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Effects of injectable trace minerals administered concurrently with a modified live virus vaccine on long-term protection against bovine viral diarrhea virus acute infection in dairy calves

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ABSTRACT

The objective was to evaluate the effects of injectable trace minerals (ITM) concurrent with modified-live virus (MLV) vaccination on protection from bovine viral diarrhea virus (BVDV) infection in dairy calves. In a previous study (Palomares et al., 2016), thirty dairy calves received two doses of a MLV vaccine subcutaneously (SC), concurrently with ITM (n = 15) or saline (n = 15), SC. Five months later, 20 of these calves received ITM (G1, n = 10) or saline (G2, n = 10) according to their previous groups and were challenged intranasally with BVDV2. Five unvaccinated calves were also challenged with BVDV2 (G3). Blood samples were collected on days 0 (BVDV challenge), 3, 5, 6, 7, 8, 9, 11, 14, 18, 21, 32 and 61 for leukocyte count, virus isolation and BVDV serum neutralizing antibodies (SNA). Mild-moderate clinical signs were observed in G3 after BVDV challenge. Group 1 showed lower sum health score and nasal score on d5 and fecal score on d8 compared to G2. Rectal temperature and leukocyte counts were not different between G1 and G2. In contrast, G3 calves had significant leukopenia and lymphopenia from d3 to d7 ($P < .05$) and higher rectal temperatures on d6 to d8, compared to values on d0 ($P < .05$). All unvaccinated calves became viremic, while viremia was not detected in G1 or G2. Average daily gain was not different between vaccinated groups, however, only G1 calves had significantly greater ($P = .04$) ADG compared to non-vaccinated calves during the first 14 days post challenge. Vaccinated calves treated or not with ITM were protected from BVDV2 infection five months post-vaccination.

1. Introduction

Bovine respiratory disease complex (BRDC) is one of the most important health issues of young livestock in the United States (USDA APHIS. Dairy Heifer Raiser, 2011). Moreover, BRDC was the second most common disease in pre-weaned dairy heifers and also a major illness affecting weaned dairy heifers. Prevalence of BRDC has been reported to be almost 6 times higher than all other common diseases affecting post-weaned heifers (digestive problems, lameness/injury and navel infection; USDA APHIS. Dairy Heifer Raiser, 2011). The pathogens most commonly involved in BRDC include 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*. Bovine viral diarrhea virus is known to cause immunosuppression potentiating infection by other pathogens (e.g. *Pasteurella multocida* and *Mannheimia haemolytica*), which contributes to disease severity. Cattle persistently infected with BVDV are the main source of BVDV transmission in domestic cattle, and play an important role in the dissemination and pathogenesis of BVDV (Moennig and Becher, 2015). Intensive efforts have been made to reduce the incidence BVDV acute infections and to eliminate persistent infections among cattle populations.

Vaccination is a powerful tool to prevent BVDV infections, and

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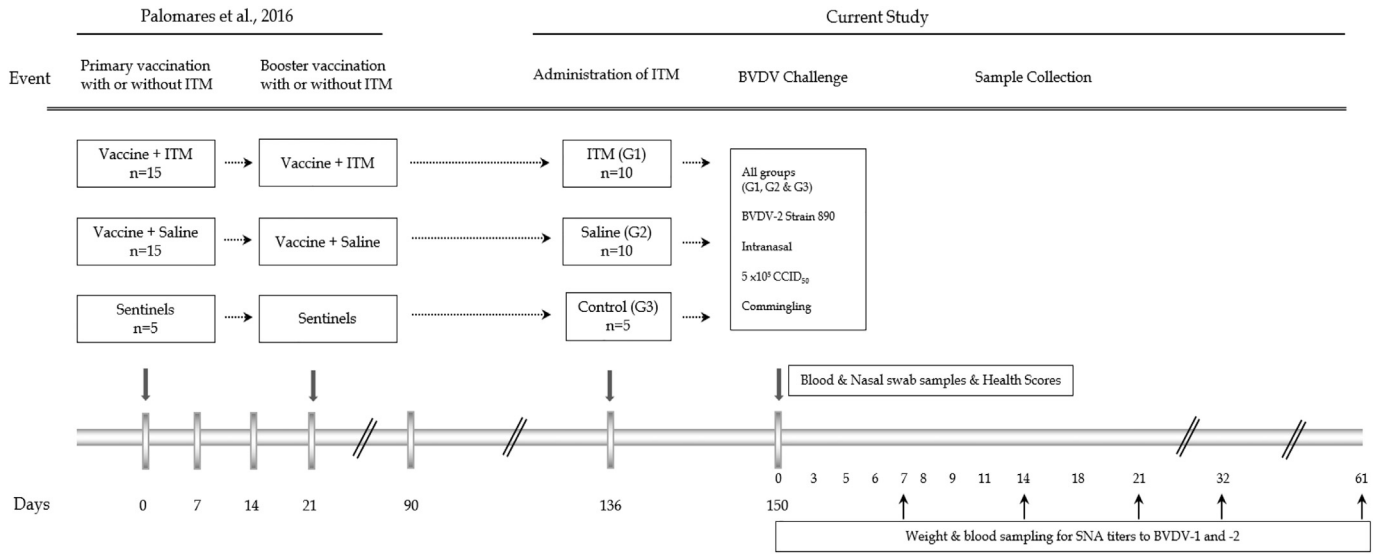

Fig. 1. Experimental design of the previous study (Palomares et al., 2016) administering injectable trace minerals (ITM) or saline concurrent with BRDC-MLV vaccination in dairy calves (at 3.5 months of age and 21 days later); and the current study administering another dose of ITM or saline 14 days before BVDV-2 challenge (approximately at 8 months of age).

modified-live virus (MLV) vaccines have the benefit of stimulating both arms of the immune system (antibody production and cell mediated immunity) enhancing all the means for the control of virus replication, viremia and shedding (Rhodes et al., 1999). Nonetheless, even with the extensive use of both inactivated and live attenuated vaccines to manage BVDV, cattle can succumb to disease (Fulton et al., 2005a; Kelling et al., 2005). Factors such as interference by transferred maternal antibodies in calves < 60 days of age (Ellis et al., 2001; Chamorro et al., 2015), the general immunological status of cattle, weather stress, antigenic mismatch between different vaccine and field viruses, vaccine handling, and level of pathogen exposure, have been documented to affect the immune response and protection after vaccination (Heininger et al., 2012). Nutritional deficiencies may also contribute to poor vaccine response in cattle (Rice et al., 1986; Thomas, 2009).

Several studies have demonstrated the impact of trace minerals on 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 essential for optimal immune function (Chirase et al., 1994; Percival, 1998; Underwood and Suttle, 1999), health status and growth in cattle (Spears and Kegley, 2002), particularly in highly stressed cattle, such as newly received feeder calves (Duff and Galyean, 2007).

Multiple factors affect trace minerals levels in cattle, including high variability in requirements and mineral intake among cattle accordingly to their production stage, inadequate trace mineral levels in soil, forages, and feedstuffs, as well as the presence of mineral antagonists in feed, water, and forages, which limit their absorption. The use of injectable trace minerals (ITM) has advantages such as delivery of a known and controlled amount of mineral and rapid and efficient absorption and storage following treatment (Pogge et al., 2012). This might be relevant for cattle having reduced dry matter intake (i.e. during transportation and receiving periods, weaning and vaccination). Thus, the use of ITM reduces the variability in trace minerals levels observed in most cattle given free choice mineral intake (Arthington and Swenson, 2004).

Previous studies have shown positive effects of ITM administration on humoral and cell mediated immune response to BRDC vaccines in dairy (Palomares et al., 2016; Bittar et al., 2018) and beef cattle (Arthington and Havenga, 2012; Roberts et al., 2016; Bittar et al., 2016). We previously demonstrated that the use of ITM concurrently with a BRDC-MLV vaccine in dairy calves resulted in earlier and more robust antibody titers to BVDV1, and stronger mononuclear cell proliferation after stimulation with BVDV1 and BRSV antigen than the control calves (Palomares et al., 2016). Additionally, ITM enhanced antibody titers to *Mannheimia haemolytica* and proliferation of mononuclear cells after *Pasteurella multocida* stimulation (Bittar et al., 2018).

Given the available data indicating that ITM can impact immune responses to vaccination, studies to determine the efficacy of the concomitant use of ITM with MLV vaccines on protection against pathogens involved in the BRDC are warranted. In the present study, we hypothesized that administration of ITM at the time of MLV vaccination and again 5 months later improves the immune response and long-term protection against experimental BVDV2 acute infection in dairy bull calves. Therefore, our objective was to assess the effects of injectable trace minerals supplementation (containing Cu, Zn, Mn and Se) used concurrently with a BRDC-MLV vaccine, and again 5 months later, on the humoral immune response and protection from a non-cytopathic (ncp) BVDV2 experimental infection five months after priming vaccination in dairy bull calves.

2. Materials and methods

2.1. Experimental design, vaccination, and treatments

The study was performed at the University of Georgia (UGA) Oconee Farm (Watkinsville-GA) from October 2014 through January 2015. The research protocol was conducted as approved by the University of Georgia, Institutional Animal Care and Use Committee (UGA-IACUC# A201402-005-Y2-A5). This study was performed using 25 weaned intact Holstein bull calves (from the commercial farm BrooksCo Dairy, Quitman GA) that were previously used in another study (Palomares et al., 2016). The experimental design of the previously reported study (Palomares et al., 2016) and the current trial are shown in Fig. 1. The animals averaged eight months of age at enrolment. During the study, calves grazed fescue grass (*Festuca arundinacea*) with free access to Bermuda grass hay (*Cynodon dactylon*), and water ad libitum. In addition, calves received daily supplementation (2.5 Kgs per calf) of a commercial ration (Cattleman's special beef; Godfreys Warehouse; Madison-GA) offered in two meals. No additional mineral supplementation was provided.

In the previous study, an initial group of thirty calves (3.5 months of

age) 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 (SC). Theses calves were randomly assigned to one of two treatment groups as follows: (1) ITM (n = 15): subcutaneous administration of injectable trace minerals (1 mL/45 Kg BW; Multimin® 90, Multimin USA Inc., Fort Collins, CO) or (2) Control (n = 15): subcutaneous injection of sterile saline (1 mL/45 Kg). Administration of ITM provided 15, 60, 10 and 5 mg/mL of Cu, Zn, Mn, and Se respectively. Three weeks after initial vaccination, calves received boosters with the same 5-way MLV vaccine, and the same attenuated-live bacterial vaccine by the SC route. Concurrent with the vaccine booster, a second subcutaneous administration of injectable trace minerals (1 mL/45 Kg; Multimin® 90) or sterile saline (1 mL/45 Kg) 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. These calves were separated from the vaccinated calves for 14 days following priming and booster vaccination in order to prevent infection with shed vaccine virus. The overall schematic of the study activities is shown in Fig. 1.

Five months after priming vaccination, ten calves belonging to either ITM and control groups were randomly selected from each group and received ITM (1 mL/45 Kg; Multimin® 90; G1, n = 10) or saline (1 mL/45 Kg; G2, n = 10) respectively, according to their previous group assignment. Additionally, the five additional herd-mate calves of the same age that did not receive vaccine or treatment and served as sentinel animals during the previous study, were used as unvaccinated BVDV infected group (G3, n = 5).

The injectable trace minerals (Multimin® 90) were administered on day −14 to the G1 calves following the manufacturer's recommendations (1 mL/45 Kg of body weight, SC). Animals in G2 received an equivalent volume (1 mL/45 Kg of body weight, SC) of a saline solution (Vetone Sterile Saline®; Nova-Tech Inc., Grand Island, NE) on day −14. All injections followed guidelines of the Beef Quality Assurance Program (Beef Quality Assurance 2010®; Centennial, CO).

2.2. Bovine viral diarrhea virus challenge

All 25 calves (10, 10 and 5 from groups G1, G2 and G3, respectively) were experimentally inoculated with an ncp type 2 BVDV isolate (strain 890). The BVDV 890 strain was originally obtained from the APHIS Center for Veterinary Biologics in 1989 (Ames, IA). It has been in frozen storage at −80 °C and is subjected to propagation in MDBK cells at least every other year to maintain viable stock. It has been produced in quantity to be available as a challenge BVDV virus. The stock BVDV2 strain 890 used in the present study was biologically cloned via successive passages by use of limiting dilutions with subsequent minimal propagation to produce an adequate amount of stock virus for characterization and animal challenge exposure studies during the six-month period prior to the challenge study. This strain has been previously characterized and causes a significant decrease in leukocyte counts (lymphocytes and neutrophils) with a mild to moderate development of classical clinical disease (Walz et al., 2001a,b).

The inoculum consisted of an infected cell culture supernatant containing 1 × 10^5 50% cell culture infectious dose ($CCID_{50}$) per mL of noncytopathic BVDV-2 strain 890. The BVDV isolate was propagated in monolayers of Madin-Darby bovine kidney (MDBK) cells using Dulbecco's Modified eagle's medium (DMEM®; Cellgro, Manassas, VA) supplemented with 10% equine serum (HyClone™ Donor Equine Serum U.S.; Fischer Scientific; Pittsburgh, PA), 1% L-alanyl-L-glutamine (Corning® glutagro 100× Liquid™, 200 mM; Corning Cellgro; Manassas, VA), 0.2% anti-fungal (Amphotericin B Liquid 250 μg/mL®; Corning; Manassas, VA) and 0.4% antibiotic (Penicillin 10,000 I.U.-Streptomycin 10,000 μg/mL Solution 100×®; Corning; Manassas, VA). After incubation for 72 h at 37 °C and 5% CO2, the BVDV-2 infected cell culture was frozen at -80 °C and thawed to disrupt the cells and release the viral particles. Once thawed, the infected cell culture was aliquoted in 50 mLs conical tubes and a 1.5 mL sample was collected for virus titration, and both were further frozen at −80 °C. The 1.5 mL sample was thawed and titrated using immuno-peroxidase staining of MDBK cell monolayers in 96-wells plates. The Reed–Muench method was used to determine the final $CCID_{50}$/mL for inoculation. The inocula remained frozen at -80 °C until the day of inoculation. One hour prior to inoculation, the frozen inocula were thawed. Then, 5 mLs of the BVDV-2 inoculum were aliquoted into individual 12 mL sterile syringes and held in ice until use. The inoculation was performed by intranasal aerosolization of 5 mL of inoculum (2.5 ml in each nostril) with 10 cm long tip-fenestrated cannula coupled to a 12 mL syringe for each calf. After inoculation all calves were comingled in an 8-acre pasture with adequate shade during the whole experimental period. One sample of the inoculum was transported on ice to the lab to determine the $CCID_{50}$/mL after all inoculations were completed.

2.3. Clinical evaluation

Health status including rectal temperature, hydration, attitude, nasal discharge and fecal consistency of the calves was evaluated using the scoring system developed at the University of Wisconsin (http://www.vetmed.wisc.edu/dms/fapm/fapmtools/8calf/calf_health_scoring_chart.pdf). Clinical examination was performed on each calf on study days −14, 0, 3, 5, 6, 7, 8, 9, 11, 14 and 18 relative to the day of BVDV challenge. Clinical signs were assessed by evaluation of six variables evaluated individually (hydration status, nasal secretion, ears and attitude, feces, eyes, and cough) on a scale from 0 to 3, with 0 representing a lack of clinical signs and 3 representing severe clinical signs. The daily sum health scores were the total sum of each individual clinical sign variable, and the daily sum health score was generated by adding all individual scores of the six variables evaluated, to the extent that the minimum sum daily health score being equal to 0 (zero) and the maximum equal to 18 (i.e. if the score of all six variables that compose the daily sum health score were the maximum value, in this case a score of 3, then, 3 times 6 is equal 18, that is the maximum score obtained). Clinical signs and health scores were assessed by three experienced veterinarians, who were not aware of calves' treatment allocation. Additionally, body weight was measured on days 0, 8, 14, 21, and 32 relative to challenge (day 0).

2.4. Sample collection and processing

From the calves in each group, blood samples were collected via jugular venipuncture using an 18 gauge × 2.5-cm single sample needle (Vacuette®; Nipro Medical Industries Ltd., Gunma, Japan) into vacuum tubes (Vacutainer®, BD Diagnosis, Franklin Lakes, NJ) with and without anticoagulant to obtain whole blood and serum, respectively. Blood samples were collected on study days −14, 0, 3, 5, 6, 7, 8, 9, 11, 14 and 18 into two individual 8.5 mL glass tubes for buffy coat collection (BD Vacutainer ACD Solution A REF364606®; BD Diagnosis, Franklin Lakes, NJ), into one 2 mL polypropylene tube for leukocyte and platelet count (BD Vacutainer K2 EDTA 3.6 mg REF367841®; BD Diagnosis, Franklin Lakes, NJ) and into two individual 10 mL glass tubes (BD Vacutainer Serum®; BD Diagnosis, Franklin Lakes, NJ) for serum mineral concentration. Additionally, blood samples without anticoagulant were collected on days −14, 0, 7, 14, 21, 33 and 63 for serum neutralizing antibodies titers (SNA) after challenge.

The blood samples containing ACD Solution A were processed for buffy coat isolation as previously described (Harpin et al., 1999) and stored at -80 °C for further VI. Clotted blood samples were spun (2500 rpm × 15 min) for serum separation. Later, serum samples were stored at −80 °C for further analysis.

Nasal swab samples for each calf were collected for virus isolation

(VI) on study days −14, 0, 3, 5, 6, 7, 8, 9, 14, and 18. An individual cotton swab (Sterile cotton tipped applicators®; Puritan Medical Products Company LLC; Guilford, ME) was inserted in each nostril scraping the nasal mucosa, and stored in a tube containing 3 mL of PBS containing antibiotics (Penicillin 10,000 I.U.-Streptomycin 10,000 µg/mL Solution 100×®; Corning; Manassas, VA) and anti-fungal (Amphotericin B Liquid 250 µg/mL®; Corning; Manassas, VA). Nasal samples were transported to the laboratory in a cooler containing ice for further processing. Tubes were mixed using a vortex. Swabs were removed and the remaining solution was filtered (Millex®GP 0.22 µm; Millipore Ireland Ltd.; Cork, Ireland) and stored at −80 °C for later VI.

2.5. Serum neutralizing antibody titers

Serum neutralizing antibody titers against BVDV1 and 2 were determined via a standard virus neutralization test at the University of Georgia Athens Veterinary Diagnostic Laboratory (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 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 DMEM 100 $CCID_{50}$ of the appropriate cytopathic BVDV1 and 2 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 (approximately 10^4 cells) of a MDBK 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. Virus isolation

For virus isolation (VI), 250 µL of buffy coat cell suspension and nasal swab samples from each animal was added to individual 25-cm^2 tissue culture flasks containing a monolayer of MDBK cells. For cell culture, DMEM medium supplemented with 10% equine serum, L-alanyl-L-glutamine (Corning® glutagro 100× Liquid™, 200 mM; Corning Cellgro; Manassas, VA) was used. After 3 days of incubation in 5% CO_2 at 37 °C, flasks were frozen at -80 °C and thawed; 50 µL of the cell suspension from each flask was then transferred into 3 wells of a 96-well plate seeded with MDBK cells (first plate). The inoculated 96-well plate was incubated for 3 days, and the culture medium from each well was transferred to the corresponding wells of a new 96-well plate seeded with MDBK cells (second plate). Both 96-well plates (first and second) were tested for BVDV antigen by use of an immunoperoxidase staining technique as previously described (Palomares et al., 2012).

2.7. Leukocytes and platelets count

Blood samples containing EDTA were transported in a cooler containing ice and analyzed for leukocytes and platelets count in the Pathology Department of the University of Georgia (UGA), College of Veterinary Medicine (CVM) in Athens-GA. The total leukocyte, platelet and differential leukocyte counts for each sample were determined by use of an automatic cell counter (HESKA® CBC-Diff, Vet Hematology System, Des Moines, IA).

2.8. Statistical analysis

Data were analyzed using the Statistical Analysis System (SAS® version 9.3; SAS Institute, Cary, NC, USA). Statistical assumptions of normality and constant variance were tested through Shapiro Wilk's and Levene's tests, respectively. A logarithmic base 2 transformation was applied to the antibody titers. For calculation and comparison of geometric mean (GM) antibody titers, back-transformed antibody titers were calculated for each group at days −14, 0, 7, 14, 21, 32 33 and 61 after BVDV infection. The changes in mean leukocyte and platelet counts and rectal temperature over time were determined comparing the values on day 0 with the values on days 3, 5, 6, 7, 8, 9, 11, 14 and 18 using repeated-measures analysis Proc-GLIMMIX model using calf as random effect, and groups and time points as fixed effects, with the Tukey test used to adjust for multiple comparisons. Means daily sum health score, rectal temperature, body weight, average daily gain, leukocyte counts, and SNA titers were compared among the treatment groups by using repeated-measures analysis Proc-GLIMMIX model using calf as random effect, and group and time as fixed effects, with the Tukey test used to adjust for multiple comparisons. For variables in which the groups did not have similar means at day 0, we used the first day measurement of each variable as covariate in the statistical model, to minimize the differences between groups at the beginning of the study, therefore allowing for a more accurate comparison among groups. For all analyses, values of $P \leq .05$ were considered significant, and $0.05 < P \leq .10$ was considered a tendency. All results are shown as least squared means (LSM) obtained from the statistical software SAS after analysis.

3. Results

Before BVDV challenge (d0), all groups had comparable mean rectal temperature, sum of health scores, mean body weight, total and differential leukocytes count, and platelet counts ($P > .05$). Mean rectal temperature significantly increased in the G3 group after BVDV challenge compared to day 0, and peaked on day 7 with values > 40 °C ($P = .001$; Fig. 2). Calves in G3 had higher ($P < .05$) rectal temperature than G1 and G2 between days 3 and 11 after BVDV challenge (Fig. 2). Rectal temperature did not increase in the vaccinated calves (G1 and G2) during the experimental period, with no significant differences between groups ($P = .71$). Sum of health scores was higher (P = .001) in G3 compared to vaccinated calves on days 7, 8, 9, 11, 12, 15 and 18 post BVDV challenge (Fig. 3). Calves treated with ITM had lower sum of health scores on day 5 ($P = .005$. Fig. 3), and lower nasal ($P = .01$) and fecal scores (P = .005) on days 5 and 8, respectively, compared to saline injected calves (data not shown). There was no significant difference regarding mean body weight among groups at any day of the study ($P > .05$; Fig. 4). Average daily gain (ADG) during the first seven days post challenge tended to be greater ($P = .08$) in G1 than G3 (Fig. 4B). Moreover, ADG within the first 14 days after BVDV challenge was significantly greater ($P = .04$) in G1 compared to G3 (Fig. 4B). During this period, G2 had intermediate ADG values, which were not significantly different from either G1 or G3 (Fig. 4B).

Mean total leukocytes counts significantly decreased (by approximately 40%) in the G3 calves between days 3 and 6 post challenge relative to day 0 ($P < .01$). These values were significantly lower than those in G1 and G2 groups (Fig. 5A). Vaccinated calves (G1 and G2) had similar mean leukocyte count along the study. Moreover, lymphocyte count decreased significantly in G3 calves (almost two fold) between days 3 and 7 post challenge compared to day 0 (Fig. 5B). Calves in G1 and G2 had a mild decrease in lymphocyte count on days 3 and 5 after BVDV challenge, with no significant difference between groups ($P > .05$; Fig. 5.B). Unvaccinated calves had a significant increase in mean granulocyte count on days 7 and 8 post challenge ($P < .05$; Fig. 5C) which was different from values observed in G1 and G2 on the same time points. A remarkable reduction in platelet counts (19–35%) was observed in G3 (between days 3 and 9) relative to the baseline levels (Figs. 5D). Further, there was a moderate reduction in platelet counts during the evaluation period in G1 (16–19%, on days 7, 8 and 14) and G2 (17–28% on days 6, 11, 14 and 18). Calves in G2 had significantly lower platelet counts on days 6 ($P = .05$) and 14 ($P = .04$) than calves in G1. Moreover, platelets counts also tended to be lower on days 11 ($P = .10$) and 18 ($P = .07$) in G2 calves compared to G1.

Serum neutralizing-antibodies against BVDV1 were high

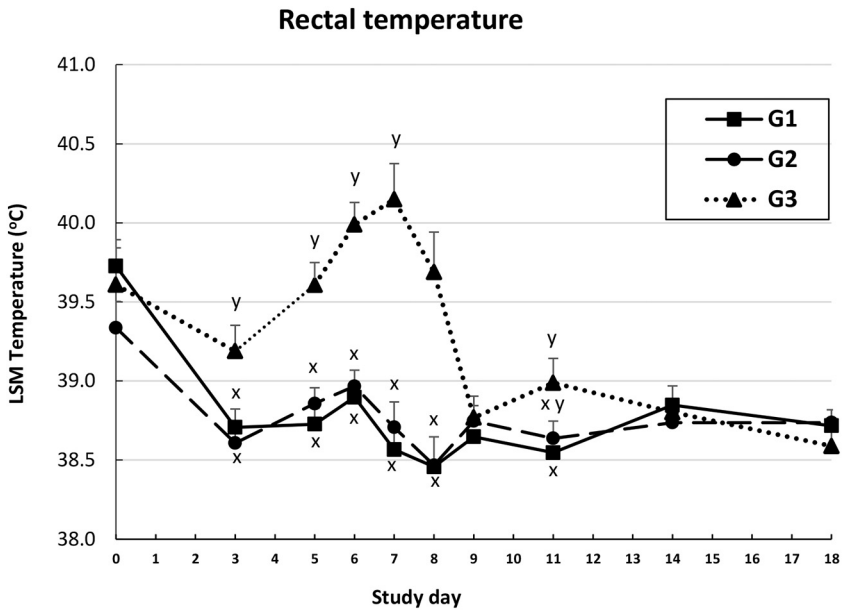

Fig. 2. Least square means (LSM) and standard error of the mean (SEM) of rectal temperature in dairy calves treated with ITM concurrent with BRDC MLV-vaccines and challenged with ncp BVDV-2890 strain 5 months later. [x,y] Significant difference between groups for each study day ($P < .05$).

(approximately 1:1028) on days −14 and 0 in G1 and G2 groups and did not increase dramatically after BVDV2 challenge (Fig. 6A). In contrast, SNA titers against BVDV2 were moderate (approximately 1:64) on days −14 and 0 and increased significantly (almost 5-fold) by 2 weeks post BVDV2 challenge (Fig. 6B). There were no significant differences in SNA titers to BVDV1 and 2 between G1 and G2 throughout the experimental period ($P > .05$). Calves in G3 were seronegative (SNA < 1:4) for BVDV1 and 2 on days −14 and 0. In this group, antibody titers against BVDV1 and 2 progressively increased reaching sero-conversion (> 4-fold increase in SNA titers) on days 32 and 61 post challenge (Figs. 6A and B).

Bovine viral diarrhea virus was not isolated from buffy coat or nasal swab samples of any of the vaccinated calves (G1 and G2) during the experimental period. Unvaccinated calves (G3) had BVDV positive buffy coat samples between days 5 to 9 after BVDV challenge. All animals in G3 were BVDV positive on buffy coat sample at least one day during the experimental period. Moreover, BVDV was isolated from leukocytes in all five calves of G3 on day 6. Interestingly, BVDV was not detected in nasal swab samples in any of the control calves during the sampling period.

4. Discussion

The present study tested the hypothesis that administration of ITM concurrently with MLV vaccination and again 5 months later enhances the immune response and long-term protection against experimental challenge with BVDV2 in weaned dairy calves. Vaccinated calves, whether treated or not with ITM were protected from acute BVDV

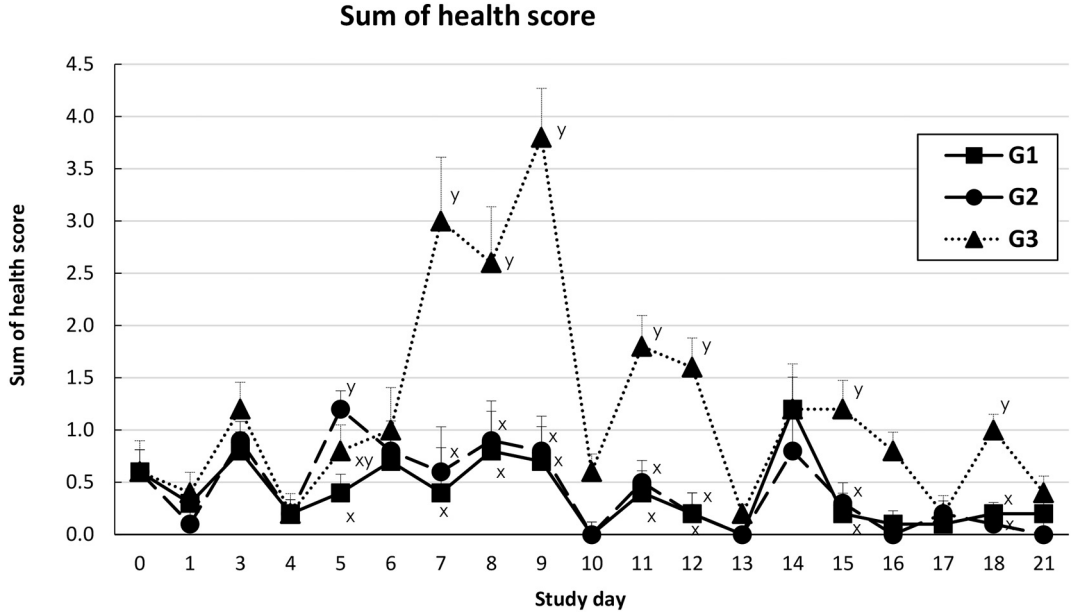

Fig. 3. Least square means (LSM) and standard error of the mean (SEM) of sum of health scores in dairy calves previously treated with ITM or saline concurrent with BRDC-MLV vaccines (Palomares et al., 2016) and challenged with ncp BVDV-2890 strain 5 months later. [x,y] Significant difference between groups for each study day ($P < .05$).

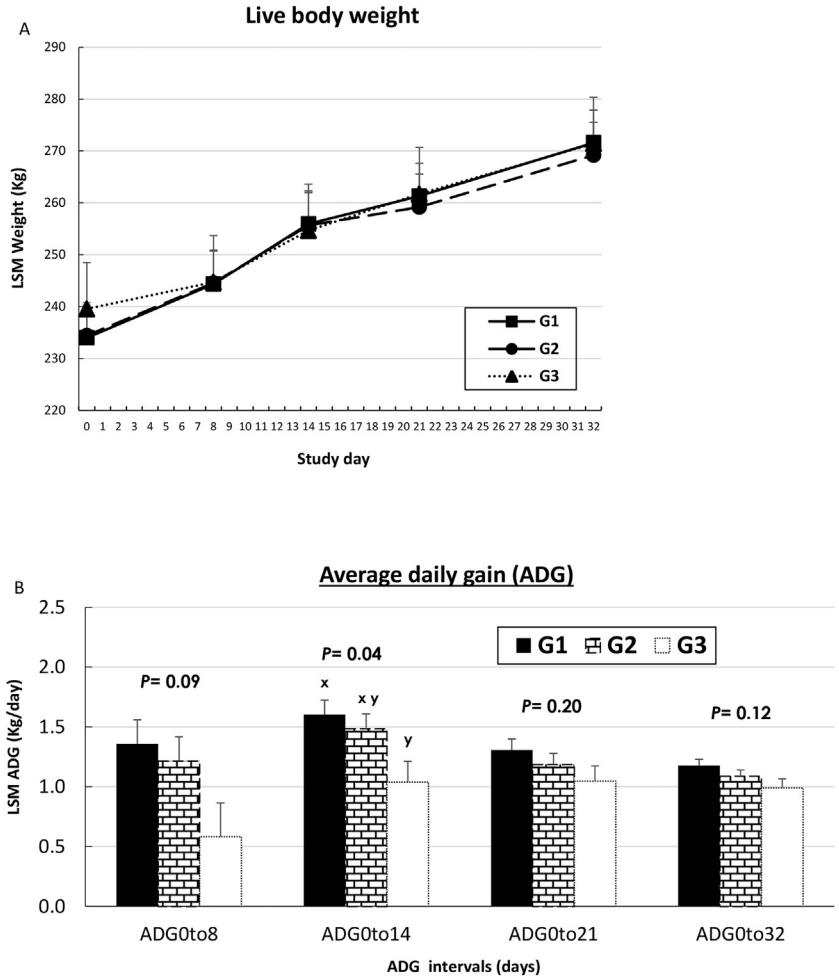

Fig. 4. A–B. Least square means (LSM) and standard error of the mean (SEM) of live body weight (A) and average daily gain (ADG, B) in dairy calves previously treated with ITM concurrent with BRDC MLV-vaccines (Palomares et al., 2016) and challenged with ncp BVDV-2890 strain 5 months later. Average daily gain was calculated for different study days (8, 14, 21, and 32) relative to baseline weight values on day 0. ^{x,y} Significant differences between groups.

clinical disease, demonstrated by the absence of fever and lower clinical scores when compared to the naïve unvaccinated calves. The immunization protocol based on priming and booster vaccination administered 21 days apart demonstrated efficacy in long-term protection (5 months after vaccination) preventing acute disease caused by BVDV challenge, reinforcing the importance of the MLV vaccines as a preventative strategy for BRDC in calves, as previously reported (Rhodes et al., 1999; Xue et al., 2010; Walz et al., 2010; Chamorro et al., 2015). Calves in the unvaccinated control group developed mild to moderate BRD. This was characterized by showing higher daily sum health score and rectal temperature compared to vaccinated calves. Clinically remarkable differences regarding the health status after BVDV challenge were not observed between vaccinated groups. However, calves treated with ITM had lower sum of health scores on day 5, and lower nasal and fecal scores on days 5 and 8 respectively (data not shown), compared to saline-injected calves. Average daily gain was not different in calves treated with ITM from saline-treated calves. Nevertheless, only calves treated with ITM had a significantly higher ADG compared to the non-vaccinated calves within the first 2 weeks after challenge. Previous studies under field conditions without experimental challenge have shown beneficial effects of ITM on growth performance and health in highly stressed, newly received beef heifers (Richeson and Kegley, 2011). In that study, the overall ADG and total dry matter intake were greater for beef heifers receiving ITM formulations compared with control animals. Additionally, animals administered ITM had reduced BRD morbidity rates and antibiotic treatment costs compared with control heifers (Richeson and Kegley, 2011).

Vaccination, whether concurrent with ITM or not protected the calves from significant BVDV-induced leukopenia and lymphopenia, a diagnostic result clearly observed in the control group. Leukopenia and lymphopenia are the main hallmarks of systemic disease occurring during BVDV infection (Ellis et al., 1998) and are believed to result in significant immunosuppression that predisposes cattle to suffer secondary infections by commensal flora and the development of bovine respiratory disease (Walz et al., 2001a; Chase et al., 2004; Palomares et al., 2012). Unvaccinated calves had a remarkable decrease in lymphocyte counts (> 45% reduction for 7 days), while a very slight and transient reduction in lymphocyte numbers was observed after BVDV challenge in the vaccinated calves (21 and 17% reduction for G1 and G2, respectively), which agrees with previous studies (Ellis et al., 1998; Falcone et al., 2003; Chamorro et al., 2015). Interestingly, calves treated with ITM had greater platelet counts than vaccinated saline-treated calves between days 6 and 18 post BVDV challenge. A recent study to determine the effect of ITM on the onset of protection elicited by a MLV vaccine in beef calves inoculated with BVDV2 five days after vaccination, showed that the use of ITM concurrent with MLV vaccine prevented a remarkable thrombocytopenia, typical of acute infection with BVDV2 strain 890 (Bittar et al., 2016).

In the current study, MLV vaccination (two doses of vaccine 21 days apart), whether in conjunction with ITM or not, prevented the viremia observed in the control calves during the week after BVDV inoculation. Viremia and viral shedding occur during the acute phase of BVDV

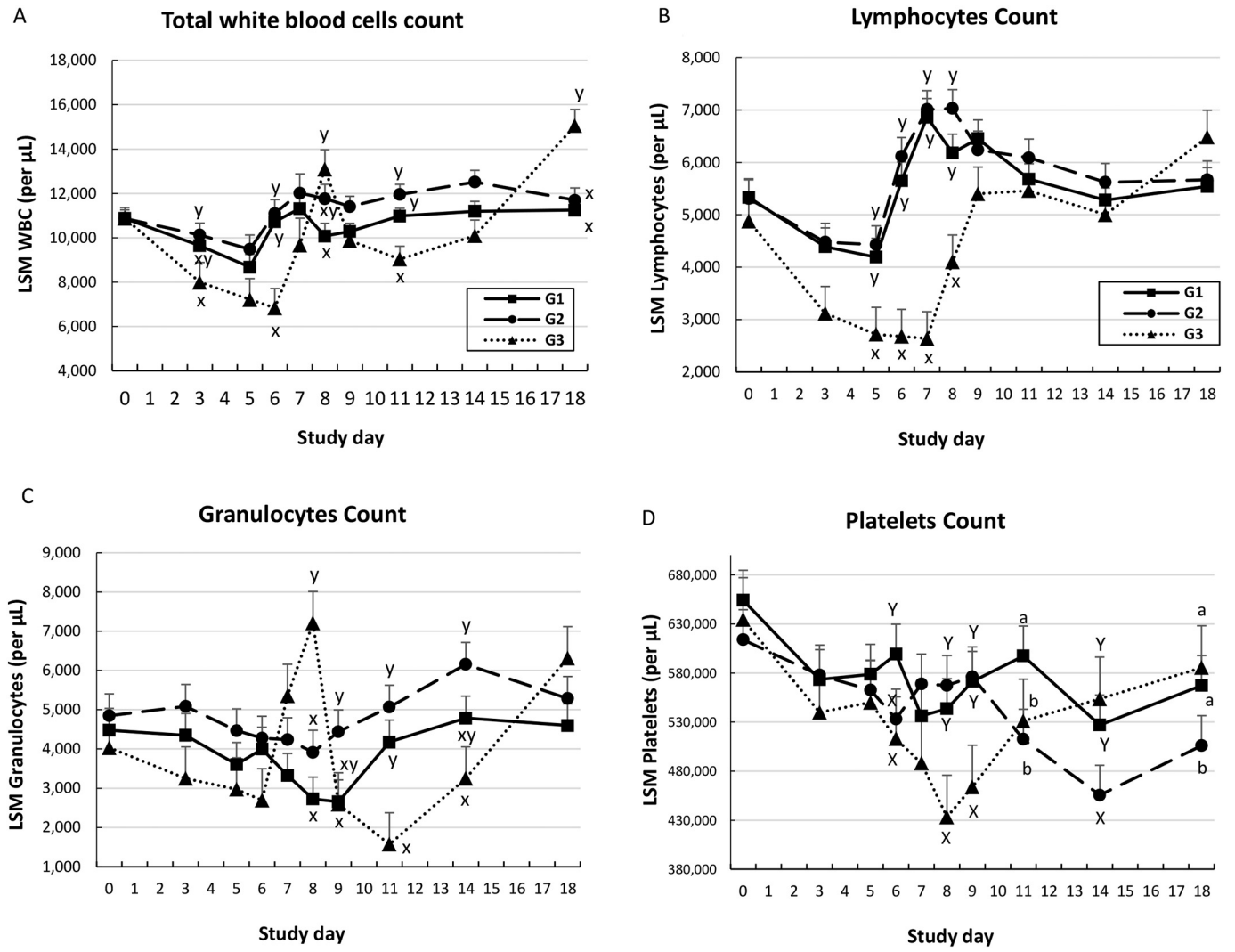

Fig. 5. A–D. Least square means (LSM) and standard error of the mean (SEM) of total white blood cells (A), lymphocyte (B), granulocyte (C), and platelet (D) counts in dairy calves previously treated with ITM concurrent with BRD-MLV vaccines (Palomares et al., 2016) and challenged with ncp BVDV-2890 strain 5 months later. [x,y] Significant difference between groups by each study day ($P < .05$). [a,b] Values tended to be different between groups by each study day ($0.05 \leq P \leq .10$).

infection, being the time when infected cattle are at higher risk of transmitting virus to herd mates (Saliki and Dubovi, 2004). Multiple vaccination challenge studies have demonstrated that most of the MLV vaccines against bovine respiratory viruses commercially available in the United States provide adequate protection from acute BVDV disease (Falcone et al., 2003; Brock, 2004; Kelling et al., 2005, 2007; Palomares et al., 2012; Chamorro et al., 2015). Further, the protective response elicited by MLV vaccination in the present study, preventing clinical signs of disease, leukopenia and viremia upon BVDV challenge is consistent with previous reports of the efficacy of MLV-BRD vaccines on long-term protection (Goyal and Ridpath, 2005; Dean and Leyh, 1999). In the present study, the use of ITM administered at the time of vaccination and 5 months later did not enhance the level of protection from clinical disease elicited by MLV vaccination. Average daily gain was not significantly different between vaccinated groups; however, only calves treated with ITM had significantly greater ADG than non-vaccinated BVDV acutely infected calves, which could suggest an additive effect of ITM use concurrently with MLV vaccination. In a previous trial, Roberts et al. (2016) showed no effect of ITM in improving performance or morbidity when the BRD incidence was low. However, the BVDV-specific antibody response to a respiratory vaccine was greater for ITM on day 14 than for the control group, as demonstrated in our earlier study (Palomares et al., 2016). It is possible that the moderate virulence BVDV strain used for challenge, the low level of stress, and the low level of infectious pressure during this study may have favored the protection from clinical disease elicited by MLV vaccination in G1 and G2, thus masking further benefits of ITM administration on animal health and performance, that have been previously described in high-risk feedlot animals (Richeson and Kegley, 2011). In addition, in our previous study, the calves treated with ITM at the time of vaccination against BRDC pathogens had earlier and greater antibody response and mononuclear cell proliferation to BVDV than vaccinated only calves (Palomares et al., 2016). This improved antibody response has been previously correlated to enhanced protection (Richeson and Kegley, 2011). Additional studies to determine the effects of ITM on clinical protection elicited by MLV vaccines against BVDV infection in high risk stressed calves are warranted.

Shedding of BVDV in nasal secretions was not consistently detected after challenge, in contrast with previous studies (Ellis et al., 1998; Falcone et al., 2003; Fulton et al., 2005a,b; Palomares et al., 2012). It can be speculated that several factors including sampling technique, viral load, replication and shedding capacity of the BVDV strain and the laboratory techniques used for viral isolation may have affected our ability to detect BVDV in nasal secretions.

The increase in serum neutralizing (SNA) antibodies specific for BVDV1 and 2 in both vaccinated groups indicated an anamnestic

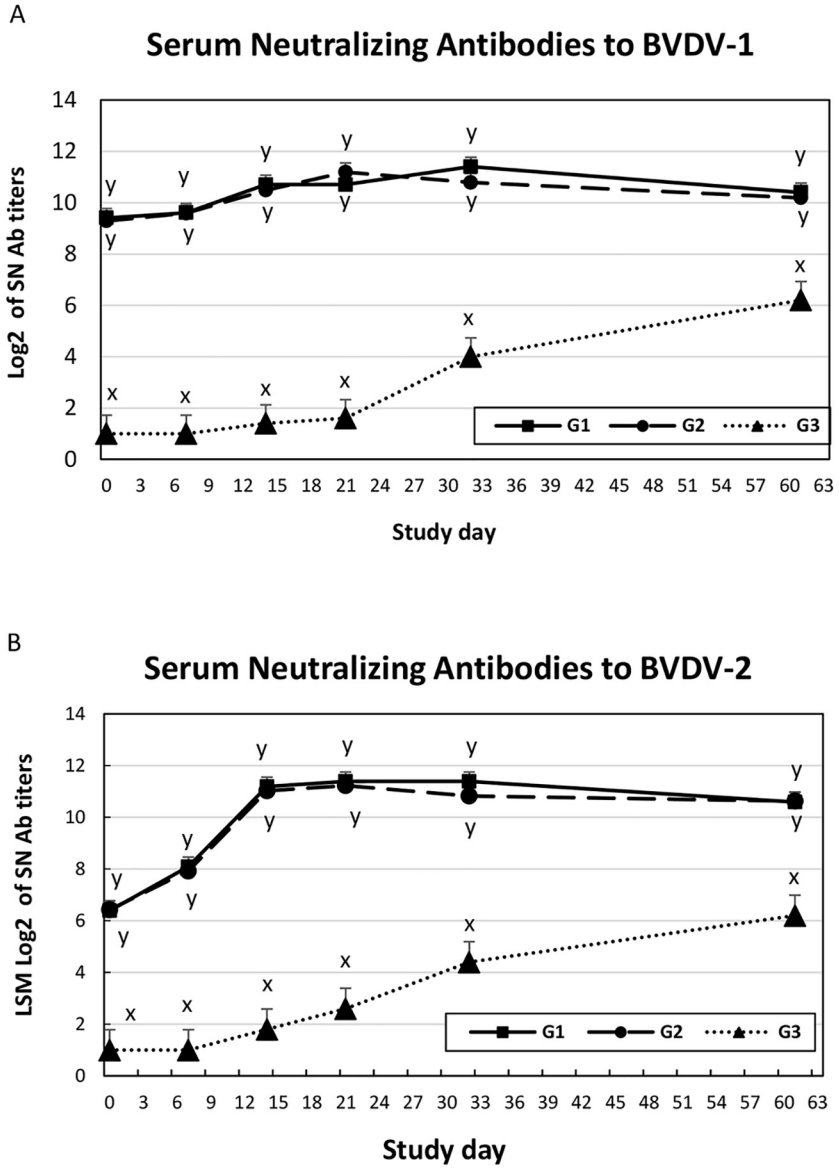

Fig. 6. A–B. Least square means (LSM) and standard error of the mean (SEM) of serum neutralizing antibody titers to BVDV-1 (A) and to BVDV-2 (B) in dairy calves previously treated with ITM concurrent with BRDC-MLV vaccines (Palomares et al., 2016) and challenged with ncp BVDV-2890 strain 5 months later. ^{x,y} Significant difference between groups for each study day ($P < .001$).

immune response after vaccination and subsequent experimental exposure. In the vaccinated calves, SNA titers against BVDV were high at the time of challenge, with higher SNA titers for BVDV1 than BVDV2. This is believed to have been induced by the homology between the BVDV1a vaccine strain used in this study and the BVDV isolate used in the SNA assay at the diagnostic laboratory (Goyal and Ridpath, 2005). The unvaccinated calves were seronegative at the time challenge, but the BVDV SNA titers increased significantly within 30 days after inoculation, with higher SNA titers to BVDV2 than BVDV1, which might be due to the exposure only to the homologous type 2 BVDV strain 890 during challenge. Further, the increase in SNA titers to BVDV1 in the unvaccinated calves reflected sufficient neutralizing cross-reactivity between BVDV genotypes (Dean and Leyh, 1999; Fairbanks et al., 2003). The absence or low SNA titers against BVDV in the unvaccinated control group within the first 14 days after challenge resulted in a greater susceptibility to BVDV infection and clinical disease, as previously reported in several studies (Falcone et al., 2003; Kelling et al., 2005; Chamorro et al., 2015).

Several favorable features are associated with an adequate trace minerals level in calves, and studies have shown benefits of ITM when combined with vaccination (Arthington and Havenga, 2012; Palomares et al., 2016; Bittar et al., 2016, 2018). Injectable trace minerals have been shown to enhance humoral immune response when injected concomitantly with multivalent vaccines in naïve dairy and beef calves (Arthington and Havenga, 2012; Richeson and Kegley, 2011; Palomares et al., 2016; Bittar et al., 2016, 2018). The group of animals used in this study are a subset of calves utilized in a previous study (Palomares et al., 2016) in which TM concentrations in liver and serum were demonstrated to be within normal ranges throughout the trial. Furthermore, administration of ITM was proven to result in a significant increase in hepatic Se, Cu and Mn levels as well as serum Se and Zn.

To the authors' knowledge, this is the first study evaluating the effects of the use of ITM concurrently with BRDC vaccines on protection against BVDV challenge in weaned calves. While we previously showed that ITM improved some aspects of humoral and cell mediated immunity (Palomares et al., 2016; Bittar et al., 2016, 2018), in this study there were few effects attributable to ITM after BVDV2 challenge (lower sum of health scores on day 5, higher platelet counts on days 6, 11, 14

and 18 compared to saline treated calves, as well as higher ADG compared to non-vaccinated calves). The low correlation between these studies may have been due to the low infectious pressure, mild virulence of the BVDV2 strain used and low stress conditions to which the calves were submitted. In conclusion, the use of ITM administered at the time of vaccination against respiratory viruses and five months later did not enhance the level of protection from BVDV2 clinical disease elicited by MLV vaccination in dairy bull calves. Further studies are necessary to effectively assess the effects of ITM on long-term clinical protection elicited by MLV vaccines against BVDV infection in high risk growing calves.

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