ANTIOXIDANT SUPPLEMENTATION FOR IMMUNITY, GROWTH, AND HEALTH OF DAIRY CALVES

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ABSTRACT

The neonatal period for dairy calves is critical for immune, metabolic, and physical development which opens a window of disease susceptibility. While the industry has relied on tools such as colostrum and vaccination to support early life immunity, there are several challenges when vaccinating neonatal calves; 1) the inability to mount an effect immune response, 2) interference with maternal antibodies, and 3) the imbalance of pro-oxidant production to antioxidant capacity, also known as oxidative stress (OS). Oxidative stress (OS) which is characterized as an imbalance of pro-oxidants to antioxidants, results in cellular oxidative damage and/or dysfunction. Oxidative stress has become a topic of interest in the neonatal period as it negatively impacts lymphocyte function which might affect vaccine response. Widely studied in mature cattle, antioxidant supplementation has the potential to improve redox balance and immune response. However, evidence supporting the use of antioxidants in neonatal calves is far scarcer yet necessary to optimize immunity and disease resistance. This thesis includes a review that summarizes research on the impact of antioxidant supplementation on calf immunity, health, and productivity and highlights remaining gaps in knowledge. Chapter 2 is a study in which expands upon current literature, determining the effect of parenteral antioxidant supplementation at birth on immunity, growth, and health in pre-weaning dairy calves, suggesting that parenteral antioxidant supplementation at birth can improve redox balance within the first 2 wk of life and improve intranasal vaccine response throughout the pre-weaning period. However, we did not find any differences between groups in growth performance or health status. Overall, micronutrient supplementation in pre-weaning and post-weaning calves improved immune responses but there is conflicting evidence supporting the subsequent positive impact on calf health and growth performance.

I dedicate this thesis to the calves. Enjoy the good life, crazy girls.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADG average daily gain

Alb. Albumin

AOP antioxidant potential

BHB beta-hydroxybutyrate acid

BHV1 bovine herpes virus 1

BRD bovine respiratory disease

BRSV bovine respiratory syncytial virus

BUN blood urea nitrogen

BW body weight

Ca calcium

Chol. Cholesterol

CP crude protein

Cu copper

Gluc. glucose

HH hip height

Ig immunoglobulin

ITM injectable trace minerals

Mn manganese

NEFA non-esterified fatty acids

OS oxidative stress

OSi oxidant status index

RONS reactive oxygen and nitrogen species

SOD superoxide dismutase

Se selenium

TP total protein

Zn zinc

CHAPTER 1: LITERATURE REVIEW: ROLE OF ANTIOXIDANTS IN CALF IMMUNITY, GROWTH, AND HEALTH

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INTRODUCTION

Despite drastic improvements in neonatal calf management in the last decades, the prevalence of morbidity and mortality remains high worldwide (Svensson et al., 2006, Windeyer et al., 2014). It is estimated that pre-weaning calf mortality is 5% in the US with the two main causes being diarrhea and respiratory disease, 32% and 14.1%, respectively (USDA, 2014b). Calf adverse health events are not only a financial burden to producers due to treatment cost but also negatively impact future production (Buczinski et al., 2021). Treatment of bovine respiratory disease (BRD), including use of antimicrobials, has increased over time and was reported to cost $42 per calf in 2016 (Dubrovsky et al., 2020).

Calves are born immunologically naive, compromising their ability to mount an effective immune response within the first weeks of life (Chase et al., 2008). Although colostrum is critical for transfer of maternal antibodies and immune cells, this contributes to the challenge of vaccinating neonates. Maternal antibodies from colostrum ingestion interfere with endogenous antibody production from parenteral vaccination (Chase et al., 2008). Intranasal vaccination is utilized to stimulate mucosal immune response for antibody production and bypass maternal antibody interference (Hill et al., 2012). However, calves experience OS during the first wk of life (Abuelo et al., 2014), which is known to reduce calves' ability to mount an immune response (Cuervo et al., 2021), regardless of route of vaccine administration. Therefore, there is critical need for strategies that can improve vaccine responsiveness in neonatal calves.

Micronutrients that have antioxidant capabilities, such as vitamins and trace minerals, have the potential to increase antioxidant capacity and improve redox balance, which might improve vaccine response. The effect of micronutrient supplementation on calves' immunity and health in an emergent area of research and has not been summarized previously. Therefore, this

review will summarize research on micronutrient supplementation in dairy calves, in the context of their developing immune system and response to vaccination. Current gaps in knowledge and future research directions will also be presented in this review.

CALF IMMUNE DEVELOPMENT AND EARLY LIFE CHALLENGES

Immune Development and Vaccination Challenges

Calves are born immunologically naïve, meaning they rely on an external source to provide necessary immunologic protection (Chase et al., 2008). Colostrum intake provides neonatal calves with antibodies, cytokines, and immune cells including leukocytes. Although the necessary immune components are present in neonatal calves, it can take up to a few months for functional competence of these cells to mature (Chase et al., 2008). Immune dysregulation is a widely studied topic in mature transition cows. Transition cows experience a decrease in their ability to mount an effective immune response due to factors such as a drastic increase in metabolic demand (Goff and Horst, 1997). During the neonatal period, calves also experience high metabolic demands to support growth, with target ADG above 0.68 kg/d (Hyde et al., 2022). Immune development and the energy demand for quick growth contributes to the window of disease susceptibility that challenges calves in early life.

Vaccination is also used to help mitigate calfhood disease within the first weeks of life. As mentioned previously, there are several challenges when vaccinating young calves including maternal antibody interference and oxidative stress. Maternal antibodies inhibit or prevent the function of host B cells which are responsible for endogenous antibody production, reducing efficacy of parenteral vaccination (Bruggemann and Rajewsky, 1982, Chase et al., 2008). To circumvent interference from maternally derived antibodies, intranasal vaccination which targets mucosal surface antibody production, has become a popular protocol to prevent respiratory

disease in neonatal calves. While utilizing intranasal vaccines directly targets the mucosal surface, acting as a first line of defense, the protection provided does not cover the full duration of disease susceptibility (Ellis et al, 2013). Aside from route of vaccine administration, OS is an emerging field of research as it might impact calves' ability to respond to pathogen exposure (Cuervo et al., 2021).

Oxidative Stress During the Neonatal Period

At birth, mammals are exposed to an oxygen rich environment for the first time, increasing endogenous production of reactive oxygen species (ROS) (Wiedemann et al., 2003). Briefly, research in humans reports that oxygen exposure at birth results in increased OS for up to 4 weeks (Vento et al., 2001). Increased OS might impact cell development and cell death (Saugstad, 2003). Similar findings have been reported in calves. Calves have higher blood concentrations of ROS in comparison to their dam after birth and prior to colostrum ingestion (Gaal et al., 2006). Colostrum intake further contributes to circulating ROS concentrations right after birth as it contains pro-oxidants (Marta and Justyna, 2008). Just as colostrum is a source of pro-oxidants, it is also a source of antioxidants. However, concentrations of antioxidants in colostrum are less than concentrations of antioxidants in milk (Marta and Justyna, 2008). Therefore, change in environment as well as colostrum intake could overwhelm the available antioxidant capacity. In fact, calves face a greater imbalance of total pro-oxidants to available antioxidant defenses than transition cows (Abuelo et al., 2014), supporting the contention that OS might play an important role in pre-weaning calf health. Oxidative stress in periparturient cattle compromises immune cell function and is an underlying factor for transition cow disease (Sordillo and Aitken, 2009). Further *in vivo* and *in vitro* experiments in calves concluded that OS

compromises lymphocyte functions essential for responding to pathogens or vaccines (Cuervo et al., 2021).

Antioxidant supplementation has the potential to improve redox balance and reduce OS by improving the antioxidant capacity. In mature dairy cattle, there is substantial evidence showing the beneficial effects of antioxidant supplementation on redox balance, health, and production outcomes (Abuelo et al., 2015). In calves, however, OS is still an emerging area of research and hitherto findings of antioxidant supplementation trials in calves have, to our knowledge, not been summarized. Thus, our aim is to collate the current knowledge of the effects of antioxidant supplementation on calf immunity, health, and growth performance.

ANTIOXIDANT SUPPLEMENTATION

Antioxidant function

The antioxidant defense system is made of both enzymatic and non-enzymatic components including antioxidant enzymes such as superoxide dismutase (SOD) as well as vitamins and trace minerals (Ighodaro and Akinloye, 2019). An antioxidant is defined as a compound that prevents oxidation of molecules and the consequent damage (Halliwell and Gutteridge, 2007). Table 1.1 summarizes the antioxidant supplementation trials conducted in calves thus far. The utilized micronutrients included selenium (Se), copper (Cu), zinc (Zn), manganese (Mn), vitamin A, vitamin E, and vitamin D. Therefore, this section will briefly review the antioxidant function of the vitamins and minerals referenced.

Vitamin E plays a critical role in providing protection against lipid peroxidation which can result from OS (Halliwell and Gutteridge, 1999). While vitamin A has several roles, the key influences are on the antioxidant defense system as well as mucosal surface structure and integrity (Jin et al., 2014). The precursor for vitamin A, beta-carotene, is also supplemented for

its antioxidant capabilities. Beta-carotene scavenges oxygen radicals, mitigating potential damage by an overabundance of ROS (Spears and Weiss, 2008). Vitamin D plays an important role in maintaining calcium homeostasis but more recent evidence suggests vitamin D is a key factor in both innate and adaptive immune signaling (Nelson et al., 2012). Selenium, a component of glutathione peroxidase enzymes, is suggested to aid in the prevention of OS by supporting neutrophil migration (Ndiweni and Finch, 1995, 1996, Mustacich and Powis, 2000) which is supported by both *in vitro* and *in vivo* studies reporting prevention of neutrophil migration to infection sites due to increased adhesion to endothelial surfaces (Ndiweni and Finch, 1995, 1996, Maddox et al., 1999). This hinderance of neutrophils to infection sites does not allow endogenous response and repair of OS. Copper is known to aid in the antioxidant defense system by its function with ceruloplasmin (Halliwell and Gutteridge, 1999). Ceruloplasmin, an acute phase protein, is a copper transport protein which is suggested to scavenge superoxide radicals (Broadley and Hoover, 1989). Copper and zinc work together as components of the Cu-Zn superoxide dismutase which is responsible for the dismutation of superoxide radicals (Halliwell and Gutteridge, 1999). Zinc also contributes to the antioxidant defense system by activating the synthesis of metallothionine which is a protein that scavenges hydroxide radicals (Prasad et al., 2004). Similar to copper and zinc, manganese is a component of a superoxide dismutase enzyme, Mn SOD, which is capable of scavenging free radicals and reducing the reactivity of ROS molecules (Weisiger and Fridovich, 1973). Studies in different species have reported increased activity of Mn SOD by supplementing dietary Mn (Lu et al., 2006). Together, the combination of these vitamins and trace minerals may be able to counteract the consequence of an overabundance of pro-oxidants such as reactive oxygen and nitrogen species (ROS).

Current literature evaluating the effect of antioxidant supplementation on calves' redox balance is scarce. (Nayak and Abuelo, 2021) supplemented dairy calves with injectable antioxidants (Se, Cu, Zn, Mn or Se, Vitamin E) at birth and assessed their redox balance through the oxidant status index (OSi), as a ratio of pro-oxidant production to antioxidant capacity (Abuelo et al., 2013). This study found that injectable antioxidants at birth improve redox balance, shown as a decrease in OSi throughout the first two weeks of life. However, this proof of principle study utilized a limited sample size. Therefore, the concept that injectable antioxidant supplementation might improve redox balance in neonatal calves should be further explored. While current evidence investigating antioxidant supplementation on redox balance is limited, total antioxidant status is often measured as a response to antioxidant supplementation. A study supplementing 7 mo-old calves with vitamins and trace minerals (Se, Cu, Zn, Mn, Vitamin A, Vitamin E) reported increased total antioxidant status in supplemented calves compared to control calves 60 d after weaning (Mattioli et al., 2020). While antioxidant status alone is an important indicator of immune response, research suggests that both antioxidant status and pro-oxidant production are essential to appropriately assess and understand redox biology (Costantini and Verhulst, 2009). Therefore, further research is required to expand upon current knowledge of redox balance in calves.

Antioxidant Supplementation Impact on Calf Immunity

Few studies have evaluated the impact of micronutrient supplementation on both innate and adaptive immune response of calves. Common immune function markers include leukocyte concentrations, white blood cell function, and antioxidant enzyme activity such as superoxide dismutase and glutathione peroxide. Bovine viral diarrhea virus directly targets platelets and therefore, platelets are often measured as immune response in viral challenge studies. In a recent

study, weaned calves (7 mo-old) supplemented with injectable trace minerals (ITM; Se, Cu, Zn, Mn) concurrent with a modified live virus vaccine against bovine herpes virus 1, bovine viral diarrhea virus types 1 and 2, bovine respiratory syncytial virus (BRSV), and parainfluenza 3 virus showed increased platelet counts post bovine viral diarrhea virus challenge compared to other groups (Bittar et al., 2020). Similarly, another study reports a tendency for increase platelet counts in association with increased antioxidant enzyme production in calves supplemented with ITM (Se, Cu, Zn, Mn) at 8 months old compared to control calves (Vedovatto et al., 2020). A decrease in platelet counts have been reported to have an association with bovine viral diarrhea virus 2 infection in calves (Rebhun et al., 1989, Walz et al., 2001, Bittar et al., 2020). Therefore, micronutrient supplementation is suggested to have improved the immune response of these calves to support the viral challenge, as platelet counts were higher in calves receiving the supplement.

A few studies also report an increase in activity and concentrations of antioxidant enzymes in both neonatal dairy and weaned beef calves supplemented with the same ITM compared to control calves (Teixeira et al., 2014, Vedovatto et al., 2020). In addition, neonatal calves supplemented with ITM (Se, Cu, Zn, Mn) exhibit increased neutrophil function, specifically a greater percent of neutrophils showing phagocytosis activity compared to unsupplemented calves (Teixeira et al., 2014, Bates et al., 2019). Overall, there is strong evidence to suggest that injectable trace mineral supplementation improves innate immunity in both neonatal calves and weaned calves.

While evidence supporting trace mineral supplementation in calves is becoming more available, research exploring vitamin supplementation is more limited. However, there is evidence to suggest positive effects of vitamin supplementation on immunity in mature cows

such as (1) enhanced neutrophil function (Hogan et al, 1990, 1992), (2) increased concentrations of antioxidant enzymes (Jin et al., 2014) and (3) increased serum concentrations of IgA (Jin et al., 2014). The extent to which these positive effects can be seen in the immature immune system of neonatal calves remains unexplored. However, there are dietary vitamin supplementation studies (Table 1.1) that suggest positive effects on calf performance, metabolism, and immunity (Reddy et al., 1985, Reddy et al., 1986, Opgenorth et al., 2020) but further research on parenteral vitamin supplementation in calves is required.

Similar to cell mediated immune responses, there is evidence suggesting that antioxidant supplementation improves humoral immunity. Two studies supplementing 3.5 mo-old bull calves with ITM (Se, Cu, Zn, Mn) concurrent with a modified live virus parenteral vaccine for bovine herpes virus 1, bovine viral diarrhea virus, and parainfluenza 3 virus and a parenteral bacterin for *Mannheimia haemolytica* and *Pasteurella multocida* reported increased and faster antibody production to bovine viral diarrhea virus 1 and *M. haemolytica* (Palomares et al., 2016, Bittar et al., 2018). Similarly, a study supplementing 7 mo-old calves with vitamins and trace minerals (Se, Cu, Zn, Mn, Vitamin A, Vitamin E) reported higher serum antibody titers to bovine herpes virus 1 (Mattioli et al., 2020). Nevertheless, there is also some contradicting evidence that found no difference in antibody production, specifically for *Salmonella* spp., in calves treated with ITM (Se, Cu, Zn, Mn, Cr) at two weeks of age compared to control calves (Bates et al., 2020). A potential explanation for this difference in antibody production reported could be the age in which calves are studied as well as the virus or bacteria of interest. Calves 3.5 mo old would be expected to have a more mature and robust immune response compared to two week old calves (Chase et al., 2008) thus resulting in noticeable serum antibody responses in calves with the more mature immune system but not in the first weeks of life. The authors claim that the ELISA

used to assess salmonella antibody titers was not appropriate due to the IgG molecule binding sites potentially being blocked by IgM and reducing optical density, therefore underestimating the immune response. However, increased nasal IgA secretions against bovine herpes virus 1 and BRSV were found in calves up to 1 mo of age supplemented at birth with injectable antioxidants (Se, Cu, Zn, Mn or Se, Vitamin E) compared to control calves (Nayak and Abuelo, 2021). This could suggest that antioxidant supplementation is capable of improving the immune responses in neonatal calves but interference of maternally-derived antibodies could have masked some of the results in studies using parenteral vaccines in newborn calves. Altogether, there is substantial evidence in the literature to support that supplementation of antioxidants can improve both innate and adaptive immunity in calves.

Antioxidant Supplementation Impact on Calf Growth and Health Status

Beyond improvements in immune parameters as a consequence of antioxidant supplementation to calves, it is important to explore the extent to which these immune changes translate into improved growth and health. Growth is commonly assessed in calves as it is an indicator of future performance and production (Van De Stroet et al., 2016). Numerous studies report no difference in ADG between calves supplemented with antioxidants (Se, Cu, Zn, Mn, Cr, Vitamin E) and control calves (Arthington et al., 2014, Teixeira et al., 2014, Bates et al., 2019, Leslie et al., 2019, Vedovatto et al., 2020). In these studies, the age at which calves first received antioxidants ranged from birth to 9 mo of age thus suggesting that antioxidant supplementation does not have an impact on growth performance during the pre-weaning or post-weaning period. Conversely, a feedlot study reported greater ADG in crossbred calves receiving ITM (Se, Cu, Zn, Mn) compared to control calves throughout the 55-d trial (Richeson and Kegley, 2011). It is possible that the difference in results presented is due to difference in

breed as well as differences in supplementation dose. Overall, there is not consistent evidence to suggest that antioxidant supplementation influences ADG in calves throughout the pre-weaning or post-weaning period.

There is also conflicting evidence on the effect of antioxidant supplementation on calf morbidity and mortality. A few studies report calves supplemented with ITM (Se, Cu, Zn, Mn) had lower prevalence of diarrhea and respiratory disease throughout the pre-weaning period (Teixeira et al., 2014, Bates et al., 2019). For example, the prevalence of diarrhea in calves supplemented with ITM within 24 h of birth was 4.9%, contrasting with the 10.6% diarrhea risk in unsupplemented control calves (Bates et al., 2019). Similarly, compared to control calves, ITM calves exhibited lower morbidity (15.6 vs. 7.5%) and mortality (3.2 vs. 1.8%) within the first 48h after birth (Bates et al., 2019). In contrast, there were no differences in mortality or pre-weaning disease treatment between calves supplemented with selenium and vitamin E at birth and control counterparts (Leslie et al., 2019). This study, however, reported a 4% decrease in the odds of diarrhea in supplemented calves but no differences in likelihood of experiencing respiratory disease. A feedlot study reports that crossbred calves supplemented with ITM (Se, Cu, Zn, Mn) one day post arrival had lower rates of BRD morbidity compared to control calves (Richeson and Kegley, 2011). As such, fewer ITM calves required a second treatment of antibiotics for respiratory disease. A few potential explanations for differences in results presented include: 1) different micronutrients supplemented, some including vitamins and others not, 2) calf management (dairy vs. feedlot), 3) prevalence of disease at each farm/region. Due to lack of evidence-based protocols, the time of supplementation and dose vary throughout the different studies, which could contribute to the inconsistencies of growth and health results. The studies referenced range from 1-39 farms used, in various geographical regions worldwide. As

such, there is a chance for great variability in the prevalence of disease in each study. Further research is required to determine appropriate supplementation strategies including frequency and dose to potentially improve calf growth, morbidity, and mortality.

FUTURE DIRECTIONS

The literature suggests that antioxidant supplementation can improve calf immunity but there are contradicting findings regarding the extent to which this translates to improved calf health and growth. Throughout the studies conducted to date (Table 1.1), there was a great variation on the type of antioxidant supplemented (e.g., vitamins, minerals, or both) as well as the age at supplementation. Currently, there are several commercial parenteral formulations containing combinations of micronutrients with antioxidant properties that could be used in calves. However, these products have not been compared to date and there is still a gap in knowledge regarding the optimal supplementation formulations and regimes for optimal calf immunity, health, and growth.

Hitherto, most of the micronutrient supplementation studies have been conducted in older, weaned calves. In dairy calves, however, the window of greater disease susceptibility is between 2-4 wk of age due to waning passive immunity concurrently with a still developing active immunity (Hulbert and Moisa, 2016). Thus, further research is required to optimize antioxidant supplementation in neonatal and pre-weaning claves to improve immunity at this critical time for calves.

Lastly, OS causes immune dysfunction in calves and antioxidant therapy improves immune responses through its mitigation of OS (Abuelo et al., 2019). However, most of the calf antioxidant supplementation studies did not adequately assess the animals' oxidant status or degree of oxidative damage, which would be required to assess the effectiveness of the

intervention. Thus, it is unclear if some of the discrepancies among studies could be due to the supplementation strategy used failing to reduce OS. Therefore, new supplementation studies should include an assessment of OS. Ultimately, more research is still needed to provide evidence-based guidance on the levels and timing of supplementation of young dairy calves that provide an effective improvement of the animals' health and performance.

CONCLUSIONS

This review summarizes the importance of antioxidant supplementation in calves. Although limited evidence is available in neonates, current literature suggest that antioxidant supplementation can improve calf immunity throughout the pre-weaning and post-weaning periods. Furthermore, there is conflicting evidence on the effect of antioxidant supplementation on calf health and growth performance. Age at supplementation, type of supplementation such as trace minerals, vitamins, or a combination, and appropriate assessment of oxidant status are potential areas of investigation to expand upon current findings. Combined efforts between researchers and veterinarians are crucial for expanding the knowledge of and appropriately utilizing antioxidant supplementation in the cattle industry.

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TABLES

Table 1.1. Reported effects of calf antioxidant supplementation on immune, health, and performance outcomes.

Study	Animals supplemented	Antioxidant supplement	Immune Outcomes[1]		Health Outcomes[1]		Performance Outcomes[1]	
			Measured	Findings	Measured	Findings	Measured	Findings
Reddy et al. (1985)	7 calves	Vitamin E 1,400 mg orally at weekly intervals	- Serum vitamin E every 2 wk for 12 wk - Serum metabolic biomarkers every 2 wk starting at wk 4-12 wk	- Serum vitamin E higher in high oral group at wk 4 - Serum vitamin E higher in injectable group at wk 2, 4, 6, 8 - No differences in creatinine, glucose, phosphorus, calcium, urea nitrogen, chloride, sodium, potassium, albumin, and total protein	- Fecal consistency twice daily	- No difference in fecal consistency	- Weekly weight	- A trend was identified for greater weight gain in supplemented calves
	7 calves	Vitamin E 2,800 mg orally at weekly intervals						
	7 calves	Vitamin E 1,400 IU injectable weekly						
Reddy et al. (1986)	7 calves	Vitamin E 1,400 mg orally at weekly intervals	- Plasma protein and packed cell volume - Lymphocyte stimulation - Infectious bovine rbinotracbeitis virus replication. - Serum antibody titers (IgG and IgM)	- Lymphocyte stimulation was higher for calves given the high amount of oral supplementation and for injected calves than for unsupplemented calves				
	7 calves	Vitamin E 2,800 mg orally at weekly intervals						

Table 1.1. (cont'd)

Study	Animals supplemented	Antioxidant supplement	Immune Outcomes[1]		Health Outcomes[1]		Performance Outcomes[1]	
			Measured	Findings	Measured	Findings	Measured	Findings
	7 calves	Vitamin E 1,400 IU injectable weekly		- No differences in concentrations of IgG1 and IgG2 among treatments - IgM was higher at wk 6 in calves given the high amount of oral supple-mentation than in all other calves - At wk 12, serum the high oral group and calves given injections inhibited infectious bovine rhinotracheitis viral replication in tissue cultures				
Richeson and Kegley (2011)	30 crossbred calves	Se (5 mg/mL), Cu (10 mg/mL), Zn (20 mg/mL), Mn (20 mg/mL) at 199 kgs			- Daily health scores - Treatment of BRD	- Rate of BRD morbidity was less - Fewer calves required second treatment for BRD		

Table 1.1. (cont'd)

Study	Animals supplemented	Antioxidant supplement	Immune Outcomes[1]		Health Outcomes[1]		Performance Outcomes[1]	
			Measured	Findings	Measured	Findings	Measured	Findings
	30 crossbred calves	Se (5 mg/mL), Cu (16 mg/mL), Zn (48 mg/mL), Mn (10 mg/mL) at 199 kgs						
Arthington et al. (2014)	75 crossbred beef calves	Se, Cu, Zn, Mn at birth	- Trace mineral status was assessed in liver biopsy samples on d 150, 200, and 250	- Greater concentrations of liver Cu and Se and lesser liver Fe concentrations compared to control			- BW was recorded at birth and on d 100, 150, 200, and 250 (weaning)	- No differences in BW gain
Teixeira et al. (2014)	395 Holstein heifers	Se, Cu, Zn, Mn at 3 and 30 days of age	- Blood samples at 7, 14, 35 days of age to measure antioxidant enzyme activity and neutrophil//monocyte function	- Increase neutrophil activity - Greater glutathione peroxide activity on day 14	- Incidence of disease in first 50 days of life	- Reduced incidence of diarrhea - Reduced incidence of combined pneumonia or Otis or both		
Palomares et al. (2016)	15 calves	Se, Cu, Zn, Mn at 3.5 months old and 3 weeks later	- Weekly blood samples to measure antibody titers to BVDV1	- Increased antibody titers to BVDV1 28 days post priming vaccination				

Table 1.1. (cont'd)

Study	Animals supplemented	Antioxidant supplement	Immune Outcomes[1]		Health Outcomes[1]		Performance Outcomes[1]	
			Measured	Findings	Measured	Findings	Measured	Findings
Bittar et al. (2018)	30 Holstein calves	Zn, Cu, Se, Mn s.c. at weaning and 3 weeks after	- Serum neutralizing antibody titers to M. haemolytica and P. multocida - Antigen induced PBMC proliferation - Interferon γ production	- Increased fold change in antibody titers against M. haemolytica - augmented PBMC proliferation upon antigen stimulation				
Bates et al. (2019)	435 Friesian-Jersey cross calves	Zn, Cu, Se, Mn, Cr s.c. within 24 of birth	-		-Morbidity -Mortality	- -Reduced morbidity and mortality from birth to 140 days	-Growth rate	- No difference in average daily rate of gain
Leslie et al. (2019)	418 Holstein calves	Se, Vitamin E at birth			- Fecal score observed at 1, 2, and 7 weeks of age - Mortality rate - Fecal pathogen excretion Odds of being treated for disease	- No treatment effect on fecal score or mortality - Protective effect against rotavirus - Reduced odds of treatment for diarrhea		
Bates et al. (2020)	15 dairy calves	Zn, Cu, Se, Mn	- Neutrophil and monocyte function - Gamma interferon release - Antibody titers - Micronutrient concentrations	- Increase in cells phagocytosing - Increase in number of bacteria ingested per cell - No difference in gamma interferon response or antibody titers No treatment effect on micronutrient concentrations				

Table 1.1. (cont'd)

Study	Animals supplemented	Antioxidant supplement	Immune Outcomes[1]		Health Outcomes[1]		Performance Outcomes[1]	
			Measured	Findings	Measured	Findings	Measured	Findings
Opgenorth et al. (2020)	8 calves	60 mL of fish and flaxseed oil and 200 mg of vitamin E in colostrum	- blood was collected on d 1, 2, 4, 7, 14, 21 to measure polyunsaturated fatty acids, oxidant status, total protein, and vitamin E concentrations - Colostrum was sampled from each calf's first feeding to assess antibody concentrations and polyunsaturated fatty acids	- No differences in serum total protein - Increased concentrations of vitamin E and decreased OSi in first wk of life	- Daily health scores	- No differences in prevalence of diarrhea or other signs of disease	- Weights and hip height weekly	- No differences in rate of growth
Vedovatto et al. (2020)	Nellore calves	Se, Cu, Zn, Mn at 8 months old	- Blood samples collected on day 0, 7, 21, 64 to assess antioxidant enzymes, leukogram, erytogram, and platelets	- Increased super dioxide dismutase on day 7 - Increased glutathione peroxide on day 7 and 21 - Greater leukocyte concentrations on day 64				
(Nayak and Abuelo, 2021)	7 Holstein calves	Zn, Cu, Se, Mn s.c. at birth	- Weekly nasal anti-BHV1 and -BSRV IgA following intranasal vaccine at birth	- Increased IgA concentrations - Faster IgA production				
	7 Holstein calves	Se & vit. E s.c. at birth						

[1] Outcomes presented compared to the unsupplemented control of the study. BHV1 = Bovine herpesvirus type 1; BRSV = Bovine Respiratory Syncytial Virus. PubMed was used to search for clinical trials and randomized controlled trials from 1946 to 2023 with the following key terms: (trace minerals OR vitamins OR micronutrients) AND (calf OR cattle OR dairy) AND (immunity OR health OR growth).

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CHAPTER 2: EFFECT OF PARENTERAL MICRONUTRIENT SUPPLEMENTATION AT BIRTH ON IMMUNITY, GROWTH, AND HEALTH IN PRE-WEANING DAIRY CALVES

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ABSTRACT

The objective of this randomized clinical trial was to determine the extent to which injectable micronutrient supplementation at birth can improve intranasal vaccine response by ameliorating oxidative stress in dairy claves from birth to weaning. For this, 120 Holstein heifer calves were enrolled at birth and randomly allocated into one of four groups. The four groups included three commercially available micronutrient supplements (Selenium, Copper, Zinc, Manganese; Selenium & Vitamin E; and Vitamins E, A, and D) and one control (saline). Calves received an intranasal vaccine against the respiratory viruses parainfluenza 2, bovine herpesvirus type 1 (BHV1), and bovine respiratory syncytial virus (BRSV) within the first wk of life. Weight (BW) and hip height (HH) were recorded, and a blood sample and nasal secretion sample were collected at birth prior to treatment and vaccine administration as well as weekly until weaning at 8 wk. Health scores, including thoracic ultrasound assessment, were recorded weekly from wk 1 to wk 8. Farm treatment records were collected after completion of the study. Serum micronutrient concentrations were determined from birth to weaning to identify micronutrient status and serum blood metabolites were analyzed as markers of nutrient utilization. Redox balance was determined in serum as a ratio of reactive oxygen and nitrogen species (RONS) to antioxidant capacity (AOP), known as oxidant status index (OSi). Intranasal vaccine response was quantified as anti-BRSV and anti-BHV1 immunoglobulin A (IgA) concentrations in nasal secretions. Linear mixed models with repeated measures were built for the following outcome variables: micronutrient concentrations, blood metabolites, redox balance, IgA concentrations, BW, and HH. Pre-planned contrasts of control vs supplemented were also built for the primary outcome of IgA concentrations. A logistic regression mixed model was built for health events and treatment of disease. Serum selenium concentrations were greater in calves receiving

supplements containing Se throughout the first 4 wk of life. However, we did not observe any consistent differences in the other micronutrients. The metabolic biomarkers indicate that supplemented calves had better energy status, as suggested by lower BHB and non-esterified fatty acids (NEFA) concentrations. Supplemented calves showed improved redox balance, indicated by lower OSi throughout the first two wk of life. Calves supplemented with antioxidants at birth had higher anti-BRSV IgA compared to control calves. Our results indicate an improved immune response to vaccines in calves supplemented with antioxidants at birth. However, this did not translate to growth and health performance as there were no differences in average daily gain (ADG) or incidence of health events throughout the pre-weaning period. This study provides evidence that improving the antioxidant capacity might improve vaccine response and further research is required to investigate appropriate frequency and dose of supplementation to improve calf growth and health.

Keywords: Antioxidants, Calves, Oxidative Stress

INTRODUCTION

Despite improvement in management including disease prevention and intervention, calf morbidity and mortality remain high (34% and 3.5-5%, respectively) in several countries (USDA, 2014b, Windeyer et al., 2014). Bovine respiratory disease is the second leading cause of morbidity and mortality in US pre-weaned heifers (USDA, 2014b). Pre-weaning disease is a financial burden on dairy farms due to treatment cost and the negative impact on future production (Dubrovsky et al., 2020). Calves are born immunologically naïve, making them most susceptible to disease within the first weeks of life as they cannot mount an effective immune response (Chase et al., 2008).

Intranasal vaccination has become a common strategy in the dairy industry to protect calves against respiratory disease by circumventing maternal antibody interaction and stimulating mucosal tissue antibody production (Windeyer and Gamsjager, 2019). This mucosal stimulation results in the production of immunoglobulin A (IgA) which act as a first line of defense to respiratory pathogens (Meeusen, 2011). However, despite route of vaccine administration, neonatal calves experience high levels of oxidative stress (OS) within the first weeks of life (Abuelo et al., 2014, Ranade et al., 2014) that negatively impacts calf lymphocyte function, including immunoglobulin production (Cuervo et al., 2021). Oxidative stress is the macromolecule damage that occurs as a result of an imbalance between pro-oxidants and antioxidants (Sordillo and Aitken, 2009). Thus, antioxidant supplementation can ameliorate OS by increasing antioxidant capacity (Abuelo et al., 2015). A previous pilot study by our group suggested positive effects of parenteral antioxidants given at birth on redox balance and intranasal vaccine response in the first month of life (Nayak and Abuelo, 2021). However, this earlier proof-of-principle study had a limited sample size and only examined the effects of

antioxidant treatment during the first month of life. Therefore, further research is needed to (i) examine the effect on vaccine response throughout the whole pre-weaning stage, and (ii) explore the impact of antioxidant supplementation at birth on health and growth outcomes.

Thus, the overall objective of this randomized clinical trial was to determine the extent to which parenteral antioxidant supplementation can improve intranasal vaccine response by improving redox balance in neonatal dairy calves. Our hypothesis is that calves receiving parenteral antioxidant supplementation at birth will have higher nasal IgA concentrations against vaccine antigens, greater ADG, and improved health compared to calves not receiving parenteral antioxidant supplements. To test this hypothesis, we compared weekly serum antioxidant micronutrient concentrations, biochemical analytes, redox balance, nasal anti-bovine-herpes-virus-1 (BHV-1) and anti-bovine-respiratory-syncytial-virus (BRSV) IgA concentrations, health scores, likelihood of treatment, and growth performance among calves receiving 3 different commercial parenteral antioxidant products or saline at birth.

MATERIALS AND METHODS

All animal use was approved by Michigan State University Institutional Animal Care & Use Committee (PROTO202000133) and animals were enrolled with the owner's consent.

Animals and Management

For this study, a total of 120 Holstein heifer calves were enrolled from two commercial dairy farms in mid-Michigan (60 calves/farm). Farm enrollment criteria included farms milking 1,000 cows or more, farms that kept their heifer calves on-site until weaning, farms that maintained electronic health records, and the farm's willingness to participate in the study. Calves were enrolled at birth by research staff. Calf enrollment criteria included female calves that required no to minimal calving assistance. Minimal calving assistance was described as up

to two h of labor with no assistance, fetal pulling using rope or chains, and/or less than 15 min of fetal manipulation or pulling. Calves stayed with their dam in the maternity pen for approximately 30 min before being processed by farm staff. Calves received 3.8 L of >23% Brix colostrum within one hour of birth via esophageal tube. A second feeding of colostrum was administered on average 6 h after the first feeding. Calves received an intranasal vaccine (Bovilis Nasalgen 3, Merk Animal Health) against the respiratory viruses parainfluenza 2, bovine herpesvirus type 1 (BHV1), and bovine respiratory syncytial virus (BRSV) within the first week of life.

At farm A, calves were housed in individual pens in the maternity barn for 24 h before being moved to the calf barns. In the calf barns, calves were housed in individual pens throughout the duration of the study (8 wk). At farm B, calves were housed in individual pens or group pens in the maternity barn for 24 h before being moved to a calf barn. For the duration of the study, calves were housed individually in individual pens or hutches inside the barn. Figure 1 provides details of milk offered throughout the pre-weaning period for both farms. Grain (Farm A, Kalmbach Calf Starter 20% CP; Farm B, Lakeshore Feed LLC 18% CP) and water were introduced at day 1 of age and offered ad libitum in both farms.

Treatment Allocation

At birth, calves were randomly allocated into one of the four treatment groups. An electronic random number generator (https://www.graphpad.com/quickcalcs/randomize1/) was used to assign a group for each calf . Farm staff was blind to the treatment allocation. The treatment groups included the three commercially available injectable products containing micronutrients with antioxidant capacity: supplement 1 (Multimin90, Multimin North America Inc.), supplement 2 (Bo-Se; Merck Animal Health), supplement 3 (Vitamins E-AD; VetOne) and

a placebo control (Saline; ICU Medical). Supplements were administered subcutaneously following label dosages: Supplement 1 contains 1.33 mg/kg of zinc, 0.11 mg/kg of selenium, 0.22 mg/kg of manganese, and 0.33 mg/kg of copper; Supplement 2 contains 0.08 mg/kg of selenium and 4.16 mg/kg of vitamin E as d-alpha-tocopherol; and Supplement 3 contains 40 IU/kg of d-alpha-tocopherol, 13,333 IU/kg of vitamin A propionate, and 1,333 IU/kg of vitamin D3. Calves in the control group received 3 mL of saline s.c. Calves did not receive further injectable antioxidant supplementation beyond assigned treatments.

Health Examinations and Sample Collection

Two hours after colostrum ingestion, research staff recorded the calf's body weight using a weight tape (Alltech) and the hip height using a tape measure, as previously validated (Heinrichs and Hargrove, 1987, Parish et al., 2012). Body weight and hip height were recorded weekly (every 7 days □ 1 day) from birth until weaning at 8 wk. Nasal secretion samples were collected prior to treatment and vaccine administration and weekly from birth to weaning as previously described (Woolums et al., 2013). Briefly, a sterilized foam plug (Jaece Industries, Inc.) was inserted into one nostril and allowed to saturate for up to 5 min. Once the foam plug was saturated, it was removed from the nostril using sterile forceps and placed into a 10 mL syringe with the plunger removed. The plunger was then reinserted to expel the nasal secretions into two 1.5 mL cryovials (Corning Inc.). A blood sample was collected prior to treatment and vaccine administration then weekly from birth to weaning via jugular venipuncture using 6 mL trace element vacutainer tubes without anticoagulant (Trace Element Serum; Becton Dickinson & Co.). Blood samples were allowed to clot for 30 min and centrifuged at 2,000 x *g* for 15 min. The serum was then aliquoted into four 1.5 mL cryovials. Both the nasal secretion samples and

serum samples were flash-frozen in liquid nitrogen at the farm upon collection, and transported to the laboratory where they were stored in -80 ºC until analysis.

Health scores were also recorded weekly from wk 1 to wk 8 using the University of Wisconsin Calf Health Scorer app (https://www.vetmed.wisc.edu/fapm/svm-dairy-apps/calf-health-scorer-chs/). Briefly, a clinical respiratory examination, including thoracic ultrasound, was performed as well as general appearance and fecal observation. The clinical respiratory exam includes nasal and ocular discharge, ear position, cough reflex, and rectal temperature. Each respiratory indicator was scored 0-3 where 0=normal and 3=severely abnormal. For example, nasal discharge is scored as follows, 0=normal, 1=small amount of unilateral, cloudy discharge, 2=bilateral cloudy or excessive mucus, 3=copious, bilateral mucopurulent nasal discharge. Calves that had a score ≥5 or ≥2 for 2 or more respiratory indicators were considered positive for respiratory disease (McGuirk and Peek, 2014). Rectal temperature 0-1 (100□F – 101.9□F) was considered normal and 2+ (≥102□F) was considered elevated. Fecal scores were also recorded as 0=normal, 1=semi-formed, pasty, 2=loose but stays on top of bedding, or 3=watery and sifts through bedding. Fecal score 0 or 1 was considered normal and ≥2 was considered diarrhea. Thoracic ultrasound using a linear 8.5 MHz probe (Easi-Scan: Go Bovine ultrasound scanner; IMV imaging) was scored on a 1-5 scale where 0-1=diffuse comet tails, 2=lobular pneumonia, 3=lobular pneumonia with 1 lobe consolidated, 4=lobular pneumonia with 2 lobes consolidated, 5=lobular pneumonia with 3 or more lobes consolidated (Ollivett and Buczinski, 2016). Health scoring was >95% performed by the first author (HC) to ensure consistency with AA filling in as needed. Treatment records were collected from the farms' herd management software (DairyComp; Valley Ag Software) after completion of the study.

To serve as controls for analysis quality control, nasal secretion samples were also collected from 5 clinically healthy Holstein cows from the Michigan State University Dairy Cattle Teaching and Research Center (East Lansing, MI), following the same protocol described for calves. For this, cows between 180-200 DIM were used as they are expected to exhibit mature and robust immune responses (Ingvartsen and Moyes, 2013). A serum sample from these cows was submitted to Cornell University Animal Health Diagnostic Center (Ithaca, NY) to confirm seronegative status against BHV-1 and BRSV via virus neutralization test. Nasal secretion samples were collected immediately prior to and one wk after administration of the same intranasal vaccine the calves received (Bovilis Nasalgen 3, Merk Animal Health). The pre- and post- vaccination samples were pooled to serve as low- and high- reference samples, respectively, for quality control during antigen-specific immunoglobulin A quantification.

Quantification of Micronutrient Concentrations

A random number generator (https://www.graphpad.com/quickcalcs/randomize1/was utilized to create a randomized subset of serum samples (7 calves/treatment/farm) that were sent to the Michigan State University Veterinary Diagnostics Laboratory (East Lansing, MI) for determination of weekly serum micronutrient concentration throughout the study. Selenium, copper, zinc, manganese, vitamin E, and vitamin A were analyzed as they were the micronutrients supplemented to the calves. Briefly, vitamin concentrations were analyzed using high performance liquid chromatography and trace mineral concentrations were analyzed using inductively coupled plasma mass spectrometry (Wahlen et al., 2005). Selenium, manganese, and vitamin A concentrations are represented as ng/mL, whereas those of zinc, copper, and vitamin E are represented as μg/mL.

Assessment of blood metabolites

Serum concentrations of BUN, BHB, calcium (Ca), cholesterol (Chol), glucose (Gluc), albumin (Alb), nonesterified fatty acids (NEFA), and total protein (TP) were quantified using commercial reagents from Catachem Inc. as biomarkers of nutrient utilization. Biomarkers were determined using a biochemistry analyzer (CataChem Well-T; Catachem Inc.) previously validated for cattle (Abuelo et al., 2020). Quality control measures included weekly calibration and the use of two-level reference samples (Catachem Inc.) at the time of calibration. Precision indicators for all biomarkers have been previously reported (Rossi et al., 2023).

Assessment of Redox Balance

Redox balance was assessed in serum within 2 mo from collection using a validated method as previously described (Abuelo et al., 2016). A commercially available fluorometric assay (OxiSelect In Vitro ROS/RNS Assay Kit; Cell Biolabs Inc.) was used to measure reactive oxygen and nitrogen species (RONS) as proxy to oxidant production. Briefly, a dischlorofluorescent dye was added to the serum sample which reacts with free radicals present, yielding a fluorescent product. This allows the fluorescent intensity to be indicative of the total RONS in the serum sample. Fluorescence was determined at 480 nm of excitation and 530 nm of emission using an automatic plate reader (Synergy H1 Hybrid; Biotek). To ensure detection of fluorescence at various concentrations, a standard curve made by 6 serial dilutions (0–10,000 n*M*) of the fluorescence probe 2',7'-dichlorodihydrofluorescein diacetate was included in each plate. All samples and standards were run in duplicates and those with a coefficient of variation of >10% were rerun. Concentrations of RONS were presented as the average relative fluorescent units (RFU) between duplicates. The inter- and intra- assay CV's were 7.3% and 5.9%, respectively.

Serum antioxidant potential (AOP) was measured using a previously described Trolox equivalent antioxidant capacity assay (Re et al., 1999). This decolorization technique directly produces a colored 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Sigma-Aldrich) chromophore by reaction with potassium persulfate (Sigma-Aldrich). Briefly, the addition of the serum sample antioxidants to the radical cation creates a reduction in ABTS, which is then compared to a known Trolox standard (Sigma-Aldrich) curve under the same conditions. Serum samples were run in triplicates and those with a coefficient of variation of >10% were rerun. Concentrations of AOP were presented as the average Trolox equivalent (TE) per microliter of sample between triplicates. The inter- and intra- assay CV's were 8.1% and 0.4%, respectively.

Redox balance was determined as a ratio of pro-oxidants to total antioxidants (RONS/AOP), known as the oxidant status index (OSi), as it accurately detects changes in redox balance in periparturient dairy cows (Abuelo et al., 2013). An increase in the ratio suggests a higher risk for oxidative stress due to an increase pro-oxidant production or depletion of antioxidant reserves.

Assessment of Antigen-Specific Immunoglobin A Concentrations

Nasal secretion samples were analyzed using a previously described ELISA assay to quantify BHV-1 and BRSV specific immunoglobulin A (Woolums et al., 2013). Briefly, prior to preparing the plates, the stock solution of each virus, BRSV and BHV-1 (National Veterinary Services Laboratory, US Department of Agriculture) were placed under a UV hood for 20 min for virus deactivation. The BRSV (strain A51908-ATCC VR-794) stock solution concentration was 600 $TCID_{50}$/mL and was diluted 1:50 with coating buffer (Thermo-Fisher Scientific). The BHV-1 (Colorado strain) stock solution concentration was 13,600 $TCID_{50}$/mL and was diluted

1:100 with the same coating buffer. Following virus dilution, 100 µL of the coating buffer containing BRSV or BHV-1 was pipetted into each well of respective plates. Plates were then incubated at 4 °C for 12 h. Subsequently, plates were washed three times with 1X ELISA wash buffer (Thermo-Fisher Scientific) using an automatic plate washer (Accuris SmartWasher; Accuris Instruments). After wash, 200 µL of blocking buffer (Thermo-Fisher Scientific) was added to each well then allowed to incubate for 1 h at room temperature. Following each incubation time, plates were washed 3 times as previously described. Once all plates were washed, they were allowed to dry for 2 h at room temperature before being stored at -20 °C in airtight bags with desiccant bags (VWR) until time of use. At time of use, plates were removed from -20 °C and washed 3 times. A 1:10 dilution of the nasal secretion samples was prepared with 1xPBS (pH 7.2) containing 0.1% Tween20 (Sigma-Aldrich). Once prepared, 100 µL of the diluted sample was added to designated wells, allowed to incubate for 1 h at room temperature, and washed 3 times. A 1:50,000 dilution of anti-bovine IgA horseradish peroxidase conjugated sheep polyclonal antibodies (Bethyl Laboratories Inc.) was prepared with 1xPBS containing 0.2% bovine serum albumin (VWR). Subsequently, 100 µL of the diluted antibody were added to all wells, allowed to incubate for 1 h at room temperature, and washed 3 times. Tetramethylbenzidine (TMB) stabilized chromogen (Thermo-Fisher Scientific) was then added to each well (100 µL) and allowed to incubate for 20 min at room temperature. Following 20-min incubation, 100 µL of ELISA stop solution (Thermo-Fisher Scientific) was added to each well. Optical Density (OD) was measured using a plate reader (Synergy H1 Hybrid; Biotek) at 450 nm within 30 min of stop solution being added to wells. OD_{450nm} is proportional to the amount of IgA in the sample. Samples were analyzed in duplicates and the low- and high-

reference samples were run alongside calf samples in all plates for quality control. The inter- and intra-assay CVs were 12.3% and 6.9%, respectively.

Statistical Analyses

Data were managed in Excel (Microsoft) spreadsheets. Statistical analyses were conducted using Rstudio (v. 2022.12.0+353, Posit Software, PBC). A linear mixed model with repeated measures was built for each outcome variable (RONS, AOP, OSi, BHV1-IgA, BRSV-IgA, BW, HH, micronutrient concentrations, and blood metabolites) using the "LME4" package (Bates et al., 2015). Treatment (Supplement 1, Supplement 2, Supplement 3, and Control), time (1-8 weeks of age), and their interaction were included as fixed effects for RONS, AOP, OSi, BW, HH, micronutrient concentrations, and blood metabolites. In addition to reporting each treatment effect, a pre-planned contrast was applied to the primary outcomes anti-BHV1 and -BRSV IgA. A linear mixed model was built for ADG at weaning with treatment as the fixed effect. Calf nested within treatment and farm were included as random effects. Week 0 measurements were included as a covariate. A likelihood ratio test was performed between the full model including all possible variables and reduced models which step-wise removed variables to determine the best model fit for each independent variable. Model assumptions were assessed by evaluation of homoscedasticity and normality of distribution of residuals. Homoscedasticity was assessed by a scatterplot of residuals. A Shapiro-Wilks test was performed, and data were consider normally distributed with a W□ 0.85. To satisfy assumptions, outliers were identified and removed if necessary and appropriate transformations were made (natural log or square root). A type III ANOVA was used to determine the model main effects, with the degrees of freedom approximated using the Kenward-Roger's method. Results are expressed as least squares means (geometric mean for transformed data) and 95% confidence

intervals. Tukey's honest significance test was used to assess post-hoc pairwise comparisons. Statistical significance was set at $P \leq .05$ and tendencies were set for $0.05 < P < 0.1$.

A logistic regression mixed model with repeated measures was built for the health score variables using LME4 package. Treatment of disease was also assessed by a logistic regression mixed model. Respiratory score, temperature score, fecal score, lung consolidation, and treatment of disease were converted to binary data. Respiratory score is represented as 0=normal and 1=positive. Temperature score is represented as 0=normal and 1=elevated. Fecal score is represented as 0=normal and 1=diarrhea. Lung consolidation is represented as 0=no consolidation and 1=consolidation. Treatment of disease is separated into treatment of diarrhea, treatment of respiratory disease, and treatment for any disease with each represented as 0=no treatment and 1=treated. Treatment (Supplement 1, Supplement 2, Supplement 3, and Control) was included as a fixed effect and calf nested within treatment and farm were included as random effects. Adjusted proportions and odds ratios were determined using the emmeans function.

Sample Size Determination

The sample size for this study was determined using JMP Pro 14. (SAS Institute) for IgA nasal secretion concentrations because intranasal vaccine response was the main outcome of the study. We determined that 30 heifers/treatment was the number required to detect a 0.25 $\log_2$ fold increase in anti-BRSV IgA concentrations considering a power of 80%, an alpha of 0.05, an attrition rate of 5%, a standard deviation of 0.24 (Swedzinski et al., 2020) and accounting for clustering in 2 herds.

RESULTS

Micronutrient Concentrations

A treatment effect was identified for serum selenium concentrations (*P*□□.001; Table 2.1). Calves receiving supplement 1 or supplement 2 had higher Se concentrations than control or supplement 3 calves at wk 1 (*P*□□0.001). The increase in Se concentration continued throughout 4 wk of age in calves receiving supplement 1 compared to control calves and supplement 3 but was only greater until wk 2 in calves receiving supplement 2. A trend was identified for calves receiving supplement 1 to have higher selenium concentration than those receiving supplement 2 at wk 6 (*P*□0.06). No treatment effects were identified for serum copper, zinc, or manganese concentrations (*P*□0.42; Table 2.1*)*. However, there was a treatment x time interaction effect identified for copper (*P*□0.013) with control calves having higher copper concentrations compared to those receiving supplement 1 at wk 6 (*P*□0.017). There were no differences between treatments identified for zinc or manganese concentrations throughout the duration of the study. No overall treatment effect was identified for vitamin E or vitamin A (*P*□0.11 and *P*□0.98, respectively). However, calves receiving supplement 3 had higher serum vitamin E concentrations (*P*□0.04) and there was a trend for calves receiving supplement 2 to have higher vitamin E concentrations (*P*□0.09) at wk 1 compared to those receiving supplement 1. A significant time effect is identified for all micronutrients (P□□0.001).

Blood Metabolites

No treatment effects were identified for Alb, BUN, Chol, Gluc, or TP (*P*□0.2, Table 2.2), however, there was a significant treatment effect identified for BHB (*P*=0.05) and a trend for NEFA (*P*=0.08) . There was a time effect identified for all blood metabolites (*P*=<0.001; Table 2.2). A significant treatment x time interaction was identified for BUN, BHB, and NEFA

(*P*<0.001, *P*=0.03, and *P*=0.01 respectively). Calves receiving supplement 3 had lower concentrations of BHB compared to supplement 1 and control calves at wk 1. Furthermore, supplement 3 calves had lower BHB concentrations compared to supplement 1, 2, and control calves at wk 4 (Table 2.2). Also, a trend was identified for supplement 2 calves to have lower BHB concentrations than control calves at wk 2 (*P*=0.07). Calves receiving supplement 3 had lower NEFA concentrations compared to supplement 1 and control calves at wk 1 (P=<0.01). Also, a trend was identified for supplement 2 calves to have lower NEFA concentrations than supplement 1 at wk 1 and 5 (*P*=0.08 and P=0.09, respectively). A significant difference was detected at wk 7 with calves receiving supplement 1 having lower NEFA concentrations compared to control calves (*P*=0.02). Calves receiving supplement 3 had lower BUN concentrations compared to control calves at wk 1 and 4 (*P*=<0.001). Calves receiving supplement 2 had lower concentrations of BUN than supplement 1 and control calves at wk 1 (*P*<0.01). Also, calves receiving supplements 1 or 2 had lower BUN concentrations compared to control calves at wk 4 (*P*=<0.01). A significant difference was identified for Alb as calves receiving supplements 1 or 3 had lower Alb concentrations compared to control calves at wk 1 (*P*=<0.05). Also, a trend was identified for supplement 3 calves to have lower Alb than control calves at wk 4 (*P*=0.09).

Redox Balance

No significant treatment effect was identified for AOP (*P*=0.83). However, a significant time effect was identified, and a trend was identified for treatment x time effect (Table 2.3). Calves receiving supplement 2 had higher AOP compared to control calves at wk 2 (*P*=0.005). Also, a trend was identified for supplement 2 calves to have higher AOP than supplement 1 calves at wk 2 (*P*=0.098). Significant treatment and time effects were identified for RONS

(*P*=0.047 and *P*=<0.001, respectively). Calves receiving supplement 2 had significantly lower RONS compared to supplement 1 and control calves in wk 1 (*P*=0.04 and *P*=0.01, respectively). Supplement 2 calves continued to have significantly lower RONS than supplement 1 calves in wk 2 (*P*=0.01). A significant time effect was identified for OSi (*P*=<0.001). Calves receiving supplement 2 had significantly lower OSi compared to control calves at wk 1 (*P*=0.03). Also, a trend was identified for supplement 3 calves to have lower OSi than control calves at wk 1 (*P*=0.09). Supplement 2 calves had significantly lower OSi compared to supplement 1 calves at wk 2 (*P*=0.002).

Intranasal Vaccine Response

No significant treatment effect was identified for either anti-BRSV or anti-BHV1 IgA (*P*=0.41 and *P*=0.54 , respectively) as well as no differences detected between treatments throughout the duration of the study (Figure 2). Therefore, pre-planned contrast results are presented here. A significant treatment effect was identified for anti-BRSV IgA (*P*=0.03; Figure 3). A trend was identified for calves receiving a supplement to have higher anti-BRSV IgA concentrations compared to control at wk 5 and 7 (*P*=0.08 and *P*=0.06, respectively). Calves receiving a supplement had significantly higher anti-BRSV IgA concentrations than control calves at wk 8 (*P*=0.03). No significant treatment effect was identified for anti-BHV1 IgA (*P*=0.35) and there were no significant differences in anti-BHV1 IgA concentrations between calves receiving a supplement or control throughout the duration of the study. A significant time effect was identified for both anti-BRSV and anti-BHV1 IgA (*P*=<0.001) in which an increase in concentrations began at wk 1 and continued throughout wk 6. Both anti-BRSV and anti-BHV1 IgA concentrations went down from wk 6 to wk 8.

Growth Performance and Health Status

No treatment effects were identified among groups for hip height or body weight throughout the duration of the study (P=0.8 and P=0.6, respectively; Figure 4). A significant time effect was identified for both HH and BW (P=<0.001). Also, there was no significant treatment effect for ADG at weaning (P=0.34). Average daily gain (□ SE) for control, supplement 1, supplement 2, and supplement 3 were 0.97 □ 0.07, 0.94 □ 0.07, 0.96 □ 0.07, and 1.01 □ 0.07 kg/d, respectively.

The risk of positive respiratory signs, fecal event, elevated temperature, and lung consolidation were 5.3%, 23.1%, 23.2%, and 21.3%, respectively. Also, the risk of being treated for any disease, diarrhea, or respiratory disease were 2.3%, 1.6%, and 0.7%, respectively. There were no significant differences in the probability of supplemented calves (Supplement 1, Supplement 2, or Supplement 3) or control calves to have positive clinical respiratory signs, a fecal event, or lung consolidation (Table 2.5). However, calves receiving supplement 3 were 1.7 times more likely to have an elevated temperature compared to control calves (P=0.05). Also, there were no differences in the probability of supplemented or control calves to be treated for any disease, diarrhea, or respiratory disease (Table 2.6).

DISCUSSION

Micronutrients and Blood Metabolites

In the current study, we analyzed serum micronutrient status throughout the pre-weaning period which indicated that micronutrient supplementation, containing selenium, at birth results in higher serum selenium concentrations throughout the first 4 wk of life (Table 2.1). There was no treatment effect identified for serum zinc, copper, manganese, vitamin E and vitamin A concentrations. However, calves receiving supplementation containing vitamin E had higher

vitamin E concentrations at wk 1. Similarly, there is a previous study that supplemented trace minerals at 3 d of age which reported higher serum selenium concentrations at 3, 14, and 35 days of age (Teixeira et al., 2014). Previous studies also reported no differences in zinc or copper concentrations (Richeson and Kegley, 2011, Bates et al., 2020). In contrast to our findings, there are reports of higher serum zinc concentrations at 14 d of age (Teixeira et al., 2014) as well as higher serum copper concentrations from week 2 to wk 8 (Bates et al., 2020). Potential explanation for differences in micronutrient concentrations include age of calves (birth-9 mo old), management type (dairy, pasture, feedlot), as well as the documented variability of serum micronutrient analysis.

Serum analysis is not the most effective to determine micronutrient concentrations, but it is commonly used in the industry to assess status (Spears et al., 2022). Copper is stored in the liver, and serum concentrations are often a misrepresentation of true copper status because of the lack of circulating copper as well as its interaction with the clotting process (Spears et al., 2022). There is no clear indication of a best method to measure manganese concentrations and it is reported that the possibility for error in serum is wide due to low circulating blood concentrations (Spears et al., 2022). It is documented that whole blood analysis of selenium is preferred because serum selenium is often increased due to hemolysis of red blood cells during serum harvesting (Spears et al., 2022). As mentioned, trace minerals are primarily stored in the liver and therefore, liver samples are the most ideal sample to determine micronutrient status. However, due to the study being implemented on commercial dairy farms, it was not feasible to include repeated liver biopsies. The primary outcome of this study was not micronutrient concentrations and therefore, serum was utilized as a convenience sample. Along with the challenges presented above, it is possible that the concentrations of parenteral micronutrients

supplemented are not sufficient to result in an increase in serum concentrations despite following label dosages. As such, further research is required to create and implement evidence-based dosing protocols to provide adequate supplementation. On the other hand, it is likely that calves supplemented with antioxidants at birth utilized the supplemented micronutrients, as evidenced by the lower RONS in the first two wk of life, and therefore, higher serum concentrations were not able to be detected.

Due to the impact oxidative stress has on metabolic status, the metabolic profile was assessed in the current study to determine the effect of parenteral antioxidant supplementation on neonatal calf metabolic status. A significant treatment x time interaction was identified for BHB, BUN, and NEFA, with calves receiving parenteral antioxidant supplementation having lower BHB, BUN, and NEFA serum concentrations compared to control calves (Table 2.2). It is of note the majority of the significant differences identified for these energy and protein biomarkers were in calves receiving supplementation containing vitamin E. This is potentially due to the role vitamin E plays in providing protection against lipid peroxidation (Halliwell and Gutteridge, 1999) and therefore, reducing pro-oxidant production and maintaining energy balance. During early life, calves utilize glucose as the primary energy source and with rumen development as well as increased starter grain intake, there is a shift in energy source to short chain fatty acids such as butyrate (Quigley et al., 1991). It is possible that lower serum BHB concentrations are due to improved utilization of BHB in calves supplemented with antioxidants at birth. Increased circulating NEFA and BHB concentrations can be indicative of fat mobilization due to negative energy balance, thus suggesting that antioxidant supplemented calves had a metabolic profile of better energy status (lower concentrations of NEFA and BHB). Due to the relation between energy biomarkers and OS (Sordillo and Aitken, 2009), it is possible that the observed improved

metabolic energy status was due to the enhanced redox balance that resulted from the micronutrient supplementation.

Redox Balance

Redox balance was the mechanism we investigated as a potential contributor to improved intranasal vaccine response. The literature on redox biology in calves is scarce and although more widely studied in mature cattle, the results cannot be directly translated to neonates (Perez-Santos et al., 2015). There are previous studies in which an increased parenteral and intranasal vaccine response was identified with parenteral micronutrient supplementation (Palomares et al., 2016, Nayak and Abuelo, 2021) but to our knowledge, this is the first study to investigate the potential for redox balance to play a role in improved immune response throughout the pre-weaning period. The current study identified a treatment effect for RONS with calves supplemented with antioxidants at birth having lower RONS throughout the first two wk of life (Table 2.3). It is likely that calves supplemented with antioxidants at birth had lower RONS due to supplementation improving antioxidant capacity and therefore counteracting over-production of RONS.

While current literature assessing antioxidant supplementation on redox balance, including both antioxidant capacity and pro-oxidant production is scarce, it is often that total antioxidant status is measured as a response to antioxidant supplementation. Therefore, the current study assessed total antioxidant capacity which resulted in calves supplemented with antioxidants at birth having higher AOP and lower OSi throughout the first two weeks of life (Table 2.3) which is supported by a previous study (Nayak and Abuelo, 2021). It is likely that calves supplemented with antioxidants at birth had better redox balance due to micronutrient supplementation and better energy status. As mentioned previously, it is possible that

supplemented calves utilized the supplemented micronutrients and therefore, an increase in serum concentrations was not detectable. Similarly, a study supplementing 7 mo-old beef calves with vitamins and trace minerals at weaning (Se, Cu, Zn, Mn, Vitamin A, Vitamin E) reported increased total antioxidant status in supplemented calves compared to control calves 60 d after weaning (Mattioli et al., 2020). While these studies show comparable results, it is important to note that calves experience OS within the first month of life (Abuelo et al., 2014) and therefore, more research is required to assess redox balance during early life. The current study expands upon findings from proof-of-principle evidence that parenteral antioxidant supplementation at birth can improve redox balance within the first two weeks of life. As such, calves supplemented with antioxidants at birth might have improved immune response during critical weeks of immune development.

Intranasal Vaccine Response

The primary outcome of this study was intranasal vaccine response, evaluated as anti-BRSV and anti-BHV1 IgA concentrations in nasal secretions. Calves supplemented with antioxidants at birth tended to have higher anti-BRSV IgA at wk 5 and 7 than control calves (Figure 3). Also, calves supplemented with commercial antioxidant products at birth had significantly higher anti-BRSV IgA at wk 8 compared to control calves. Calves supplemented with antioxidants at birth likely had improved vaccine response throughout the pre-weaning period due to micronutrient supplementation which improved metabolic energy status as well as improved redox balance during early life. Previous studies have shown improved immune response to vaccines administered parenterally concurrent with antioxidant supplementation (Palomares et al., 2016, Mattioli et al., 2020). However, not only was the route of vaccine administration different in these studies, but the study calves were also older (3.5-7 months old).

While weaning stress is a critical time for disease risk in young cattle, they have a more developed immune system at this age (Chase et al., 2008), and it is known that oxidative stress impacts neonatal calves within the first month of life (Abuelo et al., 2014, Abuelo et al., 2019). Nevertheless, these studies provide evidence that parenteral antioxidant supplementation can improve vaccine response in calves potentially by improving redox balance.

There is a study that also supplemented antioxidants at birth and evaluated response to intranasal vaccination, reporting an increase in vaccine response starting at wk 1 throughout 4 wk of age (Nayak and Abuelo, 2021). As mentioned previously, this study had a limited sample size. Therefore, our findings expand upon that study, suggesting that calves supplemented with antioxidants at birth have an increase immune response to intranasal vaccination throughout the pre-weaning period. Nevertheless, there is some contradicting evidence that found no difference in antibody production, specifically for *Salmonella* spp., in calves treated with ITM (Se, Cu, Zn, Mn, Cr) at two weeks of age compared to control calves (Bates et al., 2020). This could suggest that antioxidant supplementation at birth can improve immune response in young calves but is potentially masked by maternal antibody interference in studies that use parenteral vaccination in neonatal calves. To our knowledge, this is the first study to evaluate intranasal vaccine response throughout the entire pre-weaning period, strengthening the evidence that parenteral antioxidant supplementation at birth can improve pre-weaning calf immunity.

Growth and Health Performance

In the current study, there was no treatment effect identified for BW or HH throughout the pre-weaning period (Figure 4). Also, no difference in ADG at weaning was identified between supplemented and control calves. Similar to our findings, previous studies using 7-8-mo-old calves did not see a difference in ADG between trace mineral supplemented calves and

control calves (Arthington et al., 2014, Vedovatto et al., 2020). In contrast to our findings, however, a previous study reported an increase in ADG and final BW for calves receiving trace mineral supplementation. These calves were also older as indicated by initial body weight of 90 kg (Richeson and Kegley, 2011). Given that the current study found improved energy profile, improved redox balance, and improved immune response, we would expect to see physiological changes, i.e., greater growth in calves supplemented with antioxidants at birth compared to control calves. Trace minerals and vitamins play a positive role in energy requirements and utilization, relative to biological processes such as growth in calves. As discussed previously, the current study found lower concentrations of BHB and NEFA in calves supplemented with micronutrients at birth, suggesting better energy utilization. It is possible that the current supplementation provided enough micronutrients to improve redox balance, immune response, and energy profile but due to the direct relationship of energy metabolites and immune cell function (Calder, 2013), the magnitude of supplementation was not enough to utilize micronutrients for growth performance. It is important to note that the commercial products used throughout the literature are labeled for treatment of deficiency, contributing to the notion that evidence based supplementation strategies are crucial for optimization. A potential explanation for the difference in results presented in the literature is the difference in management and nutrition as the studies vary from dairy production, feedlot, and pasture rearing.

Health status and disease treatment are often evaluated to contextualize immunity to determine application of novel strategies. In this study, we did not find a difference in the probability of showing positive respiratory signs, diarrhea, or lung consolidation in supplemented calves compared to control calves. However, it was identified that calves supplemented with antioxidants at birth had a higher probability of having elevated temperatures

compared to the control group. Calves supplemented with antioxidants at birth had improved metabolic energy balance, improved redox balance, and improved vaccine response, suggesting that they were able to respond to natural disease exposure better than control counterparts. Elevated temperatures are often a sign of the immune system responding to pathogen invasion and therefore, it is possible that the supplements provided calves with a stronger response to pathogen exposure. However, there was no difference in the probability of calves being treated for any disease, diarrhea, or respiratory disease, suggesting that although antioxidant supplementation might have allowed for better immune response, the magnitude of treatment did not affect disease occurrence. In contrast to our findings, previous studies reported lower incidence of respiratory disease and diarrhea in calves supplemented with micronutrients compared to control calves (Teixeira et al., 2014, Bates et al., 2019). Similarly, another study reports lower odds of diarrhea in calves supplemented with selenium and vitamin E at birth than control calves (Leslie et al., 2019). However, this study also reports no difference in the likelihood of experiencing respiratory disease, which supports our findings. A noticeable difference throughout current micronutrient supplementation literature is the difference in supplementation protocol. The current study dosed micronutrient supplementation per birth weight whereas the other studies used a standardized dose for all calves, which might contribute to differences in results. It is of note that the location of study might contribute to differences in results presented. Prevalence of disease can vary per farm as well as per geographic region. In areas where disease incidence is greater, it might be easier to detect differences that might stay unnoticed in farms with lower prevalence. For example, in the current study only 0.7% of calves were treated for respiratory disease, likely masking possible treatment effects. Not only would large multi-herd studies increase the number of animals, increasing power to detect differences,

but it would also allow for more variation in management practices and likely more variation in disease prevalence. It is likely that it would be easier to detect differences due to intervention with higher disease prevalence. Therefore, study management and farm management should be considered when reviewing health and treatment data. Overall, further research is required to determine appropriate dose and frequency of micronutrient supplementation to improve calf health and growth.

Study Limitations

Given the primary outcome of this study being immune parameters, growth and health status were utilized to contextualize results. However, the sample size might have influenced the ability to detect differences in growth performance and health status as it was calculated based on mucosal IgA concentrations. Therefore, we cannot exclude the lack of differences in growth and health in this study and further multi-herd research is required to appropriately evaluate the impact of parenteral antioxidant supplementation at birth on calf health and growth.

It is important to note that the current study was completed on two farms in which had similar management practices. Thus, the results presented might not translate to the whole dairy industry. For example, the study farms feed □7 liters of milk per day until weaning whereas almost 70% of pre-weaning heifers in the US are fed □7 liters per day (USDA,2014a). Therefore, the impact of the strategies presented on calves receiving less nutrition remains to be explored and the differences in liquid feeding could potentially explain some of the differences observed throughout the literature.

CONCLUSIONS

The administration of parenteral antioxidant supplements at birth concurrent with intranasal vaccination in neonatal calves resulted in improved redox balance for the first two

weeks of life as well as improved vaccine response throughout the pre-weaning period. Furthermore, there were no differences detected among the commercial products in regard to vaccine responsiveness. Therefore, injectable micronutrient supplementation could be an appropriate strategy to improve neonatal calf immunity. However, no differences were identified in growth performance and health status and further research is required to expand upon current findings.

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TABLES

Table 2.1. Injectable micronutrient supplementation at birth effect on serum micronutrient concentrations.

	Estimated means of each time point birth to weaning																																		
	Week 1				Week 2				Week 3				Week 4				Week 5				Week 6				Week 7				Week 8				P-value		
Outcome	S.1	S.2	S.3	C	S.1	S.2	S.3	C	S.1	S.2	S.3	C	S.1	S.2	S.3	C	S.1	S.2	S.3	C	S.1	S.2	S.3	C	S.1	S.2	S.3	C	S.1	S.2	S.3	C	SE	TRT	T	TRT x T
Selenium (ng/mL)	4.5[a]	4.4[a]	4.23[b]	4.15[b]	4.33[a]	4.23[a]	4.08[b]	4.07[b]	4.27[a]	4.2[ab]	4.11[b]	4.12[b]	4.31[a]	4.23[a b]	4.2[b]	4.14[b]	4.24	4.18	4.16	4.16	4.23	4.1	4.16	4.16	4.26	4.21	4.25	4.25	4.26	4.21	4.28	4.23	0.03	<0.001	<0.001	<0.001
Copper (µg/mL)	0.79	0.71	0.69	0.69	0.74	0.70	0.73	0.68	0.66	0.74	0.73	0.75	0.81	0.83	0.80	0.74	0.83	0.84	0.86	0.82	0.81	0.86	0.87	0.98	0.90	0.87	0.94	0.98	0.91	0.93	0.94	0.90	0.05	0.96	<0.001	0.01
Zinc (µg/mL)	0.86	0.97	0.91	0.94	0.99	1.01	1	1	1	1.02	0.96	1.03	1.19	1.2	1.16	1.12	1.13	1.13	1.18	1.12	1.04	1.11	1.21	1.16	1.23	1.28	1.36	1.23	1.29	1.33	1.41	1.22	0.07	0.42	<0.001	0.89
Manganese (ng/mL)	0.67	0.67	0.74	0.61	0.64	0.75	0.67	0.68	0.52	0.66	0.53	0.57	0.65	0.57	0.66	0.57	0.76	0.73	0.72	0.61	0.83	0.75	0.77	0.71	0.74	0.89	0.87	0.81	0.69	0.87	0.89	0.66	0.08	0.46	<0.001	0.89
Vitamin A (ng/mL)	5.95	5.86	6.02	6.03	6.17	5.95	6.11	6.07	6.03	6.11	6.07	6.15	6.26	6.32	6.25	6.14	6.29	6.48	6.29	6.35	6.18	6.4	6.4	6.31	6.28	6.39	6.3	6.44	6.45	6.39	6.36	6.38	0.88	0.98	<0.001	0.33
Vitamin E (µg/mL)	0.09	1.29	1.35	1.11	1.92	2.07	1.95	1.88	2.08	2.13	2.21	2.19	2.27	2.44	2.28	2.23	2.19	2.32	2.35	2.55	2.31	2.46	2.52	2.29	2.36	2.43	2.48	2.31	2.36	2.5	2.56	2.39	0.12	0.11	<0.001	0.79

S=Supplement; C=Control; SE=Standard error; TRT=Treatment; T=Time. A linear mixed model was built and analyzed, including treatment (Supplement 1, Supplement 2, Supplement 3, Control), time (week 1-week 8), and treatment x time interaction (TrtxT) as fixed effects. Calf per treatment group and farm were included as random effects. Tukey's honest significant test was performed for pairwise comparisons. Results are presented as least squares means for each time point. [a-c] Mean values in the same row with different superscripts differ ($P \leq 0.05$) for the interaction of antioxidant supplementation and micronutrient concentration.

Table 2.2. Injectable micronutrient supplementation at birth effect on blood metabolites.

Estimated means of each time point birth to weaning																																				
	Week 1				*Week 2*				*Week 3*				*Week 4*				*Week 5*				*Week 6*				*Week 7*				*Week 8*					*P-value*		
Outcome	*Sup. 1*	*Sup. 2*	*Sup. 3*	*Con*	*Sup. 1*	*Sup. 2*	*Sup. 3*	*Con*	*Sup. 1*	*Sup. 2*	*Sup. 3*	*Con*	*Sup. 1*	*Sup. 2*	*Sup. 3*	*Con*	*Sup. 1*	*Sup. 2*	*Sup. 3*	*Con*	*Sup. 1*	*Sup. 2*	*Sup. 3*	*Con*	*Sup. 1*	*Sup. 2*	*Sup. 3*	*Con*	*Sup. 1*	*Sup. 2*	*Sup. 3*	*Con*	*SE*	*TRT*	*T*	*TRTxT*
Alb. (g/dL)	2.6	2.6	2.6	2.8	2.8	2.9	2.9	3.0	3.0	3.1	3.1	3.2	3.0	3.1	3.0	3.2	3.3	332	3.3	3.2	3.5	3.4	3.5	3.5	3.7	3.7	3.7	3.8	*3.8*	*3.7*	*3.7*	*3.7*	*0.13*	*0.2*	*<0.001*	*0.2*
BUN mg/dL)	9.0	6.9	7.9	10.5	7.8	7.1	7.9	6.7	8.7	8.9	8.4	8.2	9.0	7.5	7.7	11.2	8.9	9.8	9.1	8.9	9.6	9.8	9.6	9.4	10	10.7	10.4	10.7	*11.5*	*11.3*	*11.2*	*10.9*	*0.45*	*0.3*	*<0.001*	*<0.001*
BHB (mg/dL)	0.86[a]	0.73[ab]	0.69[b]	0.86[a]	0.72	0.61	0.68	0.76	0.8	0.82	0.81	0.83	0.78[a]	0.77[a]	0.58[b]	0.81[a]	0.82	0.77	0.82	0.84	0.84	0.8	0.78	0.76	0.75	0.76	0.71	0.79	*0.82*	*0.82*	*0.88*	*0.78*	*0.09*	*0.05*	*<0.001*	*0.03*
Chol. (mg/dL)	57.1	62.1	58.1	60.5	91.3	103.6	101.5	101.1	122.6	121.3	126.6	132.8	113.5	117.8	119.7	120.9	117.1	119.1	122.2	120.7	120.4	120.9	123.9	121.4	128.2	125.4	129.4	127.9	*126.8*	*130.3*	*130.6*	*131.8*	*0.54*	*0.78*	*<0.001*	*0.97*
Gluc. (mg/dL)	123	124	120	124	136	143	138	131	129	137	138	134	137	133	133	133	139	137	141	144	145	150	146	144	148	153	150	147	*141*	*141*	*142*	*139*	*4.05*	*0.81*	*<0.001*	*0.84*
NEFA (mmol/L)	0.58[a]	0.49[ab]	0.46[b]	0.57[a]	0.46	0.45	0.46	0.48	0.54	0.5	0.53	0.53	0.52	0.49	0.44	0.52	0.5	0.59	0.55	0.53	0.52	0.53	0.53	0.52	0.46[b]	0.53[ab]	0.53[ab]	0.56[a]	*0.72*	*0.66*	*0.66*	*0.71*	*0.04*	*0.08*	*<0.001*	*0.01*
TP (g/dL)	5.8	5.8	5.5	5.8	5.5	5.5	5.5	5.5	5.6	5.6	5.6	5.6	5.4	5.4	5.3	5.4	5.6	5.6	5.6	5.6	6.0	5.8	5.8	5.9	6.2	6.2	6.1	6.3	*6.0*	*5.9*	*5.9*	*5.9*	*0.18*	*0.7*	*<0.001*	*0.8*

Alb.=Albumin, Chol.=Cholesterol, Gluc.=Glucose, NEFA= Nonesterified fatty acids, TP=Total protein, Sup.=Supplement, Con=Control, TRT=Treatment, T=Time, TRTxT=Treatment x Time Interaction. A linear mixed model was built and analyzed, including treatment (Supplement 1, Supplement 2, Supplement 3, Control), time (week 1-week 8), and treatment x time interaction (TrtxT) as fixed effects. Calf per treatment group and farm were included as random effects. Tukeys honest significant test was performed for pairwise comparisons. Results are presented as least squares means for each time point. [a-c] Mean values in the same row with different superscripts differ ($P \leq 0.05$) for the interaction of antioxidant supplementation and blood metabolite.

Table 2.3. Injectable micronutrient supplementation at birth effect on redox balance.

	Estimated means of each time point birth to weaning																																	
	Week 1				Week 2				Week 3				Week 4				Week 5				Week 6				Week 7				Week 8				P-value			
Outcome	S.1	S.2	S.3	C	S.1	S.2	S.3	C	S.1	S.2	S.3	C	S.1	S.2	S.3	C	S.1	S.2	S.3	C	S.1	S.2	S.3	C	S.1	S.2	S.3	C	S.1	S.2	S.3	C	SE	TRT	T	TRT x T
AOP (TE/µl)	1.02	1.05	1.11	1.0	0.97	1.13	1.02	0.91	0.95	1.03	0.94	1.02	1.14	1.07	1.04	1.05	1.05	0.99	0.98	1.01	1.12	1.11	1.04	1.03	1.14	1.01	1.13	1.13	1.02	1.04	1.03	1.11	0.04	0.83	0.004	0.06
RONS (RFU)	137[a]	99.6[b]	126.4[ab]	143.8[a]	140.2[a]	106.6[b]	126.6[ab]	126[ab]	113.4	102.5	101.1	104.2	122.5	103.2	101.3	103.9	104.6	99.8	106.4	98.1	106.7	95.9	102.7	95.8	120.3	89.6	90.8	104	108.7	104.6	103.4	99.8	19.8	0.047	<0.001	0.54
OSi (Arbitrary Units)	6.59	6.18	6.34	7.36	7.48	6.0	6.5	6.79	6.51	6.29	6.35	6.05	6.04	5.93	5.85	6.05	5.92	5.87	6.37	5.95	5.94	5.62	6.03	5.71	6.08	5.65	5.48	5.74	5.94	5.9	6.11	5.58	0.57	0.28	<0.001	0.16

AOP=Antioxidant potential, RONS=Reactive oxygen and nitrogen species, OSi=Oxidant status index, TE=Trolox equivalents; RFU=Relative fluorescence units; S=Supplement; Control; SE=Standard error; TRT=Treatment; T=Time. A linear mixed model was built and analyzed, including treatment (Supplement 1, Supplement 2, Supplement 3, Control), time (week 1-week 8), and treatment x time interaction (TrtxT) as fixed effects. Calf per treatment group and farm were included as random effects. Tukey's honest significant test was performed for pairwise comparisons. Results are presented as least squares means for each time point. [a-c] Mean values in the same row with different superscripts differ ($P \leq 0.05$) for the interaction of antioxidant supplementation and redox balance.

Table 2.4. Model main effect estimates of treatments for anti-BRSV and anti-BHV1 IgA concentrations.

Variable (units)	Treatment groups				*P*-Values					
	Control	Suppl. 1	Suppl. 2	Suppl. 3	Control vs. Suppl. 1	Control vs. Suppl. 2	Control vs. Suppl. 3	Suppl. 1 vs. Suppl. 2	Suppl. 1 vs. Suppl. 3	Suppl. 2 vs. Suppl. 3
anti-BRSV IgA (log (OD_{450}))	-0.88 (-1.10 - -0.68)	-0.78 (-1.07 - -0.50)	-0.77 (-0.96 - -0.57)	-0.75 (-0.94 - -0.55)	1	1	0.78	1	1	1
anti-BHV1 IgA (log (OD_{450}))	-1.41 (-1.87 - -0.95)	-1.42 (-1.93 - -0.92)	-1.35 (-1.77 - -0.94)	-1.35 (-1.73 - -0.97)	1	1	1	1	1	1

Suppl. = Supplement ; OD_{450} = Optical density at 450 nm. A linear mixed model was built and analyzed, including treatment (Control and Supplement), time (week 1-week 8), and treatment x time interaction (TrtxTime) as fixed effects. Calf per treatment group and farm were included as random effects. Nasal secretions collected at birth, prior to vaccination were included as a covariate. Tukeys honest significant test was performed for pairwise comparisons. Results are presented as least squares means and 95% confidence intervals.

Table 2.5. Injectable micronutrient supplementation at birth effect on health outcomes.

Health Event	Contrast	Odds Ratio	95% CI	P-Value
Respiratory Event				
	Suppl. 3 vs Suppl. 2	0.99	0.22-4.49	0.99
	Suppl. 3 vs. Suppl. 1	1.23	0.22-7.07	0.82
	Suppl. 3 vs. Control	0.83	0.18-3.88	0.81
	Suppl. 2 vs. Suppl. 1	1.24	0.22-7.05	0.81
	Suppl. 2 vs. Control	0.83	0.18-3.87	0.82
	Suppl. 1 vs. Control	0.67	0.11-3.98	0.66
Temperature Event				
	Suppl. 3 vs Suppl. 2	1.10	0.61-1.92	0.79
	Suppl. 3 vs. Suppl. 1	1.10	0.56-2.15	0.78
	Suppl. 3 vs. Control	1.69	1.00-2.85	0.05
	Suppl. 2 vs. Suppl. 1	1.0	0.49-2.10	0.96
	Suppl. 2 vs. Control	1.6	0.87-2.81	0.14
	Suppl. 1 vs. Control	1.5	0.77-3.04	0.22
Fecal Event				
	Suppl. 3 vs Suppl. 2	0.69	0.35-1.39	0.31
	Suppl. 3 vs. Suppl. 1	0.87	0.44-1.71	0.68
	Suppl. 3 vs. Control	0.63	0.30-1.29	0.21
	Suppl. 2 vs. Suppl. 1	1.25	0.67-2.32	0.48
	Suppl. 2 vs. Control	0.90	0.46-1.77	0.76
	Suppl. 1 vs. Control	0.72	0.37-1.39	0.33
Lung Consolidation				
	Suppl. 3 vs Suppl. 2	0.95	0.47-1.92	0.88
	Suppl. 3 vs. Suppl. 1	1.42	0.75-2.69	0.28
	Suppl. 3 vs. Control	1.08	0.59-1.97	0.79
	Suppl. 2 vs. Suppl. 1	1.49	0.73-3.06	0.27
	Suppl. 2 vs. Control	1.14	0.58-2.25	0.71
	Suppl. 1 vs. Control	0.76	0.41-1.41	0.38

Suppl.=Supplement. A logistic regression mixed model was built and analyzed for respiratory score, temperature score, and fecal score. Treatment was included as a fixed effect and calf nested within treatment and farm were included as random effects. Odds ratio for contrasts between treatments are reported.

Table 2.6. Injectable micronutrient supplementation effect on treatment of disease.

Treatment of Disease	Contrast	Odds Ratio	95% CI	P-value
Any Disease				
	Suppl. 3 vs Suppl. 2	1.28	0.69-2.5	0.43
	Suppl. 3 vs. Suppl. 1	1.05	0.58-1.89	0.88
	Suppl. 3 vs. Control	1.15	0.63-2.12	0.64
	Suppl. 2 vs. Suppl. 1	0.82	0.43-1.53	0.52
	Suppl. 2 vs. Control	0.89	0.47-1.71	0.74
	Suppl. 1 vs. Control	1.10	0.60-2.03	0.76
Diarrhea				
	Suppl. 3 vs Suppl. 2	1.24	0.59-2.59	0.57
	Suppl. 3 vs. Suppl. 1	1.54	0.58-4.10	0.38
	Suppl. 3 vs. Control	1.00	0.50-2.01	1.00
	Suppl. 2 vs. Suppl. 1	1.25	0.46-3.42	0.66
	Suppl. 2 vs. Control	0.81	0.39-1.69	0.57
	Suppl. 1 vs. Control	0.65	0.24-1.72	0.38
Respiratory				
	Suppl. 3 vs Suppl. 2	2.44	0.05-111	0.65
	Suppl. 3 vs. Suppl. 1	0.47	0.06-3.51	0.46
	Suppl. 3 vs. Control	1.08	0.13-9.21	0.94
	Suppl. 2 vs. Suppl. 1	0.19	0.01-5.13	0.33
	Suppl. 2 vs. Control	0.44	0.02-12.72	0.63
	Suppl. 1 vs. Control	2.28	0.69-7.55	0.18

Suppl.=Supplement. A logistic regression mixed model was built and analyzed for treatment of disease (any, diarrhea, respiratory). Treatment was included as a fixed effect and calf nested within treatment and farm were included as random effects. Odds ratio for contrasts between treatments are reported.

FIGURES

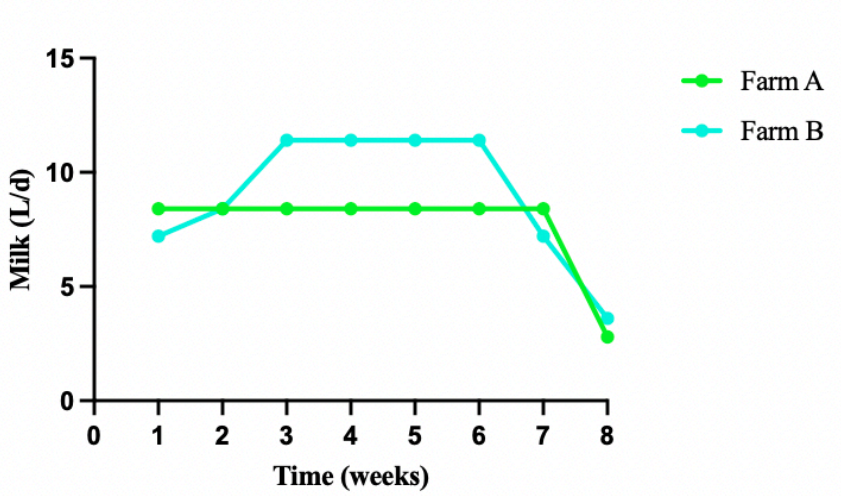

Figure 1. Calf feeding regime from birth to weaning for each farm. Milk was offered as a 1:1 mix of milk replacer and pasteurized waste milk. Quantity is presented as liters per day.

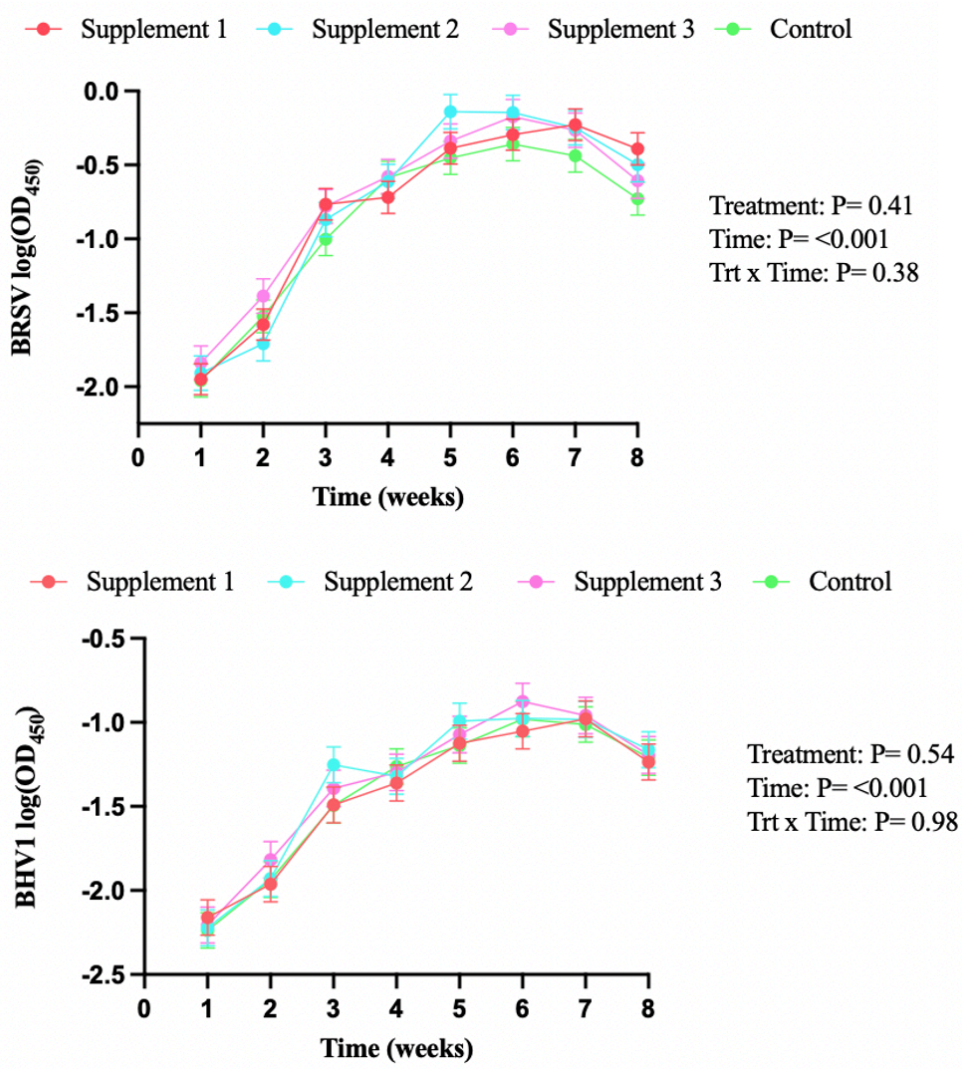

Figure 2. Concentrations of anti-BRSV and anti-BHV1 IgA throughout the pre-weaning period (week 1-week 8). A mixed model was built and analyzed, including treatment (Control, Supplement 1, Supplement 2, Supplement 3), time (week 1-week 8), and treatment x time interaction (Trt x Time) as fixed effects. Calf nested within treatment group and farm were random effects. Nasal secretions collected at birth, prior to vaccination were included as a covariate. Results are presented as least squares means and 95% CI.

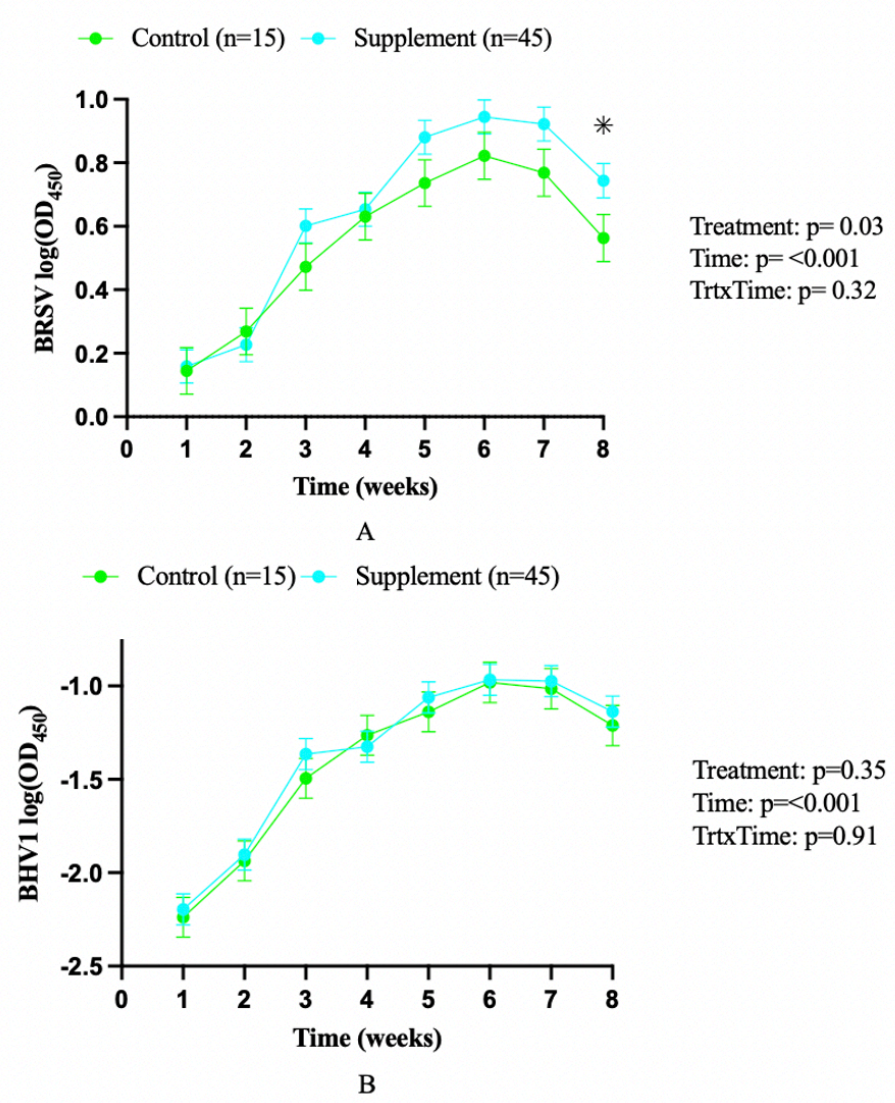

Figure 3. Concentrations of anti-BRSV (A) and anti-BHV1 IgA (B) throughout the pre-weaning period (week 1-week 8). A mixed model was built and analyzed, including treatment (Control and Supplement), time (week 1-week 8), and treatment x time interaction (Trt x Time) as fixed effects. Calf nested within treatment group and farm were included as random effects. Nasal secretions collected at birth, prior to vaccination were included as a covariate. Results are presented as least squares means and 95% CI. * denotes significant differences (P<0.05) between the control and supplement groups at given time.

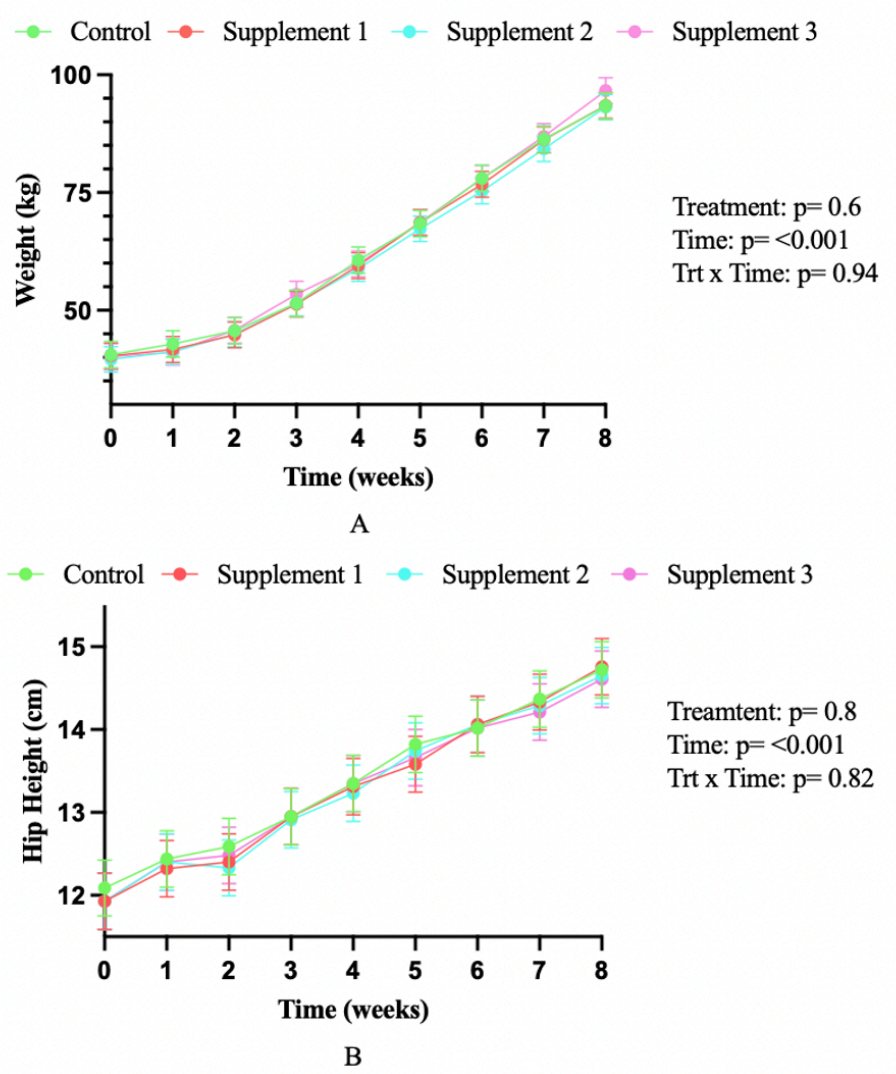

Figure 4. Body weight (A) and hip height (B) throughout the pre-weaning period (week 0 - week 8). A mixed model was built and analyzed, including treatment (Control, Supplement 1, Supplement 2, Supplement 3), time (week 0-week 8), and treatment x time interaction (Trt x Time) as fixed effects. Calf nested within treatment group and farm were included as random effects. Results are presented as least squares means and 95% CI.

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