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Effects of injectable trace minerals on humoral and cell-mediated immune responses to *Bovine viral diarrhea virus*, *Bovine herpes virus 1* and *Bovine respiratory syncytial virus* following administration of a modified-live virus vaccine in dairy calves

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ABSTRACT

Our objective was to evaluate the effect of an injectable trace mineral (ITM) supplement containing zinc, manganese, selenium, and copper on the humoral and cell mediated immune (CMI) responses to vaccine antigens in dairy calves receiving a modified-live viral (MLV) vaccine containing BVDV, BHV1, PI3V and BRSV. A total of 30 dairy calves (3.5 months of age) were administered a priming dose of the MLV vaccine containing BHV1, BVDV1 & 2, BRSV, PI3V, and an attenuated-live *Mannheimia-Pasteurella* bacterin subcutaneously (SQ). Calves were randomly assigned to 1 of 2 groups: (1) administration of ITM SQ (ITM, n = 15) or (2) injection of sterile saline SQ (Control; n = 15). Three weeks later, calves received a booster of the same vaccine combination SQ, and a second administration of ITM, or sterile saline, according to the treatment group. Blood samples were collected on days 0, 7, 14, 21, 28, 42, 56, and 90 post-vaccination for determination of antibody titer, viral recall antigen-induced IFN-γ production, and viral antigen-induced proliferation by peripheral blood mononuclear cells (PBMC). Administration of ITM concurrently with MLV vaccination resulted in higher antibody titers to BVDV1 on day 28 after priming vaccination compared to the control group ($P = 0.03$). Calves treated with ITM showed an earlier enhancement in PBMC proliferation to BVDV1 following vaccination compared to the control group. Proliferation of PBMC after BVDV stimulation tended to be higher on day 14 after priming vaccination in calves treated with ITM than in the control group ($P = 0.08$). Calves that received ITM showed higher PBMC proliferation to BRSV stimulation on day 7 after priming vaccination compared to the control group ($P = 0.01$). Moreover, calves in the ITM group also had an enhanced production IFN-γ by PBMC after stimulation with BRSV on day 21 after priming vaccination compared to day 0 ($P < 0.01$). In conclusion, administration of ITM concurrently with MLV vaccination in dairy calves resulted in increased antibody titer to BVDV1, and greater PBMC proliferation to BVDV1 and BRSV recall stimulation compared to the control group, suggesting that ITM might represent a promising tool to enhance the humoral and CMI responses to MLV vaccines in cattle.

1. Introduction

Bovine respiratory disease (BRD) has a major impact on the profitability of the dairy and beef industries in North America, resulting in substantial economic losses (Griffin, 1997; McVey, 2009). The infectious agents most consistently implicated in BRD include

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Bovine viral diarrhea virus (BVDV), *Bovine herpes virus 1* (BHV1), *Bovine respiratory syncytial virus* (BRSV), *Parainfluenza 3 virus* (PI3V), *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis*. Effective transfer of maternal antibodies against these agents in conjunction with appropriate biosecurity measures and vaccination programs are crucial to prevent and control BRD.

Several factors have been reported to affect the immune response following vaccination (nutritional status, stress, weather extremes, passive transfer of maternal antibodies, vaccination route, vaccine handling). Nutritional status, and particularly mineral levels, have been demonstrated to impact cattle health and performance (Enjalbert et al., 2006; Galyean et al., 1999; Underwood and Suttle, 1999). Trace minerals such as Zinc (Zn), Manganese (Mn), Copper (Cu), and Selenium (Se) are important for optimal immune function (Chirase et al., 1994; Percival, 1998; Underwood and Suttle, 1999) and growth (Spears and Kegley, 2002) in cattle, particularly in highly stressed, and newly received feeder calves (Duff and Galyean, 2007).

Zinc contributes with the structure and function of more than 2500 enzyme systems involved in metabolism (Andreini et al., 2009; Cousins and King, 2004). Zinc activates the enzyme superoxide dismutase, which plays a crucial role in stabilizing cell membranes against reactive oxygen species (ROS) (Bonaventura et al., 2015; Haase and Rink, 2014). Zinc is involved in DNA replication through the actions of ribonucleotide reductase, and is necessary for lymphocytes proliferation and differentiation. Zinc's major roles in the immune response involve signaling and adhesion of neutrophils and macrophages (Bonaventura et al., 2015), production of pro-inflammatory cytokines by monocytes (Rink and Kirchner, 2000), regulation of IL-2 secretion, signal transduction for T cell activation, clonal expansion, differentiation and T_H cells polarization (Haase and Rink, 2014), B-cell function, and antibody production (Pinna et al., 2002; Tomlinson et al., 2008).

Copper is important in the mitochondrial metabolic cascades for energy production to supply different organs, including those of the immune system (Failla, 2003). Copper also plays a role in superoxide dismutase activity and neutralization of ROS (Maggini et al., 2007), and contributes to the process of phagocyte killing (Linder, 1991). Ceruloplasmin is a copper-containing enzyme whose production increases dramatically during inflammation in response to the necessity of scavenging oxygen radicals released by immune cells (Percival, 1998). In rodents, copper deficiency is associated with decreased IL-2 production, lymphocyte proliferation and T cells counts (Bala and Failla, 1993; Bonham et al., 2002; Klotz et al., 2003; Linder and Hazegh-Azam, 1996; Minatel and Carfagnini, 2000; O'Dell, 1993; Pan and Loo, 2000; Percival, 1998). Similarly, studies in cattle fed a copper-deficient diet showed a significant reduction in B-lymphocytes and impaired neutrophil activity (Cerone et al., 1998).

Selenium appears to be very important to the migration of neutrophils into tissues and subsequent inflammation (Maddox et al., 1999). Selenium is a component of the enzyme glutathione peroxidase that inactivates ROS production and prevents released ROS from causing cellular damage (Maddox et al., 1999; Neve, 1991). Selenium deficiencies have been associated with depressed neutrophil migration and killing ability, and reduced B-cell response and antibody production. Moreover, Se supplementation enhanced both humoral and cell-mediated and immune responses (Maggini et al., 2007). The level of Se in tissues and blood affected the total IgM levels and BHV1-specific antibody titers after challenge (Reffett et al., 1988). Evidence that Mn plays a role in the immune system is limited. However, Mn has an essential function in removing ROS produced by active phagocytic cells (Tomlinson et al., 2008).

The benefits of administering injectable trace minerals (ITM) on animal health and performance have been previously assessed in dairy (Harrison et al., 1984; Machado et al., 2013), and beef cattle (Arthington et al., 2014; Berry et al., 2000; Genther, and Hansen, 2014; Richeson and Kegley, 2011). However, only a few studies have evaluated the effects of ITM on the immune function of cattle (Arthington and Havenga, 2012; Chirase et al., 1994; Clark et al., 2006; Droke and Loerch, 1989). Arthington and Havenga (2012) assessed the effect of administration of ITM on the humoral immune response after BRD specific MLV vaccination in cattle. That study demonstrated that the ITM given concurrently with viral vaccination enhanced the production of neutralizing antibodies to BHV1 in beef calves. Additionally, recent studies have shown that treatment with ITM concomitantly with MLV vaccination induced a faster BVDV-specific antibody response in newly received, highly stressed calves (Roberts et al., 2015).

A growing body of evidence suggests that both humoral and CMI responses are critical in protection against viral agents involved in BRD (Collen and Morrison, 2000; Howard 1990; Nobiron et al., 2003). A more complete evaluation of the immune responses induced by MLV vaccination requires the use of methods to assess both humoral (antibody response) and cellular effector mechanisms (recall antigen induced proliferation and induction of IFN-γ as the core Th1 cytokine). We hypothesized that administration of ITM improves both humoral and CMI responses to vaccine antigens in dairy calves receiving a modified-live viral (MLV) vaccine containing BVDV1 and 2, BHV1, PI3V and BRSV. The objective of this study was to evaluate the effect of an injectable trace mineral (ITM) supplement containing Zn, Mn, Se, and Cu on the humoral and cell mediated immune (CMI) responses to individual vaccine antigens in dairy calves receiving a MLV vaccine containing BVDV1 and 2, BHV1, PI3V and BRSV.

2. Methods

2.1. Study location and animal husbandry

All cattle used in this experiment were derived from a single commercial dairy farm at Quitman, Georgia. At the farm of origin, fifty calves born during the first week of February in 2014 were placed in individual hutches separated from the rest of the calves on the farm, beginning on the day of birth and maintained until the day of transportation to the experimental farm. Thirty-five healthy calves that had not suffered any clinical disease, or received any vaccines or treatments were selected and transported to the University of Georgia Rose Creek Farm in Oconee County, Georgia, USA for use in the study.

On the day of transportation, the animals received a subcutaneous (SQ) injection of tilmicosin (10 mg/kg of body weight, Micotil® 300, Elanco Animal Health, Indianapolis IN) as a metaphylaxis to prevent the occurrence of respiratory disease associated with shipping. At the experimental farm, the calves were housed in an 8-acre pasture with adequate shade. The calves were cared for in accordance with acceptable practices as outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010). In addition, the research protocol was reviewed and approved by the University of Georgia, Institutional Animal Care and Use Committee (IACUC; protocol number A2014 02-005-Y2-A5).

Throughout the study, the calves grazed Bermuda grass (*Cynodon dactylon*) and Fescue grass (*Festuca arundinacea*) with no access to mineral supplementation. Animals had access to hay (Bermuda grass and Fescue grass) and water *ad libitum*. Additionally, calves received 2.7 kg/head/day of concentrate supplement (Bulk Cattleman's Special; Godfrey's Warehouse; Madison-GA) divided into two meals.

2.2. Experimental design, animals and treatments

This study was designed as a randomized controlled trial. A total of thirty-five weaned dairy bull calves (3.5 months of age) were enrolled. All calves were clinically normal. The calves were determined to be BVDV negative by antigen capture ELISA of skin biopsies. They had variable serum neutralizing (SN) antibody titers to BHV1 (up to 1:8), BVDV1 (1:16–1:512), and 2 (1:8–1:64), and BRSV (up to 1:8), which was assessed 14 days before vaccination and treatment administration. The SN antibody titer to BVDV1 on day-14 was used to stratify the calves before random assignment to assure equivalent aggregate titers in both experimental groups. Thirty calves were administered 2 mL of a 5-way MLV vaccine containing BHV1, BVDV1 and 2, BRSV, PI3V (Express 5®, Boehringer Ingelheim, Vetmedica, St. Joseph, MO), and 2 mL of an attenuated-live *Mannheimia haemolytica* and *Pasteurella multocida* bacterin (Once PMH, Merck Animal health, Summit, NJ) subcutaneously (SQ). Calves were randomly assigned to one of two treatment groups using a software (Research randomizer,V3.0, Social Psychology Network, Middletown, CT), as follows: (1) ITM (n = 15): subcutaneous administration of injectable trace minerals (1 mL/100 Lb BW; Multimin 90, Multimin USA Inc, Fort Collins, CO) or (2) Control (n = 15): subcutaneous injection of sterile saline (1 mL/100 Lb). Administration of ITM provided 15, 60, and 10 mg/mL of Cu, Zn, and Mn (as disodium EDTA chelates) and 5 mg/mL of Se (as Na selenite). Three weeks after initial vaccination, calves received a booster of 2 mL of the same 5-way MLV vaccine, and 2 mL of the same attenuated-live bacterin SQ. Concurrent with the vaccine booster, a second subcutaneous administration of injectable trace minerals (1 mL/100 Lb; Multimin 90) or sterile saline (1 mL/100 Lb) was given to calves in ITM and control group, respectively. Five calves did not receive vaccine or treatment (ITM or saline) and served as sentinel animals to verify that no field virus exposure occurred during the study. Sentinel calves were separated from the vaccinated calves for 7 days following priming and booster vaccination in order to prevent infection with shed vaccine virus.

2.3. Sample collection

From all calves in each group, blood samples were collected via jugular venipuncture into vacuum tubes (Vacutainer®, BD Diagnosis, Franklin Lakes, NJ) with and without anticoagulant (acid citrate dextrose) to obtain whole blood and serum, respectively. Blood was collected on days-14, 0 (enrollment), 7, 14, 21, 28, 42, 56, and 90 relative to priming vaccine and ITM administration, for serum neutralizing (SN) antibody titer determination, antigen-induced *in vitro* interferon gamma (IFN-γ) production by peripheral blood mononuclear cell (PBMC), and antigen-induced PBMC proliferation. Because of the length of time required for laboratory processing, particularly to isolate PBMC and set up the assays to measure antigen-specific IFN-γ secretion, and PBMC proliferation, a subset of 15 calves per day were sampled (with the same number of calves in each subset including calves from both treatment groups) on consecutive days (Wednesday and Thursday) each week from day-14 through day 90 relative to the day of primary vaccination.

Trace mineral status of calves was assessed in liver biopsy samples (10–15 mg of liver tissue) collected from each calf on days-7, 21, and 56 relative to the day of priming vaccination. Liver biopsies were done using sterile biopsy needles (14 g and four inches long) with the assistance of ultrasonographic imaging (Ibex® Lite, E.I. Imaging, Loveland, CO) and local anesthesia (Lidocaine injectable. Aspen Veterinary Resources, Ltd. Liberty, MO).

Multiple hand-plucked samples of pasture forage, hay and feed concentrate were randomly collected on days 0, 14, 21, 28, 42, 56 and 90 for subsequent analysis for nutrient and mineral content at the Diagnostic Center for Population and Animal Health at Michigan State University, Lansing, MI (Table 1).

Table 1
Nutrient composition of feedstuffs during the experimental period (7 collections from day 0 to day 90)[a].

Item	Grass[b]	Hay[c]	Supplement[d]
––– % (DM basis) –––			
CP	14.53	13.8	17.08
ADF	38	41.5	19.41
NDF	64.47	71.7	37.45
Ca	0.48	0.49	0.06
P	0.5	0.29	0.81
Mg	0.35	0.29	0.3
K	2.8	2.2	1.28
Na	0.01	0.03	0.18
––– mg/kg (DM basis) –––			
Fe	257	354.7	121.1
Zn	25.43	38.71	49.85
Cu	7.14	10.42	5.14
Mn	94.86	48.71	18.85
Se	0.05	0.06	0.16
Mo	3.6	0.97	1.68
Co	0.47	0.38	0.36
S	0.28	0.36	0.4

[a] Values represent an average of 7 analyses from a composited mixture of several hand-grab samples.
[b] Hand-grab Fescue grass and Bermuda grass pasture samples collected in between June and August.
[c] Long-stem Fescue grass and Bermuda grass hay provided in a large-round bale at a rate of 1.0 kg/calf/day throughout the experimental period.
[d] Grain concentrate composed of soybean hulls (45.03%), Corn gluten (50.04%), molasses (2.25%), calcium (1.5%), and salt (1.0%), trace mineral (0.1%) Vitamin ADE (0.05%) was offered to calves at a rate of 2.7 kg/head/day (as-fed) throughout the experiment.

2.4. Sample processing for separation of serum and peripheral blood mononuclear cells

Within two hours after collection, blood samples were transported on ice to the laboratory. The blood samples in the tubes without anticoagulant were centrifuged at 800*g* for 15 min. The serum was then removed and stored in aliquots at −80 °C until analyzed for neutralizing antibodies against BVDV1, BHV1, and BRSV.

Blood samples with anticoagulant (4 tubes per calf) were inverted about 10 times to mix the contents well and were centrifuged at 650*g* for 10 min at room temperature. Plasma was removed and the buffy coat layer was collected from each tube and pooled into one sterile 50 mL centrifuge tube per animal. The cells were suspended to a uniform single cell state in 5 mL of Ca^{2+} and Mg^{2+} free PBS (DPBS, 10 X; Mediatech, Inc. Corning cellgro, Manassas, VA). After mixing, Ca^{2+} and Mg^{2+} free PBS was added to each tube to a final volume of 40 mL. Peripheral blood mononuclear cells (PBMC) were then isolated using a discontinuous gradient floatation procedure over 1.083 density medium (Histopaque 1083, Sigma, St. Louis, MO), and the resulting cell preparation was utilized for measurement of antigen-specific production of IFN-γ and lymphocyte proliferation. The cells were counted and assessed for viability using Trypan Blue dye exclusion (Trypan blue solution, Mediatech, Inc, Manassas, VA). Cells were suspended at 6×10^6/ml in RPMI-1640 medium with 10% gamma-irradiated fetal bovine serum for use in the assessment cultures.

2.5. Serum neutralizing (SN) antibody titers against BHV1, BVDV1, and BRSV

Serum neutralizing antibody titers against BHV1, BVDV1, and BRSV were determined via a standard virus neutralization test by the University of Georgia Athens Veterinary Diagnostic Labora-

tory (Athens, GA). Briefly, serum samples were thawed and heat inactivated at 56 °C for 30 min. Heat-inactivated serum samples in duplicate were then diluted with Dulbecco minimum essential medium (DMEM) into a serial 2-fold dilution series, starting at 1:2 in 96-well cell culture plates. To each well, an equal volume (25 μL) of Dulbecco minimum essential medium containing 100 $TCID_{50}$ of the appropriate virus was added. Addition of virus took the final starting dilution of serum to 1:4. The plates were incubated in 5% CO_2 at 37 °C for 1 h. Then 150 μL (approx 10^4 cells) of a Madin-Darby bovine kidney cell suspension in DMEM containing 10% fetal calf serum (FCS) was added to each well. The plates were incubated in 5% CO_2 at 37 °C for 4 days. An inverted microscope was used to examine the cell monolayer in each well for virus-specific cytopathic effects. The SN titer for each sample was reported as the highest dilution of serum that completely inhibited virus-induced cytopathic effects in both wells.

2.6. PBMC proliferation in response to BVDV1, BHV1, and BRSV

Cells were brought to a concentration of 6×10^6 cells/mL in RPMI + 10% gamma-irradiated (GI)-fetal calf serum (FCS−Atlanta Biologicals, Norcross, GA) +2 mM L-glutamine (MediaTec) and + 1% penicillin and streptomycin. One hundred μl of the cell suspension from each calf was aliquoted into wells of a 96-well round bottom plate in triplicate for each treatment. To each set of triplicate wells 100 μL of binary ethyleneimine-inactivated BHV-1 Cooper strain (at 6.1×10^7 TCID50 units/mL), live BVDV1 NADL (at 6.3×10^6 $TCID_{50}$ units/mL), live BRSV 375 (at 3.2×10^7 $TCID_{50}$ units/mL), Staphylococci enterotoxin SEB (Toxin Technology, Inc, Sarasota, FL, 1.0 μg/mL as positive control), or RPMI + 10% GI-FCS + 2 mM L-glutamine and 1% penicillin-streptomycin (negative control) were added. Plates were incubated at 37° C in 5% CO_2 for 5 days. After incubation, 10 μL of ^{3}H-thymidine (MP Biomedical, Cleveland, OH, 0.2 uCi/well) was added to each well and the plates were incubated at 37° C in 5% CO_2 for an additional 6 h. Well contents were then harvested onto glass fiber filters using a 96 well plate automated harvester (Filtermate, PerkinElmer, Shelton, CT), the disks were punched out and placed in 7 mL scintillation vials and the disks were allowed to dry for 12–24 h. Three ml of high efficiency (34% for tritium) scintillation cocktail (MP, Fine Chemicals, Indianapolis, IN) was added and read on a scintillation counter (Beckman-Coulter, Fullerton, CA) for determination of cell proliferation in response to each antigen or mitogen. Results were expressed as the specific incorporation calculated as the mean counts per minute for PBMC cultured with BVDV1, BHV1, BRSV, or mitogen divided by the mean counts per minute for PBMC cultured in cell culture media alone.

2.7. IFN-γ secretion by PBMC in response to BHV1, BVDV1, BRSV

Peripheral blood mononuclear isolated as described above were suspended at 6×10^6 cells/mL in RPMI + 10% GI-FCS + 2 mM L-glutamine and 1% penicillin and streptomycin. Six hundred μl of the cell suspension was aliquoted into wells of a 24-well flat bottom plate. To each set of triplicate wells were added 600 μL of stimulating agents (BHV1, BVDV1, BRSV, and SEB) as described above for the virus-induced PBMC proliferation assay. Plates were incubated at 37° C in 5% CO_2 for 72 h. The plates were removed from the incubator and centrifuged at 200*g* for 5 min. The plates were tipped to about 45° angle and 1,000 μL of supernatant was collected from each well without disturbing the cells and transferred to a 1.5 mL micro-centrifuge tube for storage at −80° C. Interferon gamma concentration in supernatants in response to stimulation with BVDV1, BHV1, BRSV, or SEB was measured by ELISA using a commercially available antibody reagent pair and recombinant standard (R&D Systems, Minneapolis, MN) at dilutions optimized in this laboratory. Values were expressed as pg/mL as determined by a standard curve run on each plate. Before analysis, the IFN-γ concentration from cells cultured in media alone was subtracted from values from cells cultured with the viruses or SEB. All PBMC supernatant samples designated for the IFN-γ assay were stored until sample collection was completed, so that all the samples could be analyzed at the same time.

2.8. Statistical analysis

Statistical analysis was performed using Statistical Analysis System (SAS, version 9.1, SAS Institute, Cary, NC). Statistical parametric assumptions of normality and constant variance were tested through Shapiro Wilk's and Levene's tests, respectively. Additionally, normal probability plots were constructed and analyzed to confirm parametric assumption. Since there was a significant departure from normality and constant variability, a logarithmic base 2 transformation was applied to the SNA and stimulation index values. The outcomes produced by the transformation were carefully examined for possible violations of the assumptions of normality. For calculation and comparison of geometric mean (GM) antibody titers, back-transformed titers for each virus tested were calculated for each group at days 0, 7, 14, 21, 28, 42, 56, and 90 after vaccination.

Stimulation index triplicates for PBMC proliferation, were Q-tested (n = 3, CI = 0.941) for outlier identification and elimination. A two independent sample *t*-test was performed to compare antibody titers, IFN-γ concentration, PBMC proliferation, and liver mineral concentrations between treatment groups for each day. Additionally, a repeated measure analysis was done to compare the antibody titers, IFN-γ concentration, and PBMC proliferation during the experimental period with the baseline on day 0. Additionally, non-parametric analysis was also applied to the untransformed data using Mann-Whitney U and Friedman tests for comparisons between treatment groups and over time, respectively. For all analyses values of $P < 0.05$ were considered significant, and $0.05 < P \leq 0.15$ constituted a tendency.

3. Results

Tissue injection-site reactions were not observed in any calves following vaccination or treatment administration. No clinical signs of BRD were observed in any of the calves during the experimental period. Concentrations of Se and Cu in most feedstuffs (except for Cu levels in hay) were lower than the suggested requirements for growing dairy calves (0.3 and 10 mg/kg of Se and Cu, respectively; NRC, 2001). Concentrations of Zn in grass and Mn in feed supplement were lower than the requirements (32 and 22 mg/kg, respectively; NRC, 2001). However, the needs for Zn and Mn were properly satisfied by the other two feed components throughout the experimental period (Table 1).

3.1. Trace mineral concentration in liver biopsy samples

On day-7 before vaccination and treatment administration, there were no differences in liver concentrations of Se, Zn, Cu, or Mn, between treatment groups ($P > 0.05$; Fig. 1A–D). All values on days-7, 21 and 56 were observed within the reference range for cattle (Herdt and Hoff, 2011), indicating that there were no trace mineral deficiencies among the group of calves utilized in this study.

There were variations in liver tissue concentrations of Cu, Mn, Zn, and Se across the three biopsy sampling days (Fig. 1A–D). Liver concentrations of Se decreased significantly in the control calves on days 21 (42.4% decrease) and 56 (60.9% decrease) compared to day-7 ($P < 0.001$; Fig. 1A). In contrast, there was a significant increase (69.5%) in liver Se concentrations in calves treated with ITM on day

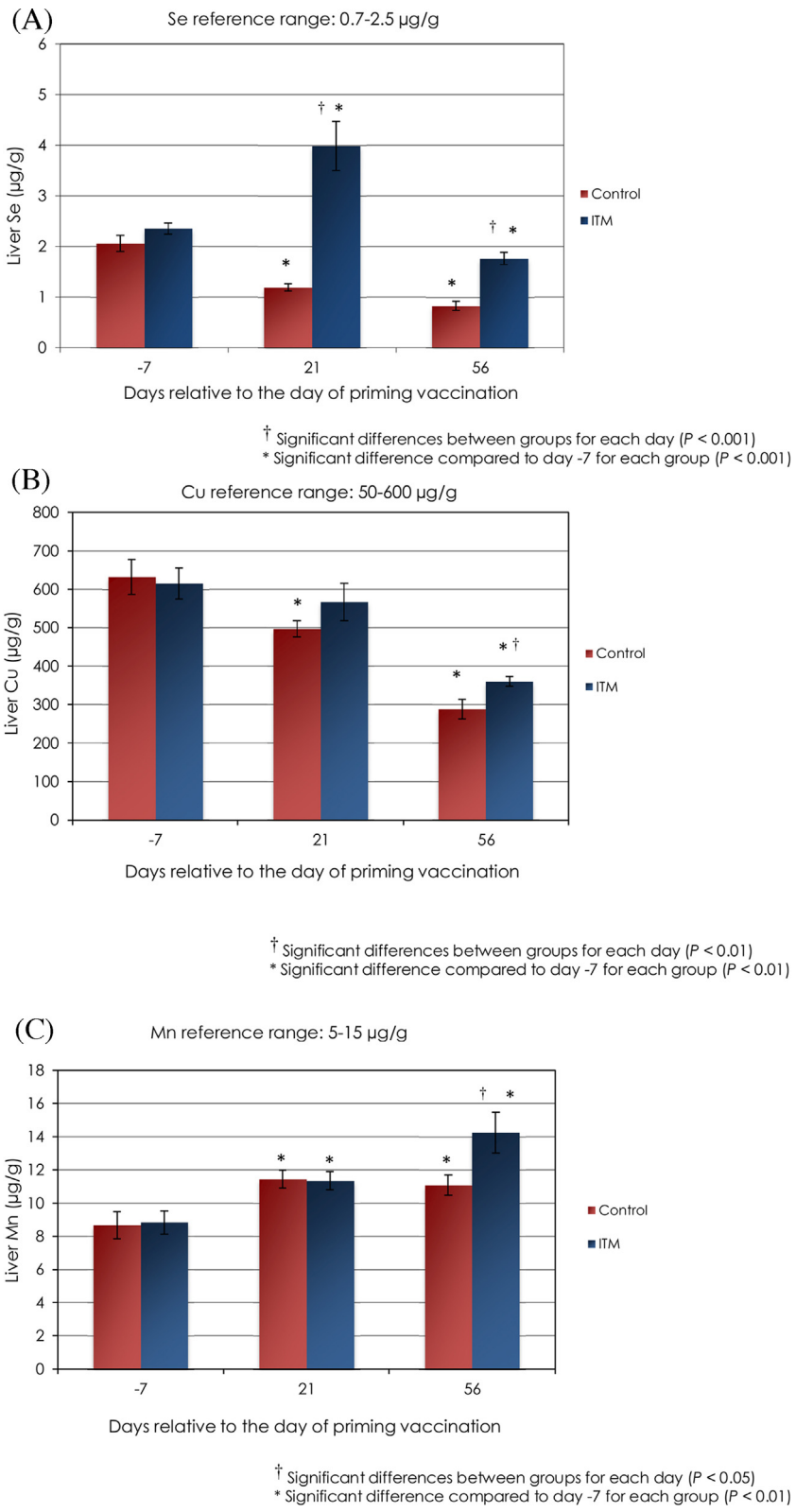

Fig. 1. Liver concentration (µg/g) of Selenium (A), Copper (B), Manganese (C), and Zinc (D) on days-7, 21 and 56 relative to the day of priming vaccination in dairy calves injected with a trace mineral supplement (ITM) or saline (Control) concurrently with administration of a modified-live virus (MLV) vaccine containing BVDV1 and 2, BHV1, BRSV and PI3V. Reference values of hepatic trace mineral concentrations: Se: 0.7–2.5 µg/g; Cu: 50–600 µg/g; Mn: 5–15 µg/g and Zn: 90–400 µg/g (Herdt and Hoff, 2011). †Significant differences between groups for each day (1A: $P < 0.001$; 1B: $P < 0.01$; 1C: $P < 0.05$) * Significant difference compared to day-7 for each group (1A: $P < 0.001$; 1B-D: $P < 0.01$).

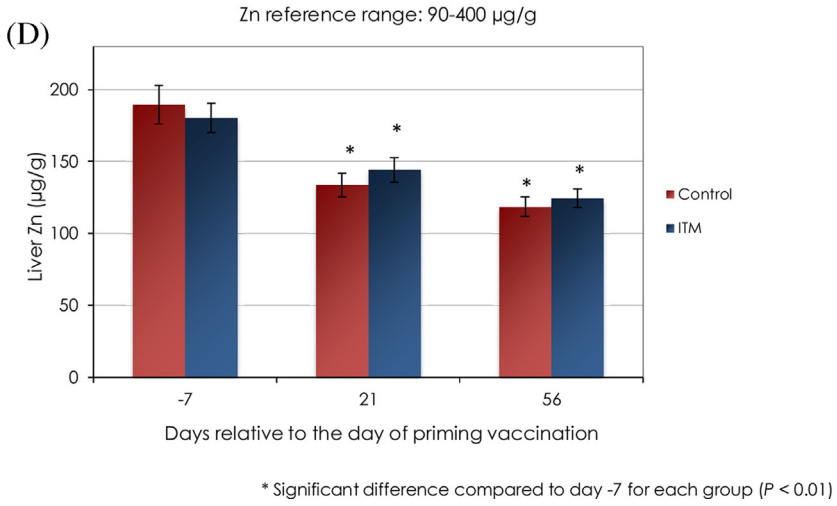

Fig. 1. (*Continued*)

21 compared to day-7 ($P < 0.001$; Fig. 1A); which then decreased on day 56 ($P < 0.001$). Hepatic Se concentrations were significantly higher on days 21 and 56 in calves treated with ITM compared to those observed in the control group ($P < 0.001$; Fig. 1A). Calves in the control group showed a significant decay in hepatic Cu concentrations on days 21 ($P < 0.01$) and 56 ($P < 0.001$) compared to day-7 (Fig. 1B). There was a slight decline ($P > 0.05$) in Cu concentration in the ITM group on day 21 compared to day-7, which was not as dramatic as that observed in the control group (decrease of 7.8% *vs* 21.5% for ITM and control groups, respectively; Fig. 1B). However, the hepatic concentration of Cu in the ITM group was significantly reduced on day 56 relative to day-7 ($P < 0.001$; Fig. 2). Significantly higher liver concentrations of Cu ($P < 0.01$; Fig. 2) and Mn ($P < 0.05$; Fig. 1C) were observed on day 56 in calves treated with ITM than

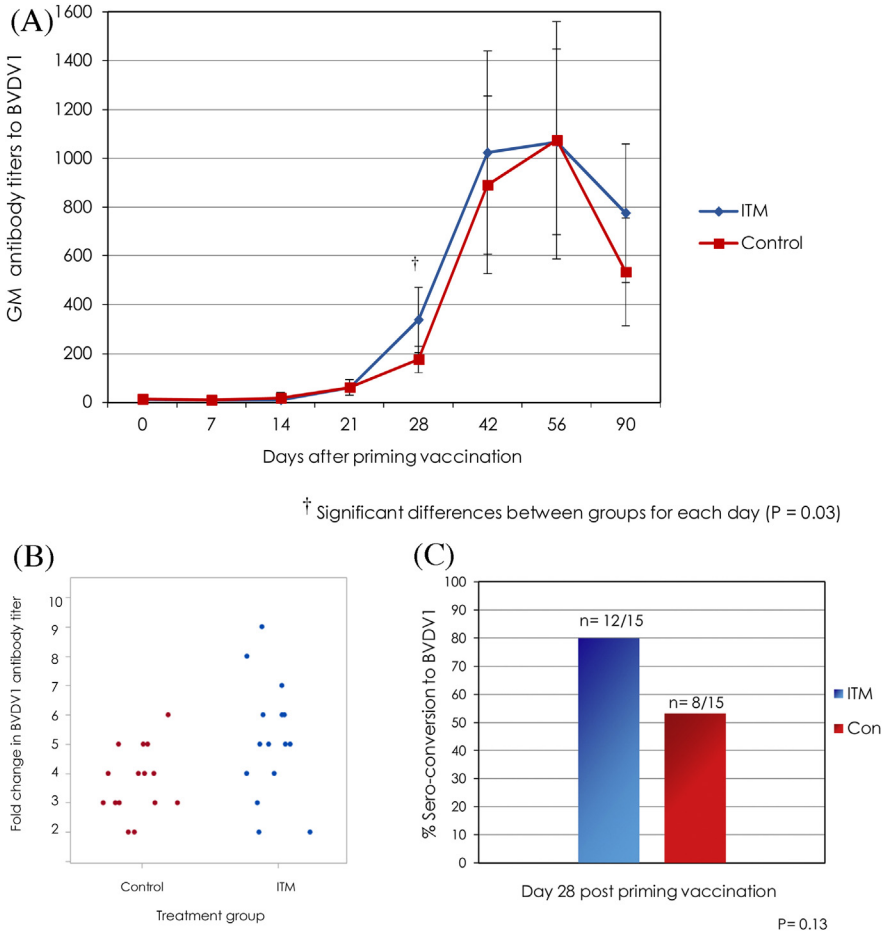

Fig. 2. Geometric means serum neutralizing (SN) antibody titers to BVDV1 (A), fold change in SN antibody titers to BVDV1 on day 28 relative to the day of priming vaccination (B), and proportion of sero-conversion or ≥4-fold increase in SN antibody titer to BVDV1 on day 28 relative to the day of priming vaccination (C) in dairy calves that received injectable trace minerals (ITM) or saline (Control) concurrently with a MLV vaccine. †Significant differences between groups for each day (P = 0.03).

the control calves. Liver concentrations of Zn decreased in both groups on days 21 and 56 compared to day-7 ($P < 0.01$; Fig. 1D). This decrease was more pronounced in the control group than in the ITM group (29.4 *vs* 20.0% decrease on day 21 for control and ITM groups, respectively; and 37.4 *vs* 30.0% decrease on day 56 for control and ITM groups, respectively).

3.2. Serum neutralizing (SN) antibody titers against BHV1, BVDV1, and BRSV

Administration of ITM concurrently with MLV vaccination resulted in higher geometric mean antibody titers to BVDV1 on day 28 following priming vaccination compared to the control group ($P = 0.03$; Fig. 2A). This higher BVDV1 specific humoral immune response was associated with both higher production of antibody and a numerical tendency of greater proportion of sero-conversion ($\geq$4-fold increase compared to day 0) to BVDV1 on day 28 in the ITM group (12/15; 80.0%) compared to the control group (8/15; 53.3%; $P = 0.13$; Fig. 2B and C). Antibody titers to BHV1 and BRSV did not increase significantly following vaccination over the experimental period in any of the treatment groups (data not shown). The unvaccinated sentinel calves remained sero-negative for the duration of the study, confirming that no exposure to a field strain of BHV1, BVDV1, or BRSV occurred.

3.3. PBMC proliferation in response to BVDV1, BHV1, and BRSV

A significant enhancement in PBMC proliferation was observed in both groups upon BVDV stimulation on different days post vaccination relative to day 0 ($P < 0.05$). Calves treated with ITM showed a more consistent enhancement in PBMC proliferation to BVDV following MLV vaccination (on days 14, 21, 42, 56 and 90 relative to day 0), compared to the control group (on days 28, 56 and 90 relative to day 0). It was interesting to note that a significant increase in PBMC stimulation index to BVDV1 occurred earlier in the ITM group (day 14) than the control group (day 28). In addition at day 14 following the priming vaccination, the PBMC proliferation response to BVDV1 tended to be higher in the ITM group than the control group ($P = 0.08$; Fig. 3A).

Both groups showed an increased PBMC proliferation response over time upon BHV1 stimulation compared to day 0 ($P < 0.05$). However, no significant differences between groups were observed in the level of BHV1 induced proliferation by treatment. The ITM group had a stronger and earlier enhancement in PBMC proliferation response to BHV1 than the control group (peak PBMC proliferation response occurred on days 28 and 56 for ITM and control respectively; Fig. 3B). Administration of ITM resulted in a more consistent PBMC proliferation response to BRSV following MLV vaccination over the experimental period than the saline injected calves (Fig. 3C). Moreover, calves that received ITM showed a significantly higher PBMC proliferation in response to BRSV recall on day 7 after the priming vaccination compared to the control group ($P = 0.01$; Fig. 3C).

3.4. IFN-γ secretion by PBMC in response to BHV1, BVDV1, BRSV

A significant increase in IFN-γ production by PBMC upon *in vitro* stimulation with BHV1 was observed on days 14, 21, 28, 42 and 56 relative to the day of priming vaccination (day 0) in both treatment groups ($P < 0.01$ Fig. 4A). Significant differences were not observed in the IFN-γ concentrations after BHV1 stimulation between groups at any time during the study. Administration of ITM concurrently with MLV vaccination induced an augmented production IFN-γ by PBMC after BRSV recall stimulation on day 21 after priming vaccination compared to day 0 ($P < 0.01$ Fig. 4B). In contrast, IFN-γ production by PBMC in response to BRSV was not enhanced for the control calves during the experimental period. *In vitro* production of IFN-γ to BVDV1 recall was not enhanced in either treatment group during this experiment (Fig. 4C).

4. Discussion

The response to MLV vaccines is a critical component in the prevention of BRD in dairy and beef cattle. Simple, practical and low cost tools to enhance the immune response elicited by respiratory virus vaccines could help to improve the overall calf health and provide a more predictable and uniform product to the feed yard and the replacement heifer program, which would result in significant economic benefits to beef and dairy producers. The results of this study indicate that the use of ITM can provide enhancement in vaccine-induced immunity, which might contribute with the development of the preventative management of respiratory disease in calf stocks.

In this study calves had adequate liver tissue concentration of all the study trace minerals assessed at all sampling dates according to standard reference values (Herdt and Hoff, 2011). Administration of ITM resulted in increased concentrations of liver Se (on days 21 and 56), Cu (on day 56) and Mn (on day 56) compared to saline injected calves. Therefore, injection of trace minerals at the beginning of the study and again 21 days later improved the Se, Cu, Zn and Mn status of the ITM calves compared to the control calves over the period of the study.

Previous studies have shown that supplementation with injectable Se, Cu, Mn and Zn improved serum and liver levels of these minerals compared to those in saline injected calves (Arthington et al., 2014; Arthington and Havenga, 2012; Pogge et al., 2012). In the current study administration of ITM contributed to increase and/or mitigate the abrupt decay in liver concentration of trace minerals in dairy calves that received two doses of the vaccines utilized. Similar to this study a significant reduction in Se, Cu and Zn concentrations has been previously reported in calves following vaccination (Arthington and Havenga, 2012; Droke and Loerch, 1989) or challenge (Chirase et al., 1994) and might be associated with the utilization of these nutritional elements to develop an immune response.

Trace mineral status and requirements may vary with the stage of growth and production (NRC, 2001). It has been documented that liver reserves of trace minerals in cattle decrease with the age, reaching significantly decreased levels by 6–7 months of age, if no mineral supplementation is provided (Puschner et al., 2004a,b). Arthington et al. (2014) reported more than 60% decrease in liver Se concentrations in calves from 150 to 250 days of age, which might have negative consequences on overall health, feed efficiency and performance. In this study, we used young calves (3.5 months of age) that were raised on an acceptable nutritional regime from immediately after birth until the end of the trial. This might explain the adequate mineral status in both groups throughout the experimental period.

Administration of ITM concurrently with MLV vaccination enhanced the ultimate level and shortened the time needed to reach expected antibody production against BVDV1, demonstrated by increased SN titers as well as a higher proportion of calves that seroconverted on day 28 after primary vaccination compared to the control calves. This quicker BVDV-specific antibody response elicited by an MLV vaccine delivered concurrently with ITM might be of significant value by conferring earlier protection following vaccination. This could be particularly important in newly received, highly stressed cattle that are at high risk of respiratory viral infections. A rapid increase in serum neutralizing antibodies against BVDV after vaccination may be beneficial to prevent infection and disease development. The antibodies may neutralize extracellu-

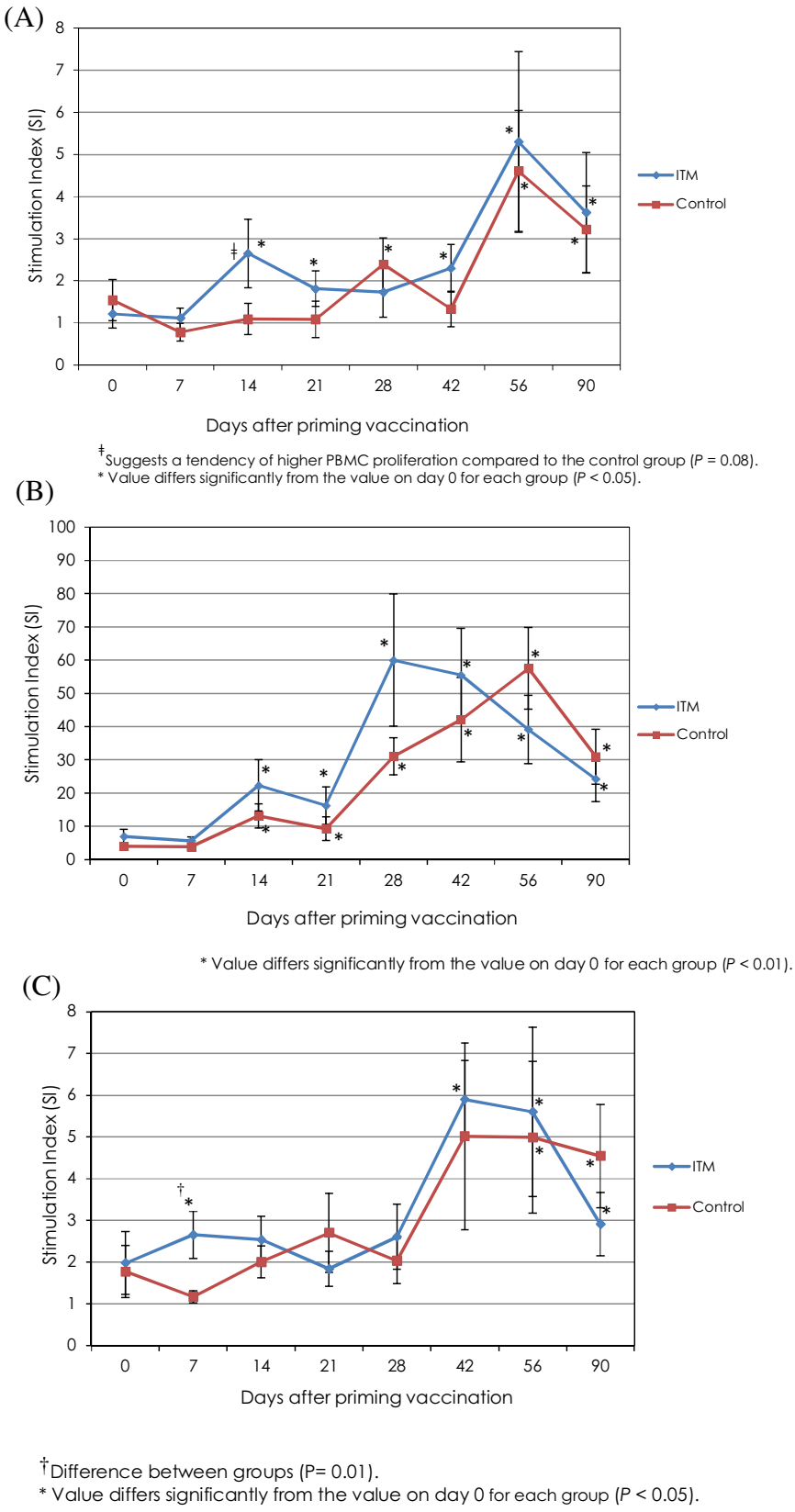

Fig. 3. Peripheral blood mononuclear cell (PBMC) proliferation (expressed as stimulation index; SI) in response to BVDV1 (A), BHV1 (B), and BRSV (C) in dairy calves injected with a trace mineral supplement (ITM) or saline (Control) concurrently with administration of a MLV vaccine. * Value differs significantly from the value on day 0 for each group (3A and C: $P < 0.05$; 3B: $P < 0.01$). ‡Suggests a tendency of higher PBMC proliferation compared to the control group (3A, $P = 0.08$). †Significant difference between groups (3C, P = 0.01).

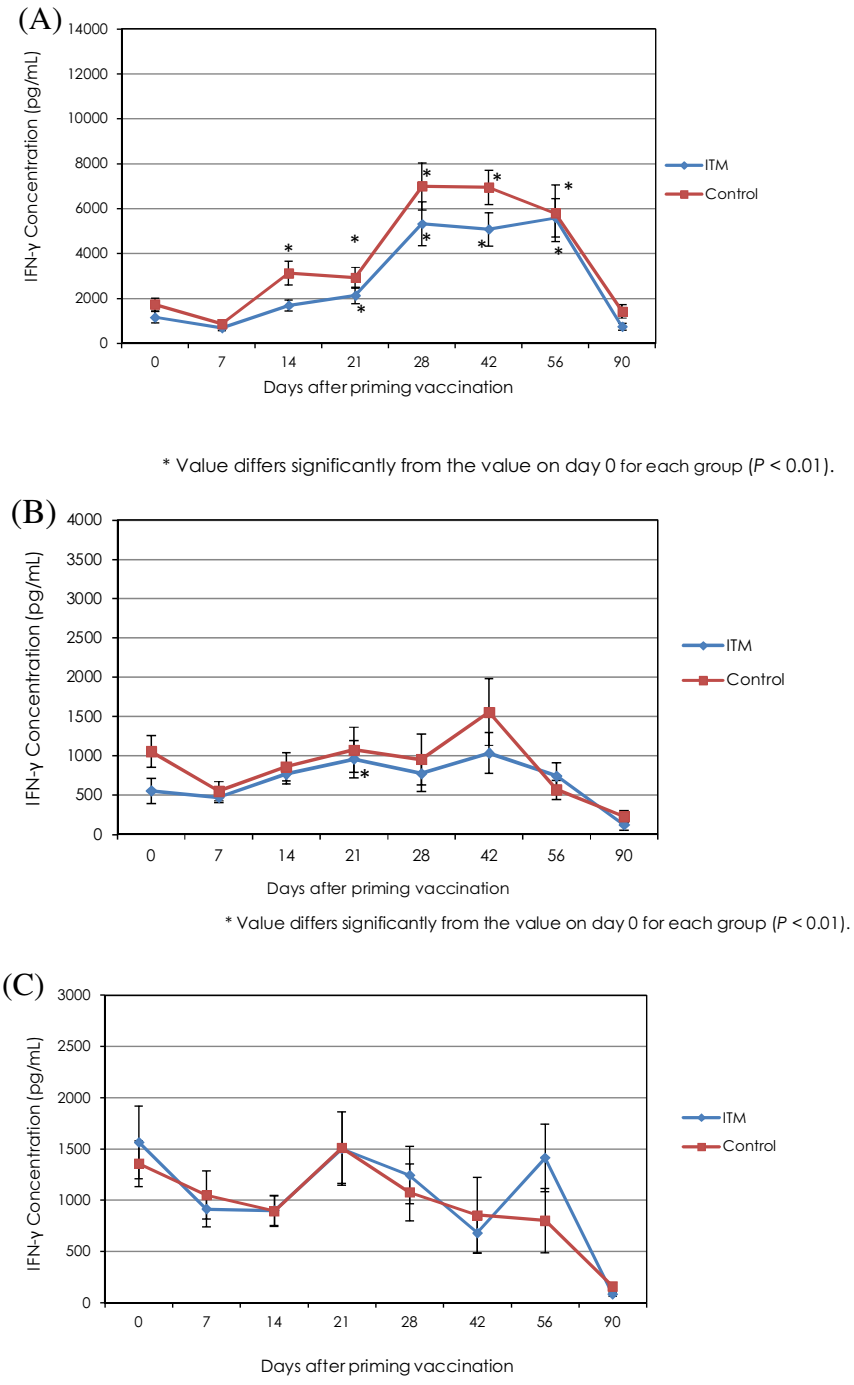

Fig. 4. IFN-γ levels (pg/mL) secreted by PBMC in response to BHV1 (A), BRSV (B), BVDV (C) in dairy calves injected with a trace mineral supplement (ITM) or saline (Control) concurrently with administration of a MLV vaccine. * Value differs significantly from the value on day 0 for each group ($P < 0.01$).

lar virus particles, inhibiting attachment of the virus to host cells, and contribute to antibody dependent cell mediated cytotoxicity (Forthal, 2014).

Similarly, previous reports have shown that ITM enhanced humoral immune response to pathogens of clinical significance in cattle production, including BVDV (Roberts et al., 2015), BHV1 (Arthington and Havenga 2012), *E. coli* (Panousis et al., 2001) and *Pasteurella haemolytica* (Droke and Loerch, 1989). In the study performed by Arthington and Havenga (2012), administration of trace minerals concurrently with an MLV vaccine to steers induced a significant increase in BHV1 serum neutralizing antibody titers on days 14, 30, and 60 post-vaccination compared to the base line titers on day 0 and to the titers in the saline injected steers.

Antibody titers to BHV1 and BRSV did not increase after priming or booster vaccination in this study. A possible reason for this lack of enhanced antibody production might be the presence of maternally derived antibodies that inhibited the development of the humoral immune response to BHV1 and BRSV. Maternal antibody did not appear to inhibit antibody production to BVDV1. Numerous studies have suggested that young calves usually do not seroconvert against many antigens following vaccination when maternally derived antibodies are present in significant levels (Ellis et al., 2001; Kirkpatrick et al., 2001; Van Donkersgoed et al., 1991; Windeyer

et al., 2015). However, similar to this study, other experiments have shown that calves that were vaccinated with certain antigens in the presence of maternal antibodies developed an anamnestic response to booster vaccination, even when antibody response to the primary vaccination was not evident (Brar et al., 1978; Endsley et al., 2003; Ridpath et al., 2003). Therefore, vaccination of calves with circulating maternal antibodies appears to elicit cell-mediated immune memory response despite an apparent lack of new antibody production at priming immunization (Endsley et al., 2003; Lemaire et al., 2000).

Injection of trace minerals concurrently with administration of a MLV vaccine improved PBMC proliferation upon stimulation with BVDV1 and BRSV. Several studies have previously shown evidence of enhancement in multiple cellular functions important to cell mediated immunity following vaccination with MLV vaccines (Platt et al., 2009; Reber et al., 2006; Sandbulte and Roth, 2003). This is the first trial showing the effect of an injectable supplement containing Cu, Se, Zn and Mn administered simultaneously with MLV vaccination on PBMC proliferation to viral recall antigen in dairy calves. In this study, two peaks of PBMC proliferation upon stimulation with BVDV1, BRSV and BHV1 were observed in each treatment group. These followed primary and booster MLV vaccination. The initial peak in PBMC proliferation in response to BVDV1 and BRSV after primary vaccination occurred 14 days earlier in calves receiving ITM compared to the controls. Furthermore, the PBMC proliferation after stimulation with BRSV (on day 7) and BVDV1 (on day 14) were significantly stronger in the ITM group compared to the control group. Even though there was no effect of treatment on the stimulation index after BHV1 stimulation, PBMC proliferation was observed to be higher in the ITM group.

Adequate supply of Zn, Cu, Se and Mn has been documented to be essential for cell signaling and cytokine production during lymphocyte activation (Puertollano et al., 2011; Spears, 2000). These trace minerals are fundamental elements in the structure and function of several metalloproteins that participate in general housekeeping processes involved in cellular clonal expansion including metabolic cascades for energy production, DNA replication and transcription, as well as protection against ROS (Failla, 2003). The enhancement of these general cellular functions might be contributing to the higher PBMC proliferation response observed in the ITM group.

A significantly increased production of IFN-γ by PBMC in response to BHV1 was observed in both treatment groups independent of ITM. Previous experiments with herpes simplex virus (HSV) have indicated that IFN-γ is produced as a crucial part of the nonspecific response to the virus infection. A marked relationship between HSV infection and induction of IFN-γ and IL-12 has been previously demonstrated (Malmgaard et al., 2000; Vollstedt et al., 2004) and seems to be critical to control of acute herpes virus infections (Ellermann-Eriksen, 2005). This serves to confirm the findings of this study. Administration of ITM concurrently with MLV vaccination induced an augmented production IFN-γ by PBMC with recall BRSV stimulation on day 21 after priming vaccination compared to day 0. Production of IFN-γ was apparently decreased by stimulation with BVDV. Previous studies have shown a down regulation of IFN-γ by *in vivo* and *in vitro* ncp BVDV infection (Rhodes et al., 1999). However, in this study both the vaccination and antigen recall were done with cytopathic BVDV.

In conclusion, administration of ITM concurrently with MLV vaccination in dairy calves resulted in earlier and increased antibody titer to BVDV1 (on day 28) and PBMC proliferation upon BVDV1 (on day 14) and BRSV stimulation (on day 7) compared to the control group. Trace minerals are crucial for the development of an adequate immune response in cattle, especially in stressed animals. Field and experimental studies focused on improving the trace mineral status of stressed feeder calves have shown positive effects of ITM improving feed efficiency, decreasing morbidity and treatment costs and improving performance and production traits (Arthington et al., 2014; Berry et al., 2000; Clark et al., 2006; Genther, and Hansen, 2014; Richeson and Kegley, 2011). The results of the current study support our hypothesis, and recapitulate the findings of previous studies demonstrating the benefits of trace mineral supplementation on the immune response to MLV vaccines in cattle. This suggests that addition of ITM to calf management protocols might represent a promising tool to improve livestock health on commercial farms.

Conflict of interest statement

The authors of this article, with the exception of LJ Havenga, declare that there are no conflicts of interest. LJ Havenga is an employee of Multimin USA.

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