EFFECT OF INJECTABLE TRACE MINERALS ADMINISTERED CONCURRENTLY WITH A NEONATAL CALF DIARRHEA VACCINE ON THE IMMUNE RESPONSE AGAINST BOVINE CORONAVIRUS AND BOVINE ROTAVIRUS IN DAIRY CATTLE

by

ADRIANA RODRIGUEZ ACEVEDO

(Under the direction of Roberto A. Palomares)

ABSTRACT

Bovine coronavirus (BCoV) and *Bovine rotavirus* (BRV) are frequently associated with neonatal calf diarrhea (NCD) in young calves, representing economic consequences in livestock. In this study, neutralizing antibody assay in serum (SNA) and colostrum (CNA) and radial immunodiffusion in serum were used to evaluate the effects of injectable trace minerals (ITM) concurrent with NCD vaccination in pregnant cattle. The results show that ITM-heifers had slightly higher geometric mean of BCoV SNA titers, without statistical differences, and higher percentage of animals undergoing seroconversion on days 21 and 35. In addition, ITM-heifers had slightly median increase of colostrum quality and higher CNA titers against BCoV than control, but without statistical significance. CNA against BRV titers in control group were higher than the titers of ITM, with no statistical difference. Calves from ITM-heifers exhibited relatively higher titers of BCoV without statistical significance. These results suggest that ITM could enhance humoral response to NCD vaccines.

INDEX WORDS: Neonatal Calf diarrhea, immune response, Injectable trace minerals, Bovine coronavirus, Bovine rotavirus.

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DEDICATION

With love and gratitude,

this thesis is dedicated to the memory of my mother,

Mariela Acevedo.

I hope I have fulfilled the promise of making you proud.

To my husband, Hector,

whose love, support, and encouragement

has inspired me to complete this research.

To my sweet daughter Sofia Restrepo-Rodriguez,

who always cheered me up,

and understood the limited time during this period.

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CHAPTER 1

INTRODUCTION

Neonatal calf diarrhea (NCD) is the most prevalent illness affecting calves during the first month of life. The disease accounts for 57% of calf mortality in unweaned calves[1,2]. Neonatal calf diarrhea is a complex disease caused by either infectious (e.g., viruses, parasites, and bacteria) and non-infectious factors. A single primary pathogen or a combination of them can be isolated from sick animals[2]. The disease is directly responsible for the increment of mortality rates in both dairy and beef cattle industry[3]. In general, this disease is characterized by a change in the regular frequency of fecal depositions, fluids and electrolytes imbalance, severe dehydration, and eventually calves' death[3]. While adult cattle can transmit the etiological agents involved in NCD, this disease affects calves less than 30 days old, due to their weaker immune system resulting in higher susceptibility to these pathogens.

Escherichia coli, Cryptosporidium, Bovine rotavirus (BRV), and *Bovine coronavirus (BCoV),* have been described as the most common microorganisms causing acute diarrhea in young calves. These pathogens account for 75-95% of enteric infections in newborn calves worldwide[4], being *Rotavirus,* the virus affecting a wider variety of young animals.

Bovine rotavirus and *Bovine coronavirus* are viruses frequently found in feces of healthy adult cattle, being a source of environmental contamination, and consequently, herd transmission[1,5]. Both viruses infect enterocytes and cause a variable degree of damage to the intestinal epithelium. The direct association between viral infection and disease development depends on the host immune status and the characteristics of the physical, chemical, and protein-based intestinal barriers that affect the virus' entry, replication, and survival in the host cells[6]. The pathogenic viral particle must be able to resist chemical barriers in the gastrointestinal tract, extreme pH ranges such as the acidity of the stomach, and the alkalinity of the intestine, proteases, and bile salts which degrade enveloped viruses. The resistance to all these factors accounts for the viral particle pathogenicity, its interaction with the intestinal environment, infection, host

immune response, and clinical signs[6]. Although both viruses can cause comparable cell damage, the association of any clinical sign or pathological lesion to a specific etiological diagnosis is not possible. However, different techniques such as ELISA monoclonal antibodies, reverse transcription (RT) Polymerase chain reaction (PCR), and immune-electron microscopy can effectively be used to detect and differentiate BCoV and BRV[2].

The epitheliochorial nature of the bovine placenta does not allow the vertical transfer of maternal antibodies from the dam to the fetus. The lack of conveyance leads to a deficiency of immunoglobins in newborn calves. Colostrum intake represents the primary source of maternal passive transfer of antibodies to the calf. When colostrum containing a high level of immunoglobulins (referred to as good quality colostrum) is supplied, it promotes early protection of the neonate by providing the necessary antibodies to bind and neutralize viruses and bacteria. Failure to provide an adequate passive transfer of antibodies, particularly IgG type, within the first hours after birth, leads to an increment of calves' susceptibility to infections[7]. Vaccination of pregnant cattle is a useful tool to increase the levels of antibodies in colostrum. Adequate immunization against these enteric pathogens during late-term pregnancy has been reported to result in an effective immune response, including antibody production that would be later transferred to the offspring[4].

Numerous factors affect the immune response after vaccination in cattle[8,9]. Dam's nutritional status is a relevant factor that may influence the protective immunity elicited by vaccination, affect the concentration of immunoglobulins in their colostrum, and determine calves' health and survival [10]. Minerals such as copper, zinc, selenium, manganese, iron, iodine, cobalt, and molybdenum have essential roles in the immune system of cattle[11–13]. Although these minerals are present in the body in small quantities, they are vital cofactors and components of multiple metabolic and antioxidant enzymatic systems[14]. It is a recommended practice to provide trace mineral supplementation to dairy and beef cattle[14]. Furthermore, the use of injectable trace minerals (ITM) offers the benefit of a known and controlled amount to be delivered with rapid and efficient absorption and storage following administration[15]. Concurrent use of ITM at the time of vaccination has shown beneficial effects on the immune response against viral respiratory pathogens

in cattle[11,16]. The reported improvements of the immune response against BVDV1, BVDV2, and BRSV after administration of ITM and modified-live virus vaccines offer a promising possibility that administration of ITM concurrent with NCD vaccination may have an effect stimulating the immune response against BCoV and BRV as well.

This thesis is based on the evaluation of the antibody response in serum and colostrum against BCoV and BRV in dairy cattle and the passive transfer to their calves after vaccination with an NCD vaccine and injection of trace minerals during pregnancy. For this purpose, a serologic test was developed and validated to evaluate the levels of neutralizing antibodies against BCoV and BRV. The knowledge about the immune response against these enteric viruses is essential to develop more effective immunization protocols to prevent and control NCD.

The results of this study will contribute to a better understanding of how the administration of ITM concomitant with NCD vaccination could improve the passive transfer of antibodies, and consequently, calves' health. This might result in decreased losses associated with NCD and increased profitability of the dairy industry. The field study methods, results, discussion, and conclusions are presented in chapter 3.

CHAPTER 2

LITERATURE REVIEW

Neonatal Calf Diarrhea

Neonatal calf diarrhea (NCD) is a major disease that has a significant impact on calves' health as well as the economy of the cattle industry. This disease is caused by diverse microorganisms, including viruses, bacteria, and parasites[1]. Moreover, NCD remains as an important cause of morbidity and mortality, accounting for 57% of pre-weaning calves' deaths[1]. Similarly, Meganck et al. (2015) reported NCD as the cause of 50% deaths in unweaned heifers calves in the United States[17]. Even though enteritis affects cattle at any age, most cases occur under one month of age, making NCD one of the most important causes of death in newborn calves and until their first four weeks of life[1]. Age-dependent adaptations and physiological differences in the immune response can explain the variation of susceptibility to viral infections at that age[6].

After birth, the first contact with the environment is through oral exploration. The oral cavity is continuously exposed to fecal contamination, which leads to the calves' gut colonization with commensal flora[3]. However, this symbiotic relationship that brings beneficial effects without causing any harm to the animal can be negatively affected when enteric pathogens colonize and invade the tract. Among the most important pathogens causing NCD in calves are *Bovine rotavirus* (BRV) group A, *Bovine coronavirus* (BCoV), *Escherichia coli*, *Salmonella spp*, *Clostridium perfringens*, and *Cryptosporidium parvum*[2]. In Addition, acute infectious with *Bovine viral diarrhea virus* early in life may also cause enteric illness in young calves. Intestinal barriers such as mucus layer, mucins, defensins, antibiotic peptides, IgA as well as innate lymphoid cells function as a protective and regulatory system that keeps the intestinal lumen free from potential pathogens[18]; however, several microorganisms can invade intestinal cells. Upon cell

infection, viral replication and cytopathic effects along with the subsequent innate and adaptive immune responses, cause damage to the enteric epithelia impairing its absorptive function[19].

In neonatal calves, the disturbance of the intestinal absorptive system by these viral infection results in an abrupt onset of liquid feces, and a quick imbalance of electrolytes. Sick calves typically suffer from dehydration, depression, loss of sucking reflex, and the ability to stand. The disease can progress to metabolic acidosis, impairment of vital organs function, resulting in an increased risk of mortality[20]. Infections of BCoV and BRV have detrimental effects on cattle industry profitability due to the costs related to veterinary service, treatment, calves' poor weight gain, and eventually calves' death[1,5].

The increment of depositions, consistency (usually of mucoid appearance), color, as well as the presence or not of blood clots, have no significant correlation with a specific enteropathogens[4]. The NCD caused by pathogenic bacteria can induce similar clinical signs. Enteric microorganisms, such as *Escherichia coli*, bind to the intestinal epithelial disrupting the tight junctions and microvilli, causing malabsorptive syndrome. Even though enteric microorganisms have different mechanisms of pathogenesis, only good quality samples and tissues can be of help for the clinical testing and differentiation of bacteria, viruses, or coinfection in enteric diseases[21]. Several enteric microorganisms with pathogenic potential have been studied for decades, but their association with NCD has been difficult due to their presence in healthy cattle[1].

Bovine rotavirus and *Bovine coronavirus* are the most important viral pathogens associated with acute diarrhea in newborn calves worldwide, but other pathogens can cause the disease as well[22]. These viruses account for 49.1% (BRV) and 40.7% (BCoV), of NCD in Australia[23]. These viruses can be detected in newborn calves as early as one day of age[21]. The NCD outbreaks associated with BCoV commonly increase during the cold season. High humidity and low temperatures had been reported as factors determining the increment of virus survival[4].

The early BCoV and BRV infection and disease occurrence during the first days of life would require an immediate immune response. Therefore, protection from these infectious agents is highly dependent on the calf's passive immunity, which is primarily represented by the presence of IgG antibodies provided by colostrum [22]. Even though dams are naturally exposed to antigens and seropositive, the titers decline soon after calving in unvaccinated animals. Dam's vaccination during the late stages of pregnancy is vital to stimulate a robust humoral immune response, represented by virus-specific IgG, produce high-quality colostrum, and prevent enteric illnesses in calves[1].

Several serological and molecular techniques can be used for the identification of NCD pathogens. Fresh or fixed specimens from different intestinal areas, depending on the agent, can be used for diagnosis. Feces and intestine specimens or intestinal content have been reported to be adequate samples for the determination of *Rotavirus*, *Coronavirus*, and *E. coli* K99[21]. PCR methods are reliable and usually very sensitive methods for the diagnosis of enteropathogens. Cross-contaminating organisms might also be identified as causal pathogens[21]. It is essential to standardize biosecurity practices to control the factors favoring the disease spread. Proper facilities management, hygiene, animal husbandry, personnel training, and dam nutrition might help to decrease the disease occurrence.

Bovine Coronavirus

Bovine coronavirus is a member of the *Coronaviridae* family and *Betacoronavirus* (β-CoV) genus[24]. It is a spherical, enveloped, and single-stranded positive-sense RNA (ss+RNA) virus[25]. The name is derived from the Latin corona, which means crown. In the electron microscope, the viral particle with its membrane-bound spike protein (S) resembles a solar crown. It is composed of 28-33kb. The large genome size and RNA type confer them with ample coding capacity and a high frequency of mutations[6,26].

Coronavirus is divided into four genera α-CoV, β-CoV, γ-CoV, and δ CoV, and in three genetically different groups. *Bovine coronavirus* belongs to group two, a group characterized by the presence of hemagglutinin-esterase[25]. This virus comprises five core proteins spike glycoprotein (S), Nucleoprotein

(N), membrane protein (M), Hemagglutinin-esterase (HE), and small membrane protein (E). The Spike protein (S), which has an essential role in receptor binding and cell entry mediator determines the host tropism[24,26].

The Spike protein, a type I fusion protein, is a critical glycoprotein that mediates the host cell binding and membranes fusion. This protein is the target for neutralizing antibodies during the immune response and a promising tool for vaccine development[27]. Thus, BCoV initiates infection by S protein engaging 9-*O*-acetylated sialic acid in host cells. The viral particle also possesses sialate-9-*O*-acetyl esterase, which functions as receptor destroying-enzymes (RDE), facilitating reversible binding[28]. S protein must adjust to the host cell receptor to induce a productive infection. In a new host, susceptible and permissive cells, as well as molecular changes in the Spike protein seem to be determinant for cross-species transmission. Coronaviruses can undergo genomic recombination, a process that results in altered tropism and host adaptation[25]. C-type lectin in dendritic cells and macrophages can recognize high mannose patterns present in viral pathogens. However, viral particles can subvert this immune response and take advantage of it to get access to the host cell[6].

Hemagglutinin-esterase is an exclusive protein in the β-CoV group and is believed to have originated from Influenza HA fusion protein. This evolution probably occurred by gene transfer and further adaptation and remodeling of the receptor binding domain[28]. Even though this glycoprotein does not participate directly in cell binding, it facilitates the reversible cell binding of *O*-acetylated sialic acids recognized by the S protein through sialate-9-*O*-acetylesterases receptor destroying-ezimes[29].

The virion - cell interaction starts with the spike protein S binding to the host cellular receptor: glycan Neu5,9Ac2 (5-N-acetyl-9- *O*-acetylneuraminic acid)[24,30], and the endocytosis of the viral particle. Following the internalization of the virion, the low pH triggers the fusion between the viral and cell membrane delivering the viral genome into the cell's cytoplasm, which is immediately translated by the host cell's ribosomes. Translation produces 1a and 1ab polyproteins, which are later processed to form several viral proteins, including RNA dependent RNA polymerase (RdRp) and other proteins that help in

the formation of cytoplasmatic double-membrane vesicles, which serve as viral factories. The polymerase completes the synthesis of RNA, and the formed (-) RNA is copied to create positive sense RNA and mRNA. The formed mRNA gives origin to structural and non-structural proteins. The three membrane-bound proteins S, E, and M are incorporated into the endoplasmic reticulum. At the same time, the (+) RNA is encapsidated by the protein N. Nucleocapsid acquires membrane proteins S, E, and M through the budding with endoplasmic reticulum-Golgi intermediate vesicles. The viral particle is finally transported in a smooth-walled vesicle, which fuses with the cell plasma membrane releasing the virus[6,30]. All this process of viral replication impacts the cell structure, resulting in cytopathic effects (CPE). Several authors have described the pattern of CPE in *Coronavirus* infection in different animal species. The CPE induced by BCoV in the MDBK cell line has been characterized as cell enlargement, swelling, detachment, granular appearance, and syncytia formation[31–33]. Products of infected cells and viral pattern recognition initiate antiviral state to clear the virus. However, the virus strategies to counteract the host proteins synthesis results in the antiviral state modulation. All these factors contribute to the virus pathogenesis and the clinical manifestations in the host[30].

Bovine coronavirus causes neonatal calf diarrhea and winter dysentery in older calves and represents a primary cause of acute respiratory disease in cattle[34,35]. BCoV has a tropism for the epithelial cells of the enteric and respiratory tracts. In most cases, BCoV infection begins in the small intestine to further expand to the colon, causing a more severe disease compared with BRV. As other enteric infections, the virus replication and cells' damage destroy the epithelia resulting in a blunting of mature enterocytes[35]. This loss of villi epithelium causes imbalances in the intestinal fluids and nutrients absorption leading to malabsorption and maldigestion[3]. Yellow muco-hemorrhagic feces resulting from the damage in the colonic mucosa can be observed preceding a much profuse watery diarrhea. The drastic imbalance rapidly lessens the body fluids leading to dehydrated and weak calves that promptly lose the sucking reflex. In untreated calves, the disease rapidly progresses into metabolic acidosis, coma, and eventually death. In some cases,

both enteritis and respiratory syndrome signs can be observed at the same time leading to a more severe prognosis[35].

The virus is widely distributed and commonly reported in North America, Australia, Europe, and East Asia cattle population[26]. The incidence of 90% makes cattle very likely to be diagnosed with BCoV[35]. It can severely affect calves during the first month of life[22]. Although this enteric virus is mostly reported as the primary agent causing neonatal calf diarrhea, some authors make reference as the second most common pathogen causing diarrhea in calves[21].

Infected cows can be the potential source of infection and contamination for the herd and the environment. Oma et al. (2016) indicated that adult animals chronically and sub-clinically infected can be the source of infection for newborns. The infected calves likewise, could harbor viral particles in the lymph nodes for weeks and also shed it in feces, affecting more calves[36]. Several factors, including the stress associated with high confinement during the winter season, make winter dysentery a common disease in northern states of the USA with reports of 50 to 100% morbidity and approximately 2% mortality rates in the cattle industry[26,35].

The BCoV-associated disease is correlated with immunological and environmental risks[37]. Winter associated stress, shipping, and sanitary factors affect the exposure and development of the disease. Transmission from dams to calves occurs during the postpartum period. Chronically infected cows can shed virus in nasal secretions and feces. Calf to calf transmission can likewise occur by close proximity and by respiratory secretions[35]. In preliminary studies, Saif and Smith et al. (1985) reported the BCoV antigen detection in the small intestine, colon, trachea, and nasal smears three hours after oral/intranasal exposure to BCoV. They suggested that the replication of BCoV in the oropharynx with the subsequent infection of the gastrointestinal tract from swallowed viral particles contributes to the substantial presence of the virus in the small intestine[38]. Reynolds at al. (1985) supported the hypothesis that shedding might occur from both tracts simultaneously. In their study, they reported events of synchronized excretion in feces and nasal swabs. However, it was not always concurrent[39]. Furthermore, they suggested that no antigenic and

pathogenic difference between the isolates was identified[39]. Hasoksuz et al. (1999), reported antigenic variation between BCoV stains; nevertheless, those variations were not always correlated with the sample origin (fecal or respiratory)[40].

Specific treatments for BCoV are not currently available. Oral and intravenous solutions are needed to provide rehydration. Vaccination of pregnant cows/heifers and passive transfer of antibodies to calves through colostrum seem to be so far the most effective method of prevention and control. Given that BCoV cannot be clinically differentiated from other enteric infections, it is important to submit the fecal samples from calves suffering NCD for laboratory testing. Fecal samples and nasal swabs can be analyzed for antigen capture ELISA employing monoclonal antibodies or for virus isolation and RNA detection using PCR[3].

Even though the fecal-oronasal route is the most common route of infection and some authors have reported fecal samples as a good source to detect the virus[37], one study reported nasal swabs as a more advantageous method for BCoV virus detection[36]. Lungs, trachea, and nasal samples can also be representative samples in post-mortem diagnosis. Several enteropathogens, including viruses, bacteria, and parasites, affect the gastrointestinal tract of calves, causing similar atrophy in the villous epithelia. In severe illness, pathophysiological findings can demonstrate the presence of coinfections or multiple-microorganisms infection that might be increasing the severity of the clinical signs[26].

Prevention and control of BCoV infections are mainly oriented to the identification and isolation of affected animals as well as application of standard biocontainment protocols to control the infection risks (calving season, high confinement, stress, and weather-related aspects). Sanitation of contaminated areas with lipid solvents is a measure to prevent and control the transmission of enveloped viruses such as BCoV. Vaccination of cows with parenteral vaccines and passive transfer of maternal immunity through colostrum represents a reliable method of prevention. In addition, immunization of neonates with an oral vaccine may also be a strategy to induce the immune response to BCoV. Nevertheless, maternally derived antibodies might interfere with the vaccine. Workman et al. reported the importance of stimulating immunity against

BCoV before calves entry to the feedlot to reduce the incidence of BCoV associated respiratory disease[41]. All these strategies combined might have a synergistic effect reducing the viral particles in the environment and herd infection.

Although Coronaviruses have been studied for decades, there is still a lack of knowledge regarding the complexity of its replication cycle, the factors involved in their pathogenesis, the interaction and manipulation of the host cells, mutations, and interspecies transmission. These facts underline the importance of expanding our understanding of Coronaviruses in order to prevent and limit their infectivity[30]. In general, viruses have an affinity for a species-specific receptor. Nevertheless, some viruses evolve to be able to bind different subtypes receptors changing their tropism or making more extensive the range of hosts. Adaptation of *Coronavirus* from animals to human hosts takes a few mutations in the spike protein (S), which represents the ability of the viral particle to bind the host cell receptor and get access to the cell machinery leading to replication and transmission to a new host. This possible adaptations in the human-animal interface represent a severe public health threat[30].

*Coronavirus*es, initially linked to cold in humans, began to gain attention after a much more lethal strain, SARS-CoV and MERS-CoV, were identified. These viruses have been classified as a viral respiratory disease that affects the lower respiratory tract. Severe Acute Respiratory Syndrome (SARS) - CoV was known in the world in 2002 when an epidemic outbreak alarmed the world. The infection affects the lower respiratory tract causing atypical and lethal pneumonia with a high fever and generalized malaise and lymphopenia. On the other hand, Middle East Respiratory Syndrome (MERS)-CoV was first isolated in 2012 and caused a 35% mortality rate. It causes acute respiratory distress, high fever, coughing, and organ failure[30].

Recently, the World Health Organization reported a new outbreak of severe acute respiratory syndrome caused by a novel strain of *Coronavirus* (SARS-CoV-2) that originated in Wuhan, China. The outbreak possibly caused by a zoonotic origin has set clear global priorities that can lead to the outbreak control. Data, as reported by March 22th, 2020, showed 81,601 cases confirmed in China, 31,573 in the

United States of America, and a total of 332,935 confirmed cases and 14,510 deaths in 189 countries areas or territories[42]. With the occurrence of this severe outbreak, the subsequent declaration of Public health emergency of international concern in January 2020, and lastly, the pandemic declaration coronaviruses became recognized as emerging pathogens capable of causing a devastating health crisis in the human population[43,44].

Studies conducted to establish the antigenic and genomic differences between coronaviruses have concluded that specific changes in the amino acid sequence in the S1 subunit can change the receptor binding and receptor specificity, increasing the host range[25,45]. Some of the circulating BCoV strains in China have been studied to determine its evolutionary recombination. Applying a phylogenic analysis based on the full-length genome, researchers were able to compare the sequences of S, M, and hemagglutinin (HE) genes. Compared with the BCoV Mebus reference strain, notorious differences in genes demonstrated that 77% (10/13) of the Chinese circulating strains were HE genes recombinant. The M genes revealed to shared 94-100% of nucleotides. Moreover, N genes revealed to have amino acid substitutions converting the Chinese strain into the evolved strain[34]. These facts underline the importance of expanding our understanding of coronaviruses to prevent and limit its impact on human and animal health.

Studies that provide information regarding effective methods of vaccination and antiviral therapy for BCoV infections in cattle may have a significant impact on the prevention and control of CoV infections in humans.

Bovine Rotavirus

Rotavirus, a member of the *Reoviridae* family, is an icosahedral, non-enveloped, double-stranded RNA virus. It is composed of 11 genome segments encoding six structural proteins (VP1, VP2, VP3, VP4, VP6, VP7), and depending on the strain, five or six non-structural proteins (NSP1, NSP2, NSP3, NSP4, NSP5, NSP6)[46]. The outer capsid structural proteins VP4 (P) and VP7 (G) are also classified into 35 (P) and 27 (G) different subtypes[47], being G6, G8, G10, P1, P5, and P11 the most common types found in cattle.

Other strains have been found with less frequency[47]. VP4 (P) and VP7 (G) structural proteins have an essential role in stimulating the host immunity, given its ability to induce the production of neutralizing antibodies by the host [48].

The viral particle and cell interaction start by the virion protein 4 (VP4) attachment to the sialic acid terminal domain in the host cells' receptors[6,49]. After that, VP4, the protease-sensitive antigen, and VP7, the glycosylated antigen, are detached from the particle[46]. The double-layered particle gets into the cytoplasm by clathrin-mediated endocytosis. In order to avoid the immune response, the viral particle is partially uncoated in endolysosomes. This location protects the dsRNA genome from the cell's antiviral state activation and degradation. The synthesis of viral mRNA begins within the core particle by the polymerase (VP1) encoded in the viral particle. The new +RNA is extruded into the cytoplasm, where it serves as a template for viral proteins synthesis and as a complementary strand for negative-sense RNA genesis. The progeny cores located at the periphery of virus factories are coated with viral protein 6 (VP6) forming the double-layered viral particle which bud across the endoplasmic reticulum acquiring VP4 and VP7 proteins that form the outermost layer of the viral particle in a mature infectious particle. Mature viral particles are later released by cell's lysis[6].

Rotaviruses are highly diverse viruses; the accumulation of mutations accounts for its wide variety; genetic mechanisms such as recombination and reassortment can likewise occur. During infection of a heterologous host, different strains belonging to the same serogroup of BRV can undergo genome' segments mixing. The exchange of gene segments can lead to an evolutionary mechanism of interspecies reassortment, presenting a potential impact of this virus in human health[47]. Genome sequencing of *Bovine Rotavirus* and *Human rotavirus* revealed a close association between their genomes, suggesting that *Human rotavirus* has a bovine origin or common coevolution that leads to an interspecies combination between both viruses[48,50].

Seven different serogroups of rotavirus have been classified (A to G) base on the VP6 inner capsid protein genotype. Groups A, B, and C have been identified in mammals and poultry. Groups D, F, and G

have been identified only in poultry, whereas group E has been found only in pigs. Group A rotavirus has been experimentally shown to cause diarrhea in different species, including cattle[48,51]. These groups can be further classified according to the outer capsid proteins. The viral proteins VP4 (P) spike protein and VP7 (G) shell protein are the basis of this binary classification[52].

Papp et al. (2013). identified the most prevalent surface antigens of rotavirus in cattle. They categorize the G6 (56.7%) and G10 (20.6%) as the most predominant in cattle, G8 (3.5%) was also found with far less frequency. VP4 (P) nonstructural protein combination was assessed as well. They found P5 with a prevalence of 25.9% and P11 with 21.5%, whereas the remaining 43.6% was untippable[47]. The three-principal combinations found between the nonstructural proteins VP4 and VP7 were G6/P5 and G6/P11 and G10/P11. Individual combinations of G and P were also identified. However, they have considerably less impact on cattle diarrheic infections[47]. The discovery of the most common circulating strains among cattle has served as a template to produce more effective vaccines.

Rotavirus affects various host species, including cattle, swine[47], piglets, birds, and humans, resulting in detrimental consequences[48]. *Bovine rotavirus* is one of the main etiological agents of neonatal calves' diarrhea worldwide. Several characteristics, including the virion particle resistance to a wide pH range, its stability in the environment, the low doses required for infectivity, and the high load in feces make its eradication more complicated[53]. The first cases of BRV were detected in 1969 when calves' diarrheic feces were analyzed to identifying the etiologic agent[48]. It was the primary rotavirus from group A isolated and confirmed in diarrheic calves[53]. Since then, BRV has been found and commonly associated with acute gastroenteritis in cattle worldwide. It was isolated in at least one calf from 63% of the farms, and in 20% of the calves with diarrhea[3]. Its transmission is mainly through the fecal-oral route. The large amounts of viral particles produced, the fecal contamination due to sick animals shedding accounts for the high incidence, and the likelihood of herd outbreaks. Similar to BCoV, BRV can infect adult cattle; however, it usually affects young calves with more severity, especially during their first month of life, given their immature innate immune system and lack of strong adaptative immunity. Calves' susceptibility to

infections and subsequent digestive tract illness by BRV decreases as they mature and get adequate innate and adaptive immunity, improving their humoral and cellular immune status[48].

Livestock industry can be severely affected by BRV infections due to the costs related to veterinary care, treatments, calves' poor weight gain, and, eventually, death. The dynamics of transmission, distribution, and persistence in the environment depends on the associated serogroup[48]. These viruses can be detected in newborn calves as early as one day of life[21] with a devastating consequence given their immature immune system.

Bovine rotavirus, as well as other microorganisms that affect the intestinal mucosa, causes malabsorptive syndrome. During BRV infection, cell lysis is required for viral particle release. Given that BRV infects mature enterocytes, the constant lysis of these cells causes the loss of surface area in the intestinal crypts, which impede the absorption of nutrients, and fluids. These changes create an osmotic imbalance, in which the fluids dragged into the lumen surpasses the absorptive capacity of intestinal villi[3].

Likewise, recent evidence suggests that the BRV non-structural protein 4 (NSP4) is responsible for secretory diarrhea. This hypothesis suggests that NSP4 produced during virus replication is released in the intestinal lumen after cell lysis. Exposure to NSP4 causes a negative impact on intestinal nutrients absorption and water balance. Upon release, NSP4 induces the Calcium-dependent chloride secretion and block the sodium-dependent glucose cotransponder SGLT1, which has a vital role in water absorption[54]. NSP4 acts as an enterotoxin with a differential effect than those produce by bacterial enterotoxins. Rotavirus NSP4 induces calcium (Ca^{2+}) release from the endoplasmic reticulum. The increment in Ca^{2+} concentrations inhibits the intestinal ability to process carbohydrates, decrease Na^{+} and glucose absorption, and increases/limits Cl^{-} secretion[55]. This imbalance alters the water reabsorption and allows accumulation of carbohydrates and other nutrients in the lumen, changing the composition and transit across from the different intestinal compartments, which facilitates the development of diarrhea[55].

Rotavirus modulation of the immune response has been consistently studied. NSP1 protein counteracts the antiviral pathways in different ways. It can degrade IFN transcription factors IRF1, IRF5, and IRF7, block activation of NF-kappaB necessary for IFN induction, and can prevent STAT1 and STAT2 nuclear translocation, affecting the antiviral state. Subverting the antiviral innate immune response negatively impacts the calf's health and increases the necessity of medical intervention to recover the normal physiological conditions[56,57].

The detrimental effects of BRV infection in cattle's health could be ameliorated by the implementation of vaccination programs in pregnant cows. Vaccination against NCD may have significant benefits for the herd's health and a substantial reduction in NCD incidence. This vaccination strategy could provide strong humoral immunity to the dams, which will be further passively transferred to the calf through the colostrum. The commercially available multiantigen inactivated virus vaccines are intended for the prevention of diarrhea caused by BRV serotypes G6 and G10, and BCoV as well as *Clostridium perfringens* and *Escherichia coli*. Adequate immunity against BRV during the first 48 hours of life can only be achieved by feeding the calves with good quality colostrum containing proper amounts of immunoglobulins (IgG). These antibodies neutralize the virus preventing the cells from infection and consequently preventing colonization and upcoming infections[53].

The diagnosis of BRV infection can be achieved using standard methods of diagnosis. Virus isolation, antigen capture ELISA, and PCR can detect BRV when a sample is collected correctly. Fecal samples must be collected directly; a sample collected from the field might not be representative of the disease given the presence of the virus in the contaminated environments. Adequate quantity, quality, transportation, and storage of the sample can be vital for a proper diagnosis. In dehydrated calves, there may not be evidence of diarrhea; however, other samples, including postmortem samples of intestinal tissues, can be used. PCR has high sensitivity and specificity. In contrast, ELISA commercial kits have shown variability[21].

Given the lack of pharmaceutical treatment to control BRV infections, the main therapy recommended in young calves is focused on the recovery fluids and electrolytes balance. In moderate to severe cases of dehydration, oral electrolytes and intravenous fluids are suggested to maintain hydration. Antibiotic treatment should only be used if a bacterial cause is identified. In non-bacterial diarrhea, the use of antibiotics as treatment may suppress the microbiota worsening the disease and allowing yeast overgrowth[58].

The likelihood of developing diarrhea and its severity can be influenced by host and pathogen factors. Therefore, prevention and control of BRV infection are directly related to the control of risk factors that increase the exposure (e.g., hygiene conditions of pens, personnel training). The implementation of the Sandhills calving system can provide standardized managing practices to prevent contact and unnecessary exposure of newborn calves, thus preventing and controlling neonatal diseases. The system avoids calves from high animal-density lots and continuous exposure to pathogens; instead, it suggests moving of pregnant cows to calving pastures and house the calves by age[59].

Several factors, such as colostrum intake, immunoglobulins concentration, and feeding time after birth, have been reported as important factors that provide protection to calves and influence the impact of NCD[60,61]. Dam's immunological status may vary according to their parity. Reschke et al. (2017) and Conneely et al. (2013) reported significant differences in IgG concentration in heifers compared with cows, especially after fourth lactation[60,61]. Interestingly, in both studies, there was a tendency of colostrum improvement as parity increases. This effect may be explained by more frequent exposure to antigens during several lactations. Further studies to quantify antigen-specific immunoglobulins after each parturition could help to elucidate this effect.

Since calves rely solely on maternally transferred antibodies during the first days of life, it is essential to improve the overall quality and quality of colostrum produced by heifers. Adequate vaccination of pregnant cattle is essential to assure enough passive transfer of antibodies, especially in heifers, which usually have lower quality and quantity in colostrum.

Immune Response

The innate immune response against viral infections is directed by type I interferon (IFN) and NK cells, whereas antibody production by B lymphocytes and cytotoxic T lymphocytes mediate the adaptive response[18]. During the early course of a viral infection, innate cells recognize viral products such as RNA or DNA or viral products generated by infected cells, activating type I IFN, which inhibits viral replication and promotes proinflammatory cytokines production. IFN-γ secretion by natural killers activates macrophages, stimulates B lymphocytes isotype switching, and T cells differentiation. All these processes lead to the killing of infected cells[62]. The adaptive response to viral infection is mediated by the production of high-affinity antibodies that neutralize the virus during the extracellular phase. They prevent the viral particle from getting into the cell or the virus attachment to a new cell after replication occurs. On the other hand, cytotoxic T lymphocytes and CD4 helper T cells recognize viral peptide antigens presented in the context of major histocompatibility complex (MHC), resulting in massive lymphocyte proliferation and cytokines production that stimulates phagocytic cells and more $CD8^+$ T lymphocytes activation and differentiation to kill infected cells[18].

Differently from other species, calves are born immunocompetent, but agammaglobulinemic. A period of time is needed for their naïve system to develop antibodies to respond to infections. Given the immunological characteristics of calves at birth, and the high sensitivity to infections, the calf's immune protection entirely depends on the molecules transferred through colostrum and their constant presence in the gut lumen[5,63,64].

Placental transfer of nutrients and immune molecules depends on the placental characteristics of each animal species. Circulation or restriction of molecules across the placenta occurs by facilitated transportation or by diffusion, which depends on the thickness and histological structure of the cellular barrier between maternal and embryonic blood. The histological characteristics of the epitheliochorial placenta in ruminants limits the permeability and transport of immune molecules, including globulins, between the dams and their developing fetuses[65].

Passively transferred antibodies through colostrum has been demonstrated to have a positive effect on preventing enteric diseases in newborn calves. The transfer of monoclonal antibodies has been described to have a protective function against reinfections with *Rotavirus* and other enteric pathogens[66]. Al-Alo et al. (2018) reported a positive correlation between maternally derived antibodies and the immune protection against calf diarrhea. They concluded that the ingestion of maternally derived components such as cells, cytokines, and immunoglobulins might contribute to calf immunity and protection against NCD[67]. Other molecules with immunomodulatory and antimicrobial properties present in colostrum, such as iron, lactoferrin, and proteins of the complement system, also have multiple immunological benefits[68,69].

The absorptive capacity of the calf's lumen depends on age. Soon after birth, the modification of intestinal tissue changes the ability of newborn calf' enterocytes to absorb macromolecules such as immunoglobulins[70]. The marked increment of intestinal tissue growth and epithelial remodeling during the first two days after birth leads to the loss of epithelial cells absorption of immunoglobulins. When colostrum containing IgG is ingested immediately after calving, the immunoglobulins are absorbed through the small intestine and transferred to calf's circulation contributing to the mucosal and systemic immune response[71,72]. Given that neonatal calf diarrhea is of rapid onset and occurs in most cases before the calf's development of antibodies, adequate amounts of colostrum-transferred antibodies must be administered during the first hours of life[67,73,74].

Mammary gland development in heifers, as well as regeneration and redevelopment in multiparous animals, begins several weeks before calving, while colostrum formation occurs a few days before calving. After calving, the secretion of the enriched mammary secretion starts. This secretion is plentiful of immunoglobulins IgG1 and IgG2, where the former accounts for 75%. However, the high concentration of IgG in colostrum decreases soon after parturition[70]. The high IgG degree of specificity and affinity makes it be considered as a critical factor in passive immunity. It performs essential effector functions, which include neutralization of antigens and activation of complement[18].

IgG concentration in colostrum fluctuates according to several factors [75]. Gulliksen et al. (2008) reported significant differences in the levels of IgG depending on parity. They found that cows in their four parity have considerably higher amounts of IgG in serum than those in the first (heifers), second, and third parity[61,75]. Moore et al. (2005) reported a drastic decline of IgG two hours after parturition[76]. Similarly, Conneely et al. (2013) reported an IgG reduction of 86% 6 to 9 hours after calving[61]. The USDA standard guidelines to reduce the impact of immunoglobulins drastic reduction and the short period of calves' intestinal absorption recommend fed calves within two hours after birth with a concentration of 50g/L of IgG, with a minimum of 100g in total, which represents approximately 3.8 liters of colostrum [7,77]. In comparison with cows, heifers have lower colostrum quality, making it difficult to achieve the recommended concentration of immunoglobulins (>50g/L of IgG) in colostrum[2,75]. This factor contributes to the failure of passive transfer in calves and increment of disease susceptibility. Vaccination on critical groups, such as pregnant heifers, can positively enhance immunity and quality of the colostrum pools and therefore reduce the likelihood of disease development in young calves.

Vaccination

The worldwide presence of BCoV and BRV, along with the environmental conditions, weather-related factors, and herd management, have led to the high prevalence of these viral infections and their negative impact on the livestock industry. BCoV infection results in a high incidence of diarrhea-related deaths in newborn calves and mild disease in adult calves and cows. It is present in more than 90% of unvaccinated cattle[3]. The 90% of incidence and the ubiquitous characteristics of BCoV make cattle very likely to be diagnosed with this virus[35]. As a result, a reliable method for acquiring active and passive immunity is required. The NCD disease prevention and control depend on several factors, including adequate nutrition, proper peripartum management, reduction of overcrowded and contaminated environments, and effective immunity through vaccination[2].

Vaccine antigens stimulate the immune system inducing neutralizing antibodies and cell-mediated immunity, protecting against specific pathogens [5]. Viral infections by BRV and BCoV have a notable health

impact on livestock, which makes it necessary to provide pregnant cows with protective immunity and the ability to produce high-quality colostrum for their calves [53]. The USDA, dairy cattle management practices report indicates that during 2013, 73.8% of all operations administered vaccines to cows, but only 49.3% of all operations vaccinate preweaned heifers against any disease. Vaccination against *Bovine Rotavirus* accounts only for a 13.4% of vaccinated heifers[77].

Several studies have reported the positive effects of vaccination against NCD[5,53,64,78]. Experimental trials evaluating the role of the immunization route in the generation of protective immunity against mucosal pathogens have reported important findings. Comparing mucosal (oral, intranasal) and systemic vaccines, Belyakov et al. (2009) concluded that mucosal vaccines might be a more effective inducing humoral and cell-mediated response, preventing and controlling gastrointestinal infections. However, only a few mucosal vaccines are commercially available[79]. Boileau and Kapil (2010) recommended vaccination of pregnant cows as a strategy to prevent BCoV enteric diseases[35].

The associations between the vaccine antigen concentration and the induced immune response have also been studied. Crouch et al. (2000) evaluated different antigen concentrations to detect the most reliable dose capable of producing a significant increase in antibody titers against BCoV. The authors reported that a single vaccine dose was capable of increasing neutralizing antibodies titers with a considerable duration of protection in cattle. In that study, different antigen units were used (vaccines: A: 7.6 antigen units, B: 38 antigen units, C: 190 antigen units) to detect the most reliable dose capable of significantly increasing the titers against BCoV[5]. Those animals treated with ≥ 38 units showed an increment in BCoV titers, and those with 190 antigen units had more extended protection. The same authors evaluated in 2001 the serological and colostral responses to a combinational antigen vaccine (rotavirus serotypes G6, P5, coronavirus, and E. coli K99, containing aluminum hydroxide as adjuvant). They concluded that a single dose in pregnant cows significantly improved the levels of passive transference in calves for at least four weeks[64].

The use of distinct adjuvants plays a critical role in the antigen uptake, and its composition seems to have comparative differences[79]. Durel et al. (2017) evaluated two NCD similar vaccines containing

different adjuvants. They suggested that squalene and squalane adjuvants may stimulate the immune system more efficiently in comparison with aluminum hydroxide and mineral oil. In the same study, cows that were previously vaccinated were revaccinated with the same or different NCD vaccine. The study concluded that both vaccines significantly increased the antibody titers at day 21 post vaccination[78].

Several vaccines against BRV and BCoV are commercially available, including Scourguard®, Rotavec k99®, Guardian®, Scour Bos™ 9, among others. Technology and knowledge have made possible manufacturing vaccines using diverse components of the viral particle. Vaccination of pregnant cows and heifers with the subsequent passive transfer of antibodies to their calves have been amply recommended. In some cases, such as the rotavirus vaccines, they can confer immunity to serotypes not included in the vaccine[53]. On the other hand, active immunity through oral administered vaccines offers protection to the small intestine in susceptible calves, although studies had reported poor efficacy[80,81]. De Leeuw et al. (1980) experimentally evaluated commercial oral vaccines in calves. A small group deprived of colostrum was vaccinated with a commercial vaccine and challenged with rotavirus isolated and purified from feces. In a larger field trial, calves were feed with pooled colostrum, vaccinated with a commercial modified-live virus vaccine, and the control group received a saline placebo. They concluded that in the colostrum deprived group, a lapse of time is needed for a full immune response. No differences between colostrum-fed vaccinated, and the placebo group was observed. They suggested that maternally derived antibodies might neutralize the vaccine and that vaccination in newborn calf might not be needed when colostrum is fed[80]. Similarly, Acres and Radostits (1976) evaluated the efficacy of a commercial modified live Reo-like vaccine (MLRV). In that study, the calves were orally vaccinated, fecal samples were analyzed for Coronavirus and Reo-like viruses. They concluded that the MLRV vaccination did not result in a significant difference between the studied groups and that the efficacy of this vaccine might vary among herds. Likewise, De Leeuw (1980) suggested that maternally derived antibodies might neutralize the vaccine[81]. Other researchers suggested that determining the appropriate vaccine route, type, and schedule can

maximize the vaccine response and minimized the immunosuppression and the maternal antibodies interference[82].

Conditions such as poor nutrition, interference by maternal antibodies, overwhelming by multiple antigens administration, and immune suppression might influence the animal response to vaccines. Other factors associated with antigen and vaccine manipulation, such as differences in circulating serotypes and vaccine serotypes, immunogenicity, duration of immunity, improper management manufacturer directions, or administration, may also affect the animal response to vaccines[83].

The secretion of immunoglobulins in colostrum and milk and its subsequent benefit for calves can be achieved through the establishment of adequate vaccination protocols. Crouch et al. (2001) reported that the antibodies elicited by vaccination and posteriorly positive passively transferred (PPT) to calves through colostrum were able to increase the level of specific antibodies in calves serum[64]. While in the 1991-1992 period, the USDA reported 59% of PPT and 41% of failure in passive transfer (FPT); in 2007, the same organization reported 80.8% of PPT and 19.2% of FPT in calves. This report showed that producers were aware of the importance of PT in heifers' general performance[7].

Oral vaccines, such as Calfguard®, are designed to provide active immunity to calves. However, in newborn calves, the lapse of time necessary for the immune system to produce enough vaccine-elicited antibodies impede the effective action of the vaccines at the time of more NCD susceptibility. The lack of immunity during this susceptibility window is supplied by the IgG antibodies present in the mammary gland secretion, which is absorbed through the calves' intestinal mucosa and transferred to the bloodstream[1,84].

The large number of cases associated with BCoV and BRV, the peak of occurrence during the nursing period, and the economic loses[85] make it essential to evaluate the current vaccination cost-effectiveness and promote the implementation of effective immunization protocols that can substantially help to prevent the impact of NCD in dairy and beef farms and cattle industry profitability. Dam's nutrition during the late stages of gestation may have a significant impact on the immune response to vaccination and efficacy on protection. The nutritional status may be improved by the administration of supplements

formulated to meet trace minerals and vitamin needs. A novel approach of supplementation of trace minerals in combination with vaccination has been proposed as a promising method to enhance the immune response after vaccination of pregnant cows, promoting a significant decrease in the herd's disease incidence[11,16].

Trace Minerals

Minerals are essential nutrients in cattle health, growth, and reproduction. Trace minerals (TM) are elements present in the body in low concentrations, usually micrograms or milligrams. Some of the most crucial TM for dairy cattle comprises copper, selenium, zinc, manganese, cobalt, iron, iodine, molybdenum, fluorine, and chromium[14]. They are essential in health and productivity and play a critical role in numerous physiologic and biochemical processes[86]

Trace minerals have multiple benefits increasing the levels of antioxidant enzymes and reducing the oxidative processes[87]. Trace minerals are involved in numerous biological processes, including enzymatic activities, hormones synthesis, reactive oxygen species (ROS) balance, transportation of oxygen to tissues, energy production, and growth and reproductive functions. The National Academies of Sciences, Engineering, and Medicine reported the nutrients requirements in dairy cattle. The list included 10 trace minerals (copper, cobalt, iron, iodine, molybdenum, manganese, zinc, selenium, chromium, and fluorine)[14] (Table 2.1). However, copper, selenium, manganese, and zinc are recognized as some of the most deficient is grazing cattle[88]

Table 2.1. Microminerals Requirements for a dry cow.

Micromineral mg/kg	Days pregnant		
	240	270	279
Selenium	0.3	0.3	0.3
Manganese	16	18	24
Zinc	21	22	30
Copper	12	13	18

Even though forage (e.g., silage, baleage, haylage) is the most common source of TM for cattle, other alternative sources of oral supplementation, such as energy and salt blocks fortified with minerals, are commercially available[12]. Injectable supplements of TM have the advantage over oral supplementation of administering a controlled and known amount of minerals.

Injectable trace minerals (ITM) produced by Multimin USA, Inc is an aqueous supplement formulated to meet the requirements of TM in cattle when used strategically on critical points during cattle management. This injection complements the grass-fed based diet (which usually has inadequate TM concentration), and oral mineral supplementation. This injectable microminerals formulation contains zinc 60mg/ml, copper 15mg/ml, selenium 5mg/ml and manganese 10mg/ml.

Selenium (Se) is a fundamental element in cattle nutrition. It is essential for the proper function of the Glutathione peroxidase enzyme (GSH-Px). GSH-Px has a protective activity preventing cells from the damage caused by reactive oxygen (ROS) species produced during phagocytosis[14,89]. It is also involved in neutrophils migration and reduction of inflammation, particularly in dairy cows, decreasing the incidence of subclinical and clinical mastitis[89]. Deficiencies of selenium are associated with a decrease in milk production and health impairment. According to Oltramari et al. (2014), dairy cattle supplemented with organic or inorganic Se did not show differences in milk production and quality. Both forms, organic and inorganic, were able to reduce the somatic cell counts in milk[89]. This report was also consistent with Machado et al[90], who found a similar association between ITM and a reduced incidence of mastitis. However, their association was only in multiparous cows[90]. Due to the wide variation of Se concentration in grass-feeding in comparatively short distances, supplementary organic or inorganic forms in a 0.1-0.3 mg/kg DM dose are recommended to meet the requirements[89].

Copper (Cu) is a critical part of cytochrome oxidase, which has multiple enzymatic activities. It functions as an antioxidant necessary to prevent DNA damage, immune system suppression, myocardial atrophy, bones, and hair alterations[91,92]. Mattioli et al. (2018) reported the effects of Cu improving weight gain and antibody titers after infection with *Bovine herpes virus-1* (BoHV-1)[93]. The deficiency of Cu was

correlated with a reduction of neutralizing antibodies against BoHV-1. Copper deficiencies might be responsible for impairing the neutrophils' response to intracellular pathogens[91]. Moreover, Cu supplementation helps cattle to reach normal concentrations of ceruloplasmin, which is vital for the absorption and transport of iron to form hemoglobin[92]. It is also an important marker of acute inflammatory stress[86].

Clinical manifestations, including alteration in osteoarticular tissue, cardiovascular and enteric diseases, poor weight gain, and poor innate and acquired immune responses, were associated with copper and zinc deficiencies[93]. Hair depigmentation, predominantly around the eyes, is a typical sign of copper deficiency as well as scours, which is a distinctive sign of cooper deficiency in rimminants[14]. Several countries, including the United States and Canada, have reported low Cu concentrations in forages, limiting its availability in grass-feeding diets[91].

Manganese (Mn) is a vital cofactor in many enzymatic processes; it may also help in glucose and insulin regulation during inflammatory processes[86]. During the immune response, phagocytes produce reactive oxygen molecules; these molecules are highly toxic to other cells. However, different enzymes control these toxic compounds to reduce cell damage. Among them, catalase, glutathione peroxidase, and superoxide dismutase (SOD)[94]. Manganese superoxide dismutase (Mn-SOD) contained in the mitochondria mediates the catalysis of superoxide anion leading to better control of oxidation and cellular damage[95].

Zinc (Zn) is an essential constituent of thymosin, a vital hormone for the regulation of cell-mediated immunity[92]. It is critical for animal health and performance during stress conditions and immune deficiencies. It is also recognized by its vital role in enzymatic processes in DNA replication and transcription and zinc-dependent signaling pathways[86,91]. Copper-zinc SOD present in the cytosol is important in oxygen metabolites detoxification[94]. Low concentrations of Zn in plasma may be associated with inflammatory reactions, hormone imbalance, and stress[93]. Supplementation with Zn has been reported to reduce somatic cell counts and prevent mastitis in dairy cows[96].

Nutritional requirements during the transition period (approx. 21 days pre-calving and 21 days post-calving) in dairy cows regularly surpass the forage/feed-based nutrients value. The priority in which cattle use minerals during this critical period can alter their availability for other processes. The reduction of TM bioavailability leads to subclinical deficiencies that might alter the immune system functions[88,97,98]. Pedernera et al. (2010) observed that the peripartum period increases the energy demands leading to a negative energy balance and increment of oxidative stress, probably due to antioxidants reduction during early lactation[99]. Critical physiological periods such as stress, fetal growth and colostrum, and milk production may result in an increasing necessity of minerals supplementation and other nutrients[90].

Although cattle can acquire some of these minerals from forage-based nutrition, the minerals content might vary in each pasture. Factors such as soil characteristics, plant species, weather conditions, and fertilization can affect the quality and mineral composition in the forage[92,97]. McDowell et al.[100] revealed that copper, zinc, selenium, among others, are the minerals most commonly deficient in ruminants grazing. Therefore, if the feed is not tested, it is impossible to predict the consumed mineral quantities. In cases in which dietary requirements are not met, it is necessary to implement adequate mineral supplementation.

Several studies have demonstrated the positive effects of injectable TM in the humoral response[11,12,16]. Administration of injectable Se, Zn, Cu and Mn concurrent with respiratory virus vaccination in dairy and beef cattle was associated with earlier and increased antibody titers and leukocyte proliferation upon stimulation with BRD pathogens, improved mucosal respiratory protection and mitigation of the reduction of $CD4^+$ and $CD8^+$ T lymphocytes after BVDV2 and BHV1 challenge dairy and beef calves. [11,12,16] Other studies reported the beneficial effects ITM concurrent with vaccination in cattle, elevating the concentrations of the antioxidant enzymes in plasma[95] and increasing the levels of neutralizing antibodies against respiratory viruses and bacteria[11].

These studies suggest that implementing vaccination concurrent with strategic TM supplementation could result in stronger immunity, which is further passively transferred to the calf through the colostrum [11,16,90,101]. The effects of TM concomitant with a NCD killed virus vaccine on the induction of antibodies to BCoV and BRV have not been characterized yet and are the subject of this research.

CHAPTER 3

EFFECT OF INJECTABLE TRACE MINERALS ADMINISTERED CONCURRENTLY WITH A NEONATAL CALF DIARRHEA VACCINE ON THE IMMUNE RESPONSE AGAINST BOVINE CORONAVIRUS AND BOVINE ROTAVIRUS IN DAIRY CATTLE

Introduction

Neonatal calf diarrhea (NCD) is a major disease that has a significant impact on calves' health as well as the economy of the cattle industry. This disease is responsible for more than 50% of pre-weaning heifer calves' deaths in The United States.[1,17] Among the pathogens most frequently involved causing NCD in calves are *Bovine rotavirus* (BRV) group A, *Bovine coronavirus* (BCoV), *Escherichia coli*, *Cryptosporidium parvum, Clostridium perfringens*, and *Salmonella spp*[2].

Bovine rotavirus and *Bovine coronavirus* are the most important viral pathogens associated with acute diarrhea in newborn calves worldwide. Fecal contamination is usually the principal source of BRV and BCoV infections. Consequently, the intestinal epithelial cells are infected and used as a site for viral replication. As these viruses replicate, they induce damage to the mature enterocytes with disruption of the intestinal epithelia[35]. Loss of villi epithelium causes imbalances in the intestinal fluids and nutrients absorption leading to malabsorption and maldigestion[3] with muco-hemorrhagic diarrhea and dehydration.

Prevention and control of BCoV and BRV infections are mainly focused on biosecurity and biocontainment protocols (i.e., sanitation of contaminated areas) to control the infection risks, vaccination of cows, and passive transfer of maternal immunity to the calves through the colostrum.

Protective immunity conferred by colostral neutralizing antibodies requires an adequate humoral immune response after vaccination of pregnant cows, immunoglobulins transportation to the colostrum,

and appropriate passive transfer to the susceptible calves[79]. The effects of the neutralizing antibodies against enteropathogens in dairy cows have been previously assessed after vaccination[5,64,102]. These studies demonstrated that vaccination of pregnant cows could result in the production of colostrum pools with a high concentration of pathogen-specific IgG and cells, resulting in the continuous presence and appropriate levels of antibodies in the calves' intestinal lumen. This lactogenic protection is necessary until calves produce age-dependent specific antibodies[5,64,102].

In comparison with mature cows, heifers produce a lower amount of colostrum with lower immunoglobulin concentration, making it difficult to achieve the proposed concentration by USDA (>50g/L of IgG)[2,7,75]. This factor may contribute to the failure of passive transfer in some calves and increase their disease susceptibility. Management tools that improve the immune response to NCD vaccination and the colostrum immunological quality, especially in first calving cows, would be of significant value for dairy herds' health programs reducing the likelihood of NCD occurrence in young calves.

Adequate trace minerals supplementation is essential for an optimal immune function and health in cattle[8,9,103–105]. Several studies have demonstrated the beneficial effects of injectable trace minerals (selenium, zinc, copper, and manganese), on the immune response to stimulate proinflammatory cytokines, and to modulate the oxidative stress in cattle, increasing levels of ceruloplasmin and haptoglobin[11,16,87]. Arthington et al. (2012) reported that ITM administration increased vaccine-induced SNA against BHV-1, BVDV-1, and BVDV-2 in seronegative steers[106]. Likewise, subsequent studies demonstrated that administration of ITM in conjunction with modified-live virus (MLV) vaccination was associated with an increased antibodies titers to BVDV-1 on day 28 post-vaccination, enhanced peripheral blood mononuclear cell proliferation upon stimulation with BVDV-1 and BRSV, and further mitigation of the CD4, CD8 T lymphocyte decay after BVDV-2 infection in dairy and beef calves[11,16].

Given the available data indicating that ITM enhances the immune response to BRD virus-bacteria vaccination, studies to determine the effects of the concomitant use of ITM with inactivated BCoV-BRV-

E. coli vaccines on immune protection against these pathogens are warranted. We hypothesized that the ITM administration concurrent with NCD vaccination enhances the serum and colostral neutralizing antibodies to BCoV and colostral neutralizing antibodies titers to BRV in pregnant cattle vaccinated 6 to 7 weeks before parturition. We also hypothesized that dairy calves fed with colostrum from cows treated with ITM have greater levels of maternally derived neutralizing antibodies against BCoV than calves receiving colostrum from control cows/heifers. Therefore, the objectives of this study were: 1.To evaluate the effect of ITM administration concurrent with NCD vaccination on the serum neutralizing titers to BCoV, and the neutralizing antibody titers to BCoV and BRV in colostrum and milk of dairy cows and heifers vaccinated 6 to 7 weeks before parturition. 2. To compare the level of BCoV antibody titers in dairy calves fed colostrum from cows/heifers treated with either ITM or control (saline) at the time of NCD vaccination

Serological techniques can be used to determine vaccine-elicited antibody titers[107,108]. Virus Neutralization is a specific and sensitive method that allows detecting functional antibodies capable of neutralizing the virus infectivity. It is commonly used to quantify neutralizing antibodies present in serum samples after vaccination[107]. Even though this technique can be used to determine the effectiveness of NCD vaccination in farms with high risk of NCD, tests for determination of SNA to BCoV and BRV are not commercially available in USA. Therefore, in the present study, we validated a reliable protocol for the determination of SNA titers against BCoV in dairy cows and calves.

Materials and methods

Experimental design, vaccination, and treatment

The study was performed at the University of Georgia Teaching Dairy Farm from January 2019 to December 2019. The study was done following a protocol approved by the University of Georgia, Institutional Animal Care and Use Committee (UGA-AUP#A2018 10-020-Y2-A3) Forty-eight Holstein pregnant cattle (21 cows and 27 heifers) were enrolled in this study when they had approximately seven months of pregnancy. Animals were confirmed to be pregnant (approximately 210 days of gestation) by transrectal palpation. The cows were induced to end their lactation (dry-off) within five days before

enrollment. Animals were progressively included in the study during a year until completing the sample size. Cows and heifers were vaccinated intramuscularly (IM) with a 2 mL dose of a commercial inactivated multivalent vaccine containing *Bovine rotavirus* (BRV; serotypes G6 and G10), *Bovine coronavirus* (BCoV), and enterotoxigenic *Escherichia coli* K99 pili adherence factor (ScourGuard® 4K, Zoetis Animal Health) 6-7 weeks before calving following manufacturer's recommendations. Animals were randomly assigned to one of two treatment groups using an electronic randomizer (www.randomlist.com), as follows: 1. ITM (n= 26; 14 heifers and 12 cows): Animals were administered injectable trace minerals (1ml/200lb BW for cows and 1ml/150 BW for heifers; Multimin® 90, Multimin USA Inc, Fort Collins, CO) subcutaneously (SC). 2. Control (n= 22; 13 heifers and 9 cows): Animals were SC injected with sterile saline (1 mL/200 Lb). Administration of ITM provided 15, 60, 10, and 5 mg/mL of Cu, Zn, Mn, and Se, respectively. In addition, animals were immunized with a vaccine containing inactivated *E. coli* and *Salmonella typhimurium* for prevention of mastitis. Three weeks after the initial vaccination and trace mineral injection, the animals received a booster of the same NCD vaccine (ScourGuard®) and a second dose of ITM or sterile saline according to the treatment groups. The NCD vaccine and ITM treatment were administered on different sides of the neck. ScourGuard® was administered IM in the right side of the neck, whereas ITM was SC administered on the left side. Animals in both groups comingled in the same pasture of approximately 4 acres. They received Bermuda hay grass, annual winter silage, whole cottonseeds, and water *ad libitum*. In addition, animals received daily supplementation with a total mixed ration (22Lb/TMR) that provided nutritional requirements for pregnant dairy heifers and dry cows, according to the National Research Council (NRC). Approximately three weeks before parturition, the animals were placed in a smaller pasture of about 2 acres, where they were monitored by the farm personnel three times a day. When animals were close to parturition, they were placed in a pen and monitored for signs of parturition using a closed-circuit camera system (ring®). After calving, the calves were immediately separated from their dams to avoid nursing. Cows were milked mechanically using a portable milking machine (Tek Motion Brand-25L buckle) located in the calving facility. Colostrum was obtained and placed in a four-pint bottle for feeding the corresponding calf. In addition, colostrum quality was evaluated using a portable digital Brix

refractometer[23]. Only animals with ≥16 Brix degrees were included in the study. The measurements represent the quantity of Ig contained in colostrum samples and directly determine the colostrum quality[72]. This instrument has been studied and recommended as it exhibits excellent characteristics for the identification of colostrum quality. Its accuracy estimating IgG quantity is not affected by the samples' temperature as it can occur in other techniques[109]. Compared with other methods, refractometry has a higher sensitivity for assessing IgG[110]. Each calf (males and females) received 2.8 L (6 pints) of colostrum from its corresponding dam within 3 hours after birth. Calves were encouraged to feed on the bottle. However, in cases in which calves did not have adequate suckling vigor, colostrum was provided via esophageal tubing. In addition, calves were fed approximately 8-12 hours later with 2L of colostrum from their corresponding dams.

Sample collection

Blood samples were collected in all adult animals via coccygeal venipuncture into vacuum tubes (Vacutainer®, BD Diagnosis, Franklin Lakes, NJ) without anticoagulant. Blood samples were collected on days 0, 14, 21, 35, and at calving for determination of BCoV SNA titers. Colostrum samples were collected on the day of calving, and transition milk samples were collected on days 3 and 7 post-calving for determination of neutralizing antibody titers against BCoV and BRV. Blood samples were also collected from the calves on days 0, 3, and 7 relative to the parturition via jugular venipuncture into vacuum tubes without anticoagulant for determination of BCoV SNA titers. Serum was centrifuged, aliquoted, and frozen at -80°C. Colostrum samples were thoroughly mixed, aliquoted, and frozen at -20°C until processing. Fecal samples from cows and calves were collected before and after calving. The samples were preserved at -80°C for further detection of BCoV and BRV. A veterinarian performed a clinical examination of calves during sampling days. The occurrence of diarrhea, duration, and severity of clinical signs and oral fluid therapy was reported by the farm manager.

Immunological Assays

Virus Neutralization Assay for BCoV

Serum Neutralizing Antibody (SNA) titers against BCoV were determined through a standard virus neutralization test. As the assay was not currently available in the US, it was validated at the University of Georgia, Athens, Veterinary Diagnostic Laboratory. Previously to the testing day, all serum aliquots were thawed and heat-inactivated at 56ºC for 30 minutes. Madin-Darbi Bovine Kidney (MDBK) cells grown in Dulbecco's Modification of Eagle's medium (DMEM) supplemented with a (10%) of Fetal bovine serum (FBS) were used for BCoV Mebus strain. Cells were incubated at 35.5ºC without CO_2. The virus was grown, aliquoted, and the 50% endpoint was calculated. Dilution for serum neutralization test at 100 $TCID_{50}$/25µl was estimated. On the test day, cells were suspended in DMEM with FBS, reaching a final concentration of 125,000 cell/ml. Then, 25µl of heat-inactivated samples were diluted with 25µl of DMEM into a serial 2-fold dilution series, starting at 1:2 in 96-well cell culture plates. 25µL of DMEM was added to each well in the virus control and 50µL of DMEM in cell control wells. Then, 25µL of BCoV 100 $TCID_{50}$ dilution (1:30) was added to each well-containing serum samples, controls, and virus control. Back titration was set by adding 25µL of DMEM, and 25µL of the four tenfold serial dilutions were added to the corresponding well (10^{-1}, 10^{-2},10^{-3}, 10^{-4}). Plates were incubated for 1 hour at 35.5ºC±2ºC in a 1% CO_2. After incubation, 150µl of MDBK cells at 125,000 cells/mL were added to each well. Plates were incubated for seven days at 35.5ºC±2ºC in an atmosphere with a 1% CO_2. After incubation, the 96-well plates were observed under an inverted microscope for the presence or absence of cytopathic effect (Table 3.1). The endpoint titer of the antibody was the highest dilution in which complete neutralization was observed. The results were reported as BCoV serum neutralizing antibody titers. Mebus BCoV positive control and germfree negative control sera were generously provided by Dr. Linda Saif at Ohio State University, College of Veterinary Medicine.

Several authors have described the pattern of CPE produced by Coronavirus infection in different animal species[31–33]. In this study, CPE was characterized by gradual cell enlargement, swelling, and

detachment, followed by a granular appearance and cell membranes fusion resembling syncytia formation (Figure 3.1). The CPE was visually detected under an inverted microscope at 5 to 7 days post-infection when all the plates were observed (Figure 3.1) and compared with the negative control (Figure 3.2).

Colostrum Neutralizing antibodies (CNA) to BCoV and BRV

Colostrum neutralizing antibody titers against BCoV and BRV were determined through a standard virus neutralization test at the Research Technology Innovation (RTI) laboratory, LLC, South Dakota. Briefly, cell lines for each virus were plated before the assay. For rotavirus, MA104 cells were plated 48 hours in advance. For coronavirus, Human rectum adenocarcinoma cell line (HRT) were plated 24 hours before the day of the assay. On the day of the CN testing, serial 1:2 dilutions of colostrum samples were made in MEM media, and an equal volume of the virus was added to the wells at a concentration of approximately 300 $TCID_{50}$. Trypsin was added to the MEM for Rotavirus. The virus was back titrated on a separate plate by using 4 ten-fold dilutions of the working stock to verify the concentration. Positive and negative control sera were run on each plate. After dilutions were made, plates were incubated at 37ºC±2ºC for approximately 1 hour. After incubation, the colostrum/virus mixture was transferred to the plates previously seeded with the cells for each virus. Plates were incubated for approximately 3 hours. After incubation, the colostrum/virus mixture was removed, and cell culture media was added. Plates were incubated at 37ºC±2ºC for 24 hours for Rotavirus, 72 hours for coronavirus until they were fixed and stained.

Serum Radial Immunodiffusion

Serum total IgG antibodies were determined at the University of Georgia, Tifton Veterinary Diagnostic, and investigational laboratory using a commercial Radial Immunodiffusion kit. The kit is intended for the quantification of Bovine IgG in calves. Briefly, on the test day wells containing the antibody/antigen formed a ring, then the precipitating rings were measure and compared with the reference standard.

Statistical analysis

For cows and heifers, the response variables were SNA titers against BCoV on days 0, 14, 21, 35 after vaccination and on calving day; colostrum quality (CQ) at calving; and CNA against BCoV and BRV on days 0, 3 and 7 relative to calving. The response variables considered for calves were BCoV SNA titers and total IgG.

Titers were transformed using the based two logarithm, which accounts for actual laboratory dilutions. Seroconversion was calculated based on the $\log_2$ transformed values as a 4-fold increase in SNA titers relative to the titer at the day of primary vaccination. Although statistical testing was conducted on the transformed values, statistical summaries and figures were presented as GM and 95% confidence intervals of the mean after a back transformation to facilitate inference and interpretation.

Data were analyzed using R version 3.6.2[111]. The statistical analysis started by the visual exploration of distributional properties of variables in order to identify the most suitable statistics to apply, i.e., parametric or non-parametric. Shapiro Wilk test for testing normality was applied as a confirmatory test statistic. Two-independent-sample t-test was applied to compare treatments (ITM and control) when the null hypothesis that the data were normally distributed was not rejected. When the null hypothesis that the data are normally distributed was rejected, the Wilcoxon test was used instead. For all analyses, values of $P \leq 0.05$ were considered significant, and $0.05 < P \leq 0.1$ was considered a tendency.

Results

Serum neutralizing antibodies (SNA) to BCoV in cows and heifers:

Overall, data of BCoV SNA were not normally distributed (Shapiro Wilk test $P < 0.05$; Figure 3.3). There was no consistency in the distributional properties of the data analyzed over time relative to primary vaccination, treatment (ITM and control), and parity condition (heifers and cows). Data were normally distributed on days 0, 14, and 21, but not on day 35 after primary vaccination ($P < 0.05$; Figure 3.3). At calving, data of BCoV SNA titers were normally distributed for the ITM group but not for the control group.

Because of the contrasting distributional properties of the data on different time points, both parametric and non-parametric tests were conducted in all cases.

At vaccination day (day 0), animals in the control and ITM groups had similar SNA titers to BCoV (Figure 3.4). The SNA titers attained their maximum level on day 14 after primary vaccination in both treatment groups with a gradual decline afterward. Heifers in the ITM group tended to have a slightly higher geometric mean (GM) BCoV SNA titers over time than control heifers (P= 0.16; Figure 3.5, Table 3.2). For cows, there were no significant differences in the GM BCoV SNA between treatment groups during the different time points (Table 3.2). However, cows in the ITM group tended to have lower SNA titers than control cows (P= 0.15; Figure 3.5, Table 3.2). Although the GM SNA titers before vaccination (day 0) were significantly lower in heifers compared to cows, on day 14 after vaccination, heifers had a significant increase in SNA titers reaching comparable titers to those for cows. Moreover, the width of 95% confidence intervals (CIs) for the GM BCoV SNA titers across time suggests that titers of heifers were more variable than those for cows.

The percentage of heifers undergoing seroconversion to BCoV on days 21 and 35 relative to day 0 was higher in the ITM group compared with the control group. Control heifers had an increased seroconversion (46%) on day 14 after primary vaccination (Figure 3.6). A small percentage of cows had seroconversion to BCoV after vaccination, without significant differences between treatment groups.

Colostrum quality (CQ)

Density plots (discriminating treatment and parity condition), and the Shapiro Wilk test indicated that CQ values were not normally distributed ($P < 0.05$; Figure 3.7). Primiparous heifers in the ITM group had slightly increased median CQ values compared to control heifers, but without statistical significance ($P > 0.05$). Even though there was no significant difference between groups of heifers, ITM-treated heifers had less variability in CQ compared with the saline-treated heifers. Multiparous cows in the control group had a significantly higher median CQ than cows in the ITM group ($P < 0.05$; Figure 3.8).

Colostrum neutralizing antibodies (CNA) against BCoV and BRV in cows and heifers

Overall, data of colostrum neutralizing antibodies to BCoV on all-time points were not normally distributed (Shapiro Wilk test $P < 0.05$; Figure 3.9, Table 3.3). At calving, CNA titers to BCoV were not normally distributed ($P < 0.05$; Figure 3.9,), whereas data had a normal distribution on days 3 and 7 ($P > 0.05$; Figure 3.9). Animals in the ITM group had higher CNA titers to BCoV than control animals (Figure 3.10). Heifers in the ITM group had a tendency of higher BCoV CNA titers at calving ($P = 0.08$) and on day 3 post-calving ($P = 0.09$) than heifers in the control group (Figure 3.11, Table 3.3). In contrast, cows treated with ITM tended to have lower BCoV CNA titers at calving than control cows ($P = 0.08$).

Colostrum neutralizing antibody titers to BRV were not normally distributed (Figure 3.12). Only at calving, CNA titers to BRV were normally distributed in the ITM group (Figure 3.12). There was no statistical difference in CNA titers to BRV between groups (Figure 3.13, Table 3.4). Saline-treated heifers had numerically higher BRV CNA titers than the heifers in the ITM group on post-calving days 3 (P= 0.08) and 7 (P=0.07). In cows, CNA titers to BRV were not different between groups (Figure 3.14).

Total IgG in calves

Values of total IgG were not normally distributed (Shapiro Wilk test $P < 0.05$; Figure 3.15). There were no statistical differences between calves born to animals treated with ITM or saline ($P > 0.05$; Figure 3.16).

Serum neutralizing antibodies (SNA) to BCoV in calves

The null hypothesis of normality for BCoV SNA titers was not rejected for most of the time points and parity condition ($P > 0.05$, Figure 3.17). Overall, the values of SNA titers to BCoV were slightly higher in calves born to heifers in the ITM group when compared to calves born to cows in the control group (Figure 3.18). Treatment with ITM had no significant effects on the passive transfer of BCoV SNA in calves ($P > 0.05$). Nevertheless, calves from heifers in the ITM group exhibited numerically higher BCoV SNA titers than calves from heifers in the control group (Figure 3.19, Table 3.5). Calves born to cows, regardless of the treatment, had similar BCoV SNA titers.

Discussion

Effective immune response to NCD vaccination that results in an adequate concentration of IgG in colostrum and subsequent passive transfer to calves is a significant critical point to reduce the impact of neonatal diarrhea in young calves. This study focused on the hypothesis that the administration of ITM concurrent with NCD vaccination results in increased BCoV- and BRV-specific antibody titers in dairy cows and heifers and enhanced passive transfer to their calves. There were no significant effects of ITM administration on the immune response to NCD vaccination and the subsequent passive transfer to the calves. However, the results indicated that heifers in the ITM group tended to have enhanced colostrum quality, and higher circulating and colostral BCoV antibody titers than saline-treated heifers, suggesting a potential positive response to ITM administration in primiparous dairy heifers.

In contrast, the use of ITM concurrent with NCD vaccination did not show any benefit on colostrum quality and serum and colostrum virus-specific antibody titers in multiparous cows. In the present study, the multiparous cows previously vaccinated were boosted with a NCD vaccine and treated with ITM at the time of dry-off (end of milking campaign). Factors such as preexisting circulating antibodies, oxidative stress, metabolic and nutritional changes at the end of lactation, and mammary gland inflammation and regeneration may have affected the immune response after vaccination and the role of trace minerals in this group of cows.

Previous studies in dairy and beef calves demonstrated positive effects of ITM administration on the immune response and protection elicited by respiratory modified-live virus vaccination. In those studies, the use of ITM was associated with enhanced SNA antibody production against BVDV1, stronger and faster lymphocyte proliferation upon BVDV and BRSV stimulation, and mitigation of the reduction in CD4 and CD8 T lymphocytes after BVDV2 infection[11,16]. Differences in age and reproductive status (calves versus pregnant females), viral antigens, vaccine type (inactivated versus MLV), and nutritional demands may account for discrepancies between the current study and previous trials.

A remarkable seroconversion against BCoV was observed in heifers but not in multiparous cows. Since cows were vaccinated against NCD on previous lactations, they had high BCoV SNA titers at the beginning of the study. These increased antibody titers might have limited their ability to seroconvert against BCoV after vaccination. Preexisting circulating antibodies induced by previous vaccination and/or prolonged natural exposure might interfere with the humoral immune response following vaccination in multiparous cows. Crouch et al. (2000) suggested that a higher amount of antigens might be needed when preexisting immunoglobulins are present. In that study, the antibody response following vaccination with an inactivated vaccine against BCoV was evaluated in a group of pregnant cows. The cows were initially assessed for preexisting antibodies to BCoV, then classified in descending order and assigned to 4 vaccination groups. Those animals in the group containing the highest level of preexisting antibodies were vaccinated with the lower antigen vaccine dose. No increment of titers was observed in that group, whereas neutralizing antibodies were detected in the groups vaccinated with higher antigen units, suggesting that the antibody response in seropositive animals is dose-dependent.

Overall, the colostrum quality (measured by refractometry) and the colostral BCoV and BRV antibody titers were lower in heifers than cows, suggesting an association between parity and colostrum immunoglobulin concentration (Conneely et al., 2013; Gulliksen et al., 2007). Parity is a parameter that affects mammary function, including colostral and transition milk yield and quality. Primiparous cows are characterized for producing colostrum with a lower concentration of immunoglobulins compared with multiparous cows[112], limiting its use for colostrum pools in dairy farms. Lactogenesis in primiparous cows is different from that in multiparous cow[113]. During the final stage of pregnancy and before calving, the mammary epithelial cells start producing and secreting milk. For multiparous cows, a significant amount of secretory tissue from previous lactations remains, and the process of mammary alveologenesis and duct development is less dramatic than for primiparous cows[113]. Management tools that improve the immune response to NCD vaccination and the colostrum immunological quality of first calving cows would be of significant value for dairy herds' health programs.

In addition, several other factors might affect the concentration of IgG in colostrum, including individual variation among animals, temperature-humidity index, quantification method, presence of high-fat content affecting refraction, differences in homogenization, among others (Conneely et al., 2013; Shivley et al., 2017; Conneely et al., 2012; Godden et al., 2008).

In this study, the BCoV- BRV-specific antibodies induced by the inactivated multivalent vaccine effectively passed from serum to colostrum in both treatment groups. Moreover, the administration of ITM at the time of vaccination tended to increase the colostral antibody concentration against BCoV in primiparous heifers, but not in multiparous cows. The concentration of BCoV- and BRV-specific IgG in colostrum remarkably decreased on post-calving days 3 and 7. This decay in colostrum IgG titers occurs typically during the transition from colostrum to milk[5,112,114]. Similar to the results of the present study, Crouch et al. (2001) observed a significant increment in BCoV and BRV colostral and milk antibody titers after immunization with an inactivated NCD vaccine (*rotavirus* serotype G6 P5, *coronavirus*, and purified *E. coli* K99). The increment of BRV was more notorious in those cows with lower natural exposure-preexisting titers. In general, the titers against both viruses decline during the first week after parturition but were able to be absorbed and detected in the calves' serum[64]. This decay in antibody concentration has been reported to account for 3.7% per hour after calving (Conneely et al., 2013).

Similarly, Mostl et al. (1988) reported intermediate antibody titers in cows previously exposed to BCoV and BRV. They were vaccinated with an experimental oil-based adjuvant vaccine containing inactivated BCoV and BRV. After parturition, calves were fed with their dams'colostrum, challenged with both viruses, and evaluated for diarrhea occurrence. Even though in that study, the viral shedding was low, the authors concluded that the lactogenic immunity was not able to provide enough protection against BCoV and BRV after day 7 post calving[115].

Maternally derived virus-specific IgGs have the ability to bind and neutralize BCoV and BRV involved in NCD. In coronavirus, the spike (S) glycoprotein, which mediates the attachment and virion entry, can be blocked by high-affinity neutralizing antibodies[116]. The blockade of this obligatory docking

site impedes the access to cell machinery, and consequently, no viral replication occurs[107]. The passive transference of high specificity antibodies (IgG1) against BRV through colostrum supplemented with titers of IgG1 of 4096 was reported to provide high protection against the BRV[117]. The vaccination of pregnant cows could result in the production of colostrum with a high concentration of pathogen-specific IgG, cells, and cytokines resulting in appropriate levels of antibodies with immunomodulatory properties in the calves intestinal lumen. This lactogenic protection is necessary for the period of high susceptibility to enteric diseases until calves are able to produce [5,64,102].

In the current study, all calves had a positive passive transfer, demonstrated by high serum IgG concentration 3 days after colostrum intake. The median serum concentration of total IgG at day 3 of age tended to be numerically higher (without statistical difference) in calves born to ITM heifers compared to calves born to control heifers. In contrast, total serum IgG concentration appeared to be numerically lower in calves born to multiparous cows treated with ITM when compared to calves born to saline-treated cows. Calves born to primiparous heifers treated with ITM exhibited numerically higher BCoV SNA titers than calves from heifers in the control group.

In addition to the sanitary conditions of the calving facilities and hutches areas, vaccination against NCD and efficient passive transfer of maternal IgG represent critical points to protect calves from infections during the first days of life. Evaluation of the association between vaccination and risk of NCD indicated that calves from vaccinated dams had a significantly lower risk of diarrhea compared with calves from unvaccinated controls[17,53]. In the study by Meganck et al. (2015), there was a significant decrease in the incidence of diarrhea in the calves fed with colostrum from vaccinated cows (14.3%) compared with control calves (39.7%). Still, there were no effects of vaccination on the length of diarrhea [17].

Despite the lack of statistical significance, the results of the present study showed that heifers that received ITM concurrent with administration of an inactivated multivalent NCD vaccine tended to have greater colostrum quality, and higher circulating and colostral antibody titers against BCoV, compared to vaccinated control heifers. Moreover, calves born to primiparous heifers treated with ITM also tended to

have an enhanced passive transfer of total IgG and greater BCoV-specific SNA titers compared to calves born to control heifers. Altogether, the results may suggest that the use of ITM might represent a tool to improve the colostrum quality of primiparous animals in dairy herds. This study had the limitation that it was performed in the University of Georgia dairy teaching farm with a low sample size (27 heifers and 21 multiparous cows). Further studies, including a greater sample size in a larger dairy herd under commercial management conditions, are required to elucidate the significance of the observed tendencies.

Conclusion

The results of this study showed that the administration of ITM combined with NCD vaccination (Bovine coronavirus, Bovine rotavirus, and *E. coli* K99) in pregnant dairy cattle did not have significant effects on the antibody response against BCoV and BRV. However, pregnant heifers treated with ITM concomitant with NCD vaccination tended to have increased serum and colostrum titers of BCoV-specific antibodies. In addition, calves born to heifer treated with ITM tended to have higher titers of BCoV neutralizing antibodies than calves born to control primiparous heifers. In contrast, this trend was not observed in multiparous cows, in which ITM did not enhance the level of neutralizing antibodies.

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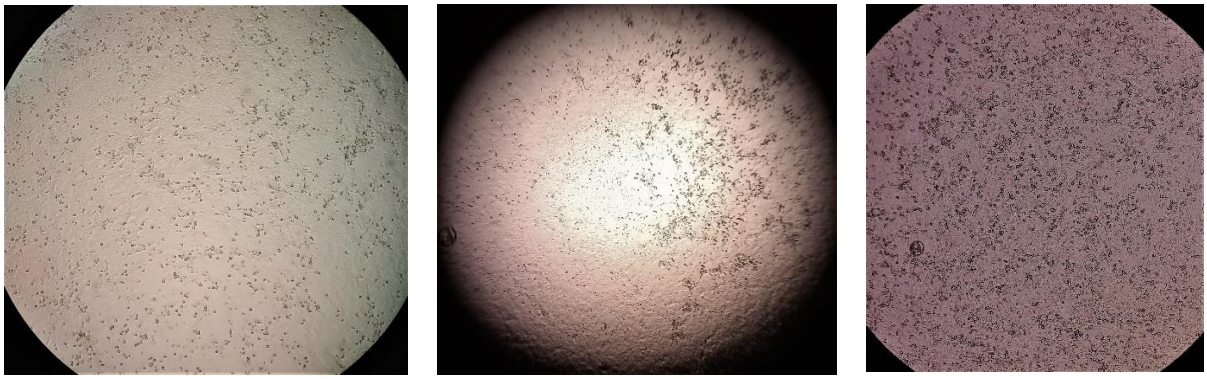

(a) (b) (c)

Figure 3.1. Cytopathic effect of BCoV: (a) low, (b) moderate, and (c) high. Cell enlargement, swelling, and detachment, followed by a granular appearance, and syncytia formation.

Figure 3.2. No cytopathic effect in a 100% confluent MDBK. The cells forming monolayer, no detachments, swelling, and grouping observed.

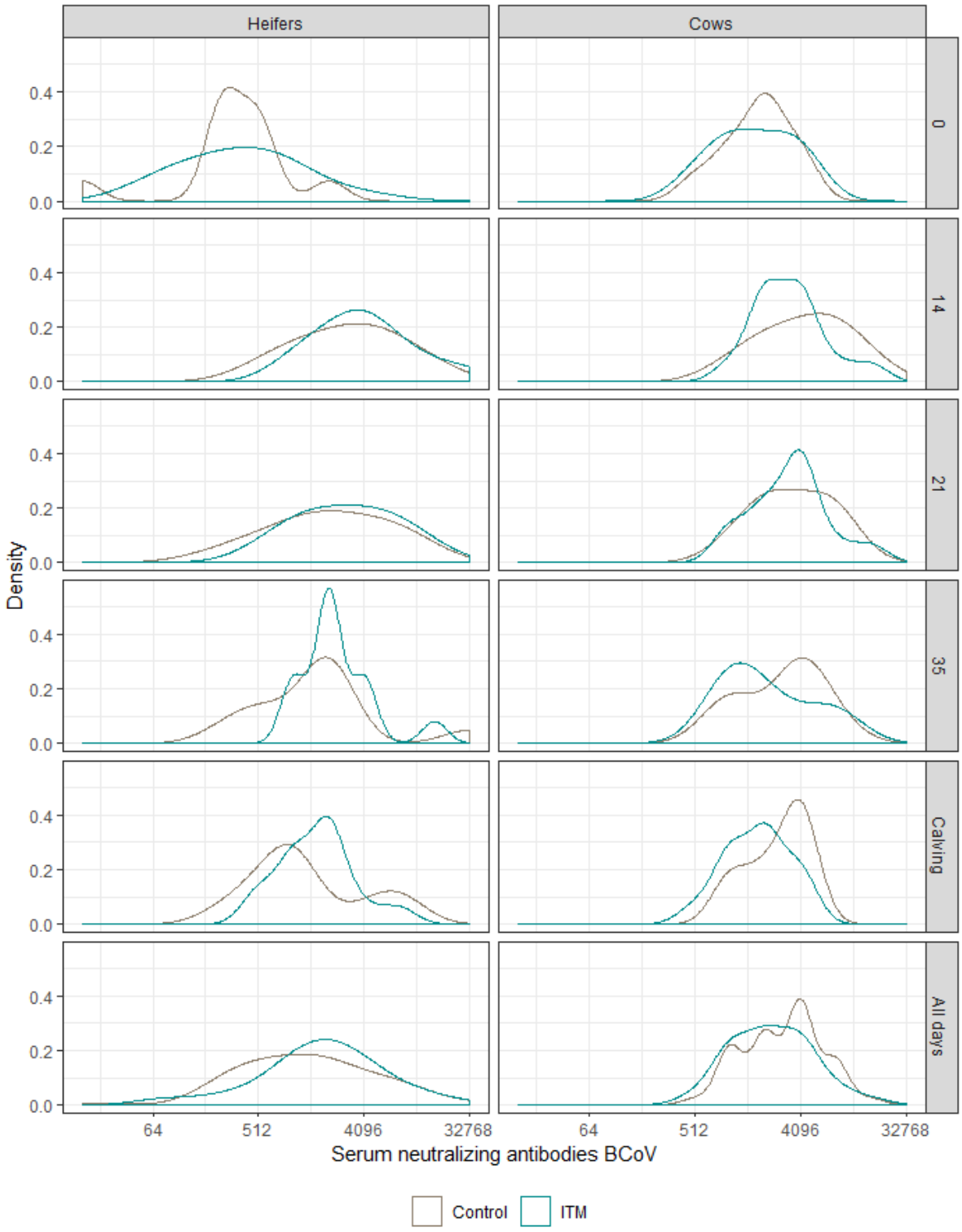

Figure 3.3. Density plot of serum neutralizing antibody (SNA) titers against Bovine coronavirus (BCoV) sort by treatment, days after vaccination, and parity condition. Density is the standardized relative frequency of animals with a particular SNA titer that the area under each curve equals one.

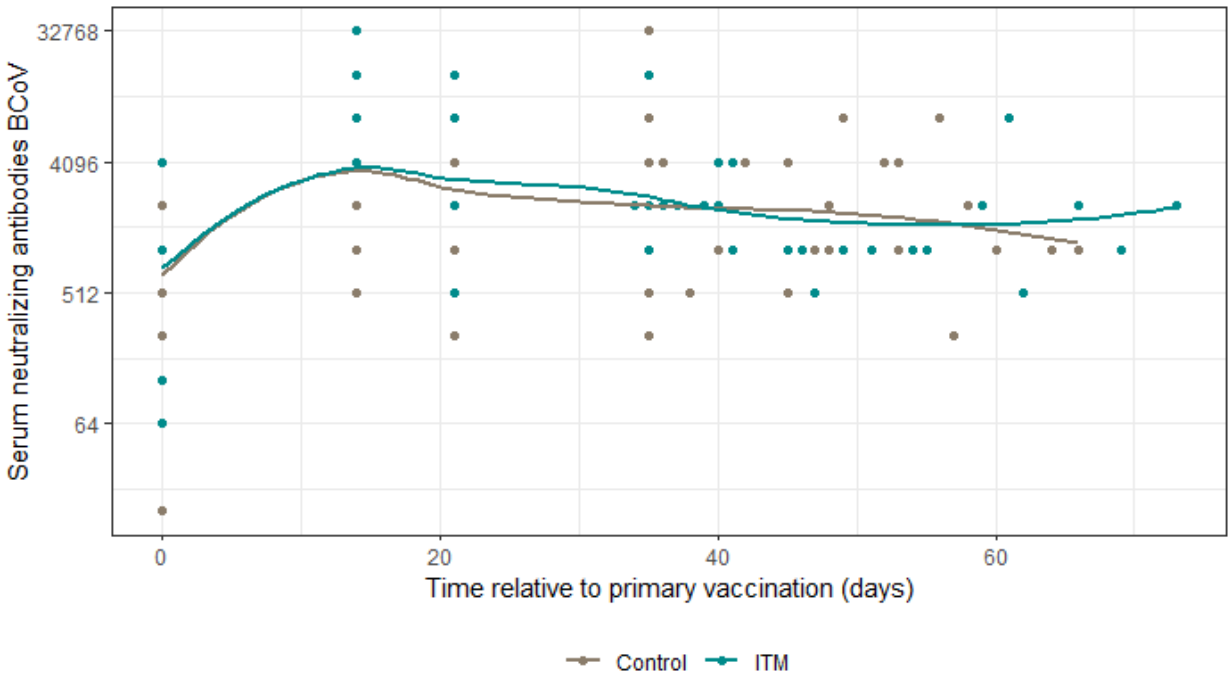

Figure 3.4. Serum neutralizing antibodies to BCoV during post vaccination period. The line is a smooth loess estimation.

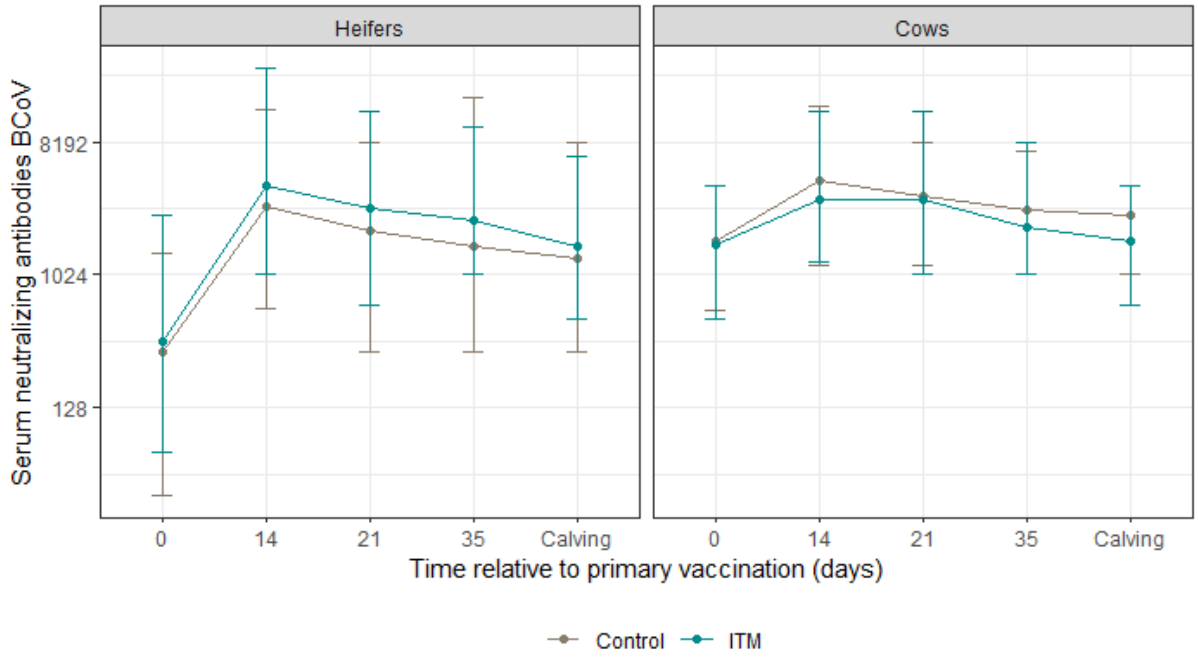

Figure 3.5. Serum neutralizing antibodies against Bovine coronavirus after neonatal calf diarrhea (NCD) vaccination and administration of injectable trace minerals (ITM). The points represent the geometric mean, and the error bars represent the 95% confidence interval of the geometric mean.

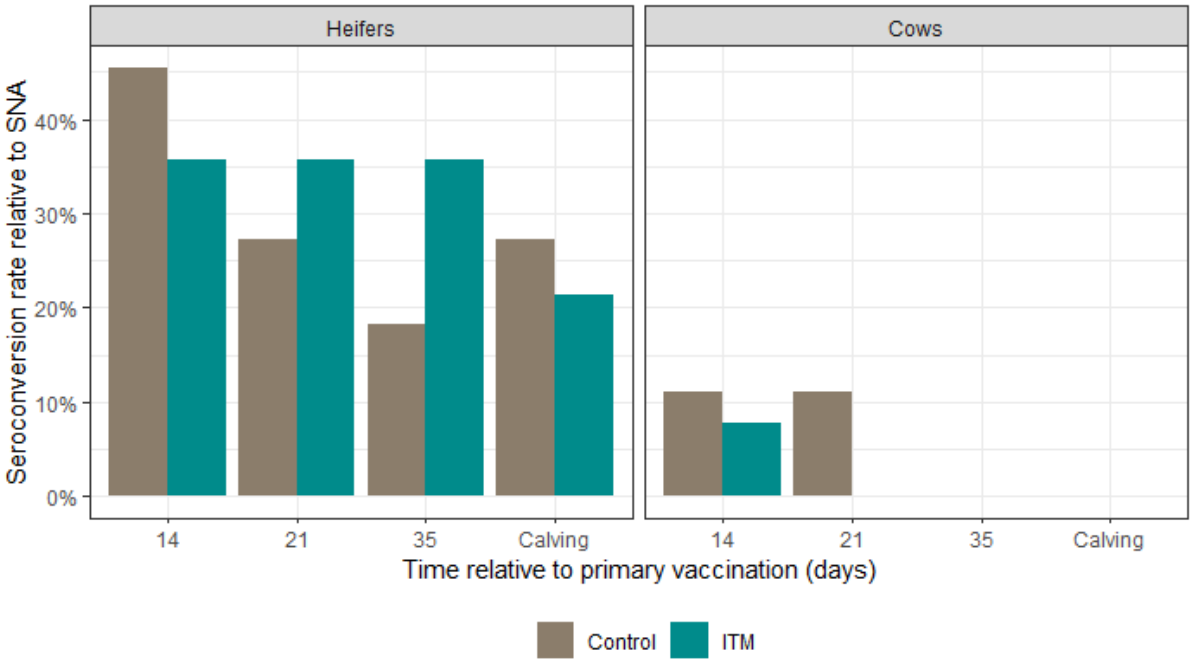

Figure 3.6. Percentage of seroconversion (4-fold increase) against BCoV. Based on serum neutralizing antibodies (SNA) titers against BCoV coronavirus relative to SNA titers at vaccination day.

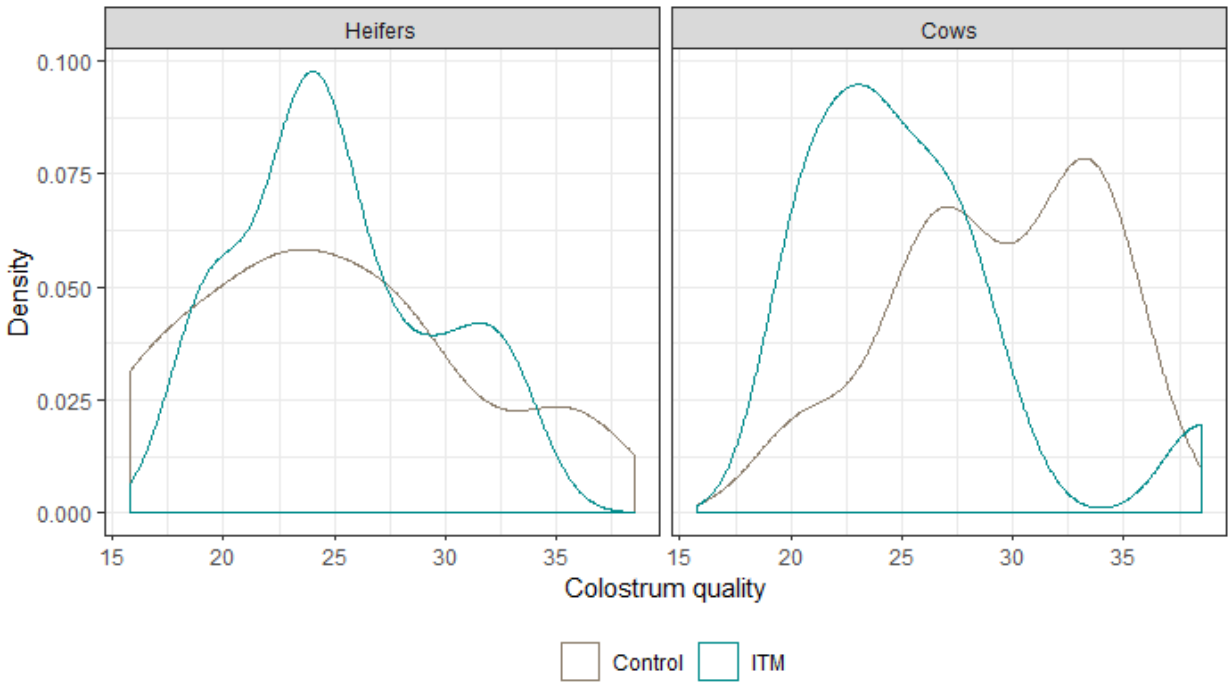

Figure 3.7. Density plot of colostrum quality sort by treatment, days after vaccination, and parity condition. Density is the standardized relative frequency so that the area under each curve equals one.

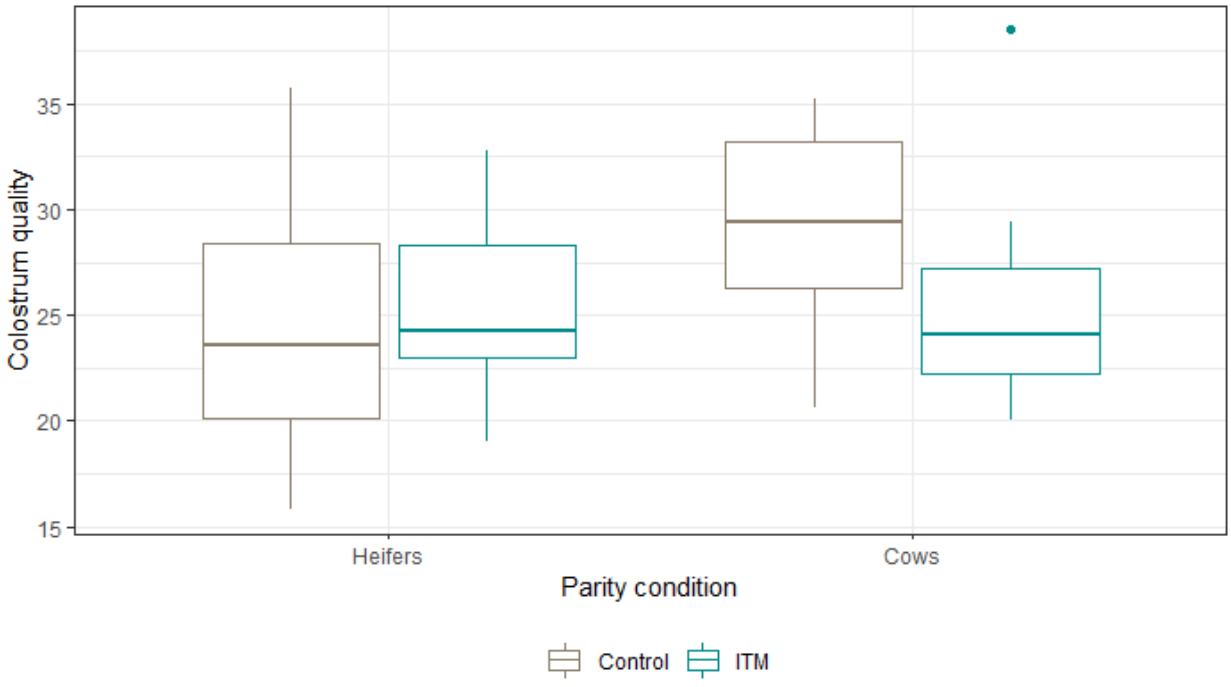

Figure 3.8. Colostrum quality by treatment and parity condition.

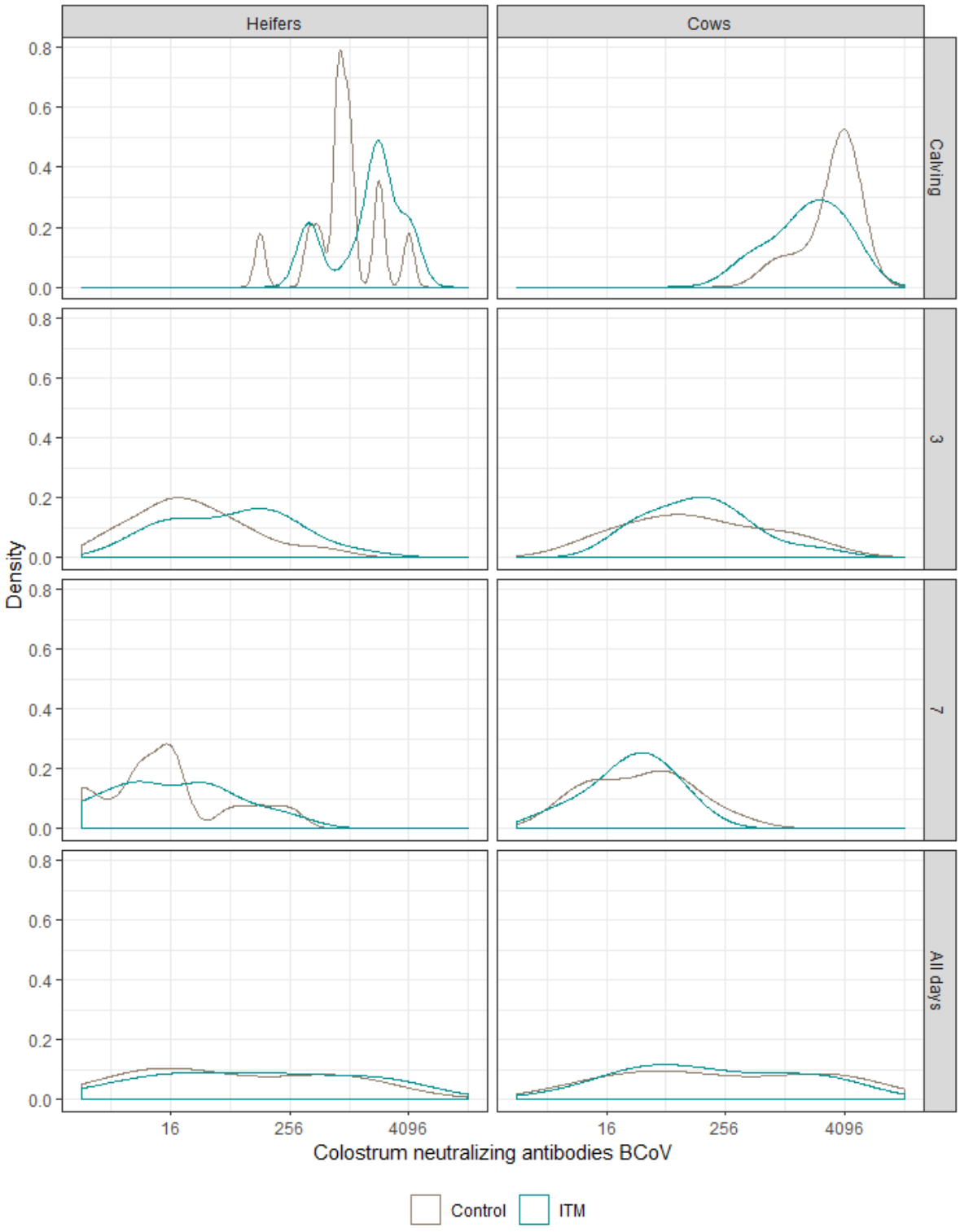

Figure 3.9. Density plot of colostrum neutralizing antibodies (CNA) against Bovine coronavirus (BCoV) sort by treatment, days after vaccination, and parity condition. Density is the standardized relative frequency so that the area under each curve equals one.

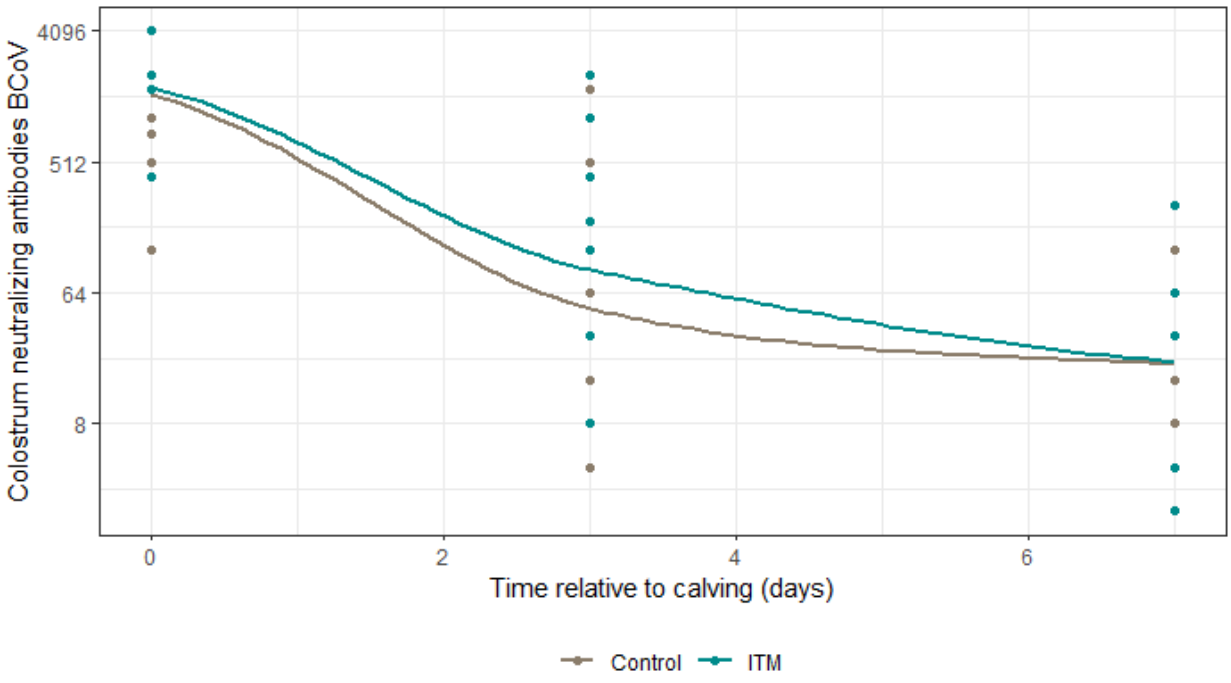

Figure 3.10. Colostrum neutralizing antibodies to BCoV at calving and on days 3 and 7 post calving. The line is a smooth loess estimation.

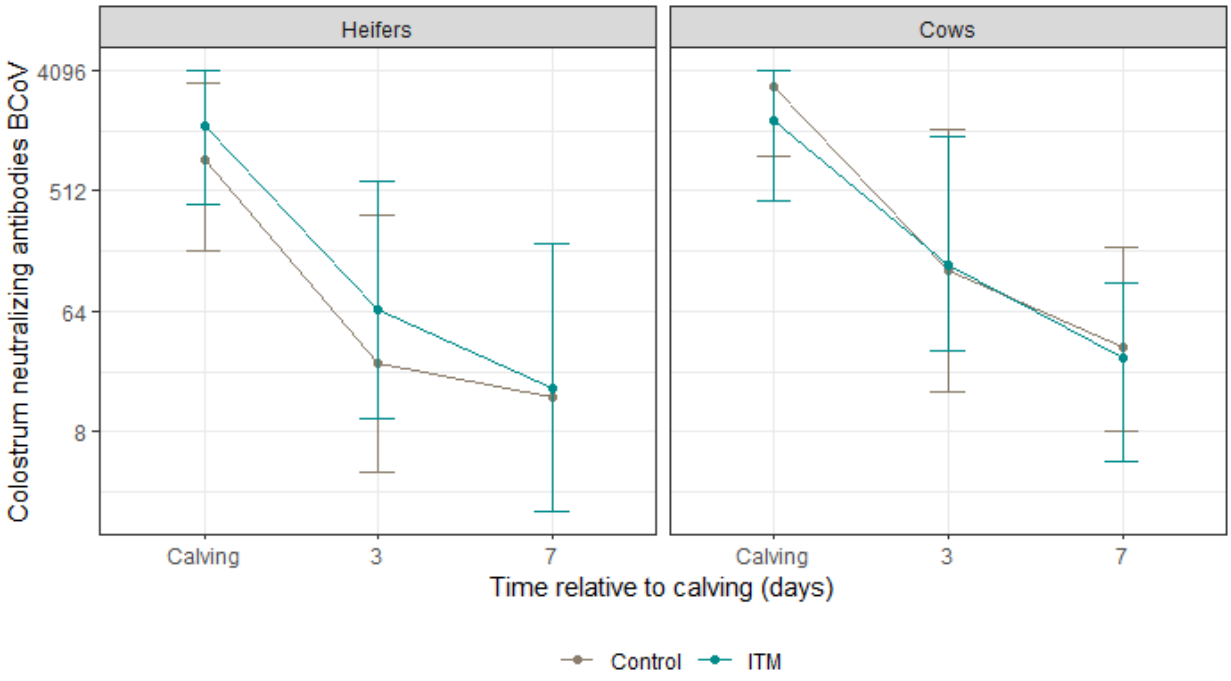

Figure 3.11. Colostrum neutralizing antibodies against Bovine coronavirus after neonatal calf diarrhea (NCD) vaccination and administration of injectable trace minerals (ITM). The points represent the geometric mean and the error bars represent the 95% confidence interval of the geometric mean.

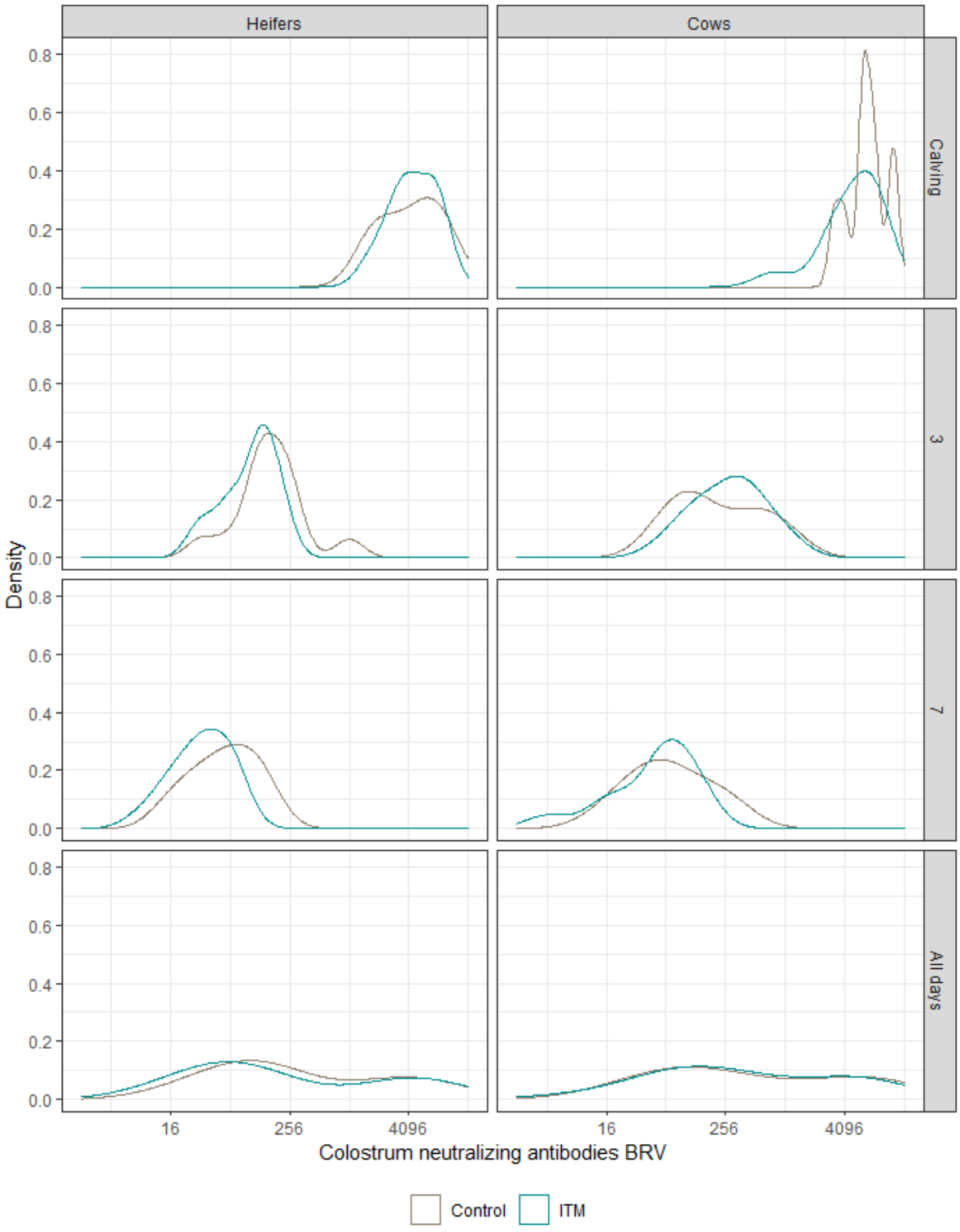

Figure 3.12. Density plot of colostrum neutralizing antibodies (CNA) against Bovine rotavirus (BRV) sort by treatment, days after vaccination, and parity condition. Density is the standardized relative frequency so that the area under each curve equals one.

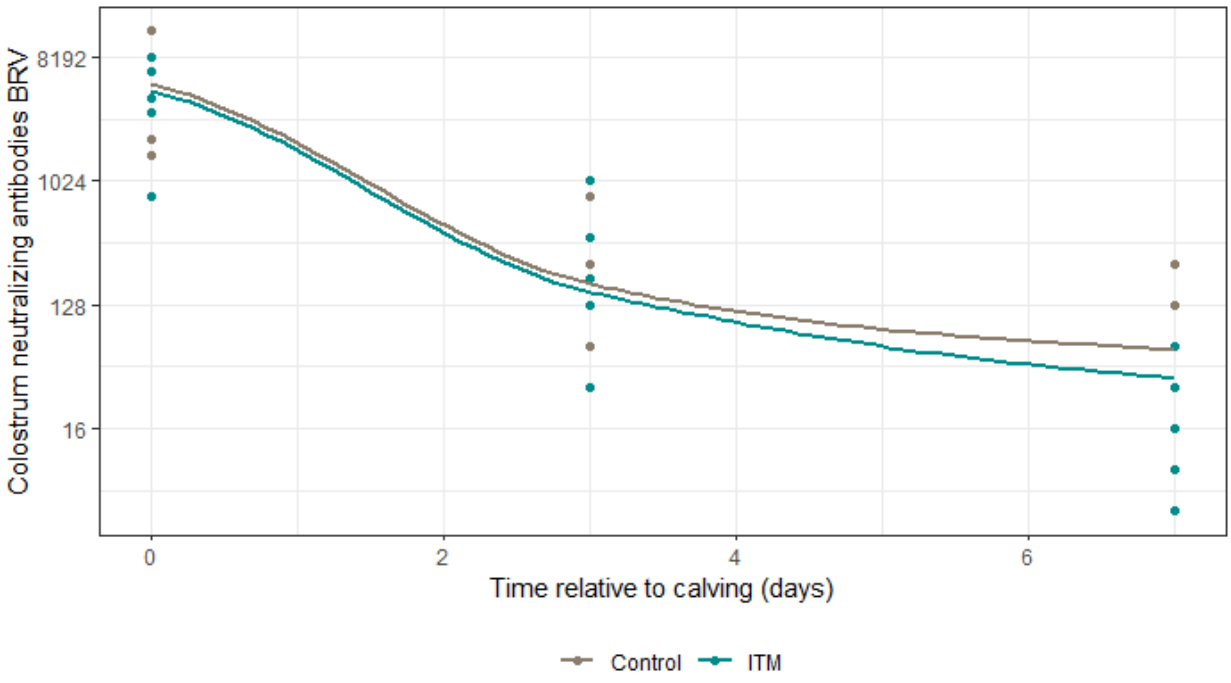

Figure 3.13. Colostrum neutralizing antibodies to BRV during post-vaccination period. The line is a smooth loess estimation.

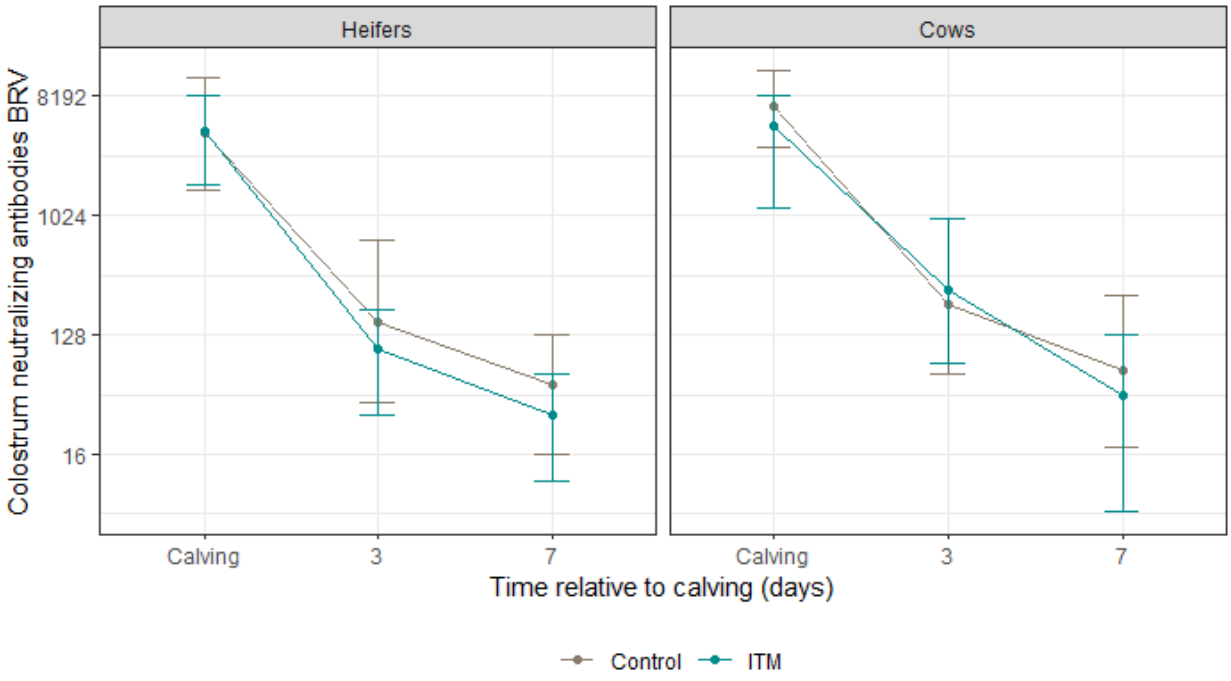

Figure 3.14. Colostrum neutralizing antibodies against Bovine rotavirus after neonatal calf diarrhea (NCD) vaccination and administration of injectable trace minerals (ITM). The points represent the geometric mean and the error bars represent the 95% confidence interval of the geometric mean.

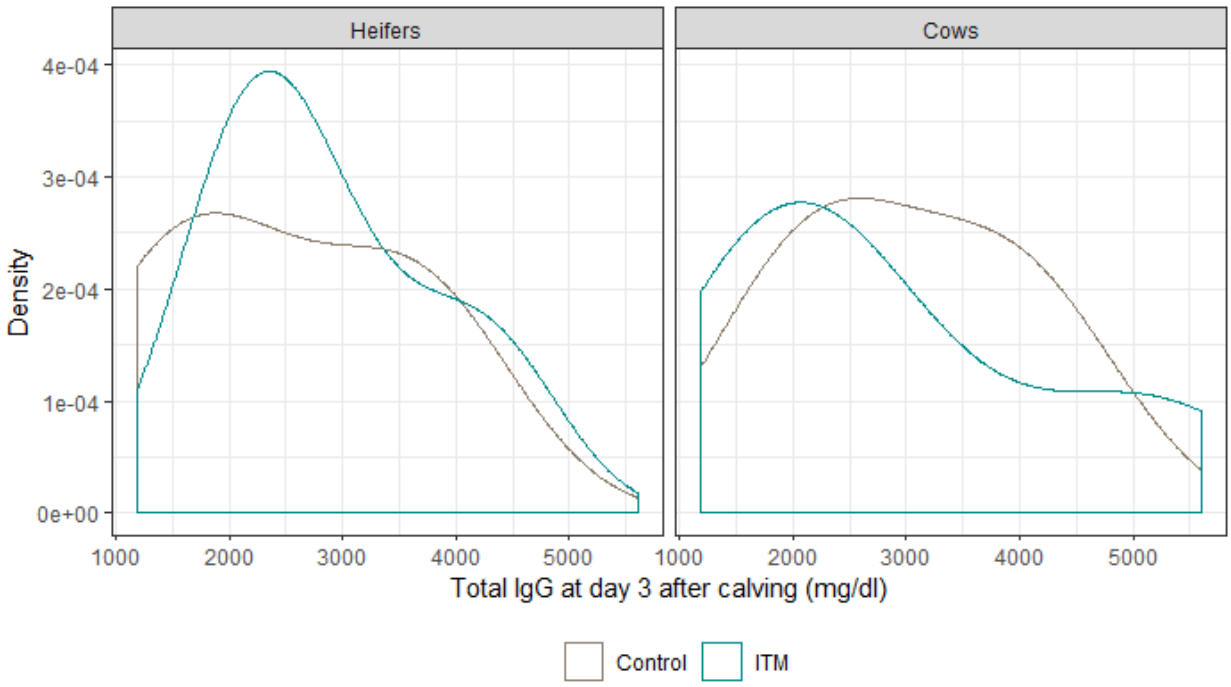

Figure 3.15. Density plot of total IgG at day 3 after calving in calves sort by treatment, and mother parity condition. Density is the standardized relative frequency so that the area under each curve equals one.

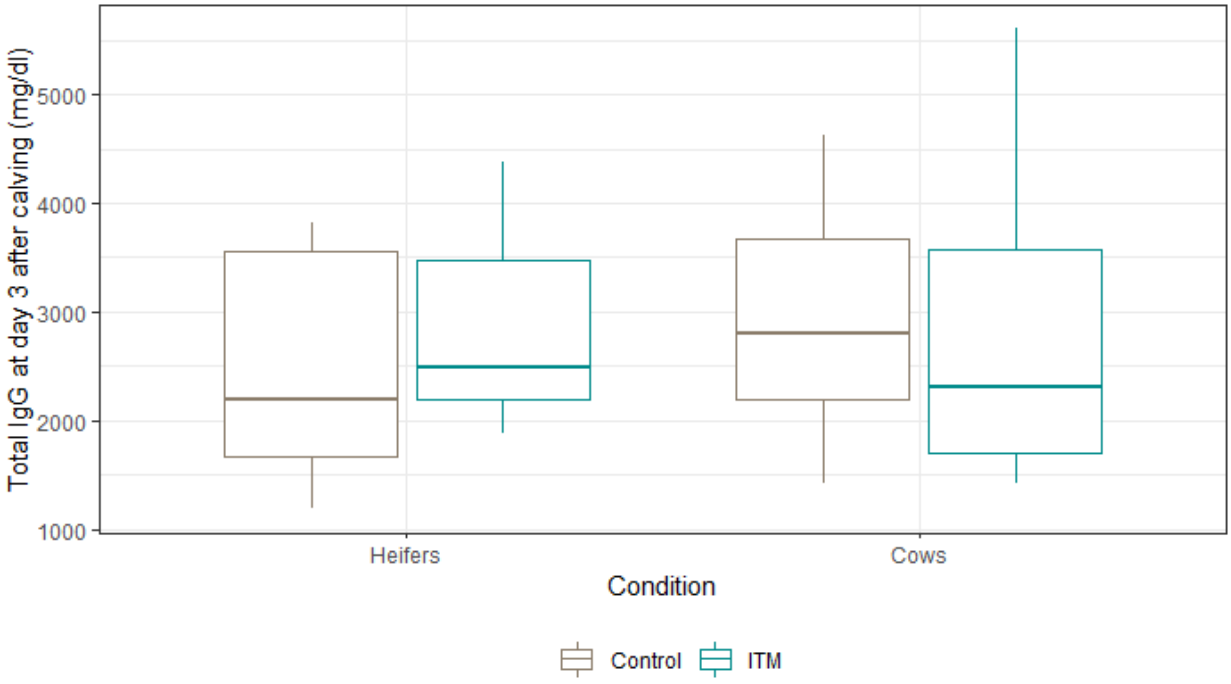

Figure 3.16. Total IgG by treatment and parity condition.

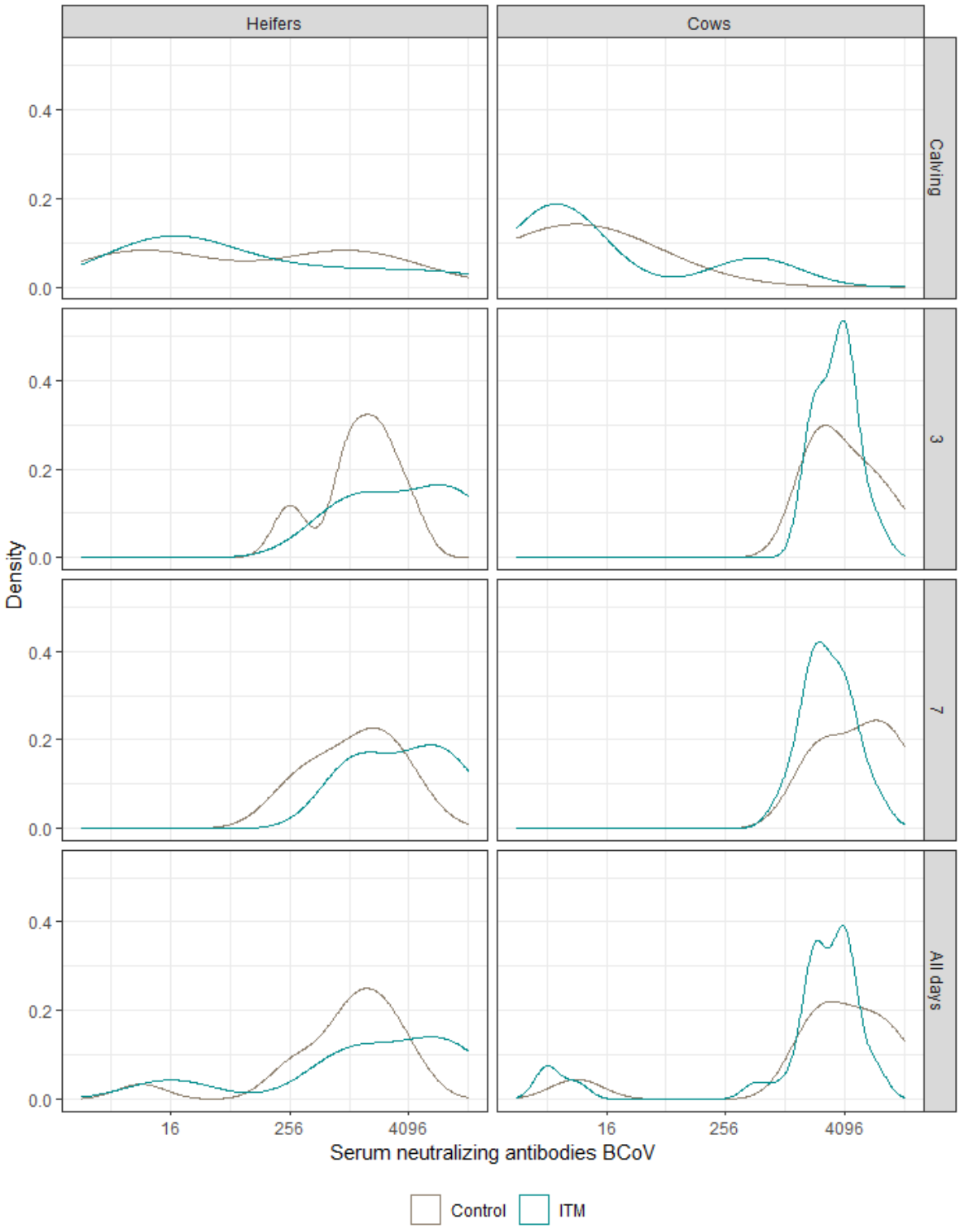

Figure 3.17. Density plot of serum neutralizing antibodies (SNA) against Bovine coronavirus (BCoV) in calves sort by treatment, days after calving, and mother parity condition. Density is the standardized relative frequency so that the area under each curve equals one.

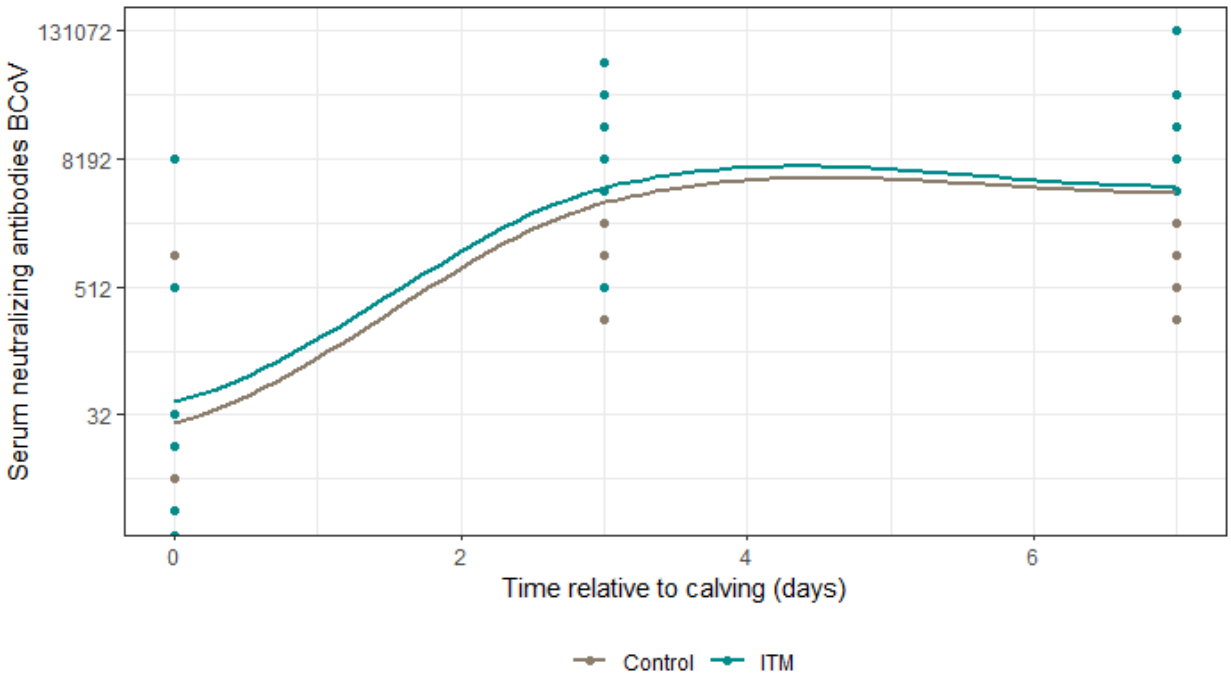

Figure 3.18. Calf serum neutralizing antibodies to BCoV after neonatal calf diarrhea (NCD) vaccination and administration of injectable trace minerals (ITM) to the mothers. The line is smooth loess.

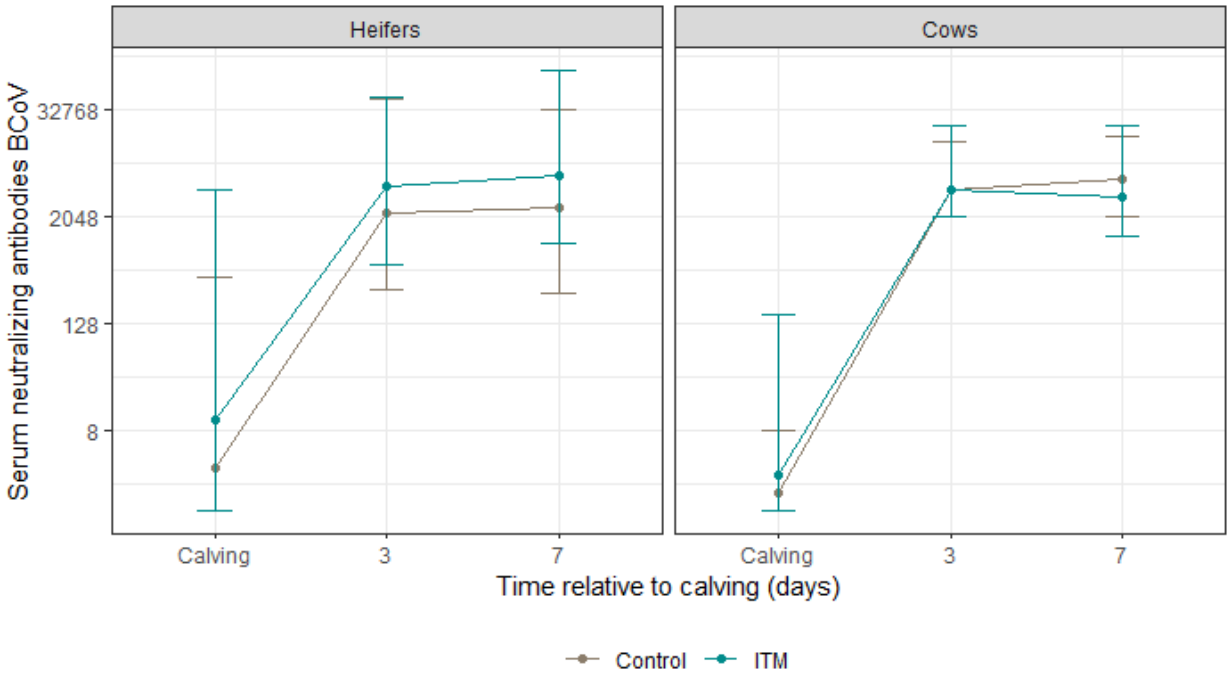

Figure 3.19. Calf serum neutralizing antibodies BCoV after neonatal calf diarrhea (NCD) vaccination and administration of injectable trace minerals (ITM) to the mothers. The points represent the geometric mean, and the error bars represent the 95% confidence interval of the geometric mean.

Table 3.1. Reading chart.

Analyte	Cell effect	Result	Interpretation
Serum samples Positive Control Negative Control	CPE	NEGATIVE	Looking for Antibodies: Neutralizing Antibodies present in samples and controls will protect the cells from the cytopathic effect of the virus. CPE will not be observed.
	NO CPE	POSITIVE	
Back Titration Virus Control	CPE	POSITIVE	Looking for the virus: When the virus is present and neutralizing antibodies absent, the virus effect will be reflected by the presence of CPE.
	NO CPE	NEGATIVE	

Notes: for validation purposes cells control cannot exhibit any CPE, and Negative control must be negative.

Table 3.2. Statistical testing for serum neutralizing antibodies against Bovine coronavirus, $\log_2$ transformed, of the difference between control and ITM by time relative to primary vaccination (days) and condition.

Time	Condition	t statistic	t (p-value)	Wilcoxon statistic	Wilcoxon p-value
0	Heifers	-0.334	0.741	74.500	0.910
	Cows	0.191	0.850	61.000	0.890
14	Heifers	-0.769	0.451	66.000	0.556
	Cows	0.820	0.425	71.500	0.387
21	Heifers	-0.785	0.442	65.000	0.521
	Cows	0.179	0.860	62.000	0.835
35	Heifers	-0.944	0.360	54.500	0.193
	Cows	0.716	0.483	68.000	0.527
Calving	Heifers	-0.490	0.631	61.500	0.396
	Cows	1.459	0.162	78.500	0.170
All days	Heifers	-1.197	0.234	1644.500	0.157
	Cows	1.388	0.168	1693.000	0.148

Table 3.3. Statistical testing for colostrum neutralizing antibodies against Bovine coronavirus, log_2 transformed, of the difference between control and ITM by time after calving.

Time	Condition	t statistic	t (p-value)	Wilcoxon statistic	Wilcoxon p-value
Calving	Heifers	-1.776	0.088	55.500	0.083
	Cows	1.851	0.080	77.000	0.083
3	Heifers	-1.733	0.096	57.000	0.097
	Cows	-0.105	0.918	50.000	0.801
7	Heifers	-0.276	0.785	85.000	0.787
	Cows	0.400	0.695	59.000	0.743
All days	Heifers	-1.100	0.275	696.500	0.247
	Cows	0.424	0.674	515.000	0.690

Table 3.4. Statistical testing for colostrum neutralizing antibodies against Bovine rotavirus, log_2 transformed, of the difference between control and ITM by time after calving.

Time	Condition	t statistic	t (p-value)	Wilcoxon statistic	Wilcoxon p-value
Calving	Heifers	-0.191	0.850	88.500	0.921
	Cows	1.436	0.167	68.000	0.323
3	Heifers	1.717	0.100	125.500	0.086
	Cows	-0.548	0.592	46.500	0.610
7	Heifers	1.937	0.065	126.500	0.078
	Cows	0.971	0.344	63.000	0.532
All days	Heifers	0.684	0.496	925.500	0.313
	Cows	0.340	0.735	501.500	0.834

Table 3.5. Statistical testing for calf serum neutralizing antibodies to coronavirus of the difference between control and ITM by time relative to calving and condition.

Time	Condition	t statistic	t (p-value)	Wilcoxon statistic	Wilcoxon p-value
Calving	Heifers	-1.015	0.325	30.500	0.232
	Cows	-0.757	0.459	49.000	0.688
3	Heifers	-0.914	0.380	27.500	0.335
	Cows	0.000	1.000	54.000	1.000
7	Heifers	-1.034	0.318	32.000	0.337
	Cows	1.251	0.227	72.000	0.196
All days	Heifers	-1.018	0.314	304.000	0.208
	Cows	0.007	0.995	519.500	0.640