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Supplementation with selenium, iron, and vitamin E in calves under immunological challenge

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Introduction: The management and nutrition of young calves are important for the production system due to their great impact on future milk production potential. This study was to evaluate the performance and health of calves in the suckling phase, with supplementation of selenium, iron, and vitamin E while undergoing immunological challenge with *Anaplasma marginale*.

Methods: An experiment was conducted on an experimental farm using 42 newborn Holstein male calves, aged up to 60 days, in a completely randomized design, and they were assigned to the following three treatments: C (control milk replacer, N = 14); SeVitE (milk replacer supplemented with 0.6 mg organic selenium/kg + 100 IU vitamin E, N = 14); SeVitEFe (milk replacer supplemented with 0.6 mg organic selenium/kg + 100 IU vitamin E + 200 mg Fe chelate/kg, N = 14).

Results: Selenium, iron, and vitamin E intakes were higher in supplemented animals. The mean blood count of *Anaplasma marginale* was lower in supplemented calves compared to controls and increased with time after inoculation. Increased serum selenium was observed in supplemented calves, without vitamin E and iron changes. Plasma lactate concentration was lower in supplemented animals and those with SeVitEFe had lower urea concentration at 60 days compared to control. Treatments did not influence hematological parameters, while some only changed with the age of animals. Plasma glutathione peroxidase concentrations were higher in supplemented animals, with the interaction between treatment and time, where higher concentrations were observed at 40 days of experiment for supplemented calves compared to control. Treatments did not influence the performance of animals. Animals with SeVitEFe showed a lower incidence of diarrhea in the first thirty days of the experiment.

Discussion: Supplementation of selenium, iron, and vitamin E showed effectiveness in improving the animals' oxidative metabolism, altering biochemical and hematological parameters, and reducing the pathogens of *Anaplasma marginale*, and the incidence of diarrhea, but did not enhance the animals' performance.

KEYWORDS

antioxidants, performance, immunology, immunonutrients, nutrition, ruminant

1 Introduction

The suckling phase, which encompasses the period from birth to weaning, is critical for calves, and mortality and morbidity rates can be high, compromising the efficiency of the dairy cattle production system.

At birth, calves have developed but still immature immune systems. The synepitheliochorial placenta of cattle does not allow the passage of immunoglobulins and other serum proteins (Reis et al., 2007). Soon after birth, calves are challenged by the contaminated environment with harmful viruses and bacteria, as well as by the climate and other external factors (Reis et al., 2007). At birth, they acquire maternal immunoglobulins through the consumption of colostrum, a process called passive immunity transfer, and thus can respond to infections in the first weeks of life (Uyama et al., 2022).

Care and nutrition during the early phase are crucial for the growth and future milk production potential of replacement heifers. Certain nutrients, such as selenium, iron, and vitamin E, are essential for the development of the animal's immune system. Selenium is an essential micronutrient in ruminant nutrition, being part of the glutathione peroxidase enzyme, an important component in the cellular antioxidant system (NRC, 2001). In addition, as it is involved in the arachidonic acid metabolism, it improves the ability of neutrophils to eliminate pathogenic microorganisms when it is supplemented (NRC, 2001). Like selenium, Vitamin E is involved in inter and intracellular antioxidants (NRC, 2001). In recent years, vitamin E has been shown to play an important role against damage caused by free radicals and in improving the immune system response (McDowell, 2000). Iron is a component of the heme group, a component of enzymes participating in the electron transport chain, and is also found in hemoglobin and myoglobin. Its deficiency results in hypochromic microcytic anemia due to the inability to produce hemoglobin, associated with depressive immune responses. As a result, calves become lethargic, and feed intake and weight gain are reduced (NRC, 2001). The current recommendations of these nutrients for dairy calves in milk replacer are 0.3 mg of selenium/kg of dry matter (DM), 85 mg of iron/kg of DM, and 50 IU of vitamin E/kg of DM for animals between 35 to 125 kg of live weight.

According to the U.S. Department of Agriculture (2015), around 1.9 million calves were lost due to non-predator causes in 2015 in the

United States; the main causes being respiratory problems (26.9%), followed by losses related to birth problems (17.8%) and digestive problems (15.4%). The percentages of sick calves during the suckling phase in Brazil are on average 43% for diarrhea, 21% for pneumonia, and 7% for parasitic sadness (Azevedo et al., 2024). In an evaluation carried out in 1,188 rural properties in Brazil, the survival rate of calves up to one year of age was 86.47%, with a still worrying mortality rate of 13.26% (IDEAGRI, 2021). When evaluating the main causes of calf mortality, among the 11,000 deaths that occurred in 69,000 calves evaluated, 21% were due to diarrhea, 20.43% were due to tick fever, and 14.20% were due to pneumonia (IDEAGRI, 2019). Bovine tick fever is composed of two diseases caused by protozoa called babesiosis (caused by *Babesia Bigemina* and *Babesia Bovis*) and anaplasmosis (caused by *Anaplasma Marginale*), which are transmitted by ticks and flies, has and have a major impact on livestock in several countries, as it compromises animal productivity (Ferreira et al., 2022). The prevalence of *A. marginale*, and *Babesia* spp. in Latin America was reported as 48.9% and 39.8% (95% CI: 24.6–57.2%), respectively. The prevalence of *A. marginale* was higher due to the high prevalence of this agent (67.1%) in Mexico (Ferreira et al., 2022).

Newborn calves, adequately fed with colostrum, supplemented with selenium, iron and vitamin E throughout the suckling phase, can have an improved health status in this critical phase, since these nutrients support immune system activity, reduce the incidence of diseases, and consequently improve performance. This study aims to verify the effects of supplementation with selenium, iron, and vitamin E, on the incidence of diarrhea, oxidative metabolism, blood metabolites, hematological parameters, and performance of calves under immunological challenge with *Anaplasma marginale* in the suckling phase.

2 Materials and methods

The project was approved by the Animal Use Ethics Committee of the Animal Science Institute, with protocol number CEUA/IZ 268-18. We confirm that this study was carried out in compliance with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines and all methods were performed following the relevant guidelines and regulations.

2.1 Location and experimental design

The experiment was carried out on the experimental farm of the Animal Science Institute of Ribeirão Preto/SP using 42 male Holstein calves (initial weight 38.33 ± 4.09 kg) for 60 days. Calves were acquired from a commercial farm with 2 to 6 days of age. At the farm, calves were properly fed with the provision of 4 liters of high-quality colostrum (Brix > 22%) within four hours after birth and then another meal of 2 liters from the second milking. Calves received transition milk from the second milking (6 L per day) on the second and third day of age. After the fourth day of life, they received 6 liters of milk a day until they were transported to the farm where the experiment was carried out.

On the day of arrival at the experimental farm, calves remained for 12 hours in a collective pen, with wood shavings bedding and heating with infrared light lamp, and received 2 liters of oral hydration (Calf Care[®], De Laval) per animal via bottle. After this period, animals were allocated to individual tropical hutches, with sand bedding, and individual access to starter and drinking water. Supplementation began one day after calves arrived at the experimental farm, considered the first day of the experiment, and all received treatments for 60 days.

The design was completely randomized, with calves allocated to the following treatments: C (control milk replacer, N = 14); SeVitE (milk replacer supplemented with 0.6 mg organic selenium/kg + 100 IU vitamin E, N = 14); SeVitEFc (milk replacer supplemented with 0.6 mg organic selenium/kg + 100 IU vitamin E + 200 mg Fe chelate/kg, N = 14). Each calf was considered an experimental unit.

Supplementation was administered through capsules previously manipulated for each treatment and added to the milk replacer individually at the time of its daily preparation in the bucket with the respective bottle for each calf. The sources of supplemented nutrients were amino acid selenium (YES - Mineral Selenium, 5,000 mg/kg), ferrous glycine hydrate chelate (BASF - Iron Glycinate, 22%), and alpha-tocopherol acetate (LUTAVIT E 50, BASF, 500,000 IU/kg). The amount for capsules was calculated to ensure that the animals received at least 0.6 mg of Se; 200 mg of Fe and 100 IU of vitamin E (149 mg) per kg of dry matter, considering the amount of the respective nutrients from the intake of calf starter and milk replacer according to the dietary program adopted.

2.2 Management and experimental parameters

Calves were fed for 60 days, receiving 6 liters of milk replacer/day until 30 days from the beginning of the experiment and 4 liters of milk/day, from 31 to 60 days of the experiment. The commercial milk replacer (Sprayfo[®] Violeta, Trouw Nutrition) was diluted in drinking water preheated to 39°C to reach a dry matter concentration of 14%. The grounded calf starter was offered *ad libitum*, always in small amounts, being gradually increased according to the animal's needs. The milk replacer and calf starter composition are shown in Table 1.

The intake of milk replacer and calf starter was daily monitored, recording the volume of the liquid diet consumed and weighing the calf starter offered and the following day's leftovers.

Water was offered *ad libitum* to calves throughout the experimental period and had selenium concentration of 0.008 mg/L and iron concentration of 0.010 mg/L.

2.3 Immunological challenge and oxidative metabolism parameters

An immunological challenge was performed on all calves on day 40 of the experiment and inoculation was carried out by applying 1 mL via intramuscularly puncture of low-virulence isolates of *Anaplasma marginale* (1×10^7 red blood cells infected, registered in GenBank under codes EU676176 (CEPA UFMG1). Blood samples inoculated with the pathogen (CEPA UFMG1) were kept in liquid nitrogen with dimethyl sulfoxide cryoprotectant until the date of inoculation. On the respective days of inoculation, the inocula preserved in liquid nitrogen in multiple Eppendorf tubes were thawed, and 25 microliters of the inoculum were mixed with 1 mL of the refrigerated inoculation solution in a sterile environment. The final solution was then placed in Eppendorf tubes inside a thermal box with ice and immediately transported to the calf pen, located 30 meters from the laboratory, for inoculation. The calves were always inoculated in the morning after milk management.

TABLE 1 Starter ingredients and chemical composition of the ground starter and milk replacer were offered to the calves during the experiment.

Composition (%)	Starter	Milk Replacer
Ground corn	36.0	–
Wheat bran	20.0	–
Soybean meal	40.0	–
Salt	1.0	–
Premix ¹	3.0	–
Nutrients (%)		
Dry matter	90.30	89.93
Crude protein	25.67	20.53
Ether extract	3.88	8.81
Neutral detergent fiber	17.81	2.56
Acid detergent fiber	7.20	2.23
Lignin	1.05	1.34
Non-fibrous carbohydrates	45.94	60.42
Total digestible nutrients ²	70.55	89.09
Selenium (mg/kg)	0.48	0.17
Iron (mg/kg)	115.03	191.96
Alpha tocopherol (mg/kg)	37.54	82.38

¹ Maximum level - calcium: 270 g; cobalt: 25 mg; copper: 840 mg; fluoride: 500 mg; phosphorus: 50 g; iodine: 50 mg; magnesium: 15 g; manganese: 2,500 mg; selenium: 25 mg; sodium: 55 g; virginiamycin: 800 mg; vitamin A 333,330 IU; vitamin D3: 33,330 IU; vitamin E: 1,210 IU; Zinc: 3,360 mg.

² $NDT = (0.98 \times CNF) + (0.93 \times PB) + 2.25 \times (EE-1) + 0.75 \times (NDLPb - Lig) \times [1 - (Lig/NDLPb)0.667] - 7$ (NRC, 2001).

Blood samples were collected from the jugular vein before inoculation and 20 days after to quantify the activity of the glutathione peroxidase enzyme (GPx), total antioxidant capacity (TAC), and reduced glutathione (GSH).

From the date of the immunological challenge, daily monitoring of rectal temperature was performed at 7:30 AM and 4:30 PM, as well as observation of the eye score and hematocrit measurements.

To certify the success of inoculation and its effectiveness in the study, blood smears were prepared on days 46, 53, and 60 of the experiment for *Anaplasma marginale* quantification, and blood samples were collected 20 days after inoculation (60 days of the experiment) to perform indirect fluorescent antibody test (IFAT) and PCR for *Anaplasma marginale*. As the period of clinical manifestation of the disease generally occurs on average 20 to 38 days after the calf has contact with the pathogen, blood was collected on a fixed date of 20 days after inoculation (60 days of experiment) to maintain equal inoculation days among animals. Clinical signs of the disease were considered when animals had rectal temperatures above 40°C and hematocrit below 60% of the basal value of each calf. To obtain the basal hematocrit level of each calf, a blood sample was collected before inoculation and the red cell count was read. No animal showed clinical signs of the disease during the experiment.

2.4 Blood metabolism parameters and blood count

Blood samples were collected from calves by jugular puncture on the first day, 40 and 60 days of the experiment, two hours after offering the milk replacer, for analysis of selenium, iron, blood count, ferritin, and the following biochemical parameters: β -hydroxybutyrate, glucose, total protein, urea, creatinine, creatinine kinase, lactate dehydrogenase (LDH), lactate, albumin, and IgG. For vitamin E analysis, blood samples were collected from animals on the first day and at 60 days of the experiment.

For selenium, iron, ferritin, and vitamin E measurements, blood samples were collected in vacutainer tubes without anticoagulant and centrifuged at 3,000 RPM to obtain serum, stored frozen in plastic tubes. The tubes used for vitamin E analysis were protected with aluminum foil and the serum was stored in amber plastic tubes.

2.5 Stool consistency score and incidence of diarrhea

The stool consistency score (0 = normal, well-formed stools; 1 = abnormal, but not yet showing diarrhea, little pasty stools; 2 = moderate diarrhea, pasty stools; 3 = severe diarrhea; very pasty or liquid stools) was daily assessed. Fecal scores 2 and 3 were considered cases of diarrhea. The incidence of diarrhea, presented as a percentage, was calculated according to the following equation:

Incidence of diarrhea

$$= (\text{sum of the total occurrence of stool scores 2 and 3} \\ \times 100) / \text{total number of days of stool score observation} .$$

Calves with a score of ≥ 2 , received 4 L of daily oral electrolytes, divided into two meals, two hours after offering a milk replacer and a lactobacilli-based probiotic, until the consistency of the feces reduced the score. Intravenous electrolytes were administered, when necessary until body rehydration occurred. Calves were medicated with parenteral antimicrobials, a combination of trimethoprim and sulfamethoxazole, and non-steroidal anti-inflammatory drugs with the active ingredient flunixin meglumine, as advised by the veterinarian.

Health status and presence of clinical cases of other diseases were daily observed, and daily environmental temperature and relative humidity data were recorded.

2.6 Performance

All calves were weighed on a digital scale, and measurements of height at the withers, heart girth, hip width, and body length were recorded at the beginning of the experiment, and every 15 days until the end of 60 days. Height measurements were performed using an appropriate ruler, fixed to a right-angled support, measuring the vertical distance between the ground and the withers. Heart girth, hip length, and width measurements were carried out using a measuring tape. Body length was measured by measuring the distance from the scapula to the beginning of the tail insertion. Hip width was measured by measuring the distance between ischia.

2.7 Laboratory analysis methodologies

2.7.1 Analysis of calf starter, milk replacer, and water

Ground calf starter and milk replacer samples were collected weekly and frozen to prepare a monthly pool for chemical analysis, carried out at the Laboratory of Bromatology - FMVZ/USP. Samples were ground in a mill with a 1 mm sieve for the analysis of dry matter (DM, method 930.15), crude protein (CP, method 984.13), ether extract (EE, method 920.39), mineral matter (MM, method 942.05), acid detergent lignin (method 973.18), according to [AOAC \(1996\)](#). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) analyses were also carried out, according to [Van Soest et al. \(1991\)](#) and [Goering and Van Soest \(1970\)](#), respectively.

To calculate non-fibrous carbohydrates (NFC) and total digestible nutrients (TDN), the following [NRC \(2001\)](#) formulas were used:

$$\text{NFC} = 100 - (\text{crude protein} - \text{ether extract} \\ - \text{ash} - \text{neutral detergent fiber})$$

$$\text{TDN} = (0.98 \times \text{NFC}) + (\text{DC} \times \text{CP}) + 2.25 \times (\text{EE} - 1) + 0.75 \times (\text{NDF}_{\text{cp}} - \text{lignin}) \times [1 - (\text{lignin}/\text{NDF}_{\text{lp}})0.667] - 7$$

where DC = digestibility coefficient (93 % for milk replacer, 86.45 % calf starter),
 CP = crude protein, NDF_{FCP} = neutral detergent fiber free from crude protein.

Water samples were stored in 300-mL plastic bottles and frozen at -6°C for later analysis of selenium and iron (SMEWW, 2017). Selenium and iron analysis of calf starter, milk replacer, water, and mineral supplement were carried out in the Laboratory of Minerals of the Faculty of Animal Science and Food Engineering at USP/Pirassununga. The samples underwent wet digestion with nitric-perchloric acid using an adapted fluorimetric method (Olson et al., 1975), and iron concentration was analyzed using the colorimetric method (Fe liquiform, Labtest kit). Analysis of alpha-tocopherol in the calf starter, milk replacer, and vitamin supplement were carried out at the CBO Laboratory (Valinhos, SP) using the methodology with HPLC (Isocratic, fluorescence detector) and reverse-phase column separation technique, detection at 326 nm emission and 294 nm excitation and quantification by external standard (Horwitz and Larimer, 2005).

2.7.2 Blood analysis

Concentrations of GPx, GSH, TAC, and IgG were determined at the FMVZ/USP laboratory. Vacuum-packed total blood samples were collected in two 8.5-mL tubes containing lytic heparin as an anticoagulant for GPx and TAC analyses. Samples were centrifuged at 3,000 rpm for 10 min at 4°C with slow deceleration, with plasma and buffy coat being collected. After the first centrifugation, an aliquot of plasma was removed, stored in amber Eppendorf tubes, and kept frozen at -80°C . The total antioxidant capacity (TAC) was determined using a commercial kit (RANSEL[®] Laboratories, Randox, Crumlin, UK) according to the protocol described by the manufacturer. Centrifugation was repeated two more times with phosphate buffered saline (PBS 10%) until the supernatant was completely clear. The red blood cell mash obtained was packaged in amber microtubes and frozen in a freezer at -80°C for subsequent GPx analysis. Serum GPx activity was determined on a Randox automatic biochemical analyzer (model RX Daytona) using Randox[®] commercial kits (RANSOD and RANSEL) to determine the values and corrected by the hemoglobin concentration measured by spectrophotometry.

To determine GSH, whole blood collected in vacuum tubes containing EDTA was used. In a test tube, 200 μL of whole blood was hemolyzed with 1,800 μL of distilled water, then, 3 mL of precipitating solution (3.34 g of metaphosphoric acid, 0.44 g of EDTA $\text{Na}_2\text{H}_2\text{O}$, 60 g of NaCl and distilled water q.s.p 200 ml) were added to the hemolysate. The solution remained at rest for 5 minutes and was then centrifuged for 5 minutes at 3,000 rpm. The intermediate content was collected, stored in amber Eppendorf tubes, and kept in a freezer at -80°C until analysis. To determine the GSH concentration, a 200- μL aliquot of the supernatant was added to 800 μL of Na_2HPO_4 solution (300 mmol/l). After homogenization, 100 μL of 0.05% DTNB (2-dinitrobenzoic acid) solution was added to the system, with reading being performed at

412 nm within 30 seconds after the addition of DTNB. From the standard curve values, the straight-line equation was calculated and the absorbance values of analyzed samples were converted into mg/dL.

The blood smear technique to obtain the number of *Anaplasma marginale* was performed with 5 μL of blood and using the GIEMSA dye. Blood smears were taken from all calves at 46, 53, and 60 days of the experiment. There was a problem with the storage of these smear slides, and it was decided to keep only slides in excellent readable condition. Due to this fact, the number of slides used to describe the results were 15 (C=4, SeVitE =4, SeVitEFe=7).

For blood collected for PCR analysis, the tube containing EDTA as an anticoagulant and frozen at -20°C was used. For the indirect fluorescent antibody test (IFAT), blood was collected in a tube without anticoagulant, centrifuged and the serum was frozen at -20°C until sent for analysis. Both analyses were carried out at the Laboratory of Veterinary Protozoology/VET/UFMG. The presence of anti-*A. marginale* antibodies were detected using IFAT (Silveira, 2012) and anti-bovine monoclonal antibody (AbD Serotec) conjugated to fluorescein isothiocyanate (FITC). The antigens used were produced by the laboratory (IICA, 1987) and the strains utilized in this study were *A. marginale* strain UFMG1 (Bastos et al., 2010). For each reaction, positive and negative control serum was used (Bastos et al., 2010). Samples were considered positive if they showed fluorescence at dilution of 1:40.

Molecular analysis was conducted (Silveira et al., 2016). DNA was extracted from 300 μL of whole blood using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's instructions. The nested PCR was performed in two stages: conventional PCR followed by nPCR. An aliquot of the first amplified PCR product was used for a second PCR reaction with a second set of primers. These primers were chosen to amplify the target sequence of the first PCR reaction, thereby increasing sensitivity. The following sets of primers were used to detect the *A. marginale* msp4 gene. First reaction (872 bp): MSP45: 5GGG-AGCTCCTATGAATTACAGAGAATTGTTTAC3 and MSP43: 5CCG-ATCC TTAGCTGAACAGGAATCTTG3 (de la Fuente et al., 2008); second reaction (294 bp): AnapF 5_CGCCAGCA AACTTTTCCAAA3 and AnapR 5ATATGGG GACACAGGCA AAT3. Twice-distilled water was used as the negative control (noDNA). As a positive control for the amplification of the msp4 gene fragment, DNA extracted from 300 μL of whole blood from a calf experimentally infected with the UFMG1 strain of *A. marginale* was used (Bastos et al., 2010).

The first reaction mixture comprised 1.2 μL dNTPs (2.5 mM), 0.15 μL Taq polymerase (0.05 U), 1.5 μL reaction buffer IB (1x), 0.6 μL of a solution containing the mixed primers (10 mM), and 10.05 μL sterile ultra-pure water. A 1.5 μL aliquot of the DNA template was added to the reaction mixture to give a final volume of 15 μL . The second reaction mixture comprised 2.0 μL dNTPs (2.5 mM), 0.25 μL Taq polymerase (0.05 U), 2.5 μL reaction buffer IB (1x), 1.0 μL of a solution containing the mixed primers (10 mM) and 16.75 μL sterile ultra-pure water. An aliquot (2.5 μL) of the amplicon obtained from the first reaction was added to the reaction mixture to give a final volume of 25 μL . Amplification was carried out under the conditions described. Amplification was performed using an Eppendorf

Mastercycler[®] thermocycler (Eppendorf, São Paulo, SP, Brazil). The protocol used to amplify *A. marginale* was as follows: 94 °C for 5 min (initial denaturation step); 30 cycles of 92 °C for 1 min (denaturation), 54 °C for 1 min (annealing), 72 °C for 2 min (extension); and a final extension step at 72 °C for 8 min. Following amplification, the reaction mixtures were maintained at 12 °C. PCR amplicons were separated by electrophoresis on 1% agarose gel (40 min, 100 V), stained with gelred (Biotium, São Paulo, SP, Brazil), and visualized under ultraviolet light.

Selenium and iron analysis in serum were carried out in the same laboratory as those in feed and used the same analysis methodologies.

To perform blood cell count, blood samples were collected using vacutainer tubes containing EDTA as an anticoagulant. Immediately after collection, samples were refrigerated and transported to the veterinary laboratory for analysis. Blood samples were analyzed in an automated pochi-100ivdiff[®] cell counter, which uses impedance and spectrophotometry techniques. The following parameters were analyzed: erythrocyte, hematocrit, and hemoglobin count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), leukocyte count, and platelet count.

Serum ferritin was analyzed by two-site enzyme immunoassay (sandwich), the chemiluminescent substrate (Lumi-Phos* 530) was added to the container and the light generated by the reaction was measured with a luminometer.

Analysis of biochemical parameters were conducted at the Metabolism Laboratory at ESALQ/USP. Blood samples were collected with the aid of a vacuum tube containing sodium fluoride to obtain plasma and tubes without anticoagulants to obtain blood serum. Samples were centrifuged at 3,000 rpm for 15 minutes. Specific commercial enzymatic kits from Labtest Diagnóstica S.A. (Lagoa Santa, MG, Brazil) were used to analyze plasma glucose (Ref. 85), serum total protein (Ref. 99-250), plasma urea (Ref. 104), plasma creatinine (Ref. 35), plasma creatinine kinase (Ref. 96), plasma lactate dehydrogenase (Ref. 86), plasma lactate (Ref. 116), and serum albumin (Ref. 19). The concentration of plasma β hydroxybutyrate (BHB) using the commercial kit RANBUT (Randox Laboratories, Life Sciences Ltd., Crumlin, United Kingdom). All biochemical metabolites were analyzed in an automatic biochemical system (SBA-200; CELM, Barueri, SP, Brazil).

To estimate the concentration of serum proteins, Megabrix[®] portable manual refractometer (IONLab Barigui, Brazil) was used. Blood samples were centrifuged in a laboratory centrifuge model CEOO1-B1[®] - Kacil (Recife, Brazil), at a speed of 2,500 rpm for 5 minutes.

The quantity of serum IgG samples from the calves was measured using a sandwich ELISA, according to the procedures previously published (Reber et al., 2008). Briefly, rabbit anti-bovine IgG antibody (capture antibody, B5645; Sigma, St. Louis, MO) diluted 1:400 in sodium carbonate buffer was used to coat Immulon 4HBX plates (Thermo Corp., Milford, MA) at 4–8 °C overnight. After, all plates were washed three times using the wash buffer. For this assay, plasma samples were diluted 1:1 000 000. The

samples and a standard dilution of bovine gamma globulin preparation were placed in duplicate wells and incubated for 1 h at 20–26 °C. The plates were washed three times. Bound IgG was detected using a rabbit anti-bovine IgG conjugated to horseradish peroxidase (detection antibody, A5295; Sigma, St. Louis, MO). The detection antibody was diluted 1:1000 and incubated for 30 min. The plates were washed three times. The quantity of bound detection antibody was measured using the substrate 2,20-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, A-9941; Sigma, St. Louis, MO) containing 20 μ L of 30% hydrogen peroxide per 11 mL ABTS (preconditioned at RT for 15 minutes) and incubated 30 min. The color signal was measured using a plate reader with a 405 nm filter. An immunoglobulin standard (commercial bovine gamma globulin, I5506; Sigma, St. Louis, MO) was prepared as a serial dilution with a range of 50–0.39 ng/mL. The intra-test coefficient of variation was 11.0% and the inter-test coefficient was 13.1%.

For serum alpha-tocopherol analysis, samples were saponified to hydrolyze and solubilize fats and proteins and extracted with hexane. Serum alpha-tocopherol analyses were performed using high-performance liquid chromatography with a fluorescence detector (Arnaud et al., 1991).

2.8 Statistical analysis

Results were submitted to statistical analysis according to a completely randomized design using PROC MIXED from SAS 9.4 (SAS, 2013) according to the model below:

$$Y_{ij} = \mu + D_i + A_j(D)_k + \varepsilon_{ijk}$$

Where Y_{ij} = dependent variable; μ = overall average; D_i = diet effect ($i = 1$ to 3); $A_j(D)_k$ = random effect of the animal within treatment ($k = 1$ to 3); ε_{ijk} = assumed random error $NID \sim (0, \sigma^2)$.

Blood variables were analyzed as repeated measures over time, verifying the normality of residuals and homogeneity of variances by PROC UNIVARIATE procedure. The model collected over time according to the previous design considering the physiological stage of calf growth or weeks of collection (time, 0, 40, and 60 days), diets (Control, SeVitE, SeVitEFe), and the interaction between time and treatment using PROC MIXED from SAS, according to the following model:

$$Y_{ijklm} = \mu + D_i + A_j (D)_k + \varepsilon_{ijk} + T_m + T_m \times D_i + \omega_{ijklm}$$

Where Y_{ijklm} is the dependent variable value, μ is the overall mean, D_i is the fixed effect of diet 1 ($i = 1-3$), $A_j(D)_k$ is the random effect of the animal within of diet, ε_{ijk} is the residual error. T_m is the fixed effect of sampling time ($m = 1-3$), $T_m \times D_i$ is the fixed interaction effect between sampling time and diet, and ω_{ijklm} is the random experimental error. All means were obtained using LSMEANS. The Akaike information criterion (AIC) was used to determine the structured covariance matrix. Degrees of freedom were defined according to Kenward and Roger (1997). All means were estimated by the LSMEANS procedure and compared by the Tukey test, considering 0.05 as the probability of type-I error. Treatment responses were tested and a significance level of 0.05 was adopted.

3 Results

3.1 Feed and micronutrient intake

The dry matter intake of the feeds and total dry matter intake were not different among treatments. However, the intakes of selenium, iron, and vitamin E, were higher in supplemented animals throughout the experimental period compared to control treatment ($P < 0.03$, Table 2).

3.2 Oxidative metabolism in the immunological challenge and *Anaplasma marginale* blood count parameters

Regarding oxidative metabolism, glutathione peroxidase activity was higher in supplemented compared to control calves ($P=0.03$, Table 3). Interaction between treatment and time was observed ($P < 0.01$, Figure 1), with higher glutathione concentrations in the plasma of supplemented calves after 40 days of experiment

compared to control. Reduced glutathione and total plasma antioxidant activity were not different among treatments ($P > 0.05$).

The mean numbers/mL of *Anaplasma marginale* were lower in supplemented calves compared to controls ($P = 0.0408$, SEM = 17.02, Figure 2) and increased with time after inoculation ($P = 0.0408$, SEM = 17.39, Figure 3).

The IFAT analyses in the serum of the calves were all reactive for serology and the PCR were all positive for *Anaplasma marginale*.

3.3 Blood metabolism parameters and blood count

Increased selenium concentration in the serum of supplemented calves ($P < 0.01$) compared to control was observed (Table 4). However, vitamin E ($P=0.57$) and iron ($P=0.59$) concentrations in the serum were not different among calves. Plasma lactate dehydrogenase (LDH) was lower in calves supplemented with SeViEFe ($P=0.01$) and lactate was lower in supplemented animals ($P=0.03$) compared to control.

TABLE 2 Dry matter intake and of micronutrients by calves supplemented with different micronutrients during the experimental period.

Variables	Treatments ¹			SEM ²	P-value
	Control	SeVitE	SeViEFe		
Feed intake from 1 to 30 d					
Milk replacer dry matter (kg/d)	0.55	0.56	0.56	0.02	0.96
Starter dry matter (kg/d)	0.06	0.06	0.05	0.01	0.86
Total dry matter (kg/d)	0.61	0.62	0.61	0.02	0.92
Selenium (mg/d)	0.12 ^b	0.22 ^a	0.21 ^a	0.009	<0.01
Vitamin E (mg/d)	47.74 ^b	86.25 ^a	85.28 ^a	2.84	<0.01
Iron (mg/d)	112.92 ^b	115.12 ^b	144.70 ^a	4.82	<0.01
Feed intake from 31 to 60 d					
Milk replacer dry matter (kg/d)	0.50	0.50	0.49	0.0007	0.71
Starter dry matter (kg/d)	0.66	0.57	0.53	0.06	0.40
Total dry matter (kg/d)	1.16	1.07	1.02	0.06	0.38
Selenium Intake (mg/d)	0.40 ^b	0.52 ^a	0.49 ^a	0.03	0.03
Vitamin E intake (mg/d)	66.09 ^b	130.22 ^a	127.57 ^a	2.71	<0.01
Iron intake (mg/d)	172.04 ^b	162.44 ^b	212.43 ^a	7.98	<0.01
Feed intake from 1 to 60 d					
Milk replacer dry matter (kg/d)	0.53	0.53	0.52	0.01	0.96
Starter dry matter (kg/d)	0.37	0.32	0.30	0.03	0.46
Total dry matter (kg/d)	0.89	0.86	0.83	0.04	0.57
Selenium (mg/d)	0.27 ^b	0.36 ^a	0.35 ^a	0.02	<0.01
Vitamin E (mg/d)	57.26 ^b	108.37 ^a	106.44 ^a	2.56	<0.01
Iron (mg/d)	143.52 ^b	139.77 ^b	179.23 ^a	6.08	<0.01

¹ Treatments: Control = Milk replacer Control, n=14; SeVitE = Milk replacer supplemented with organic selenium and vitamin E, n=14; SeViEFe = Milk replacer supplemented with organic selenium, vitamin E, and iron chelate, n=14. Different letters indicate statistically significant differences.

² SEM, standard error of the mean.

TABLE 3 Blood oxidative metabolism during immunological challenge of calves supplemented with micronutrients.

Variables ⁴	Treatments ¹				Time (days)			P-value ³		
	Control	SeVitE	SeVitEFe	SEM ²	40	60	SEM	Treat	Time	Treat*Time
GPx (U/g Hb)	1,082.15 ^b	1,256.96 ^a	1,408.62 ^a	83.47	1,235.88	1,262.60	60.86	0.03	0.72	<0.01
GSH (mg/dL)	18.73	18.38	22.51	1.56	19.62	20.12	1.06	0.13	0.65	0.19
TAC (mmol/L)	0.82	0.83	0.79	0.01	0.79	0.88	0.01	0.62	0.08	0.41

¹ Treatments: Control = Milk replacer Control, n=14; SeVitE = Milk replacer supplemented with organic selenium and vitamin E, n=14; SeVitEFe = Milk replacer supplemented with organic selenium, vitamin E, and iron chelate, n=14. Different letters indicate statistically significant differences.

² SEM, standard error of the mean.

³ Treat, treatment effect; Time, effect of time; Treat*Time, interaction between treatment and trial time.

⁴ GPx, glutathione peroxidase; GSH, reduced glutathione; TAC, total antioxidant capacity.

Blood selenium, vitamin E, glucose, and beta-hydroxybutyrate concentrations increased during the experiment ($P < 0.05$, Table 4). Ferritin was higher at 60 days compared to previous periods ($P = 0.04$). Total protein decreased from the beginning of the experiment until 40 days, then increased, but at lower values compared to the initial value ($P < 0.01$). There was an interaction between treatment and experimental time on plasma urea concentrations ($P = 0.05$, Figure 4), and it was observed that the values among treatments were very close at the beginning of the experiment and 40 days of supplementation, but there was a difference at 60 days when animals supplemented with SeVitEFe showed a lower concentration compared to control animals. Plasma creatinine, lactate, albumin, and IgG concentrations decreased during the experimental period ($P < 0.01$).

The other plasma concentrations of metabolites were not affected by supplementation. None of the other blood metabolite variables had a significant interaction between treatment and time.

Mean corpuscular hemoglobin ($P = 0.05$) and its concentration ($P = 0.04$) decreased from the beginning to 40 days of supplementation

and increased at 60 days to values similar to the initial ones. The percentage of eosinophils ($P < 0.01$) increased from the beginning to 40 days of supplementation and decreased at 60 days. Platelet count decreased during the experiment ($P < 0.01$, Table 5).

3.4 Stool consistency score and incidence of diarrhea

Animals supplemented with SeVitEFe had a lower incidence of diarrhea ($P = 0.03$) compared to the other treatments in the first 30 days of supplementation (Table 6), with no change in stool consistency scores during the experiment.

3.5 Performance

Performance and feed efficiency variables were not different among treatments (Table 7).

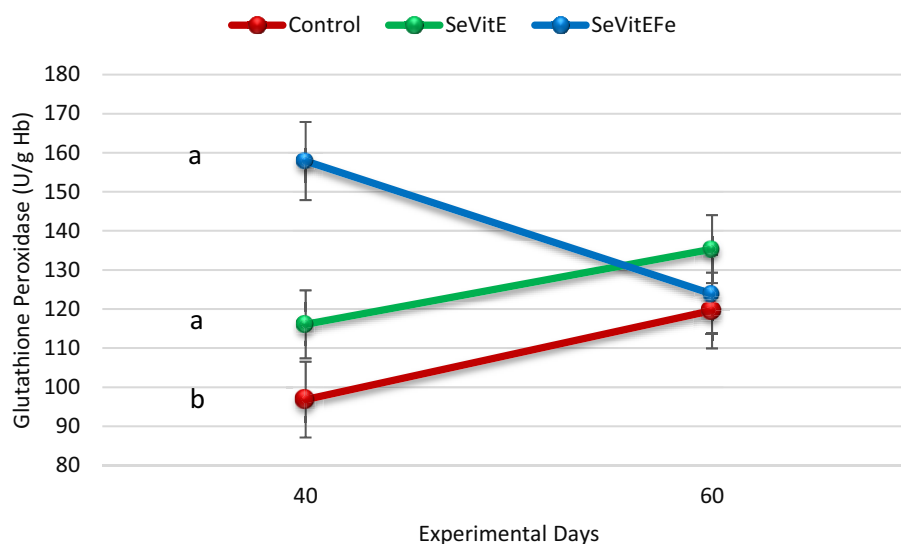


FIGURE 1

Glutathione peroxidase concentration in the blood plasma of calves in the different treatments (Control = Milk replacer Control, N=14; SeVitE = Milk replacer supplemented with organic selenium and vitamin E, N=14; SeVitEFe = Milk replacer supplemented with organic selenium, vitamin E, and Iron chelate, N=14) on the days of experiment (Different letters indicate statistically significant differences, treatment and time interaction effect, $P < 0.01$, SEM = 6.62).

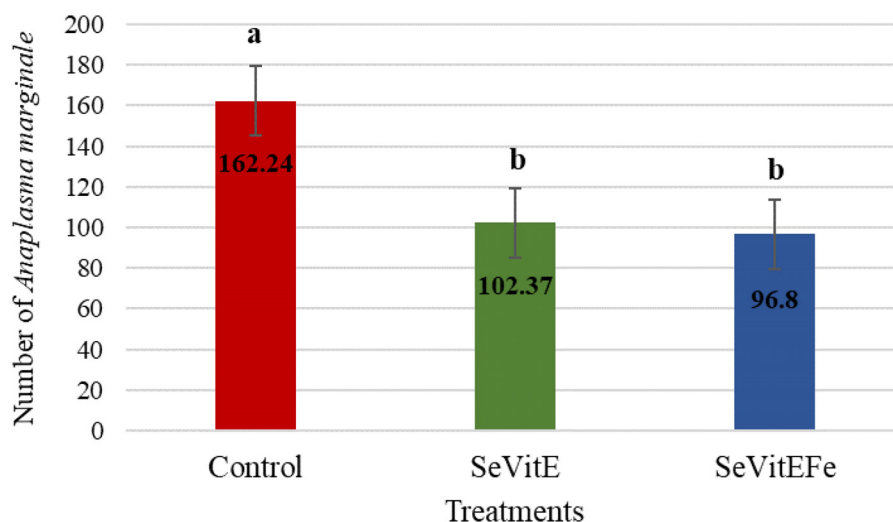


FIGURE 2

Average number of *Anaplasma marginale* in treatments (Control = Milk replacer Control; SeVitE = Milk replacer supplemented with organic selenium and vitamin E; SeVitEFe = Milk replacer supplemented with organic selenium, vitamin E, and Iron chelate) at 46, 53, 60 days of experiment (Different letters indicate statistically significant differences, $P = 0.0408$, SEM = 17.02).

4 Discussion

Selenium, iron, and vitamin E intake were increased in supplemented animals throughout the experimental period and changes in the concentrations of blood metabolites of animals were observed, indicating their supportive role against pathogens. Selenium is a cofactor necessary for the function of the glutathione peroxidase enzyme (Goff, 2018), which plays a unique role in the battle against oxidative stress. The increased selenium intake in supplemented calves resulted in higher plasma selenium and glutathione peroxidase concentrations. The glutathione concentration in supplemented calves before the immunological challenge was on average 19.26 U/g Hb (20%) for SeVitE and 61.06

U/g Hb (63%) for SeVitEFe, more than in control animals. Glutathione is an important antioxidant that can prevent cellular damage caused by various oxidative stressors (Wangsanut and Pongpoom, 2022), a can for instance protects hemoglobin from oxidative degradation and inhibits inflammation (Brigeliuss-Flohé and Flohé, 2020). We can infer that with a greater amount of glutathione in the blood of SeVitEFe calves, there was better systemic support to protect from the pathogen *Anaplasma marginale*, since SeVitE and SeVitEFe supplemented calves had a lower blood pathogen count. There was an increase in the blood pathogen count after inoculation for all calves since they received the same amount of *Anaplasma marginale* during inoculation. The initial corpuscles of this pathogen, when inoculated into cattle,

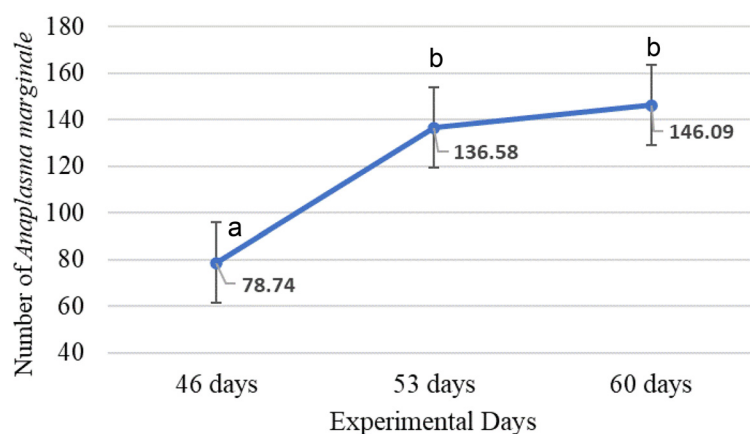


FIGURE 3

Mean number of *Anaplasma marginale* at 46, 53 and 60 days of experiment (Different letters indicate statistically significant differences, $P = 0.0353$, SEM = 17.39).

TABLE 4 Blood metabolites of calves in the different treatments and during the experimental period.

Variables ⁴	Treatments ¹			SEM	Time (days)			SEM ²	Probability ³		
	Control	SeVitE	SeVitEFe		0	40	60		Treat	Time	Treat*Time
Selenium (µg/mL)	0.066 ^c	0.075 ^b	0.090 ^a	0.002	0.074 ^b	0.080 ^a	0.078 ^a	0.002	<0.01	0.04	0.12
Vitamin E (µg/mL)	5.22	5.73	4.50	0.82	4.76	.	5.55	0.53	0.57	0.13	0.21
Iron (µg/dL)	176.26	158.54	157.76	14.43	127.95 ^b	182.02 ^a	182.60 ^a	12.17	0.59	0.01	0.49
Ferritin (ng/mL)	0.25	0.27	0.21	0.05	0.21 ^b	0.18 ^b	0.35 ^a	0.05	0.77	0.04	0.80
Total Protein (g/dL)	6.08	6.21	6.43	0.11	7.16 ^a	5.59 ^b	5.96 ^c	0.10	0.11	<0.01	0.44
Glucose (mg/dL)	124.08	122.89	122.00	5.72	115.67 ^b	127.95 ^a	125.35 ^a	4.38	0.96	0.04	0.15
BHB (mm/L)	0.090	0.095	0.094	0.006	0.073 ^c	0.084 ^a	0.122 ^b	0.006	0.89	<0.01	0.69
Urea (mg/dL)	19.01	19.62	16.98	0.88	19.70 ^a	17.31 ^b	18.60 ^a	0.74	0.09	0.03	0.05
Creatinine (mg/dL)	1.25	1.20	1.12	0.04	1.27 ^a	1.19 ^b	1.12 ^c	0.03	0.16	0.01	0.25
LDH (U/L)	1,352.78 ^a	1,327.11 ^a	1,098.53 ^b	62.22	1,266.35	1,174.68	1,337.39	59.85	0.01	0.15	0.22
Lactate (mg/dL)	18.22 ^a	13.48 ^b	12.05 ^b	1.74	19.50 ^a	12.98 ^b	11.26 ^b	1.72	0.03	<0.01	0.13
Albumin (g/dL)	3.01	3.01	2.93	0.04	3.04 ^a	2.91 ^b	3.00 ^a	0.03	0.43	0.01	0.39
IgG (mg/mL)	21.41	23.36	20.07	2.05	28.94 ^a	17.60 ^b	18.30 ^b	1.50	0.53	<0.01	0.43

¹ Treatments: Control = Milk replacer Control, n=14; SeVitE = Milk replacer supplemented with organic selenium and vitamin E, n=14; SeVitEFe = Milk replacer supplemented with organic selenium, vitamin E, and iron chelate, n=14. Different letters indicate statistically significant differences.

² SEM, standard error of the mean.

³ Treat, treatment effect; Time, effect of time and Treat*Time, interaction between treatment and trial time.

⁴ BHB, β-hydroxybutyrate; LDH, Lactate dehydrogenase.

adhere to erythrocytes and multiply and invade other erythrocytes. Supplemented calves presented the lowest blood count of pathogens indicating that the immune system of these animals was better able to cope with the infection. Additionally, calves supplemented with SeVitEFe performed more effectively to bacterial challenges, with a lower incidence of diarrhea than animals maintained on control diets, proving the immunostimulant effect of the micronutrients. In

the literature, there are no reports to date of a specific interaction between iron metabolism with selenium and vitamin E (Ensley, 2020; NASEM, 2021). There may be an interaction between these nutrients during supplementation, particularly when the physiological state differs from deficiency or marginal supplementation. Further research is needed to explore this interaction in greater depth.

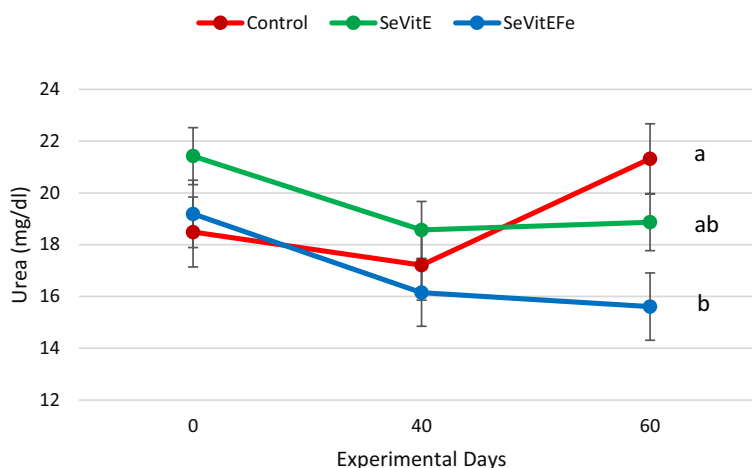


FIGURE 4

Urea concentration in the blood serum of calves in the different treatments (Control = Milk replacer Control, N=14; SeVitE = Milk replacer supplemented with organic selenium and vitamin E, N=14; SeVitEFe = Milk replacer supplemented with organic selenium, vitamin E, and Iron chelate, N=14) on the days of the experiment (Different letters indicate statistically significant differences, interaction effect of treatment and time, P=0.05, SEM = 0.67).

TABLE 5 Blood count of calves in different treatments and during the experimental period.

Variables	Treatments				Time (days)				P-value		
	Control	SeVitE	SeVitEFe	SEM	0	40	60	SEM	Treat	Time	Treat*Time
Erythrogram											
Erythrocyte (millions/mm ³)	8.42	8.12	7.29	0.33	8.14	7.75	7.94	0.29	0.06	0.62	0.40
Hemoglobin (g/dL)	9.85	9.57	8.68	0.39	9.83 ^a	8.86 ^b	9.41 ^a	0.33	0.11	0.07	0.23
Hematocrit (%)	30.79	30.21	27.17	1.16	30.96	28.24	28.97	1.09	0.08	0.17	0.59
Corpuscular Volume (u ³)	36.62	35.81	36.84	0.69	37.39 ^a	35.51 ^b	36.39 ^a	0.55	0.55	0.02	0.95
Corpuscular Hemoglobin (pg)	11.70	11.44	11.65	0.22	11.79 ^a	11.19 ^b	11.81 ^a	0.20	0.68	0.05	0.26
Corpuscular hemoglobin concentration (g/dL)	31.96	31.75	32.06	0.31	31.81 ^b	31.43 ^b	32.52 ^a	0.31	0.77	0.04	0.15
Leukogram											
Leukocytes (thousand/mm ³)	8,907.71	7,418.88	8,857.62	1,127.44	7,425.84	9,863.01	7,895.36	1,102.40	0.57	0.24	0.95
Segmented (%)	45.66	48.67	45.38	1.56	45.79	51.57	42.34	4.10	0.85	0.24	0.34
Eosinophils (%)	0.80	0.94	0.81	0.17	0.39 ^b	1.56 ^a	0.60 ^b	0.18	0.82	<0.01	0.25
Typical lymphocytes (%)	51.07	43.30	50.78	2.58	48.09	45.93	51.14	2.44	0.06	0.30	0.90
Monocytes (%)	4.81	4.22	4.59	0.89	4.24	4.33	5.06	0.89	0.89	0.77	0.94
Platelet count (thousand/mm ³)	589,522	69,664	564,688	392,652	696,539 ^a	636,61 ^b	517,697 ^c	38,052	0.06	<0.01	0.14

¹ Treatments: Control = Milk replacer Control, n=14; SeVitE = Milk replacer supplemented with organic selenium and vitamin E, n=14; SeVitEFe = Milk replacer supplemented with organic selenium, vitamin E, and iron chelate, n=14. Different letters indicate statistically significant differences.

² SEM, standard error of the mean.

³ Treat, treatment effect; Time, effect of time and Treat*Time, interaction between treatment and trial time.

Supplementation with vitamin E, iron, and selenium increased the selenium concentration in the serum of animals, with no effect on Fe and Vitamin E. There are few studies reporting the effect of selenium and vitamin E supplementation on calves during the suckling phase.

There are studies in literature assessing Se and vitamin E supplementation in peripartum cows and the concentration of these nutrients in the colostrum and blood of calves in the first days of life, that is, newborns (Abdelrahman and Kincaid, 1995;

TABLE 6 Average fecal score and incidence of diarrhea in calves in different treatments during the first 30 days, from 31 to 60 days, and throughout the experimental period.

Variables	Treatment ¹			SEM ²	P-value
	Control	SeVitE	SeVitEFe		
1 to 30 days					
Fecal score	1.97	1.77	1.62	0.11	0.10
Incidence of diarrhea (%)	66.89 ^a	60.24 ^a	51.12 ^b	4.06	0.03
31 to 60 days					
Fecal score	0.18	0.11	0.12	0.02	0.23
Incidence of diarrhea (%)	4.91	2.18	2.72	1.01	0.14
1 to 60 days					
Fecal score	1.06	0.94	0.90	0.06	0.25
Incidence of diarrhea (%)	35.66	31.14	28.07	2.51	0.11
Oral hydration (L)	33.62	31.00	37.80	–	–

¹ Treatments: Control = Milk replacer Control, n=14; SeVitE = Milk replacer supplemented with organic selenium and vitamin E, n=14; SeVitEFe = Milk replacer supplemented with organic selenium, vitamin E, and iron chelate, n=14. Different letters indicate statistically significant differences. Different letters indicate statistically significant differences.

² SEM, standard error of the mean.

TABLE 7 Performance of calves in different treatments and during the experimental period.

Variables	Treatment ¹			SEM ²	P-value
	Control	SeVitE	SeVitEFe		
Initial weight (kg)	38.77	38.43	37.78	0.63	0.81
Final weight (kg)	67.18	65.43	61.79	2.38	0.27
Initial height (cm)	76.71	77.25	76.28	1.06	0.81
Final height (cm)	86.53	87.71	86.11	0.83	0.37
Initial length (cm)	62.64	61.00	61.00	1.13	0.50
Final length (cm)	73.89	74.11	72.00	0.88	0.19
Initial chest circumference (cm)	80.14	79.50	79.60	0.79	0.82
Final chest circumference (cm)	95.39	93.92	92.61	1.39	0.37
Initial croup width (cm)	29.07	29.57	29.07	0.43	0.65
Final croup width (cm)	36.00	35.71	34.93	0.60	0.44
Gain from 1 to 30 days					
Weight (kg)	0.27	0.29	0.29	0.03	0.91
Height (cm)	0.11	0.11	0.12	0.02	0.98
Length (cm)	0.17	0.19	0.14	0.02	0.36
Chest circumference (cm)	0.17	0.17	0.16	0.02	0.96
Croup width (cm)	0.09	0.08	0.11	0.01	0.31
Food efficiency	0.43	0.44	0.47	0.04	0.82
Gain from 30 to 60 days					
Weight (kg)	0.69	0.63	0.54	0.04	0.09
Height (cm)	0.21	0.19	0.21	0.01	0.67
Length (cm)	0.27	0.22	0.23	0.02	0.33
Chest circumference (cm)	0.36	0.34	0.28	0.03	0.21
Croup width (cm)	0.14	0.12	0.08	0.01	0.10
Food efficiency	0.58	0.56	0.51	0.02	0.22
Gain from 1 to 60 days					
Weight (kg)	0.48	0.45	0.41	0.03	0.32
Height (cm)	0.16	0.16	0.17	0.01	0.99
Length (cm)	0.21	0.22	0.19	0.01	0.37
Chest circumference (cm)	0.26	0.24	0.22	0.02	0.47
Croup width (cm)	0.12	0.10	0.09	0.01	0.41
Food efficiency	0.54	0.53	0.48	0.02	0.36

¹ Treatments: Control = Milk replacer Control, n=14; SeVitE = Milk replacer supplemented with organic selenium and vitamin E, n=14; SeVitEFe = Milk replacer supplemented with organic selenium, vitamin E, and iron chelate, n=14.

² SEM, standard error of the mean.

Gunter et al., 2003; Jaaf et al., 2020; Kamada et al., 2007; LeBlanc et al., 2002; Ranches et al., 2017). Calves that were supplemented with 0.80 mg of organic Se/day during the suckling phase, presented an increase in the selenium concentration in the serum (0.068 µg/ml) compared to control animals (0.051 µg/ml) and increased phagocytic activity of macrophages (Salles et al., 2014). Selenium glyceride was offered orally

on the second day of life to calves at doses of 0.5 or 1 mg of Se/kg of body weight and blood parameters were monitored until the fourteenth day of life (Żarczyńska et al., 2021). An increase in serum Se from 0.063 to 0.184 µg/mL at the dose of 0.5 mg/kg BW, and of 0.200 µg/mL with 1 mg/kg was observed on the second day. However, the blood glutathione peroxidase concentration only

increased after ten days of supplementation, with calves receiving a dose of 0.5 mg/kg BW having values of 180 U/gHb and those receiving 1 mg Se/kg having 220 U/gHb, compared to control animals (130 U/gHb) (Żarczyńska et al., 2021). It was observed that with an increase in the selenium supply, there is also an increase in the amount of glutathione peroxidase. These authors worked with higher supplementation doses, compared to the present study, and reported that it was effective in improving Se status, and that the biochemical parameters monitored did not indicate a negative effect on the functioning of liver and kidneys (Żarczyńska et al., 2021).

Calves are deficient in selenium at birth, and supplementation at this phase of life can contribute to the development of their immune system (NASEM, 2021) and/or prepare the defense system to act against pathogens. In the present study, supplemented calves ingested on average of 0.09 and 0.08 mg/kg more selenium and obtained an increase in serum level of 0.009 and 0.024 µg/mL and an increase in glutathione peroxidase of 17.48 and 32.65 U/g Hb, respectively for SeVitE and SeVitEFe to control. It was demonstrated that even with the increase in glutathione peroxidase in SeVitE in relation to control calves, there was no effective action against *Anaplasma* pathogens and in the incidence of diarrhea. However, when calves were supplemented together with iron (SeVitEFe), they showed a 1.86-fold increase in glutathione concentration in relation to SeVitE and had an effective reduction in the number of *Anaplasma* pathogens. Furthermore, combined supplementation of micronutrients resulted in a 10% reduction in the incidence of diarrhea in the first 30 days of life, a phase in which the disease appears most frequently.

The purpose of selenium supplementation above requirements recommended by NRC (2001) and maintained in Nasem (2021), would be to increase the concentration of the nutrient in the blood of calves, and consequently increase and maintain higher glutathione peroxidase concentrations throughout the entire suckling period. So, if the calf faces any stress, pathogen, or disease, the enzyme would be available to act immediately. The increase in the blood glutathione peroxidase concentration depends on the increase in serum selenium (Thompson et al., 1981), and this increase in amount and time depends on the dose of supplemented selenium. In the study in which high doses were offered to calves (1 mg Se/kg BW resulting in 0.200 µg Se/mL in the blood) an increase in the concentration of the enzyme was observed 10 days after starting supplementation (Żarczyńska et al., 2021). On the other hand, with doses of 0.113 mg Se/kg of dry matter, resulting in 0.030 µg Se/mL, it took almost two months to observe an increase in the concentration of the enzyme in the calves' blood (Thompson et al., 1981).

Regarding vitamin E and iron supplementation, although their intake increased, 107.40 vs 57.26 mg/day of vitamin E for supplemented and control animals, and 179.23 vs 141.64 mg/day of Fe for SeVitEFe and non-supplemented calves; values remained unchanged in the calves' blood (5.11 vs 5.22 µg/mL of vitamin E for supplemented and control animals, and 157.76 vs 167.40 µg/dL of Fe for SeVitEFe and non-supplemented animals). When Jersey calves were orally supplemented with high DL-alpha-tocopherol acetate doses (two grams daily) from birth to 45 days of age, their serum vitamin E concentrations increased over time, rising from 1.55 to 4.38

µg/mL (Reis et al., 2007). These authors observed that calves, between birth and the first twenty days of life, presented very low serum vitamin E concentrations, which suggests the need for supplementation of this vitamin in the newborn's diet (Reis et al., 2007). In the current study, even offering double (108.37 and 106.44 mg/day for SeVitE and SeVitEFe) the daily vitamin E recommendation (44.7 mg/day), which are lower than the values used in the study with Jerseys calves (Reis et al., 2007), it was not sufficient to change the concentration of the nutrient in the blood of supplemented calves; however, vitamin E supplementation improves the ability to kill neutrophils and increases macrophage function in cows (Politis et al., 1995; Xiao et al., 2021). The biochemical actions of Se and vitamin E complement the body's physiological mechanism. Vitamin E increases Se retention and prevents the auto-oxidation of lipids within cell membranes, preventing the formation of peroxides; however, only GPx can destroy the peroxides already formed (Lippman et al., 2009; Pak et al., 2002).

A study was carried out to understand the need for iron supplementation for the development of suckling calves and reported that blood iron concentrations in newborn calves before colostrum feeding were equal to those in their mothers (166 µg/dL) and decreased up to two months after birth to values of 141.52 µg/dL (Atyabi et al., 2006). In the present study, there were no differences in iron concentration among treatments, but iron values increased in serum over the experimental period (127.95, 182.02, and 182.60 µg/dL, for initial value, 40 and 60 days, respectively) due to iron supplementation since the iron concentration in the blood of calves tends to fall shortly after birth (Getty et al., 1968). Iron is a microelement and the main component of hemoglobin. Newborn mammals exhibit physiologically low hemoglobin values, and it is known that milk is deficient in iron and can lead a nursing animal to iron deficiency relatively easily (Getty et al., 1968). One of the ways in which the nutrient is stored is in the form of ferritin and hemosiderin proteins (McDowell, 2000), with ferritin being stored in the liver and spleen, and when required by the body, it is mobilized (Grotto, 2010). There are still few studies reporting the influence of chelated minerals, especially iron, on ruminant production. Unlike what occurs with monogastric, in which there is emphasis and recommendation on supplementation, there is little discussion about iron supplementation for ruminants because deficiency in adult animals is rare. The new recommendations of nutritional needs for dairy cattle indicate that iron deficiency in calves is associated with low immune response (NASEM, 2021), however, there is no greater approach to supplementation and/or increased supply of the mineral in the first phase of suckling.

It was expected that calves supplemented with iron would show an increase in erythrogram and ferritin, but this result was not observed in the current study. However, the general erythrogram parameters of calves indicate that they were not in an anemic state, since there was no decrease in plasma hemoglobin and other parameters such as mean corpuscular volume and mean corpuscular hemoglobin remained within normal values. Fe supplementation together with the other micronutrients contributed to improving oxidative metabolism, changing some

biochemical parameters and the immune system, with reduced the number of *Anaplasma marginale* pathogens and the incidence of diarrhea compared to calves supplemented only with Se and VitE.

Among blood metabolites, animals supplemented with SeVitEFe had lower urea, lactate, and LDH values. Urea was lower in SeVitEFe animals at 60 days of experimentation. Urea is one of the main indicators of the animal's protein metabolism, and its change in the blood is faster than albumin when there is a protein deficit in the animal's diet (Lima et al., 2012). Blood lactate is related to rumen lactate, which is produced in the rumen when animals begin to ingest solid feed, and the rumen fermentation process becomes more active. The dry matter intake of the animals did not differ between treatments, although the SeVitEFe treatment was numerically slightly lower, but not to the point of perhaps influencing the levels of urea, LDH and lactate. This reduction may be more related to the defense metabolism of the animals against pathogens, which was more efficient in this treatment. Urea, LDH and lactate values are close to those found by other authors evaluating the metabolite in calves during the suckling phase (Feitosa et al., 2007; Lima et al., 2012).

As there was no difference among treatments for feed intake, another approach in addition to nutritional to explain these biochemical and hematological changes in the blood is the fact that control and SeVitE calves presented a higher incidence of diarrhea. Biochemical parameters were evaluated in neonatal calves with diarrhea, and it was reported higher urea and creatinine concentrations in animals with diarrhea compared to healthy ones, probably because of dehydration (Akyüz and Kükürt, 2021; Kozat et al., 2021).

One of the indices to determine the diagnosis of metabolic diseases in ruminants is the total activity of lactate dehydrogenase and its isoenzymes, and there is an increase in LDH activity when clinical symptoms of the disease are present (Klein et al., 2020). In sick calves, a slight increase in LDH and a significant increase in LDH1 activity were detected (Klein et al., 2020). In the present study, it was observed that calves in the control and SeVitE groups had higher LDH concentrations and higher incidences of diarrhea when compared to those in the SeVitEFe group.

It is already known in practice and in the literature that calves at this early stage of life are likely of having higher incidence of diseases and are also submitted to the critical stress period of weaning management. Therefore, immunonutrient supplementation would be a strategy to assist in the metabolism of calves at this early stage of life. The results show that glutathione peroxidase for supplemented calves was higher before inoculation with *Anaplasma marginale* (CEPA UFMG1), showing higher values of the enzyme in SeVitEFe calves. This made the oxidative metabolism of these animals to be more active to combat pathogens during immunological challenge and combat diarrhea-causing pathogens. This is a very important response for the production system, because the costs associated with treating diarrhea at this stage of life are high. In the present study, each case of diarrhea costs around US\$ 22.00 (antibiotic, antipyretic, probiotic and hydration), and many calves had recurrence. This decrease in the incidence of diarrhea in SeVitEFe supplemented calves reduces costs on medicines and labor and can positively impact the adulthood

productivity. The occurrence of diarrhea and other neonatal parameters such as time of birth, birth weight, and ease of birth are associated with detrimental effects on subsequent production and reproductive performance (Aghakeshmiri et al., 2017).

5 Conclusion

Supplementation of the micronutrients selenium, iron and vitamin E together showed effectiveness in improving the animals' oxidative metabolism, altering biochemical and hematological parameters, reducing the blood count of the *Anaplasma marginale* and the incidence of diarrhea, but did not enhance performance. Although this study demonstrated the importance of immunonutrient supplementation for calves at this early stage of life, additional studies should be conducted for nutritional recommendations for these supplements.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Animal Use Ethics Committee of the Animal Science Institute, with protocol number CEUA/IZ 268-18. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MS: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. FF: Data curation, Investigation, Writing – review & editing. CB: Investigation, Methodology, Writing – review & editing. VG: Conceptualization, Methodology, Writing – review & editing. RM: Methodology, Writing – review & editing. JS: Conceptualization, Investigation, Writing – review & editing. EF: Conceptualization, Methodology, Writing – review & editing. JF: Formal analysis, Writing – review & editing. AS: Conceptualization, Investigation, Methodology, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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