
10
12
14
Days relative to BVDV2 challenge
ITM - IN
SAL - IN
ITM - SC
SAL - SC
UNVAC

G

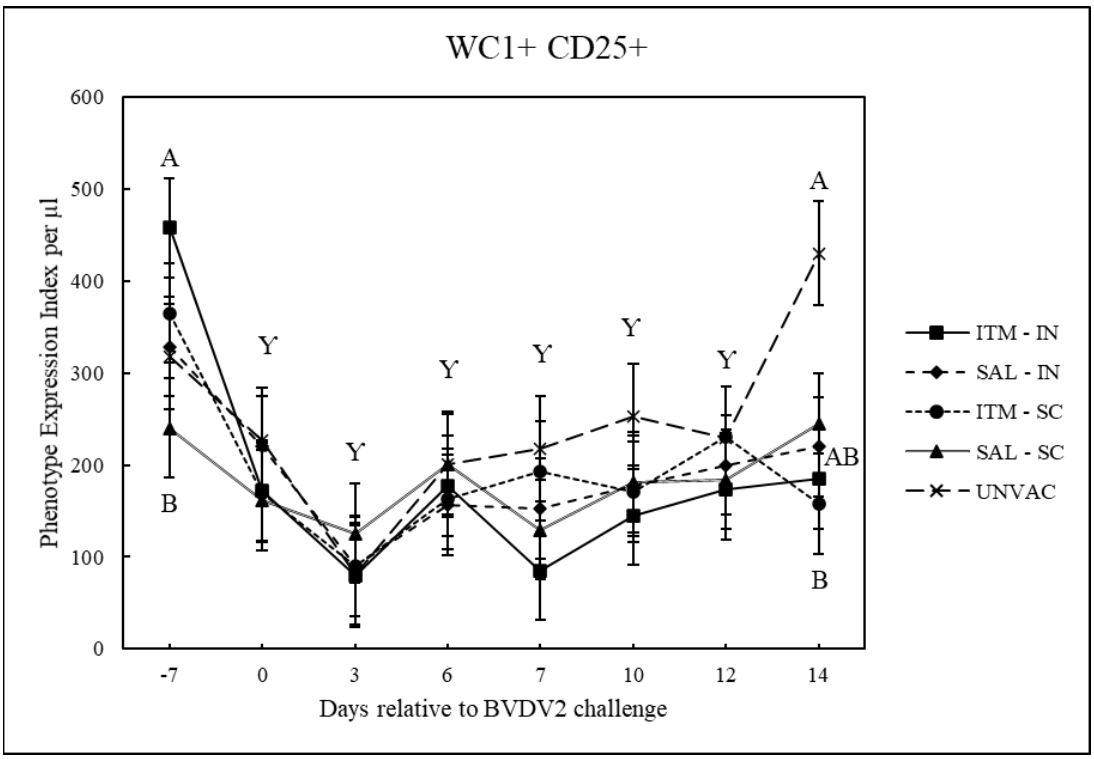
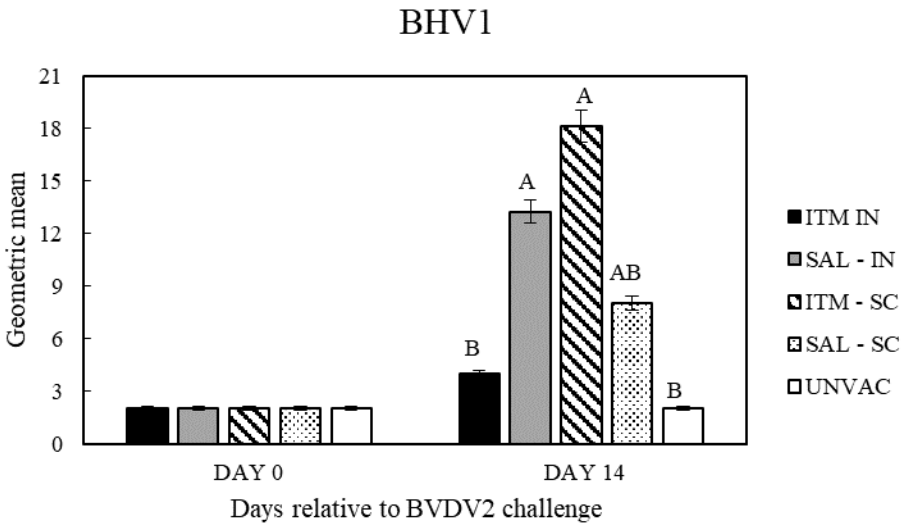


Figure 4.4 – Least squeare means (LSM) in T cell phenotype population estimated as Expression Index per µl. There was an effect of time ($p < 0.001$), groups ($p < 0.001$), and interaction between vaccination route plus treatments (groups) * time ($p = 0.01$) for $CD25^+$, and $CD8^+$. There was an effect of time ($p < 0.001$), and the interaction between vaccination route plus treatments (groups) * time ($p = 0.01$) for $CD4^+$, $CD4^+CD25^+$, $CD8^+CD25^+$. There was an effect of time ($p < 0.001$) for $WC1^+$, and $WC1^+CD25^+$. ^{A,B,C} Significant difference between groups on the respective day ($p < 0.05$). $^{\gamma}$ Difference among groups when compared with baseline ($p < 0.05$).

A



B

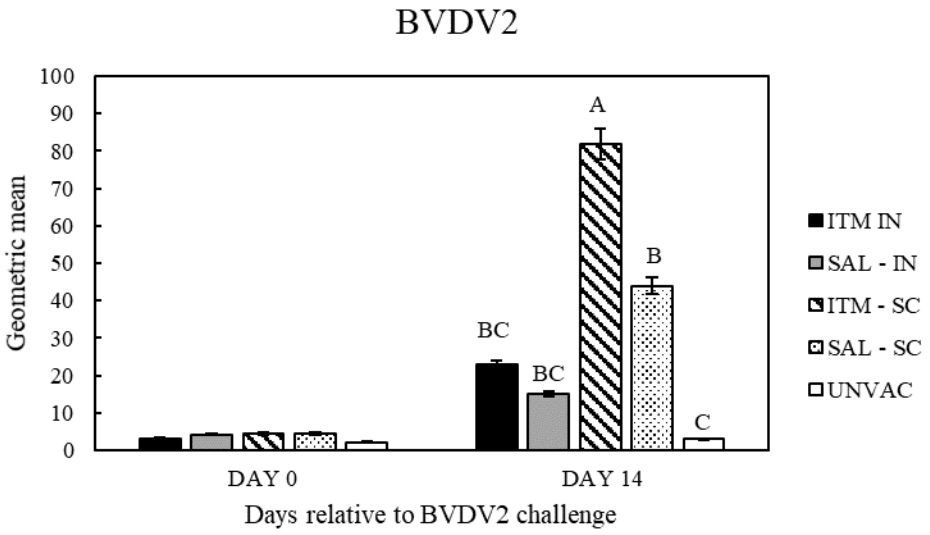


Figure 4.5 – Serum neutralizing antibodies against BHV1 and BVDV2 on days 0 and 14 after BVDV2 challenge. There was an effect on time ($p < 0.001$), group ($p < 0.001$), and group * over time ($p < 0.001$) for both BHV1 and BVDV2. A) Serum titers against BHV1 were significantly higher on day 14 for ITM-SC and SAL-IN when compared with UNVAC and ITM-IN calves (*p*

< 0.0001). There was not significant difference between ITM-IN and UNVAC; between SAL-SC and SAL-IN, and between SAL-SC and ITM-IN. B) Serum titers against BVDV2 were significantly higher in ITM-SC on day 14 when compared with groups ($p < 0.05$). There was not significant difference between UNVAC, ITM-IN, and SAL-IN; and between SAL-SC, ITM-IN, and SAL-IN. [A,B,C] Significant difference between groups on the respective day ($p < 0.05$).

Table 4.1 – Nutrient composition of feed concentrate supplement, grass, and hay bailage. Calves received feed twice a day (AM/PM), water *ad libitum*, and new hay rolls every 3 days.

Item	[a, b] Baseline grass	[a, c] Grass day 42	[d] Hay bailage	[e] Square bales	[f, g] Baseline feed	[f, h] Feed day 42
-- % (DM basis) --						
CP	13.1	13.3	15.1	21.1	19.8	21
TDN	61	60	54	64	75	75
ADF	39.2	42.5	39.6	29.9	18.2	18.4
NDF	12.1	8.7	5.4	17.1	37.2	36.5
-- mg/kg (DM basis) --						
Ca	3758	5994	5590	3871	1352	1690
P	2759	3942	2344	2410	8393	8189
Mg	2246	3032	4223	1578	3780	3886
K	10172	11181	15271	15188	16572	17681
Na	1150	1320	786	1032	1178	1420
S	3589	3677	894	1560	3889	3737
Fe	1653	1140	110	84	230	257
Zn	132.1	147.1	34.9	82.5	59.2	68.2
Cu	9.4	16.9	7.7	8.5	6.1	6.3
Mn	279.9	124.7	25.8	118.5	26.2	29.7
Se	0.094	0.088	0.174	0.047	0.170	0.165
Mo	< 0.5	< 0.5	< 0.5	< 0.5	2.1	2.1
Co	< 1	< 1	< 1	< 1	< 1	< 1

[a] Calves grazed in pastures with fescue grass (Festuca arundinacea) and Bermuda grass (*Cynodon dactylon*).

[b] Grass was hand plucked in 7 different regions of the pasture on the day of the booster vaccination, and submitted to the Diagnostic Center for Population and Animal Health at Michigan State University, Lansing, MI.

[c] Grass was hand plucked in 7 different regions of the pasture 42 days after booster vaccination.

[d] Bermuda grass hay (Cynodon dactylon) produced in a local farm close to UGA River Bend.

[e] Calves were feed with rye grass square bales once a week.

[f] Grain concentrate composed of soybean hulls (45.03%), corn gluten (50.04%), molasses (2.25%), calcium (1.5%), and salt (1.0%), trace mineral (0.1%) Vitamin ADE (0.05%). Offered to calves at a rate of 2.7 kg/head/day (as-fed) throughout the experiment.

[g] Godfrey's Feed ™– Cattlemen's special®

[h] Godfrey's Feed ™– super calf with Bovatec®

CHAPTER 5

CLINICAL STATUS AND ENDOSCOPY OF THE UPPER RESPIRATORY TRACT OF DAIRY CALVES INFECTED WITH BOVINE VIRAL DIARRHEA VIRUS 2 AND BOVINE HERPES VIRUS 1 AFTER VACCINATION AND TRACE MINERALS INJECTION[3]

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Abstract

The objectives were to compare assessment tools defining clinical protection [health status, endoscopic assessment of the upper respiratory tract (URT), leukocyte count, viremia, and virus shedding] following serial BVDV2 and BHV1 challenge relative to a matrix of four combinations of modified-live virus (MLV) booster vaccination given in the presence or not with injectable trace minerals (ITM) in dairy calves. Forty-eight dairy calves (1 months of age) that received a modified-live virus (MLV) intranasal (IN) vaccine containing BHV1, BRSV, and BPI_3V co-administered with randomly assigned subcutaneous (SC) administration of ITM (containing Se, Cu, Zn & Mn; ITM, n=24) or saline (SAL, n=24). Ten weeks later, the calves received a second dose of ITM, or saline, as previously assigned and were randomly received booster vaccination with the same IN vaccine [ITM-IN (n=12), SAL-IN (n=12)] or a SC MLV vaccine containing BHV1, BRSV, BPI3V, BVDV1 & 2 [ITM-SC (n=12), SAL-SC (n=12)]. Additionally, 12 calves did not receive vaccine or treatment and served as a negative control group (UNVAC, n=12). Forty-nine days after booster vaccination, all calves were challenged intranasally BVDV2; then seven days later challenged with BHV1. Health status, leukocyte counts, level of viremia and viral shedding were measured. Randomly chosen subsets of calves from each group were selected for endoscopic evaluation of the URT. Endoscopic scores (ES) were calculated and compared among the groups. Data were analyzed using SAS® and Stata®. Health scores indicated disease in UNVAC on days 6, 10 and 12 after BVDV2 and BHV1 challenge relative to the vaccinated groups. Unvaccinated calves had the highest URT ES after BHV1 challenge. Calves that received the SC booster vaccination (with ITM or not) had lower URT ES after BHV1 challenge than the unvaccinated calves. Calves receiving ITM-IN had significantly lower URT ES after BHV1 infection than the SAL-IN and UNVAC calves. In

conclusion, booster vaccination either IN or SQ was similarly effective in protecting calves from the serial BVDV2 and BHV1 challenges. All calves that received SC vaccination had significantly lower URT ES after BHV1 challenge than the unvaccinated calves. The administration of ITM concurrent with IN booster vaccination was associated with reduced URT inflammation after the serial challenge BVDV2 and BHV1.

Key words: Bovine respiratory disease viruses, BVDV2, BHV1, BRSV, BPI_3V, modified-live virus vaccination, trace minerals, endoscopy

Introduction

Bovine respiratory disease (BRD) is a major illness that affects the cattle industry worldwide (Griffin, 1997, McVey, 2009). Viral agents such as *Bovine viral diarrhea virus* (BVDV), *Bovine respiratory syncytial virus* (BRSV) *Bovine herpes virus 1* (BHV1), and *Bovine parainfluenza 3 virus* (BPI3V) are frequently involved in BRD (Grissett et al., 2015, Timsit et al., 2017, Smith et al., 2020). Infections with BVDV and BHV1 are commonly subclinical (Campbell, 2004, Jones, 2003, Jones and Chowdhury, 2010). However, these viruses cause immunosuppression by altering several mechanisms of the immune response, increasing animals' susceptibility to infections by other viruses or bacteria, which contributes to disease severity (Walz et al., 2010, Ellis, 2009, Levings and Roth, 2013). Significant inflammation and damage of the upper respiratory tract characterized by rhinitis, laryngitis and tracheitis have been reported after BHV1 infections in cattle (Tikoo et al., 1995, Gershwin et al., 2015).

The use of multivalent vaccines combined with well-designed biosecurity measures and a focus on early clinical diagnosis and treatment of affected animals are strategies commonly used

to control BRD (Platt et al., 2009, Gorden and Plummer, 2010, Palomares et al., 2016). Vaccination to prevent BRD has been used in almost all dairy operations with variable results in preventing disease and economic loss (Gorden and Plummer, 2010, Chamorro and Palomares, 2020) . The level of maternally transferred antibody impact the amount of interfere with the induction of the humoral immune response observed following parenteral BRD vaccination (Kimman et al., 1989, Endsley et al., 2004, Chamorro et al., 2016). Intranasal (IN) vaccination appears to represent a better route for immune priming of young dairy calves. It appears that IN vaccination often evades vaccine antigen neutralization due to the action of circulating maternal antibodies (Chamorro et al., 2016, Ellis, 2017). In addition, IN vaccination may also stimulate a respiratory mucosal response against specific pathogens in the form of secretory IgA (sIgA). The production of sIgA binds invaders in the respiratory tract to protect against viral binding and infection by the respiratory route (Vangeel et al., 2009, Hill et al., 2012, Hill et al., 2019). Several studies have reported the benefits of priming with IN vaccine but using a parenteral vaccination as a booster dose (Kardani et al., 2016, Ellis, 2017, Erickson et al., 2020). However, research comparing routes of booster vaccination administration (IN versus SC) on the induced immune response and the level of protection induced in young dairy calves are still lacking. The use of injectable trace minerals (ITM) concomitant with BRD parenteral vaccination has demonstrated to have beneficial effects on the induction of the immune response and in inducing protection against disease caused by respiratory pathogens (Palomares et al., 2016, Bittar et al., 2020). However, there are no previous reports examining the effects of the administration of ITM on the induction of protection by IN vaccination in young calves.

Prompt clinical diagnosis of BRD appears to contribute to disease control. Early diagnosis allows for earlier isolation and earlier treatment of sick animals (Gorden and Plummer,

2010, Buczinski and Pardon, 2020). This may reduce the level of pathogen transmission, the level of morbidity and the cost of mortality (Ollivett et al., 2015). Several health scoring systems have been developed for use in the clinical diagnosis of BRD (McGuirk, 2008, Love et al., 2014). However, those methods tend to fail to identify cases of subclinical pneumonia (Ollivett et al., 2015) . The use of thoracic ultrasonography is useful in detecting lung consolidation and sub-clinical BRD (Buczinski et al., 2014, Ollivett et al., 2015). However, it does not permit detection of significant inflammation of the upper respiratory tract (URT) before the bronchi and the lungs are compromised. Endoscopy is a minimally invasive and atraumatic diagnostic tool that permits the evaluation of the bovine URT (Anderson et al., 1994) . Endoscopic examination may allow identification of inflammatory changes and mucosal damage during the early stages of disease. This would be before bronchial and lung lesions develop. Thus, early identification of sick animals by endoscopy may have a significant impact on disease management. This diagnostic method has been widely used in horses for evaluation of several respiratory disorders (Holcombe et al., 2001, Slovis, 2004, Sheta, 2017). Yet, endoscopic evaluation of the respiratory tract is not routinely performed in cattle with BRD.

The present clinical trial was part of a larger randomized study that was aimed at determining the effects of the route of vaccination (IN versus SC) in combination with the administration of trace minerals (ITM or a s saline control) at the time of booster vaccination on the induction of protective immunity following a serial BVDV2 and BHV1 challenge in dairy calves. In this portion of the trial, we further evaluated the clinical presentation using multiple modalities: health status scoring, endoscopy of the URT, comprehensive leukocyte count with differential, level of viremia in circulation and viral shedding in nasal secretion following the

serial BVDV2 and BHV1 challenge. We compared these in animals that had MLV booster vaccination either IN or SC and were treated with ITM or saline in dairy calves.

Materials and methods

Farms and calves husbandry

This study was conducted at the University of Georgia River Bend Farm located in Athens, GA using 60 male dairy calves (3.5 months of age) derived from a commercial dairy farm located in Quitman Georgia, from August to October 2017. The calves were cared for in accordance with acceptable practices as delineated in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010). In addition, the experimental animal use, and biosafety protocols were reviewed and approved by the University of Georgia, Institutional Animal Care and Use Committee (IACUC; AUP# A2016 11-012-Y3-A3) and the Institutional Biosafety Committee (IBC#2017-0006), respectively.

At the farm of origin calves were managed in individual outdoor pens with hutches (1.5 m wide by 2 m long) separate from the rest of the calves and the adult herd, from the day of birth until the day of transportation to the experimental farm. The individual pens were bedded with sand. The calves were fed 4L of moderate-high quality pooled colostrum (≥22.0% Brix refractometer) from primiparous and multiparous cows within 1 hour after birth using a bottle with a nipple. After 12 hours of birth, calves were given another 2 L of colostrum. If the calves were not able to suckle, the colostrum was provided via esophageal tube feeder. Moderate-high quality colostrum was pasteurized by using Perfect Udder ® Combi pasteurizer 60G (Dairy tech, Inc. ®), and frozen until use. Moreover, the calves were fed in the first week a total of 8.5L of milk divided three times per day (06:00, 12:00, and 17:00 h), increasing the volume of milk until

week 6 (11.5L). After 6 weeks of life, the amount of milk offered was reduced until weaning. Water and calf starter diets were offered *ad libitum* starting at day 3 of life to all calves. Calves were weaned at 2 months of age and four weeks later were transported to the University of Georgia experimental farm. On the day of transportation, all the calves were administered a dose of Tilmicosin (10 mg/kg of body weight, Micotil®300, Elanco Animal Health, Indianapolis IN) subcutaneously as metaphylaxis to prevent the occurrence of respiratory disease associated with shipping. At the experimental farm the calves were housed in 8-acre pastures of Bermuda grass (*Cynodon dactylon*) and fescue grass (*Festuca arundinacea*) with shade and no access to mineral supplementation. In addition, animals had access to hay (Bermuda grass and fescue grass) and water *ad libitum*. All calves received 2.7 kg/head/day of concentrate supplement (Bulk Cattleman's Special; Godfrey's Warehouse; Madison-GA) divided into two meals.

The calves were confirmed to be BVDV-naïve using a standard virus neutralization test for serum neutralizing antibody (SNA) titers against BVDV1 and 2, and an ear notch biopsy for BVDV antigen by antigen capture ELISA. These laboratory analyses were performed at the University of Georgia, Athens Veterinary Diagnostic Laboratory (Athens, GA).

Sample size calculation

This trial is part of a larger study aimed to determine the effects of the route of booster vaccination and administration of trace minerals on protective immunity (antibody titers and cell-mediated immunity) against a BVDV2 challenge followed 7 day later by a BHV1 challenge. The sample size was calculated on the basis of humoral and cell-mediated immune outcomes resulting from two previous studies (Palomares et al., 2016 and Bittar et al., 2020). For the current study, we assumed that calves treated with ITM would have increased geometric means

in neutralizing antibody titers after BVDV2 + BHV1 infection (geometric means of 176 vs. 337 for control and ITM, with a standard deviation of 120), and an increased fraction of circulating $CD4^+$ and $CD8^+$ T cells would be measured after the serial virus challenge (means of 7.8 vs. 5.6 for ITM and control, respectively, with a standard deviation of 1.5). Considering a significance at α=0.05 and assuming a power of 80%, 8 to10 calves were needed for testing each treatment group. Allowing for 15% attrition, 60 animals were enrolled in the study (12 calves in each treatment group and 12 controls). With this sample size, we were able to detect differences in serum neutralizing antibody titers, number of $CD4^+$ and $CD8^+$ T cells (data not shown herein) as well as endoscopic scores of the URT among the groups.

Experimental design, vaccination, and treatments

The experimental design of this study corresponds to a complete randomize clinical trial with treatment (ITM or saline) and route of booster vaccination (IN or SC) as main factors. The calves were initially used for a clinical study at the farm of origin (Hoyos-Jaramillo et al., 2017). In that study calves (1 month of age) received an intranasal (IN) modified-live virus (MLV) vaccine containing BHV1, BRSV, BPI3V (Inforce 3® Zoetis Animal Health), and were randomly assigned by the team leader and corresponding author (RAP) to subcutaneous (SC) administration of injectable trace minerals (ITM, n=24) containing Se, Cu, Zn & Mn (Multimin®90) or saline (SAL, n=24), using a random number generator (www.random.org). In addition, 12 calves of similar age and physically separated from the vaccinated calves, remained unvaccinated and did not receive ITM or saline (UNVAC, n=12), and served as a sentinel group in the previous trial (Hoyos-Jaramillo et al., 2017) and for the study described here (Figure

5.1A). The calves remained in the farm of origin for a total of 12 weeks. Then they were transported to the experimental farm at approximately 3 months of age.

Ten weeks after priming vaccination (two weeks after transportation and arrival at the experimental UGA farm), the calves (approximately 3.5 months of age) within the initial group assignment (ITM and SAL) were again randomly allotted (using www.random.org) to the following four subgroups for booster vaccination and additional treatment administration (Figure 5.1A):

1. ITM-IN (n=12): Calves received 2mL of IN MLV vaccine (Inforce 3®) 60 days after primary vaccination and a second dose of ITM (Multimin®90; 1mL/100 lb SC).
2. ITM-SC (n=12): Calves were administered 2mL of a SC MLV vaccine containing BHV-1, BRSV, PI3 and BVDV-1&-2 (Bovi-shield Gold® 5 Zoetis Animal Health®) 60 days after primary vaccination and a second dose of ITM (Multimin®90; 1mL/100 lb SC).
3. SAL-IN (n=12): Calves received 2mL of Inforce 3® IN 60 days after primary vaccination and a second injection of saline (1mL/100 lb SC).
4. SAL-SC (n=12): Calves were given 2mL of a SC MLV vaccine (Bovi-shield Gold® 5) 60 days after primary vaccination and a second injection of saline (1mL/100 lb SC).

Calves in the IN vaccinated groups (ITM-IN and SAL-IN) also received 2 mL of a SC MLV vaccine containing BVDV-1&-2 (Bovi-shield Gold® BVD, Zoetis Animal Health®) in order to provide the same complement of antigens at boosting as the SC groups, with only the route of administration of the boosting dose of BHV1, BRSV, and BPI3V differing.

The 12 sentinels calves of the same age that were not prime vaccinated at the farm of origin during the initial study remained as unvaccinated group (5. UNVAC, n=12). These calves were isolated from the vaccinated calves in a different pasture before the day of booster vaccination

until the day of challenge, in order to prevent infection with vaccine virus shed by the vaccinated calves.

Administration of ITM provided 15, 60, 10 and 5 mg/mL of Cu, Zn, Mn, and Se, respectively. All injections were performed in accordance with the guidelines of the Beef Quality Assurance Program (Beef Quality Assurance 2010®; Centennial, CO).

After MLV vaccination, attenuated viruses can replicate in the upper respiratory tract and be shed in nasal secretions. Therefore, calves in IN and SC groups were physically separated for two weeks in two different pastures immediately after booster vaccination. This separation avoided nose to nose contact and a possible transmission of vaccine viruses between vaccinated groups.

Bovine viral diarrhea virus and Bovine herpes virus 1 intranasal challenge inoculations

Forty-nine days following booster vaccination and treatment administration, all the calves were intranasally inoculated with a noncytopathic (ncp) BVDV2 (strain 890). The day of BVDV2 challenge was considered day 0 (Figure 5.1A). The BVDV2 isolate was initially obtained from the Animal and Plant Health Inspection Service (APHIS) Center for Veterinary Biologics in 1989 (Ames, IA), where it was maintained and propagated in Madin-Darby Bovine Kidney (MDBK) cell culture. The BVDV2 strain 890 was subjected to successive passages in MDBK cells monolayers as previously described (Bittar et al., 2018a). The BVDV2 inoculum consisted of an infected cell culture supernatant containing 1×10^5 50 % tissue culture infectious dose ($TCID_{50}$) per ml. The challenge was performed by intranasal aerosolization of the inoculum (5mL per calf; 2.5 mL in each nostril) using individual 10 cm long tip-fenestrated cannulas attached to the 12 mL syringes containing the BVDV-2 inoculum (one cannula-syringe set for

each individual animal). Following challenge inoculation, all calves commingled in an 8-acre pasture with adequate shade, until the end of the study.

Seven days after BVDV2 challenge, the calves were challenged with BHV1 (Strain Colorado-1). The BHV1 isolate was obtained from American Type Culture Collection (ATCC; Manassas, VA USA), where it was maintained and propagated in MDBK cells. The day of BHV1 challenge was considered day 7 (Figure 5.1A).

The inoculum consisted of an infected cell culture supernatant containing 8×10^6 $TCID_{50}$ per mL of the BHV1 Strain Colorado-1 (1 VR-864) grown in monolayers of MDBK cells. The experimental challenge was performed by intranasal aerosolization of the inoculum (5mL per calf; 2.5 mL in each nostril) using the same inoculation system utilized for the BVDV2 challenge. After BVDV2 challenge all calves were mixed and commingled in the same pastures throughout the course of the study.

Clinical status and health score system

General health status was evaluated relative to the BVDV2 challenge. On days -7, 0 (BVDV2 inoculation), 1, 2, 3, 4, 5, 6, 7 (BHV1 inoculation), 8, 9, 10, 11, 12, 13, and 14 employing the University of Wisconsin health scoring system (McGuirk, 2008), while the animals were commingled in the pen. A complete health status evaluation included assessment of rectal temperature, monitoring the position of the head-ears (attitude), hydration (by skin fold), ocular and nasal secretions, and the fecal consistency for each individual calf was performed on study days -7, 0, 3, 6, 7, 10, 12 and 14. This was done while the calves were restrained in a chute for other sample collection. Clinical signs were evaluated on a scale from 0 to 3 (where 0 was a lack of clinical signs and 3 represented severe clinical signs). The daily sum of the health score

was calculated by adding all individual score values for all clinical variables collected, with a possible maximum score of fifteen. This is as previously reported (Bittar et al., 2018a). In addition, body weight was measured on the day of booster vaccination (day - 49) and on days 0, 7, and 14, relative to BVDV challenge (day 0) to allow calculation of average daily gain (ADG). Assessment of clinical signs and health scores was performed by three experienced veterinarians. In addition, they performed the virus challenge administration and performed the nasal and blood sample collection. They were blinded to the treatment allocation of the calves.

Endoscopic evaluation of the upper respiratory tract (URT)

The evaluation was performed using a mobile video-endoscopy system (Tele Pack Vet X LED-Karl Storz TN 100 EN) equipped with a flexible videoscope (5.9 mm x 110 cm, Karl Storz 60511 NKS). Endoscopic examinations were performed on days -2 (before BVDV2 challenge), 6 (six days after BVDV2 challenge and the day before BHV1 challenge) and 12 (5 days after BHV1 challenge).

Random subsets of 2-5 calves were selected from each treatment group (using www.random.org) for endoscopic evaluation of the URT. Individual calves were only evaluated at once. Different calves chosen for the additional two URT assessment to preclude the potential for carryover effects of any damage or additional inflammation resulting from the endoscopic examination. Calves were lightly sedated using acepromazine (0.1 mg/kg) I.V, then restrained in a chute in standing position for approximately 10 minutes during the endoscopic assessment. In addition, 5 mL of lidocaine was sprayed into each nostril approximately two minutes before introduction the probe to avoid discomfort and reduce the probability of sudden movement of the head.

The scope was manually guided into either the right or the left nostril of each calf. Each segment of the URT (nasal cavity, nasopharynx, larynx, trachea, and bronchi) was visually assessed for vascularization, mucosa integrity and type, quantity, and viscosity of the secretions (Figure 5.1B and C). An endoscopic score (ES) from 0 to 3 was assigned for each characteristic as follows: a. pattern of vascularization and the color of the mucosa (0: normal, pink color, 1: mildly edematous and/or congested, 2: moderately edematous and/or congested, reddish, 3: severely edematous or congested, red); b. mucosa integrity (0: lack of lymphoid hyperplasia or ulcers, 1: mild lymphoid hyperplasia, 2: moderate lymphoid hyperplasia or ulceration 3: severe lymphoid hyperplasia, ulceration or necrosis); and c. type and abundance of secretions (0:serous secretions, 1:scarce mucous secretion, 2:abundant mucous or scarce muco-purulent secretion, 3: purulent secretion). Images and videos of each endoscopic assessment were recorded for further evaluation. The endoscopic procedures as well as the evaluations were done independently by three evaluators who were blinded to the treatment group assignment. The median of the three assessment scores was calculated for each characteristic and URT segment. An overall ES was calculated for each calf by adding all the individual scores for each characteristic in all the segments of the URT, with a possible maximum score of forty-five.

Sample collection and processing

Blood samples were collected from each calf on days shown that are relative to the BVDV2 challenge. On days, -7, 0 (day of BVDV2 challenge), 3, 6, 7 (day of BHV1 challenge), 10, 12 and 14, blood was collected using jugular venipuncture with a 20 gauge×1-inch single sample needle (Vacuette®; Nipro Medical Industries Ltd., Gunma) into vacuum tubes

(Vacutainer®, BD Diagnosis, Franklin Lakes, NJ) with and without anticoagulant to obtain whole blood and serum, respectively.

Blood samples were collected into 2 mL tubes for leukocyte and platelet counts (BD Vacutainer K2 EDTA 3.6 mg REF367841®; BD Diagnosis, Franklin Lakes, NJ) on days, -7, 0, 3, 6, 7, 10, 12 and 14 Samples were collected into four individual 8.5 mL glass tubes for buffy coat separation (BD Vacutainer ACD Solution A REF364606®; BD Diagnosis, Franklin Lakes, NJ) on days 0, 7, and 14. Blood was collected into two individual 10 mL tubes with no additives (BD Vacutainer Serum®; BD Diagnosis, Franklin Lakes, NJ) for serum neutralizing antibody (SNA) titers on days 0 and 14 after BVDV challenge (data not shown herein). Blood samples were transported on ice to the laboratory within two hours following collection. Peripheral blood leukocytes (buffy coats) were isolated from blood samples with anticoagulant as previously described (Harpin et al., 1999) and stored at -80 °C for further BVDV2 and BHV1 *q*RT-PCR analysis.

Nasal swab samples were collected from each calf for detection of BVDV2 and BHV1 nucleic acids on days 0, 7 and 14 using a multiplex *q*RT-PCR. An individual cotton swab was introduced in each nostril, gently scraping the nasopharyngeal mucosa. The swabs were then placed in individual tubes containing 3 mL of PBS with penicillin-streptomycin and amphotericin, and then transported to the laboratory for processing. The samples were vigorously mixed, the swabs were removed, and the solution was filtered and stored at −80 °C for succeeding *q*RT-PCR.

Hepatic trace mineral levels

Trace mineral status of each calf was determined in liver biopsy samples on days -5, and 21 relative to the day of booster vaccination and trace mineral injection. Mineral content in liver samples was determined at the Diagnostic Center for Population and Animal Health at Michigan State University, Lansing, MI. Liver biopsies (approximately 10 mg of liver tissue) were collected using sterile surgical semi-automatic biopsy needles (16 g and four inches long; BIOPSYBELL S.R.L.; Mirandola, MO Italy). Transcostal ultrasound guidance (with a probe of 5-8 MHz; Evo Ibex®Lite, E.I. Imaging, Loveland, CO) was used to verify the location of the liver. The right caudodorsal thoracic area was clipped and scrubbed using an iodophor detergent followed by an anti-infective agent prior to the biopsy procedure. Local anesthetic (2 mL Lidocaine injectable; Aspen Veterinary Resources, Ltd. Liberty, MO) was used to desensitize skin and underlying body wall approximately 15 cm from the dorsal midline, in the 10^{th} intercostal space. After scrubbing the skin, a small incision (1 cm) was made in the skin parallel to the ribs to admit the biopsy needle. The entire procedure required less than 10 minutes. Calves were able to walk immediately after the biopsy. Calves were visually observed for the following 2 hours to evaluate their general health status and to detect any sign of discomfort. Meloxicam (1mg/kg; 7.5mg/ tablet) was administered orally (16 tablets) 12-18 hours before liver biopsy and a second dose immediately before the biopsy.

Hematological assessment

Blood samples containing EDTA were transported in a cooler with ice for determination of total white blood cell, lymphocyte, granulocyte, monocyte, and platelet counts using an automatic cell counter (HESKA5® CBC-Diff, Vet Hematology System, Des Moines, IA). Blood

samples were kept stirring for 10 min at room temperature on an orbital shaker before analysis. Only the results of total leukocyte and lymphocyte counts are reported in this manuscript as a measure of vaccine protection.

BVDV2 & BHV1 qRT-PCR nucleic acid detection

Buffy coat and nasal swab samples that had been stored at −80 °C were thawed at room temperature to be used for *q*RT-PCR. Detection of BVDV2 and BHV1 nucleic acids in peripheral blood leukocytes using *q*RT-PCR was performed at the Athens Veterinary Diagnostic Laboratory, University of Georgia, Athens GA. Total RNA was extracted from buffy coats using RNeasy Mini Kit (QIAGEN, Germantown, MD) and a BVDV1 and 2 multiplex *q*RT-PCR was performed as previously described (Letellier and Kerkhofs, 2003). For amplification of the conserved 5'untranslated region (UTR) of BVDV1 and 2, the primer pair F2: 5' CTCGAGATGCCATGTGGAC 3' and PESTR: 5' CTCCATGTGCCATGTACAGCA 3' were used. Two probes differing by three nucleotides were utilized: the 5' FAM CAGCCTGATAGGGTGCTGCAGAGGC TAMRA 3' probe was specific for BVDV1, whereas the 5' VIC CACAGCCTGATAGGGTGTAGCAGAGACCTG TAMRA 3' was used for the detection of BVDV2. For detection of BHV1 (gC sequence) in buffy coat samples the primers F: 5' ATGTTAGCGCTCTGGAACC 3', and R: 5' CTTTACGGTCGACGACTCC 3', and the probe: 5' Cy ACGGACGTGCGCGAAAAGA BHQ2 3' were used (Lovato et al., 2003). The *q*RT –PCR protocol included 10 minutes at 95 ˚C, 45 cycles of 15 seconds at 95 ˚C and 1 second at 60 ˚C. Samples with a *q*RT-PCR result equal or less than the threshold cycle (Ct) of 35 were considered positive for the presence of BVDV2 or BHV1 nucleic acids. Only results of BVDV2 detection are reported in the present manuscript.

Bovine viral diarrhea virus and BHV1 nucleic acid detection in nasal swab specimens was performed at the Animal Disease Research and Diagnostic Laboratory, South Dakota State University. Total RNA/DNA were extracted from buffy coats and nasal swab sample solutions utilizing Ambion MagMax™ 1836-A viral RNA/DNA isolation kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The Multiplex *q*RT-PCR was performed to detect the conserved 5'UTR for BVDV viral genome, using the primers pair F: 5'-GGG NAG TCG TCA RTG GTT CG -3' and R: 5'-GTG CCA TGT ACA GCA GAG WTT TT-3' and the probe 5'-FAM-CCA YGT GGA CGA GGG CAY GC-BHQ1-3'. In addition, the BHV-1 PCR was performed using primers and probe specific for the gC region (F: 5'-GGC ACT GTG ACC CTC GTG TT-3'; R: 5'-TTG ATC TCG CGG AGG CAG TA-3 and the probe 5'-TAMRA-CCG CGT GCC TCT GCT ACC CCT TC-3' – BHQ2. The PATH-ID MPX One Step kit master mix (Thermo Fisher Scientific, Waltham, MA, USA) was used as one step multiplex RT-PCR. All reactions were conducted in a real time thermocycler (ABI 7500, Thermo Fisher Scientific, Waltham, MA, USA) following the cycling parameters: 50 °C for 10 minutes and 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds.

Statistical analysis

The data were analyzed using the Statistical Analysis System (SAS version 9.3®; Cary, NC, USA) and Stata® (version 16®, College Station, TX, USA). Normality and constant variance assumptions were assessed by using Shapiro Wilk's and Levene's tests, respectively. Mean health scoring at the chute, ADG, hepatic trace mineral concentrations, and total leukocyte and lymphocyte counts were compared among treatment groups by using a repeated measures analysis model (Proc-GLIMMIX of SAS®), using calf ID as a random effect, and the interaction

of treatment group and time as fixed effects. Summed endoscopic scores were compared between treatment groups and times using a general linear model with fixed effects for group, time, and the group x time interaction. Post hoc tests were performed via the Tukey procedure to limit the type I error rate to 5% for all comparisons. Qualitative PCR results for BVDV-2 and BHV-1 on buffy coats were analyzed using a frequency procedure and compared by Chi-square test. The results were considered significant when $p \leq 0.05$, and a statistical tendency was considered when $0.05 < p \leq 0.10$.

Results

All groups had comparable ADG and summed health scores before BVDV2 challenge. The sum of the health score significantly increased on day 7 in both the ITM-IN and SAL-IN calves, and on days 10 and 12 in all experimental groups relative to the baseline values before BVDV2 challenge. In the unvaccinated calves, this increase in health scores started on day 6 (Figure 5.2B). The sum health score was significantly greater for the unvaccinated calves on day 10 after BVDV2 challenge compared to all vaccinated groups. There were no remarkable clinical differences regarding the summed health scores among the four vaccinated groups after challenge. Average daily gain was significantly reduced on day 14 after serial BVDV2 -BHV1 challenge in the unvaccinated calves compared to the vaccinated groups ($p < 0.001$, Figure 5.2A). Neither the route of booster vaccination nor administration of ITM affected the ADG.

There was a significant interaction between the effects of treatment and time ($p = 0.020$) with respect to URT ES. This indicated that the effect of treatment was dependent on the time after treatment (Figure 5.3). The URT ES was uniformly low and not found to be different among the experimental groups before BVDV2 challenge ($p = 0.82$, Figure 5.3A.1). Most of the calves

had scarce serous or sero-mucous nasal and nasopharyngeal secretions, with pink, smooth and highly vascularized mucosa, at the beginning of this trial (Figure 5.4). The intranasal challenge with BVDV2 strain 890 did not cause significant alteration of the URT mucosa (Figure 5.4) or increased ES in any group on day 6 after BVDV2 challenge relative to the baseline values ($p > 0.4$, Figure 5.3A.2). No significant differences were observed in the ES scores on day 6 after BVDV2 challenge among experimental groups ($p = 0.63$, Figure 5.3A.2). Endoscopic evaluation of the subsets of calves examined revealed a remarkable increase in the URT ES 5 days after BHV1 challenge in all experimental groups relative to the ES before BVDV-2 challenge ($p < 0.05$, Figure 3A.3). Unvaccinated calves had the highest URT ES (median: 27). They showed marked mucosal congestion, severe lymphoid hyperplasia and ulcers of the pharynx and larynx with abundant purulent secretions along the URT. This was compatible with moderate-severe rhinitis, pharyngitis, laryngitis, tracheitis, and bronchitis (Figures 5.3A.3 and 5.6). Calves treated with ITM at the time of vaccination by either route of administration (IN or SC) had significantly lower ($p < 0.01$) URT ES compared with the UNVAC group. The vaccinates had milder inflammation and damage of the URT mucosa (Figure 5.3A.3). In addition, calves in the SAL-SC group had lower URT ES than the unvaccinated calves ($p < 0.05$). Endoscopic patterns of mild URT inflammation were frequently observed in the ITM-IN, ITM-SC and SAL-SC calves and are demonstrated in Figure 5.5. The group of calves that received an IN MLV booster with saline solution (SAL-IN) had a greater ($p < 0.05$) URT ES than the ITM-IN and ITM-SC groups. The values were not significantly different from those observed in the unvaccinated calves (Figure 5.3A.3). These two groups (UNVAC and SAL-IN) showed moderate-severe inflammation of the URT mucosa 5 days after BHV1 challenge.

There was a significant decay in the hepatic concentrations of Se 21 days post booster vaccination in the vaccinated, saline-treated calves and the unvaccinated group, compared to the baseline concentrations (5 days before booster vaccination) for each group ($p < 0.01$, Figure 5.7A). In addition, Cu concentrations in the liver decreased in both of those groups, but a significant reduction was only observed for the SAL-IN group ($p < 0.01$, Figure 5.7B). Administration of injectable trace minerals (ITM-IN and ITM-SC groups) resulted in significantly higher hepatic concentrations of Se on day 21 post-booster with trace mineral injection compared to the values measured 5 days before booster ($p < 0.05$; Figure 7A). In these groups, hepatic Se concentrations following booster vaccination were significantly greater ($p < 0.0001$) than those observed in the vaccinated, but saline-treated (SAL-IN and SAL-SC) or unvaccinated groups. In addition, calves treated with ITM showed a slight increase, that was not statistically significant, in liver copper concentrations on day 21 after booster with trace mineral administration relative to the values determined before vaccination. Hepatic Zn concentrations declined in all groups after booster vaccination (Figure 5.7C). However, this reduction was less pronounced in the ITM-treated calves than the saline-treated calves (16 versus 23% reduction in ITM-SC and SAL-SC, respectively and 23 versus 32% reduction ITM-IN and SAL-IN, respectively). Manganese concentrations in liver increased in all groups on day 21 after booster vaccination (Figure 7D). Significant differences were not observed in the hepatic concentrations of Zn, Cu and Mn at any time point among groups (Figure 5.7).

Mean total circulating leukocyte and lymphocyte counts significantly declined between days 3 and 7 following BVDV challenge in the unvaccinated group (Figures 5.8A and B) These values were significantly lower than values observed among the vaccinated groups ($p < 0.05$). In contrast, total and differential leukocyte counts were not altered after the serial BVDV2 and

BHV1 challenges in calves receiving any route or mineral relative to MLV vaccination. No significant difference was observed among the vaccinated groups.

All calves in this study were BVDV2- and BHV1-negative (via *q*RT-PCR) for buffy coat assessment (Table 5.1) and by testing nasal swab samples (Table 5.2) on day 0 before challenge and commingling. *Bovine viral diarrhea virus 2* was detected by *q*RT-PCR in 10 of 12 buffy coat samples and 12 of 12 nasal swab specimens collected from the unvaccinated calves seven days after BVDV2 challenge (Tables 5.1 and 5.2). In contrast, all vaccinated calves (except for one calf in the ITM-SC group) had BVDV-negative results for buffy coat assessment and testing nasal swab samples collected seven days following BVDV2 challenge. *Bovine herpes virus 1* nucleic acids were detected in nasal swab samples in some calves (one calf in SAL-IN, one calf in SAL-SC and seven calves in UNVAC) on day 7 (before the BHV1 challenge). This virus was most likely of vaccine origin (Table 5.2). Further, BHV1 was detected in nasal swab samples of all animals tested on day 14 (following BHV1 challenge). In contrast, BHV1 was not detected in buffy coat specimens from any of the calves following BHV1 challenge.

Discussion

Modified -live vaccination against bovine respiratory viruses protected the calves from most indicators of disease following serial BVDV2 and BHV1 challenge. This was documented by lower summed clinical scores and URT endoscopic scores, a general absence of leukopenia, reduced BVDV viremia and shedding from the vaccinated calves as compared to the unvaccinated calves. In this study, calves receiving booster vaccination by either the IN or SC route two months after primary IN vaccination concurrently with ITM or saline induced comparable clinical protection from disease.

Endoscopic evaluation of the URT of the calves showed: 1. differences in the appearance of the respiratory mucosa before and after the serial challenge with BVDV2 + BHV1; 2. a clear contrast between vaccinated and unvaccinated calves in the URT damage observed, and 3. demonstrated positive effects of delivery of trace minerals concurrent with IN vaccination on the protection of the URT mucosa. This is the first study assessing the use of endoscopy as an ancillary tool to evaluate the damage caused by BVDV2 + BHV1 co-infection on the disease characteristics in the upper respiratory tract mucosa directly. Endoscopy was used to assess the clinical protection provided by vaccination concurrent with trace mineral injection in a more detailed way than previously possible. Experimental challenge with BHV1 seven days after BVDV2 challenge resulted in the development of high URT ES, characterized by moderate to severe rhinitis, laryngitis and tracheitis in the unvaccinated calves. One of these calves did not survive the dual infection, having severe pharyngeal and laryngeal lymphoid hyperplasia, ulceration of the pharynx and larynx, abundant purulent secretions along the URT, extending to the trachea and bronchi. Further, calves in this group also had significantly increased summed health scores, ongoing BVDV viremia and lasting BVDV/BHV1 shedding, which confirmed the disease pathogenesis of the challenge model by all measures taken. A previous study to develop and characterize experimental infection protocols for BRDC pathogens, described similar lesions in calves experimentally infected with BHV1 (Gershwin et al., 2015). In that study, ulcerative rhinitis, laryngitis, and tracheitis were observed on day 6 post BHV1 challenge. In addition, further histopathological evaluation demonstrated lympho-plasmocytic bronchitis, bronchiolitis, alveolitis, and disseminated bronchopneumonia (Gershwin et al., 2015).

In general, three of the vaccinated groups (ITM-IN, ITM-SC, and SAL-SC) had less URT mucosal inflammation and damage (via endoscopy) after serial BVDV2 + BHV1 challenge than

the non-vaccinated calves. Multiple studies have documented the efficacy of parenteral or intranasal MLV vaccination preventing classical clinical signs of disease and documenting mucosal damage associated with experimental BVDV or BHV1 challenge post-mortem (Chamorro and Palomares, 2020). Calves that received SC booster vaccination concurrent with ITM, or with saline, had significantly lower ES (associated with mild inflammation) five days after BHV1 challenge than the unvaccinated calves. In contrast, calves receiving an IN-booster vaccine with saline had higher ES (five days after BHV1 challenge) compared with the other vaccinated group. This IN-SAL group had similar ES to the unvaccinated calves. It is possible that immunization of young dairy calves with the homologous vaccine IN priming - IN booster vaccination protocol in the present study may have induced an less optimal anamnestic immune response that resulted in more prominent signs of local inflammation (congestion, edema, lymphoid hyperplasia and muco-purulent or purulent respiratory secretions) after BHV1 inoculation, compared with the calves that received the heterologous vaccines in an IN priming - SC booster vaccination protocol.

Bovine herpesvirus 1 replicates primarily in mucosal epithelium, and induction of local mucosal immune response contributes with virus clearance from the URT soon after infection or vaccination (Todd et al., 1972, MacLachlan and Rosenquist, 1982, van Drunen Littel-van den Hurk et al., 1994). In addition, activation of innate immune cells at the local mucosal tissue results in apoptosis of the infected host cells and activation of the adaptive cell-mediated immunity. Further, upon recurring viral exposure, antigen recognition and amplified activation of T lymphocytes would contribute to the recall of an adequate anamnestic immune response to promote elimination of virus infected cells (Martinon et al., 2009, Mahan et al., 2016).

The observed high URT ES five days after BHV1 challenge in the SAL-IN group was not associated with impaired health status, increased levels of viremia or virus shedding during the experimental period. In this trial, the animals were not evaluated beyond seven days post BHV1 challenge. Therefore, the clinical implication of the observed high ES in this group on the impending health status of the calf is uncertain and deserves further investigation. Several studies have demonstrated that IN MLV vaccination is a safe and effective way to induce an immune response and confer protection against disease in dairy calves (Kimman et al., 1989, Vangeel et al., 2009, Xue et al., 2010) . A previous study showed that an IN- booster vaccination 35 days after IN priming resulted in an enhanced memory immune response characterized by high IgA production in nasal secretions (Hill et al., 2012; Mahan et al., 2016). These studies assessed the effectiveness of an IN vaccine containing BHV1, BRSV and BPI3V on protection from BHV1 challenge. In those studies calves were administered an IN vaccine containing BHV1 or without BHV1. BHV1-vaccinated calves had less severe clinical signs, lower BHV1 shedding and higher antibody production than the calves that received the BHV1 free vaccine. That trial demonstrated that administration of an IN vaccine elicited a significant systemic anamnestic immune response and provided protection against clinical BHV1-induced disease.

Treatment with ITM was associated with a significantly lower ES after serial BVDV2 + BHV1 challenge in calves that received the IN-booster vaccination compared with intranasally vaccinated, saline-treated calves or the unvaccinated group. Supplementation with trace minerals resulted in improved Se and Cu liver status and helped mitigating the observed reduction in Zn concentrations of the saline treated calves. Further, trace mineral injection was associated with reducing the mucosal inflammation following BHV1 challenge in the ITM-IN group. Selenium, manganese, copper and zinc have multiple functions in the immune system of cattle, particularly

as structural components of antioxidant enzymes (e.g., superoxide dismutase and glutathione peroxidase) that scavenge the excess of reactive oxygen species resulting from oxidative stress (Rotruck et al., 1973, Sordillo, 2013, Teixeira et al., 2014). The increased levels of these free radicals during an oxidative imbalance induced by injury or disease can cause significant damage to plasma membranes, cytoplasmic organelle membranes and protein arrays, and nucleic acids in many types of cells, including leukocytes, that may result in immunosuppression (Sordillo and Aitken, 2009). Moreover, zinc is an integral component of enzymes involved in nucleic acid replication, transcription, and translation (e.g., polymerases, transcriptases, ribonucleotide reductases, among other). Therefore, zinc is essential for the structure and function of tissues that require active cell mitosis (e.g., skin, respiratory and gastrointestinal epithelium, testicular seminiferous tubules, secondary lymphoid structures, and foci). Supplementation with these microelements (particularly Zn, Cu, and Se) has been associated with strengthening of the respiratory epithelium integrity, improvement of the muco-ciliary clearance (Sartori et al., 2016, Doleski et al., 2017a, Doleski et al., 2017b), enhancement of antiviral immunity by decreasing viral replication, and attenuation of excessive inflammatory response, which may reduce tissue damage and secondary bacterial infections (Wessels et al., 2020). Further, in cattle, the administration of ITM has been documented to enhance humoral and cell-mediated immune responses and protection after parenteral vaccination against virus and bacteria that cause BRD (Arthington and Havenga, 2012, Palomares et al., 2016, Bittar et al., 2018a, Bittar et al., 2018b, Bittar et al., 2020). In addition, assessment of circulating T lymphocyte populations in the calves utilized in this trial (data not shown herein) revealed that treatment with ITM at the time of IN booster vaccination resulted in greater proportion of circulating $CD4^+$ and $CD8^+$ T cells following BVDV challenge compared to the unvaccinated calves and calves vaccinated IN with

saline, respectively (Hoyos-Jaramillo et al., 2018). This finding supports the results of previous studies showing positive effects of trace minerals administration on the cell-mediated immune response of vaccinated calves (Palomares et al., 2016, Bittar et al., 2020). It is possible that trace mineral supplementation in the calves given IN vaccine in the present study may have contributed to healing of the protection of the respiratory epithelium after serial BVDV2 + BHV1 challenge. This is in addition to the previously reported enhanced circulating T lymphocyte populations (Hoyos-Jaramillo et al., 2018, Bittar et al., 2020) . These collective actions may explain the reduced damage to the URT mucosa and the lower endoscopic scores after BHV1 challenge in the calves treated with ITM concurrent with IN MLV booster vaccination.

To the authors' knowledge there are no previously published studies about the use of endoscopy to evaluate the pathology of BVDV2 and BHV1 co-infections on the respiratory tract mucosa. Anderson et al. (1994) performed endoscopic evaluations of the respiratory tract of cattle to describe the anatomy and normal appearance of the nasopharynx, pharynx, and larynx of adult dairy cows. In that clinical study pharyngeal lymphoid hyperplasia was identified in 32 % of the cows evaluated (Anderson et al., 1994), This finding was associated with exposure and the resulting local immune response to respiratory pathogens (Casteleyn et al., 2008). Other reports concerning clinical endoscopy of cattle have shown other respiratory obstructive pathological conditions (Cohen et al., 1991, Mattoon et al., 1991, Gamboa and Shoemaker, 1992). However, they have indicated that morphological abnormalities of the bovine respiratory tract are unusual (Anderson et al., 1994). As technology develops, endoscopy may become a valuable diagnostic tool to detect infection/inflammation of the upper respiratory tract (when respiratory signs start) before bronchitis or bronchopneumonia may develop in the field. The barriers of cost, time and

how to improve the ease of use in the field remain. This study was not intended for validation of endoscopy as an ancillary diagnostic procedure for clinical BRD. Further, a limitation of the present study was the small sample size of the individual treatment subsets of calves evaluated by endoscopy at each time point. This is further complicated by the fact that unequal numbers of cattle from each treatment group were evaluated. Thus, further studies for validation of endoscopy as a diagnostic tool for detection of URT inflammation in the pathogenesis of respiratory disease in cattle are warranted.

Conclusions

Vaccination, when administered SC or IN concurrent with ITM or saline proved to be effective in protecting calves from serial BVDV2 and BHV1 challenge compared unvaccinated calves. Endoscopic evaluation of the URT allowed visualization of the inflammation and damaged at multiple depths in the URT caused by a serial BVDV2 + BHV1 challenge and demonstrate the protective effects of vaccination in preventing damage to the URT mucosa following the serial BVDV2 + BHV1 challenge. This study showed how abnormal the URT mucosa looks in calves co-infected with BVDV2 and BHV1 that have only mild clinical signs of respiratory disease. Calves that received SC booster vaccination (with ITM or saline) had significantly lower ES after BHV1 challenge than the unvaccinated calves. Administration of ITM concurrent with MLV vaccination enhanced the hepatic Se and Cu status, and was associated with lower ES, a lower level of inflammation and mucosa damage following serial BVDV2 + BHV1 inoculation in calves receiving IN vaccine compared with the IN vaccinated, saline-treated calves or the unvaccinated calves.

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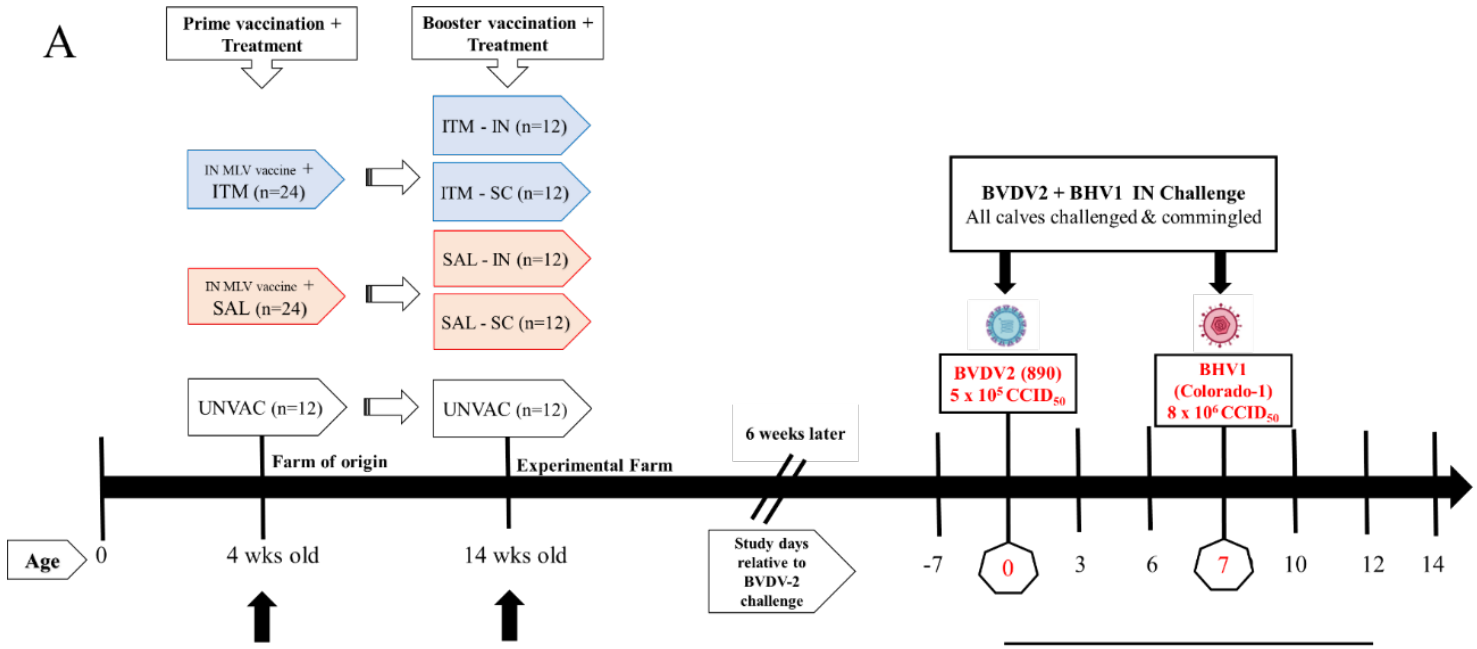

A
Prime vaccination + Treatment
Booster vaccination + Treatment
IN MLV vaccine + ITM (n=24)
IN MLV vaccine + SAL (n=24)
UNVAC (n=12)
ITM - IN (n=12)
ITM - SC (n=12)
SAL - IN (n=12)
SAL - SC (n=12)
UNVAC (n=12)
BVDV2 + BHV1 IN Challenge
All calves challenged & commingled
BVDV2 (890)
5 x 10^5 CCID50
BHV1
(Colorado-1)
8 x 10^6 CCID50
Farm of origin
Experimental Farm
6 weeks later
Age
0
4 wks old
14 wks old
Study days relative to BVDV-2 challenge
-7
0
3
6
7
10
12
14
Clinical status and health score for BRD
Prime Vaccination:
• Inforce3® (BHV1, BRSV, PI3) 2 ml via intranasal (IN)
• ITM: Multimin 90 ®: 1ml/100 lb., via subcutaneous (SC)
• Saline: 1 ml/100lb, SC
Booster Vaccination:
• Inforce3®, 2 ml IN + Bovi-shield Gold ® BVD (BVDV1 & 2), 2 ml SC
• Bovi-shield Gold ® 5 (BHV1, BRSV, PI3, and BVDV1 & 2), 2 ml SC
• ITM: Multimin 90 ®: 1ml/100 lb, SC
• Saline: 1 ml/100lb, SC

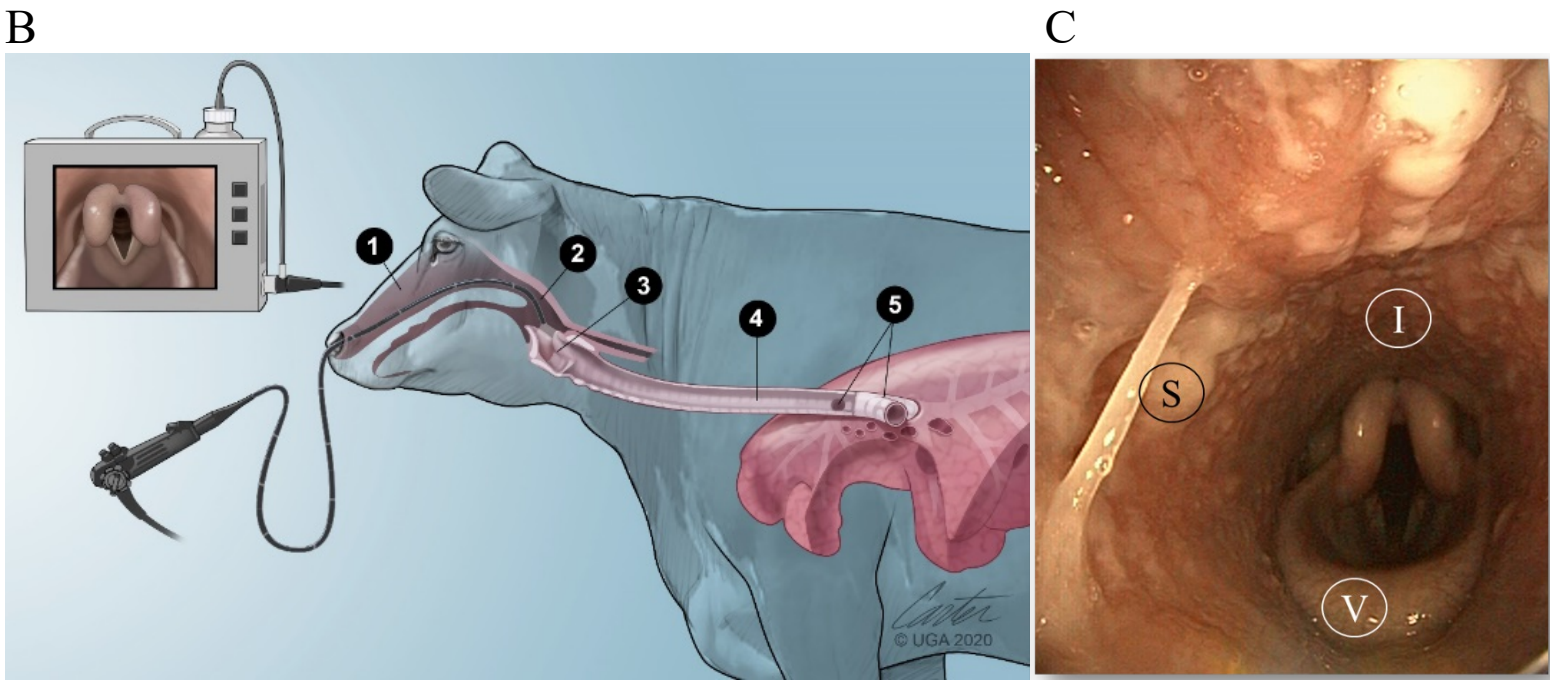
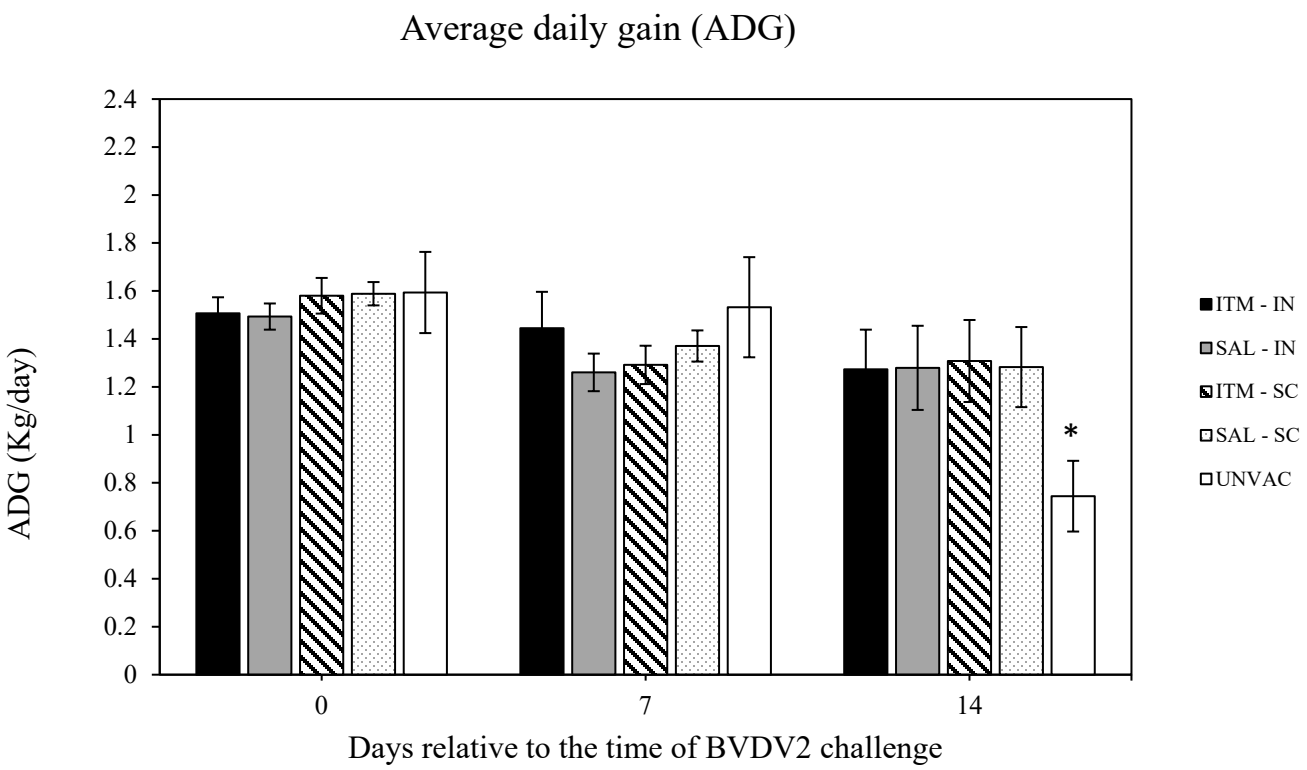


Figure 5.1. Experimental design and timeline (**A**)**.** At one month of age, calves were randomly assigned to receive IN MLV vaccination (Inforce3®) plus either injectable trace minerals (ITM, n=24; Multimin®90) or Saline (SAL, n=24). Unvaccinated group (UNVAC, n=12) did not receive vaccine nor treatment. Before booster MLV virus vaccine, all calves were transported to the experimental farm (Riverbend farm, UGA facility). At the time of booster vaccination, each vaccinated-treated group received its second treatment dose of ITM or saline, furthermore, each vaccinated-group was allotted into SC or IN vaccination route. All calves were serially intranasally inoculated with BVDV2 and BHV1 seven and eight weeks after booster vaccination, respectively. Endoscopy of the URT was performed on days -2 (two days pre BVDV2 challenge), 6 (six days post BVDV2 challenge) and 12 (5 days post BHV1 challenge) on separate

subsets of calves from each treatment. Endoscopic evaluation of the URT and scoring criteria (**B-C**). Five sections of the URT (**B**): Nasal cavity (1), pharynx (2), larynx (3), trachea (4), and bronchi (5) were evaluated for each characteristic (**C**): vascularization (V), mucosal integrity (I) and secretions (S), then given a score from 0 to 3, representing absence, mild, moderate, or severe lesions, respectively. Endoscope: Tele pack Vet X led-Karl Storz®. IN: Intranasal; SC: Subcutaneous; ITM: Injectable trace minerals; $CCID_{50}$: Cell culture infectious dose 50%. URT: Upper respiratory tract.

A


Average daily gain (ADG)
ADG (Kg/day)
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
7
14
*
Days relative to the time of BVDV2 challenge
ITM - IN
SAL - IN
ITM - SC
SAL - SC
UNVAC

B

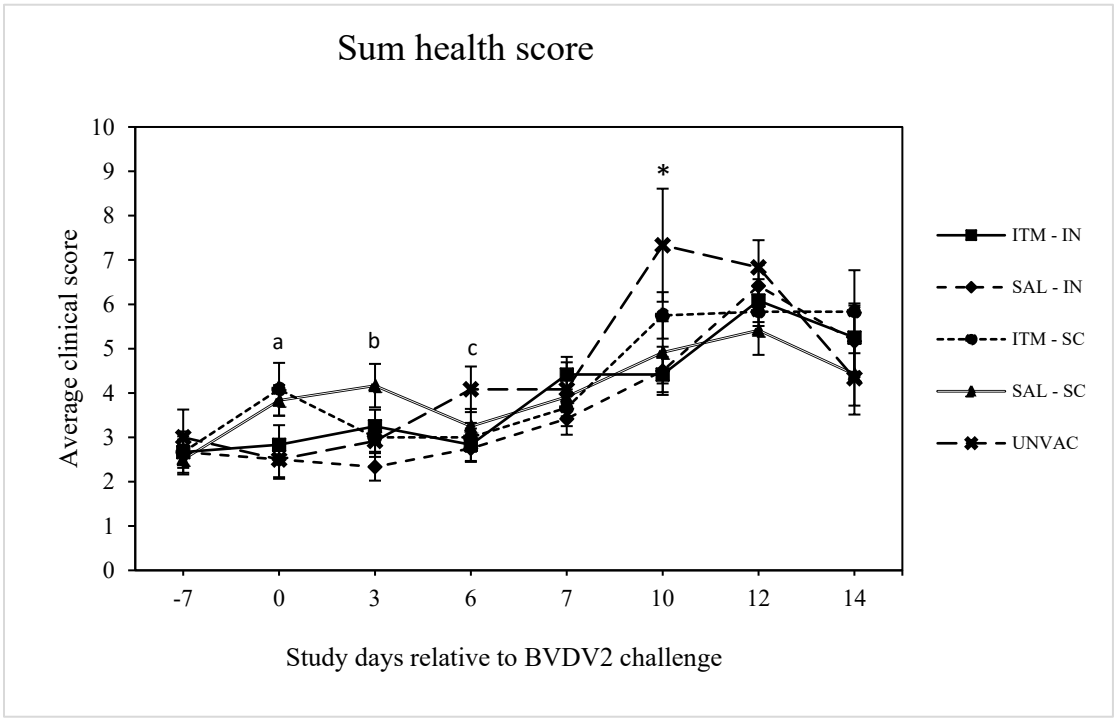

Sum health score
Average clinical score
10
9
8
7
6
5
4
3
2
1
0
a
b
c
*
-7
0
3
6
7
10
12
14
Study days relative to BVDV2 challenge
ITM - IN
SAL - IN
ITM - SC
SAL - SC
UNVAC

Figure 5.2. A: Mean average daily gain after BVDV2 + BHV1 challenge in dairy calves treated with ITM or not at the time of MLV vaccination. Average daily gain (ADG) was calculated at different study days (0, 7, and 14) relative to initial baseline weight on the day of booster vaccination (49 days before BVDV2 challenge). * ADG on day 14 was significantly lesser ($p < 0.001$) in UNVAC compared with the vaccinated groups. Significant differences were not observed among vaccinated groups. B: The sum of the health score in calves challenged with BVDV2 (day 0) and BHV1 (day 7) following MLV vaccination (IN or SC) concurrent with trace mineral (ITM) or saline (SAL) injections. Error bars represent the standard error of the means (SEM). [a] Values in ITM-SC and SAL-SC groups were significantly greater ($p < 0.05$) than those in UNVAC calves on day 0. [b] Sum health score in SAL-SC group was significantly greater ($p < 0.05$) than that in the UNVAC group on day 3. [c] The sum of the health scores was greater in the unvaccinated calves on day 6 after BVDV2 challenge compared to SAL-IN ($p =0.03$), and tended to be greater than ITM-IN ($p =0.06$), and ITM-SC ($p =0.08$). * The sum of the health scores was significantly greater ($p < 0.05$) on day 10 in the UNVAC group compared with all vaccinated groups.

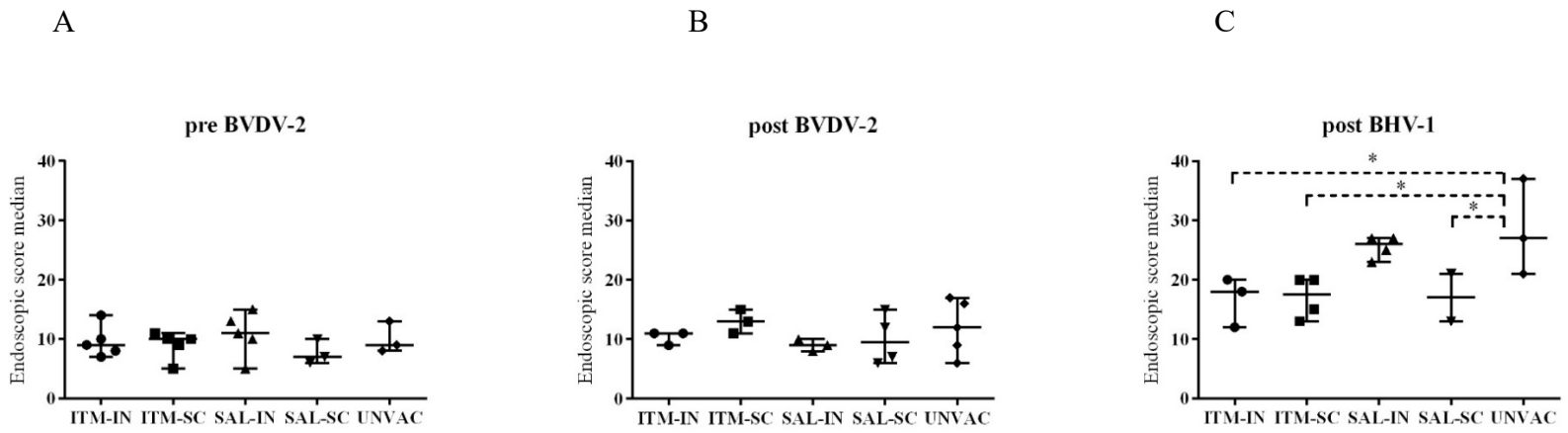


Figure 5.3. A: Endoscopic score (ES) of the URT for calves' subsets of each experimental group two days before BVDV2 challenge (A), six days post BVDV2 challenge (B) and five days post BHV1 challenge (C). An overall ES was calculated for each calf at each time point. The medians for each group are represented by the middle horizontal line of the dot plots. An increase in the URT ES was observed 5 days after BHV1 challenge in all experimental groups compared with the ES at the baseline evaluation ($p < 0.05$). * Values differ significantly from values in the UNVAC group ($p < 0.05$).

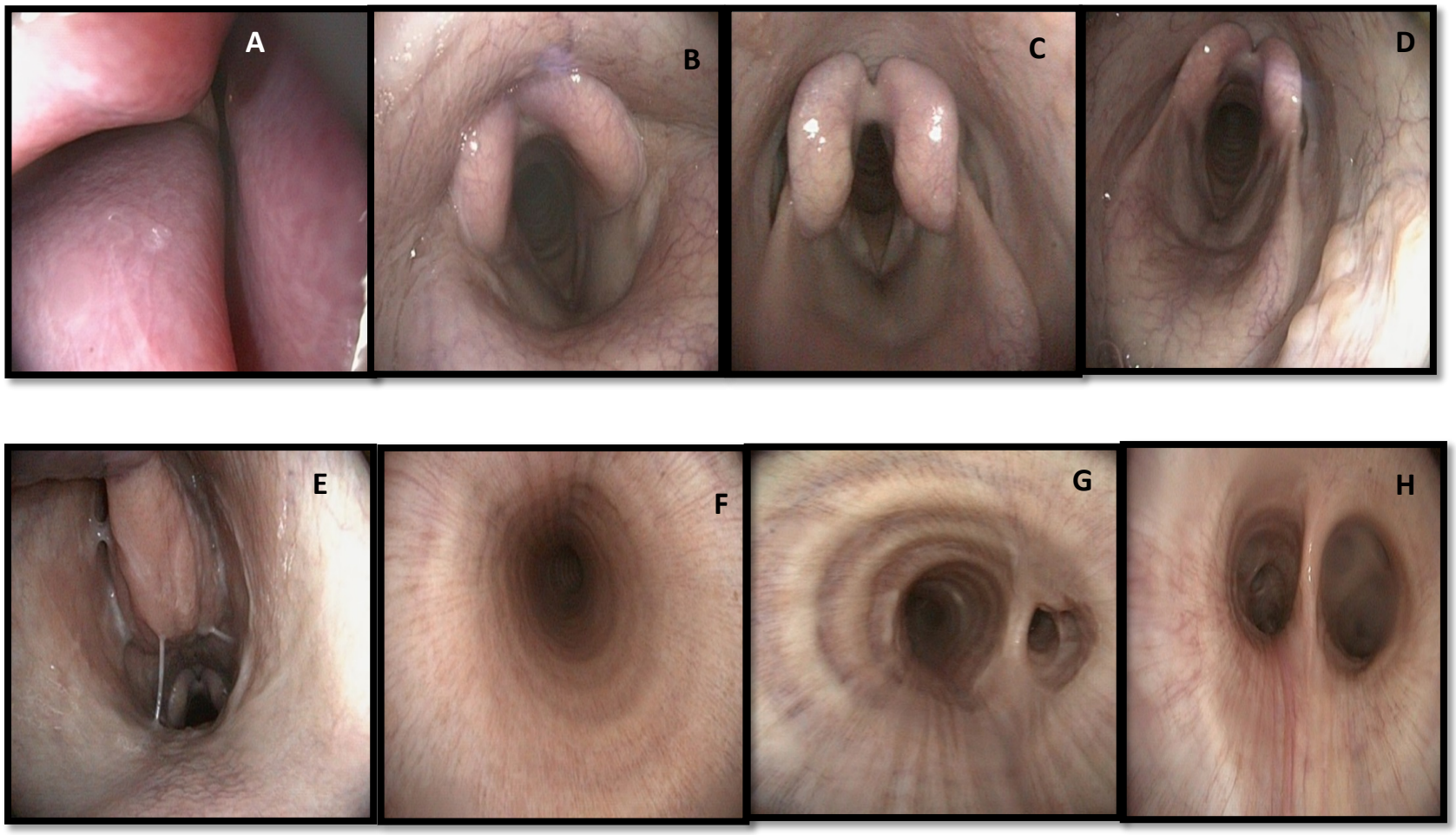


Figure 5.4. Endoscopic view of normal upper respiratory tract in dairy calves vaccinated and treated or not with injectable trace minerals (ITM) or unvaccinated calves before and after BVDV2 challenge. Nasal cavity (A) and naso-pharynx before (B) and after

(C) BVDV2 challenge. Notice the pink color of the vascularized mucosa, smooth mucosal lining, and normal sero-mucous secretions. Larynx before BVDV2 challenge (D). Laryngeal cartilages look non-edematous and normally pale-pink color. Pharynx (E), trachea (F) and bronchi (G, H) after BVDV2 challenge in MLV vaccinated calves. The endoscopic assessment of the URT appearance before and after BVDV challenge resulted in low endoscopic scores. BVDV challenge did not significantly affect the appearance of the respiratory mucosa. In most animals, mucosa color and integrity remained unaltered and clean without mucous secretions. There were no significant differences in the endoscopic scores among experimental groups before or after BVDV2 challenge.

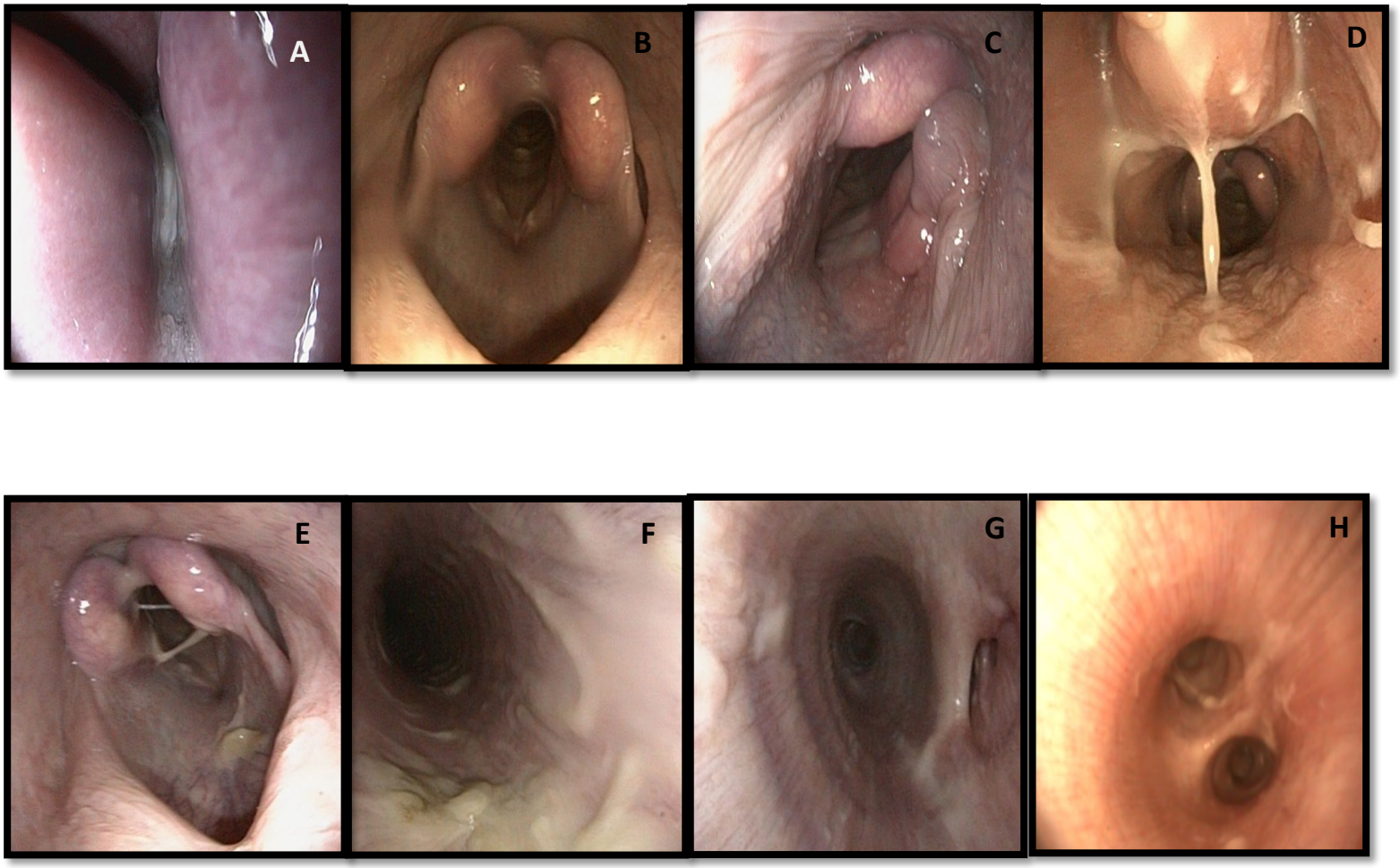

A
B
C
D
E
F
G
H

Figure 5.5. Endoscopic evaluation of the upper respiratory tract showing mild inflammation following BVDV2 and BHV1 challenge in dairy calves that received MLV vaccine and were treated with or without injectable trace minerals (ITM). Nasal cavity showing mild congestion and inflammation and scarce mucous or mucopurulent secretions (A). Naso-pharynx with mild congestion and edema of arytenoid cartilage (B), lymphoid hyperplasia (C) and mucous secretions (D). Larynx with mild edema of the arytenoid process and mucopurulent secretions after challenge with BHV1 (E). Trachea (F) and bronchi (G, H) of vaccinated calves after BHV1 challenge. The tracheal mucosa shows moderate congestion, reddish tracheal rings and scarce mucous or mucopurulent secretions. Bronchi also had scarce amounts of mucous secretions. In general, the appearance of the URT mucosa revealed higher endoscopic scores (ES between 15 and 22) compared to values before BHV1 challenge (ES around 10). Vaccination prevented pathogenic effects of BHV1. These endoscopic pattern of mild URT inflammation were frequently observed in the ITM-IN, ITM-SC and SAL-SC calves.

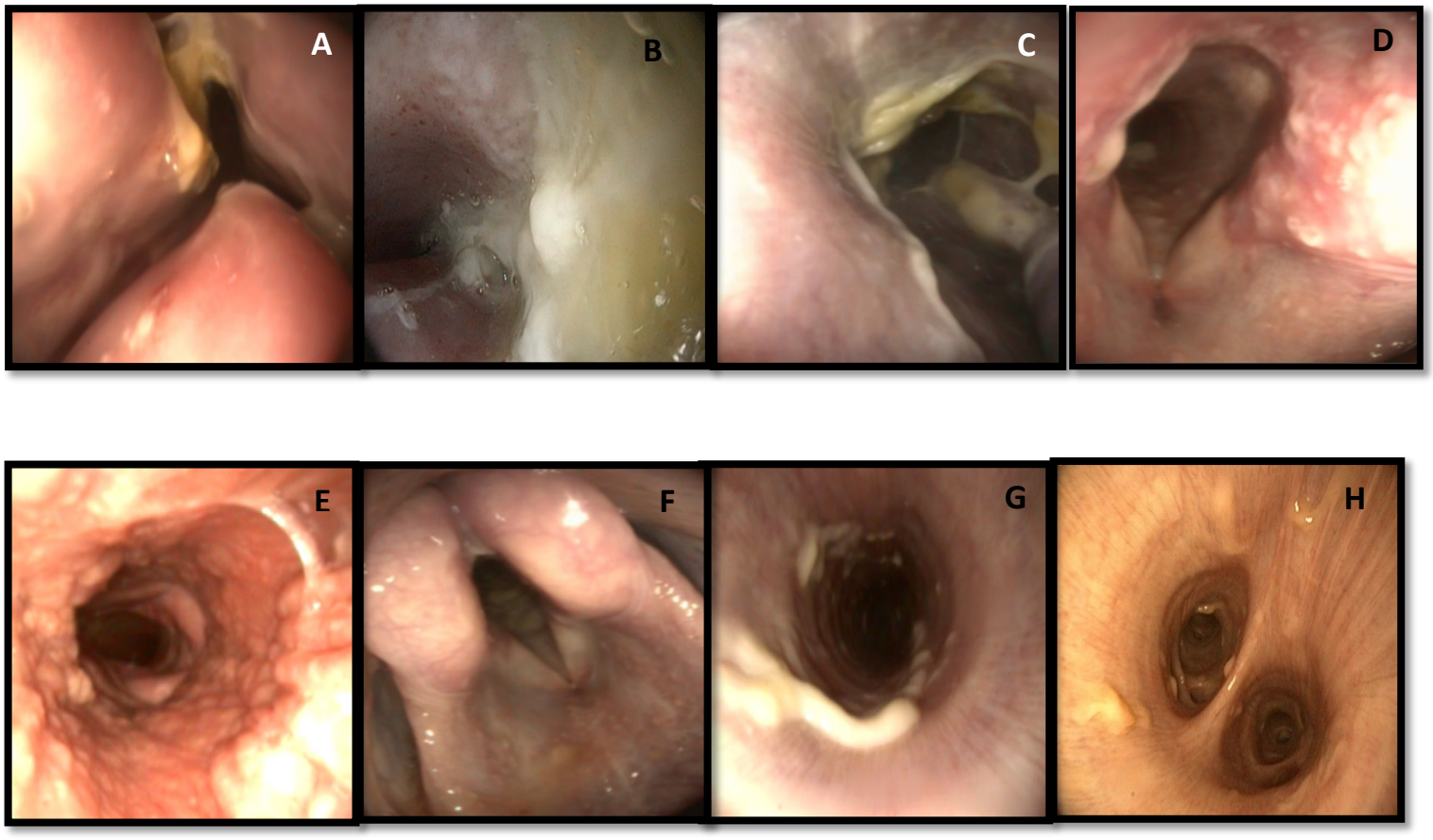
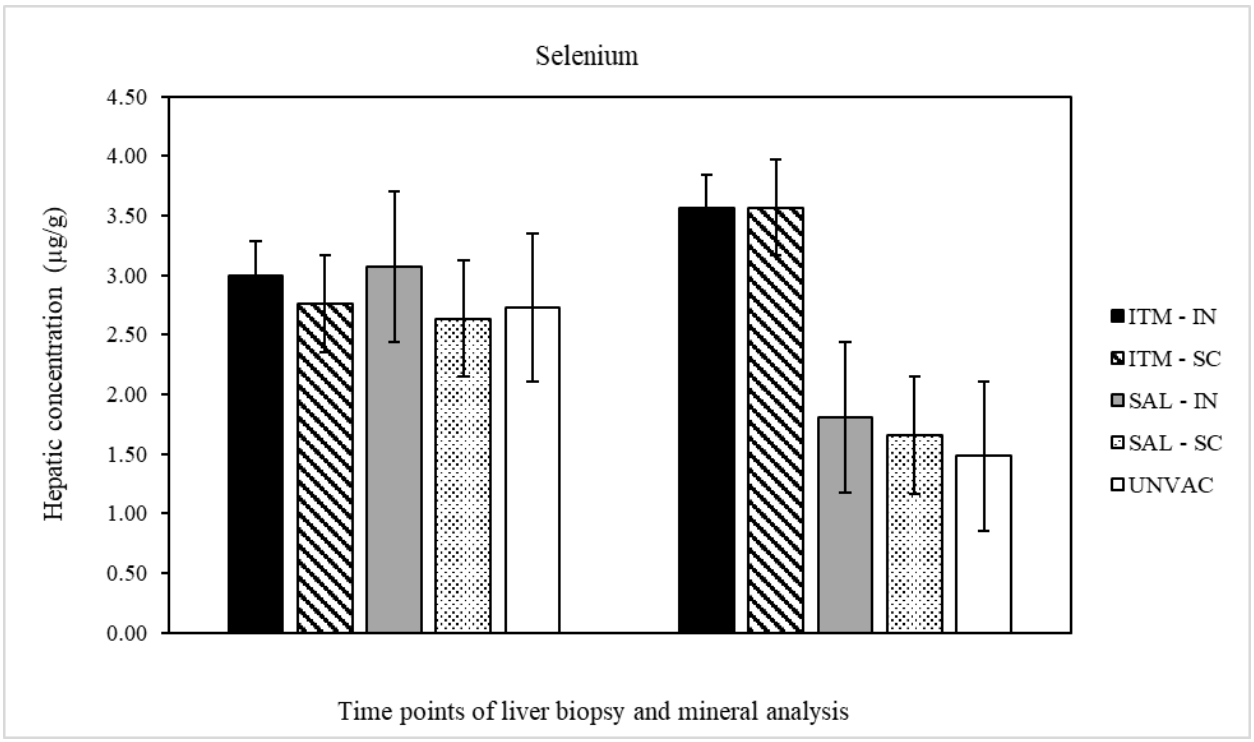
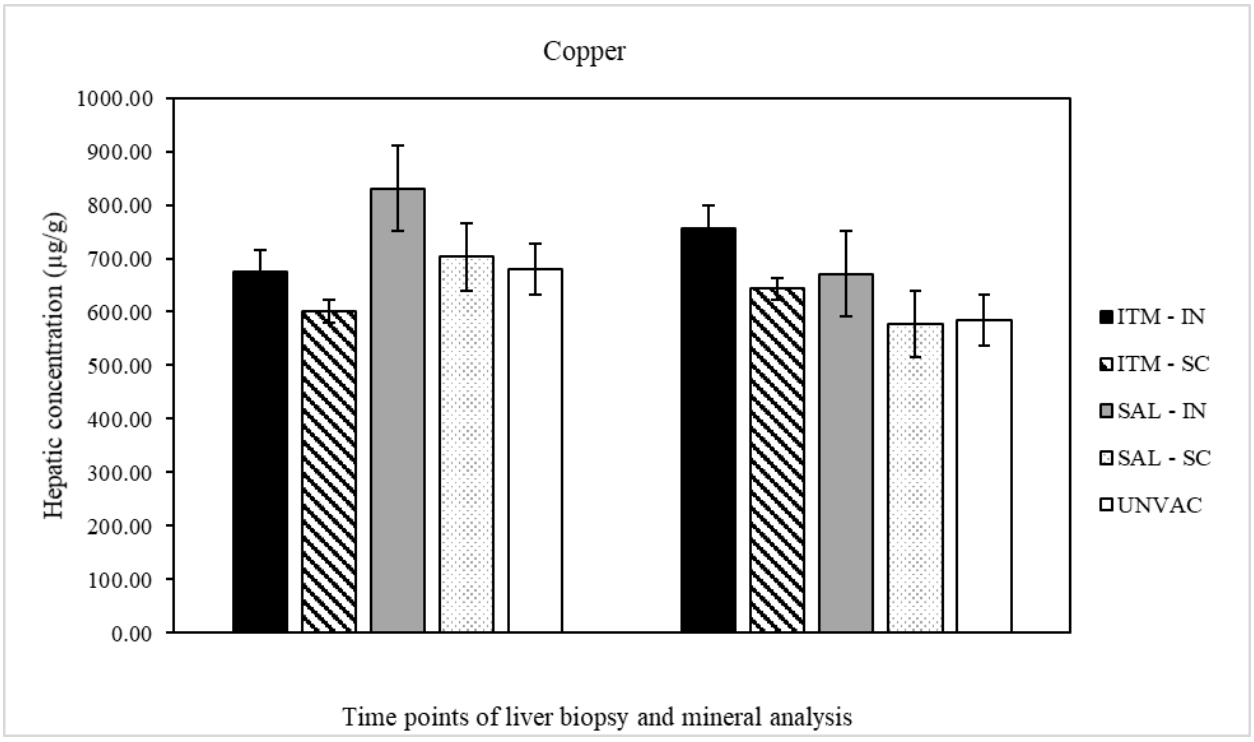
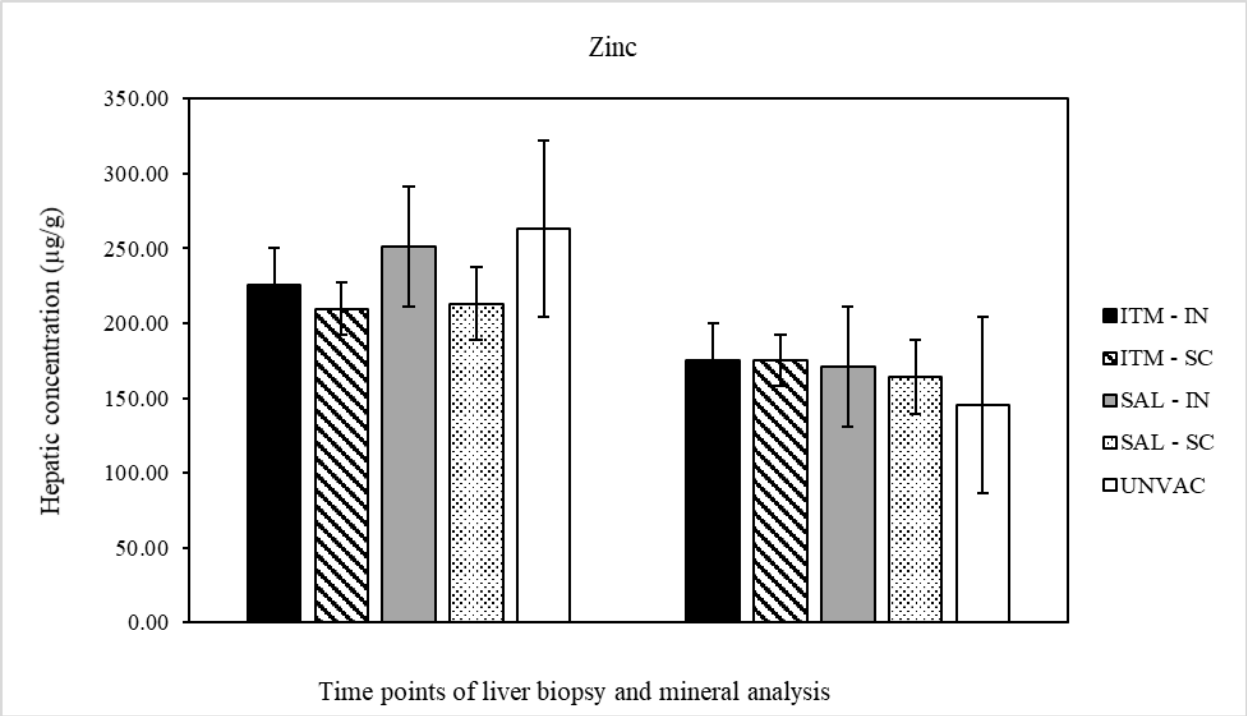

A
B
C
D
E
F
G
H

Figure 5.6. Endoscopic view of calves serially challenged with BVDV2 and BHV1. The calves shown moderate to severe rhino-tracheitis. Severe rhinitis showing abundant purulent secretions with severe congestion, inflammation, and ulcers (A, B). Severe pharyngitis with lymphoid hyperplasia and ulcers (C-E). Severe edema of the larynx with lymphoid hyperplasia and ulcers (F). Purulent secretions in trachea (G) and bronchi (H). These lesions were frequently observed in unvaccinated, untreated calves 5 days after BHV1 challenge. Calves receiving IN vaccination and saline injection also had moderate inflammation of the URT after BHV1 challenge.


Selenium
Hepatic concentration (µg/g)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
ITM - IN
ITM - SC
SAL - IN
SAL - SC
UNVAC
Time points of liver biopsy and mineral analysis


Copper
Hepatic concentration (µg/g)
1000.00
900.00
800.00
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
ITM - IN
ITM - SC
SAL - IN
SAL - SC
UNVAC
Time points of liver biopsy and mineral analysis


Zinc
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
Hepatic concentration (µg/g)
ITM - IN
ITM - SC
SAL - IN
SAL - SC
UNVAC
Time points of liver biopsy and mineral analysis

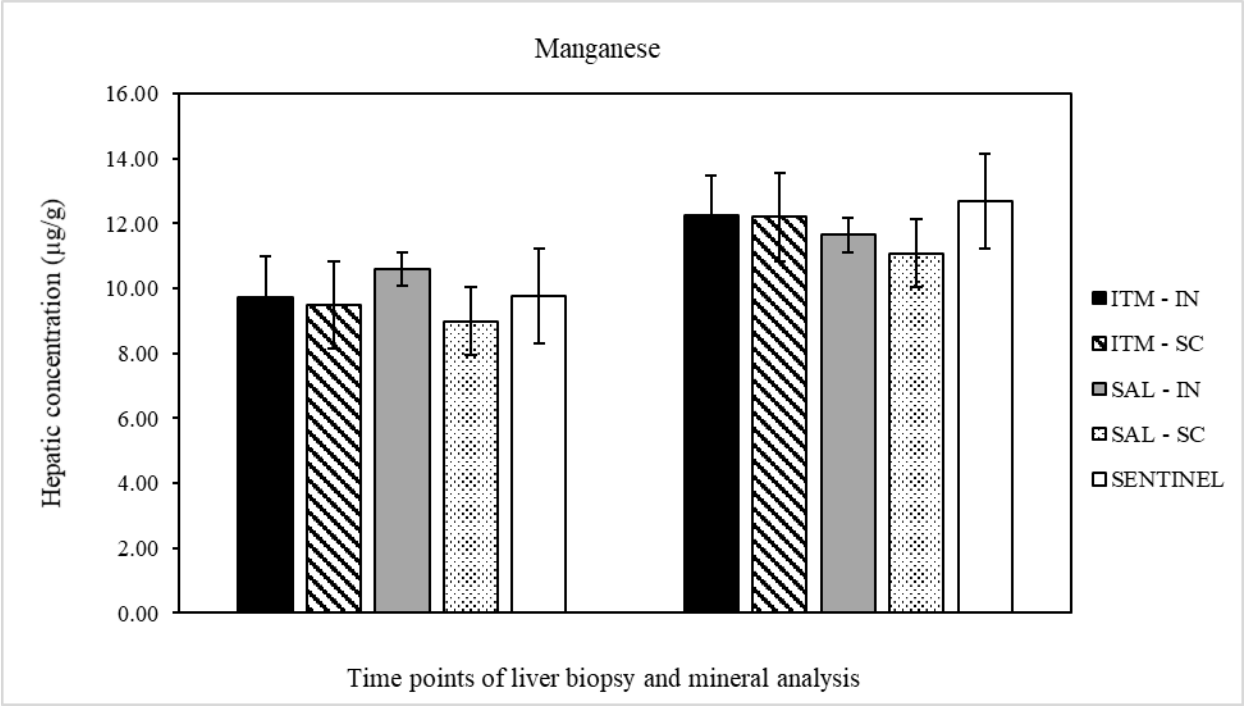
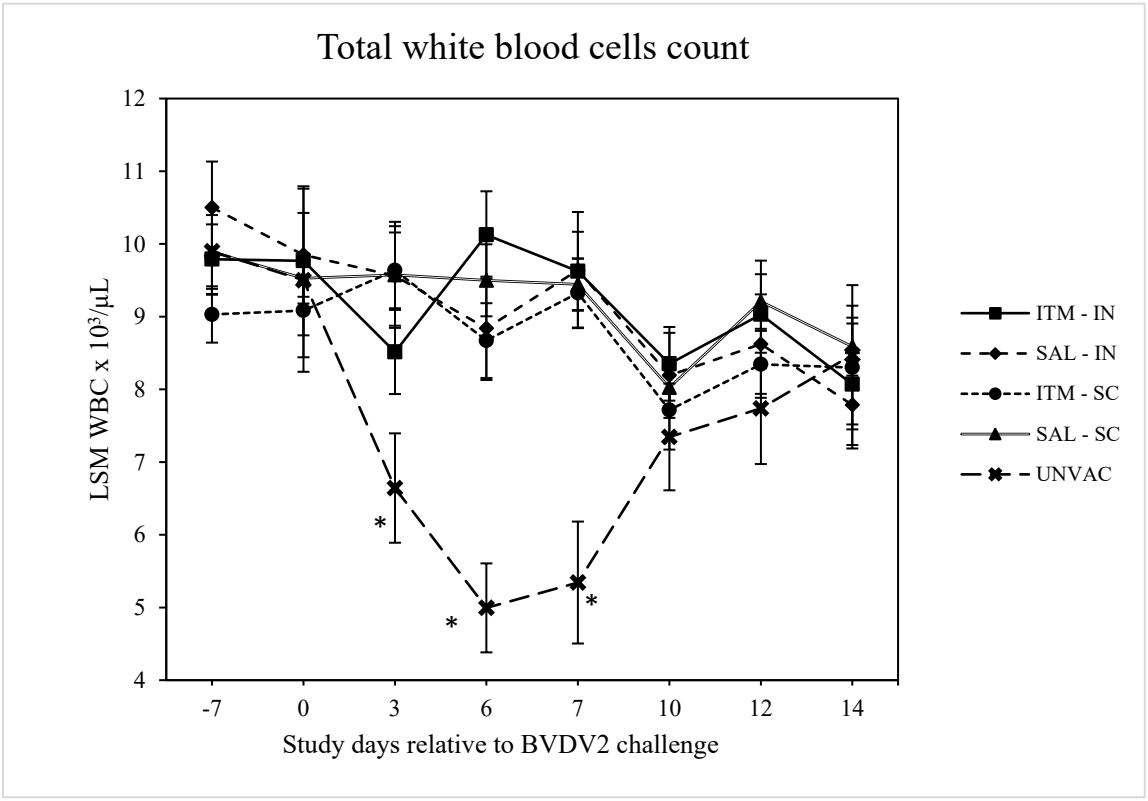

Manganese
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Hepatic concentration (µg/g)
ITM - IN
ITM - SC
SAL - IN
SAL - SC
SENTINEL
Time points of liver biopsy and mineral analysis

Figure 5.7. Mean hepatic concentrations (μg/g) of selenium (**A**), copper (**B**), zinc (**C**), and manganese (**D**) in dairy calves that received MLV booster vaccination (IN or SC) concurrent with trace mineral (ITM) or saline (SAL) injection. Error bars represent the standard error of the means for each group. Liver biopsies were collected 5 days before and 21 days after booster vaccination. Unvaccinated calves did not receive vaccine or treatment. [*]Significant differences compared to the mineral concentration 5 days before booster vaccination ($p < 0.01$).
[a,b] Significant differences between groups on liver biopsies collected 21 days after booster vaccination ($p < 0.05$).

A


Total white blood cells count
LSM WBC x 10³/µL
12
11
10
9
8
7
6
5
4
-7
0
3
6
7
10
12
14
Study days relative to BVDV2 challenge
ITM - IN
SAL - IN
ITM - SC
SAL - SC
UNVAC
*
*
*

B

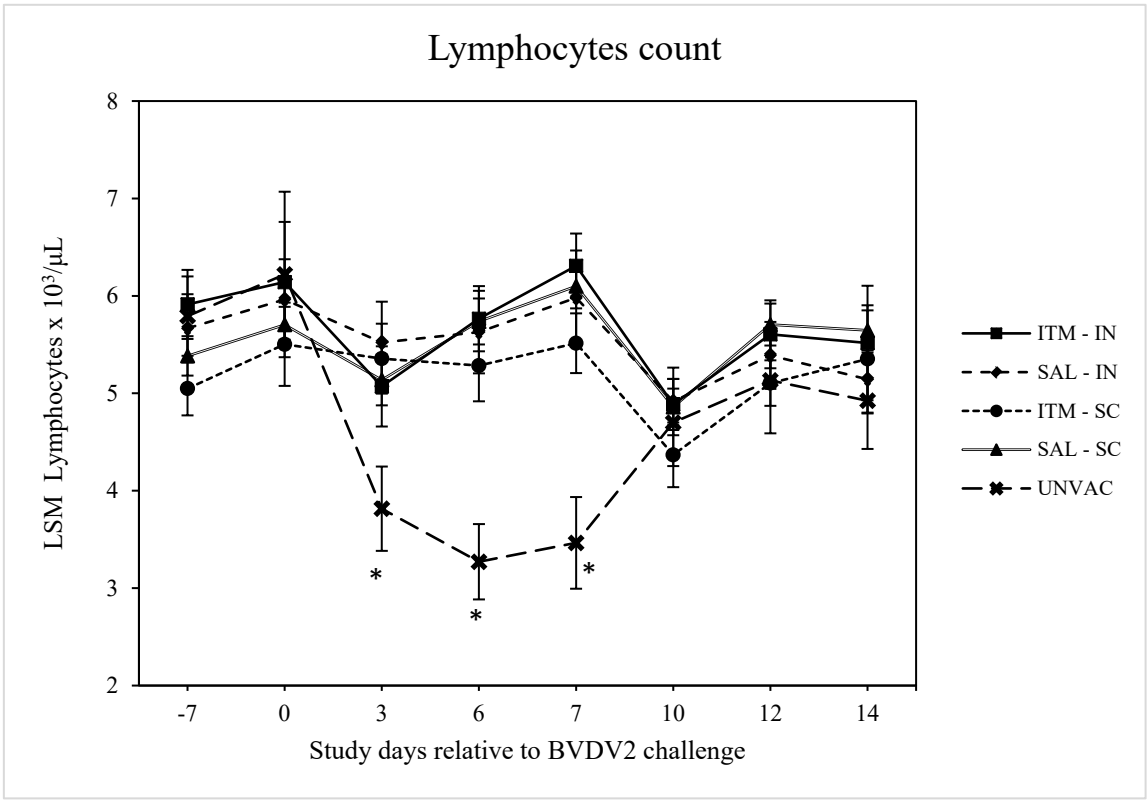

Lymphocytes count
LSM Lymphocytes x 10³/µL
8
7
6
5
4
3
2
-7
0
3
6
7
10
12
14
Study days relative to BVDV2 challenge
ITM - IN
SAL - IN
ITM - SC
SAL - SC
UNVAC
*
*
*

Figure 5.8. Least square means (LSM) of total white blood cells (WBC, **A**) and lymphocytes (**B**) in dairy calves treated with ITM or saline concurrent with MLV vaccination and serially challenged with BVDV2 (day 0) and BHV1 (day 7). *Significantly lower values on days 3, 6 and 7 after BVDV2 challenge compared to the vaccinated groups ($p < 0.05$).

Table 5.1. Percentage (number) of positive results for BVDV2 via *q*RT-PCR of buffy coat samples of dairy calves boostered IN or SC and treated with injectable trace minerals (ITM) or saline (SAL). Calves neither treated nor vaccinated (UNVAC) served as double negative control. All calves were individually serially challenged with BVDV2 (49 days after booster vaccination) and BHV1 (56 days after booster vaccination).

		Groups				
Pathogen	Days relative to BVDV2 infection	ITM-IN (n=12)	ITM-SC (n=12)	SAL-IN (n=12)	SAL-SC (n=12)	UNVAC (n=12)
BVDV2	0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	7	0 (0)	8.3% (1)	0 (0)	0 (0)	83.3% (10)

Table 5.2. Percentage (number) of positive results for BVDV and BHV1 via *q*RT-PCR from nasal swab samples of dairy calves boostered by either IN or SC and treated with injectable trace minerals (ITM) or saline (SAL). Calves neither treated nor vaccinated (UNVAC) served as double negative control. All calves were individually challenged with BVDV2 (49 days after booster vaccination) and BHV1 (56 days after booster vaccination).

		Groups				
Detected Pathogen	Days relative to BVDV2 Challenge	ITM - IN MLV (n=12)	ITM - SC MLV (n=12)	SAL - IN MLV (n=12)	SAL - SC MLV (n=12)	UNVAC (n=12)
BVDV	0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	7	0 (0)	0 (0)	0 (0)	0 (0)	100% (12)
	14	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
BHV1	0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	7	0 (0)	0 (0)	8.3% (1)	8.3% (1)	58.3% (7)
	14	100% (12) [a]	100% (12) [a]	100% (12) [a]	100% (12) [a]	100% (12) [b]

[a,b] Although all calves were BHV1-positive in nasal swab samples collected seven days post BHV1 challenge (day 14), vaccinated calves had consistently greater PCR cycle threshold (Ct) values compared to the unvaccinated calves. The vaccinated calves (n=47, except one calf in ITM-IN) had Ct >18, while 58.3% (n=7) of the calves in UNVAC had Ct ≤ 18. This may suggest a lower specific amplicon concentration (BHV1-PCR product), and possibly lower viral load in the vaccinated calves.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

The major objectives of this dissertation were to evaluate the effects of trace minerals delivered concurrently with modified live virus vaccines on the immune responses against the viral agents involved in bovine respiratory disease. This was achieved by conducting two interconnected clinical trials. One measuring immunity developed at the time of prime vaccination. A second examining the immunity activated and the protection induced by booster vaccination through the SC or IN route in conjunction with co-delivery of ITM or saline. The efficacy of these four vaccine protocols was assessed using an experimental serial challenge with BVDV2 and BHV1. This double challenge was intended to; first, mimic field infections in which BVDV and BHV1 infections are common. Both agents are endemic and generally present thigh infection pressure. Both represent persistent or latent infections. Persistent infected BVDV cattle are a lasting source of the agent in herds, and stress-induce reactivation from BHV1 latency is a ready source of infectious virus. Second, the trial was designed to challenge the activation of immune system with a virus that targets the recruitment and activation of multiple leukocyte populations, and particularly require the activation of the memory clones of several T cell subsets following BVDV2 exposure. This serial challenge was designed to cause clinical disease that was most evident by the highly cytopathic BHV1 virus able to disrupt the respiratory mucosal epithelial cells. Further, the effects of the route by which booster vaccination were administered was assessed for the capacity to prevent severe disease by using a completely randomized clinical trial in calves following both IN priming and booster (SC or IN) vaccination. It is normal

in practice on the commercial dairy farm to prime vaccinate young calves with an intranasal vaccine. This approach is utilized in an attempt to overcome the vaccine response interference by maternal antibodies transferred from colostrum and circulating for at least two months in the calf (Windeyer and Gamsjäger, 2019). In vaccines are also used to stimulate the mucosal epithelium for local production of specific IgA antibodies recognizing the major bovine respiratory disease (BRD) viral agents; BRSV, BHV1, and BPI_3V, but not BVDV for technical reasons (Hill et al., 2012 & 2019).

The first study described in chapter three, was designed as a clinical trial examining the effects of prime vaccination on a commercial dairy. Thus, all calves were vaccinated IN and randomly allocated into two treatment groups: subcutaneous administration of trace minerals (n=30) or saline solution (n=30). This trial allowed us to evaluate the effects of trace minerals (Se, Cu. Zn, and Mn, Multimin90®), delivered at the time of the priming vaccination with a modified live-virus vaccine (MLV) on the development of neutralizing antibodies in serum (SNA) recognizing BHV1, BRSV, and PI_3V and the development of mucosal specific IgA recognizing BHV1 in nasal secretions. Moreover, we also evaluated the enhancement of genes for pro and anti-inflammatory cytokine after that priming vaccination. Although, it has been reported that IN MLV vaccination does not induce significant levels of *denovo* SNA in young calves, there has been evidence of a clearly measurable increase in BHV1 and BRSV specific IgA in nasal secretions (Palomares et al., 2021). Moreover, trace mineral supplementation resulted in a slower decay of maternal BRSV SNA activity 14 and 28 days after vaccine delivery. This was combined with reduced BRD morbidity and mortality among the vaccinated-ITM calves. Apparently, calves receiving trace mineral supplementation had a better levels and activity of functional anti-oxidants. This was associated with an improved health condition and

may be associated with a better controlled immune response. Stressful conditions associated with handling, weaning, high environmental temperature, and a high pathogen load are major factors favoring BRD disease in calves. Previous evidence demonstrated that adequate trace minerals available at time of high stress (i.e., handling and vaccination), help promote faster and stronger development of humoral and cell-mediated immune response (Sordillo and Aitken, 2009). In the present studies, the nutritional requirements of the calves (including trace minerals) were all met. There were no clinical signs associated with any nutritional deficiency. observed Vaccination presents the calf a high metabolic demand. The presence of trace minerals is essential for development and activation of immune function; minerals play a role in; antibody production (Cu, Zn, Mn and Se), T cell proliferation (Zn), leukocyte migration (Se) and phagocytic and killing function of innate cells (Se, and Cu). Thus, an adequate level of trace minerals prevents mineral depletion during the activated physiologic processes that exerts higher demands on reserves. In this study, trace mineral treatment was associated with a more balanced and better regulated expression of pro and anti-inflammatory cytokine expression. In general, intranasal MLV vaccination delivered with trace minerals proved to be an adequate method to reduce the impact of respiratory disease in young calves with high levels of maternally derived antibodies.

The second study is covered in chapter four. This set of experiments was designed as a completely randomized clinical study achieved by randomly dividing the two groups from the previous experiment into four groups for further assessment as following: ITM- IN MLV vaccine (n=12), ITM- SC MLV vaccine (n=12), SAL- IN MLV vaccine (n=12), SAL- SC MLV vaccine (n=12). In addition, one last group of calves was added that served as unvaccinated control (UNVAC, n=12). This experiment was intended to evaluate the effects of trace minerals in conjunction with the route of MLV booster vaccine administration route on weaned dairy calves

(3.5 months old). It followed these parameters: differential leukocyte count, circulating fraction of several T cells subsets, and the level of protection conferred after serial viral challenge. Vaccinated calves were protected from a serial BVDV2 then BHV1 challenge. The level of protection was documented by showing that the following were reduced or prevented: leukopenia, lymphopenia, and neutropenia relative to the unvaccinated calves based on samples taken at three to ten days after BVDV2 challenge. There were no significant differences in leukocyte counts among the vaccinated calves that received trace minerals or saline. On the other hand, all fractions of circulating lymphocyte subpopulations measured ($CD4^+$, $CD8^+$ and $WC1^+$) were significantly reduced soon after the BVDV2 challenge. This was more prominent in the unvaccinated group. Nevertheless, vaccinated calves showed a slower decay in the circulating fraction of $CD4^+$ and $CD8^+$ in samples taken on days three to seven compared to the unvaccinated group. Previous research has reported a similar outcome. This appears to be part of the BVDV pathogenesis. BVDV appears to target circulating peripheral T cells and to deplete those located at lymphoid organs (Liebler-Tenorio et al., 2002, 2003, & 2004; Walz et al., 2010). This led to leukopenia and lymphopenia that is believed to be caused by apoptosis, necrosis, and trafficking of lymphocytes to viral replication zones. With respect to the route of vaccination, calves receiving SC MLV booster had a significantly a higher circulating fraction of $CD4^+$ T cells three days after BVDV2 challenge. Calves receiving trace minerals and IN MLV booster had a significantly higher fraction of circulating $CD8^+$ T cells six to seven days after BVDV2 challenge. There is evidence that the use of heterologous vaccines and a differential route of vaccination may elicit differential protection against BRD pathogens (Vangeel et al., 2007; Lu, 2009, Palomares et al., 2021). There was a decay in the circulating fraction of γδ T cells seen in all groups after BVDV2 challenge. While this is the major T cell population in young cattle it is

readily trafficked, particularly to mucosal tissues. Thus, it may be a result of movement from the circulation to the point of viral infection. Therefore, further research is warranted to understand the roles of gamma-delta T lymphocytes during viral infections. Activated T cells ($CD4^+CD25^+$, $CD8^+$ $CD25^+$, and $WC1^+$ $CD25^+$) were significantly increased in unvaccinated calves 14 days after BVDV2 challenge (seven days after BHV1 challenge). This increase may be associated with activation of T regs, T helper cells, and cytotoxic T cells. By this point in the dual infection the immune system is trying to overcome the active infections needs to regulate the inflammatory response to minimize damage from host activities. However, more research is needed to unveil the mechanisms behind the dynamic of activated cells after experimental viral inoculation. Serum neutralizing antibodies against BPI_3V, BRSV, BVDV1&2, and BHV1 were measured after booster vaccination for all groups (data not shown). There was a significant effect of time after vaccination on the SNA titers for all viruses except for BRSV in all groups. A pronounced decrease in SNA titers was evident on days 14, 28 and 42 after booster vaccination (data not shown). Moreover, SNA against BHV1 were increased seven days after BHV1 challenge in calves receiving IN MLV booster with trace minerals, and in calves receiving SC MLV booster with saline (data not shown). Serum neutralizing titers for BVDV2 were increased seven and fourteen days after BVDV2 challenge in all vaccinated calves. This was not seen in the unvaccinated calves where the SNA titers remained unchanged during that time period. With respect to their mineral status, all calves had adequate levels of all trace minerals measured in liver tissue. The levels of hepatic Cu and Se were increased 21 days after booster vaccination in the calves receiving trace minerals compared to their baseline (five days before booster). Moreover, all groups had a decrease in hepatic Zn, and an increase in hepatic Mn levels. This finding might explain the effect of the trace minerals on some aspects of the immune response

following vaccination. Overall, MLV vaccination protected calves against serial virulent viral challenge by preventing leukopenia and lymphopenia. In addition, there was a significant effect of SC MLV booster vaccination in mitigating the BVDV2-induced reduction of the circulating fraction of $CD4^{+}$T cells. Further, trace minerals given at the time of IN MLV vaccination contributed to reduction in the fraction of circulating $CD8^{+}$ T cells after BVDV challenge. Thus, trace minerals given concurrently with booster vaccination can be a good strategy in growing calves. This combination may generate an improve cellular immunity and its associated memory development, that will better prepare calves for future infections with respiratory viral pathogens.

The third study was a continuation of the experiment described in Chapter 4. This study is covered in the chapter five. This is the first study we are aware of using endoscopy as a tool to assess the damage to the upper respiratory mucosa following serial viral challenge with BVDV2 and BHV1. The main purpose of this study was to offer a further evaluation of the clinical protection induced by MLV vaccination with concurrent with trace minerals. This was achieved by evaluation of both conventional health status measures and an endoscopic assessment of the upper respiratory tract. To this viremia, and viral shedding were added as measures of efficacy. The endoscopic evaluation of the upper respiratory tract allowed us to observe differences in the respiratory mucosa before and after serial BVD2 and BHV1 challenges. The evident changes in the mucosa caused by BHV1 infection in unvaccinated calves were compatible with extensive ulcerative rhinitis, moderate to severe multifocal tracheitis, moderate to severe extensive lymphoid hyperplasia, moderate focal laryngitis, and multifocal ulcerations across the nasopharynx, larynx, and trachea. There was evidence of significant differences in endoscopic scores between calves receiving vaccines with trace minerals, saline, or no vaccine or ITM treatment. Vaccinated calves had less damage from the challenge, resulting in improved mucosal

health in calves that was enhanced by the delivery of trace minerals with the booster. The calves receiving IN MLV booster with trace minerals had lower respiratory scores than the rest of the calves five days after BHV1 challenge. This effect might be mediated by an enhanced micromineral bioavailability, thus by improving the antioxidant (e.g., superoxide dismutase and glutathione peroxidase) and mucosal tissue repair mechanisms (e.g., by Zinc) to help the respiratory mucosal epithelium recover after injury caused by systemic Lymphocytopathogenic effect of BVDV2 (Ridpath et al., 2008). It may also impact the damage and recover of mucosal cytopathic effects of BHV1 (Gershwin et al., 2015). The enhanced mineral availability may enhance the regulation of the local inflammatory response dampening the damage that can be caused by recruited granulocytes. Calves receiving SC MLV booster with trace minerals or with saline had lower endoscopic scores compared to unvaccinated calves five days after BHV1 challenge. This clinical protection may be associated with the superior protective effect conferred by the heterologous vaccines used in the 'IN prime- SC booster' vaccination protocol, as was previously reported (Lu, 2009; Palomares et al 2021). In contrast, calves receiving homologous vaccine treatments in the IN-priming IN-booster without ITM had higher endoscopic scores than the other vaccinated groups. These were comparable with the unvaccinated calves. This response might be induced by reduced induction of elements needed for a robust anamnestic response and full regulation of inflammation due to repeated use of the more limited IN vaccine. However further research is warranted to evaluate the effects of intranasal prime and booster vaccination on local and systemic inflammation, and if it has a relationship with the stability of the bacterial microbiome. Endoscopic evaluation of the upper respiratory tract was simply used as an ancillary tool. It was not intended to be promoted as a daily diagnostic scoring method in the field. Unvaccinated calves presented the highest endoscopic scores after serial BVDV2 + BHV1

challenge. In addition, this group had very high clinical scores, compatible with BVDV viremia and shedding, and with BHV1 shedding. This confirms the effectiveness of the dual challenge model for inducing significant disease. Overall, endoscopy allowed the direct visualization of the upper respiratory tract during viral challenge and documentation of local changes that were induced..

The findings presented in this dissertation may contribute to changes in some management strategies for BRD prevention in pre-weaned and weaned dairy calves. The evidence provided for enhanced immune response using prime and boost vaccination concurrently with trace mineral should be useful to the producer. Multivalent MLV vaccination, including am IN priming vaccination at early age (four weeks of age) with a booster vaccination somewhat later protected calves from significant disease induced by the dual viral challenge using BVDV2 and BHV1.This challenge series, seven and eight weeks after booster vaccination, provided a good model of disease development in the controls and allow a reasonable platform for assessment of protection and some possible elements that mediate it. Including trace minerals concurrent with vaccination programs can lead to improved immune response. This is true even for calves meeting the NRC nutritional requirements. Vaccination involves an increased metabolic demand for the immune system due to the cost of antigen presentation, lymphocyte clonal expansion base proliferation and the cost of functional differentiation. Arming T-cells to function, promoting the activity of innate immune cells and epithelial cells in the tissues and antibody production are also costly in the use of energy. The clinical evidence provided in this dissertation shows how trace minerals are typically depleted after vaccination. However, in calves receiving trace mineral supplementation a greater hepatic concentration, and/or reduced decay of these mineral was seen compared with saline treated calves. The results of this study

using different on the optimization of the route of booster vaccination after IN priming recapitulate findings from previous studies. It also confirmed the benefits of using ITM concomitant with parenteral vaccination in dairy and beef calves. This study contributes to generate baselines for the application of alternatives vaccination protocols to improve the immune response against viral components of BRD and enhance the health status of dairy calves. Finally, decreasing the prevalence of BRD by improving the overall immune response should be one of the targets to improve animal health and welfare, reduce the use of antimicrobials, and increase productivity.