Neutrophil function and antibody production during the transition period: Effect of form of supplementary trace minerals and associations with postpartum clinical disease and blood metabolites

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ABSTRACT

Our objectives were to evaluate the effect of the form of supplementary trace minerals—inorganic salts (STM: Co, Cu, Mn, and Zn sulfates and Na selenite) or organic (OTM: Co, Cu, Mn, Zn proteinates, and selenized yeast)—fed at 100% of recommended levels in both pre- and postpartum diets on in vitro phagocytic activity of neutrophils, and in vivo IgG responses to an ovalbumin challenge during the transition period. In addition, we investigated the associations of these immunological responses with incidence of postpartum clinical diseases and the dynamic changes of metabolic markers during the transition period. Pregnant heifers and cows (n = 273) were enrolled at 45 ± 3 d before expected calving, blocked by parity and body condition score, and allocated randomly to STM or OTM supplementation. Cows in both treatments were fed the same diet, except for the form of supplementary trace minerals. Automatic feeding gates were used to assign treatments to individual cows. Blood was collected on d −7 ± 3 and 7 ± 3 relative to calving in a subgroup of cows (n = 131 and 133, respectively) to measure phagocytic activity of neutrophils in vitro using flow cytometry. Subcutaneous immunization with 0.5 mg of chicken egg ovalbumin was performed in a subgroup of cows (n = 181) on d −45, −21, and 3 relative to calving. Concentration of anti-ovalbumin IgG in serum was measured by ELISA on d −45, −21, 3, 7, and 21. Trace mineral concentrations in blood were measured by inductively coupled plasma mass spectrometry on d −45, −21, −7, 0, 3, 7, 10, 14, and 21 relative to calving. Treatment did not affect the percentage of neutrophils performing phagocytosis on d −7 or 7 but the median fluorescence intensity of phagocytosis on d 7 was greater for OTM than STM. We found no differences between treatments in the level of anti-ovalbumin IgG in serum on any of the sampling days. Changes in neutrophil function from prepartum to postpartum were associated with incidence of postpartum clinical disease, postpartum feed intake and milk production, concentrations of Ca, K, Se, Mn, Co, and total protein in serum. Immunoglobulin G responses to ovalbumin injections were not associated with incidence of postpartum clinical disease but were associated with body weight, feed intake, energy balance, and concentrations of nonesterified fatty acids, aspartate aminotransferase, gamma-glutamyl transpeptidase, albumin, Na, P, and Cu in serum. In conclusion, replacement of STM by OTM improved one measure of phagocytic capacity of neutrophils in vitro, which was also greater in cows that did not develop postpartum clinical disease. The associations of innate and acquired immune responses with feed intake, energy balance, and circulating concentrations of key macro and micronutrients reinforce the importance of nutritional management for the health of dairy cows during the transition period.

Key words: organic trace minerals, neutrophil phagocytosis, humoral responses, postpartum disease

INTRODUCTION

The transition from late gestation to lactation requires major adjustments to the metabolism of a dairy cow to support the final stages of fetal growth and milk synthesis (Bauman and Currie, 1980; Bell, 1995). Nutrient requirements are only partially met by the reduced feed consumption around calving (Drackley, 1999). Mobilization of body reserves reduces the periparturient imbalances of energy, protein, and minerals, but often is not sufficient to avoid metabolic problems, oxidative
stress, and immune dysregulation (Sordillo and Aitken, 2009). In fact, a large portion of postpartum cows are affected by metabolic problems and clinical diseases, which cause short- and long-term losses in production and survival (Chapinal et al., 2011; Carvalho et al., 2019; Pinedo et al., 2020). Thus, developing strategies that consistently improve the nutritional status of dairy cows, especially during the transition period, is important to improve immune capacity, health, welfare, and performance.

Trace minerals (TM) represent a small but important component of a dairy cow’s diet. Despite their importance, knowledge regarding TM absorption and utilization in dairy cattle is still limited. Inorganic salts of TM (STM; e.g., sulfates, carbonates, or oxides) are often supplemented in diets of dairy cattle, but their absorption is compromised by antagonistic interactions within the gastrointestinal tract that reduce their bioavailability (Spears, 2003; Suttle, 2010; Goff, 2018). Organic sources of TM (OTM) are an alternative form of supplementation, in which trace elements are complexed with organic molecules (e.g., AA, peptides, or saccharides) to minimize antagonistic interactions in the gastrointestinal tract and increase bioavailability (Brown and Zeringue, 1994; Goff, 2018). Multiple studies have evaluated the effect of supplementing OTM to dairy cows with variable results. The benefits of using OTM sources or the best method and timing of including this type of supplement in dairy cattle diets are unclear, largely due to considerable heterogeneity in designs of studies (Rabiee et al., 2010; Suttle, 2010).

Two of the well-known biological functions of TM are immune cell function and oxidative balance, which have great potential to benefit health of transition cows (Spears and Weiss, 2008). Osorio et al. (2016) reported the benefits of partially replacing STM (Co, Cu, Mn, and Zn) with OTM, supplemented by oral boluses, during the periparturient period on DMI, milk production, and phagocytic activity of neutrophils at 30 DIM. Nemec et al. (2012) reported benefits of complete replacement of STM (Cu, Mn, and Zn) by OTM in mid-lactation cows for humoral responses to a rabies vaccine. Nevertheless, the effect of complete replacement in transition cow diets of all 5 traditionally supplemented TM (Co, Cu, Mn, Se, and Zn) on measures of innate and acquired immunity has not been evaluated.

Multiple assays and tests to evaluate bovine immune cell function in vitro or in vivo have enhanced understanding of immunobiology in cattle, especially in transition dairy cows (reviewed by LeBlanc, 2020; Vlasova and Saif, 2021). Nonetheless, most studies have focused on addressing specific aspects of immunobiology and not on the relationship between immune function and susceptibility to disease. In fact, studies often exclude cows that develop disease. Some studies, however, have evaluated immune function in a large number of cows and associated the outcomes of immune function assays or tests with susceptibility to common postpartum health disorders (Cai et al., 1994; Kimura et al., 2002; Hammon et al., 2006; Galvão et al., 2010; Martinez et al., 2012; Thompson-Crispi et al., 2012; Chebel, 2021).

Of the large studies available, most are focused on in vitro measures of neutrophil biology, either prepartum or postpartum, and other immune functions, such as acquired immunity, are less studied. Moreover, less emphasis has been given to how immune responses change from prepartum to postpartum, and how they are associated with the dynamics of cow metabolism during the peripartum period.

Our objectives were to test whether complete replacement of STM with OTM in both pre- and postpartum diets improves measures of innate and acquired immunity in transition cows. In addition, we evaluated whether in vitro measurements of neutrophil phagocytic activity and in vivo measurements of IgG responses to ovalbumin (OVA) challenge are associated with incidence of postpartum clinical disease and with the patterns of selected markers of metabolism during the transition period. We hypothesized that greater bioavailability of OTM compared with STM would result in enhanced phagocytic activity of neutrophils and anti-OVA IgG responses in transition cows. In addition, we hypothesize that cows that developed postpartum clinical disease would have reduced neutrophil function and IgG responses than cows that did not develop postpartum clinical disease, regardless of dietary treatments. Moreover, we hypothesize that cows with enhanced immune function during the transition period would have better energy and mineral statuses and lower inflammation than cows with reduced immune function.

**MATERIALS AND METHODS**

All procedures performed in this study were approved by the University of Guelph Animal Care Committee (Animal Utilization Protocol #4064).

**Animals, Housing, and Experimental Design**

This study was conducted at the Ontario Dairy Research Centre (Elora, ON, Canada) and was part of a larger companion study (Mion et al., 2022), in which pregnant heifers and pregnant dry cows (n = 273) were enrolled 45 ± 3 d before the expected calving date in small weekly groups (2–8 cows) between December 2018 and February 2020. At enrollment, cows were blocked by
parity and BCS, and randomly assigned to receive STM or OTM sources of supplementary TM. Briefly, cows assigned to the STM group received supplementation of Co, Cu, Mn, and Zn sulfates and Na selenite. Cows assigned to the OTM group received supplementation with Co, Cu, Mn, and Zn proteinates (Bioplex, Alltech) and selenized yeast (Sel-Plex, Alltech). Cows enrolled in a specific treatment remained in the same treatment in both prepartum and postpartum periods. Within a period, inclusion levels of TM in both treatments were identical. In the prepartum diet, inclusion levels were 0.25, 13.70, 40.00, 0.30, and 40.00 mg/kg for Co, Cu, Mn, Se, and Zn, respectively, for both treatments. In the postpartum diet, inclusion levels were 0.25, 15.70, 40.00, 0.30, and 63.00 mg/kg for Co, Cu, Mn, Se, and Zn, respectively, for both treatments. Inclusion levels were based on scientific recommendations available at the time when the experimental design was developed, and attempted to anticipate the new guidelines to be published in NASEM (2021). Basal levels of TM in feed ingredients were considered in diet formulation for supplementary inclusions of Cu, Mn, and Zn, but not for Co and Se. After discounting the estimated basal levels (established in the preparation phase) from the final target levels, the supplementary contributions of Cu, Mn, and Zn were 7.8, 13.9, and 17.3 mg/kg, respectively, in the prepartum TMR, and 9.2, 18.0, and 37.4 mg/kg, respectively, in the postpartum TMR.

Cows were housed in a freestall barn with mattress beds and equipped with automatic feeding bins (Insentec B.V.) that collect real-time measurements of feed intake and behavior. Similar numbers of cows from both treatments were kept in each pen, and each cow had access to an individual feeding bin from enrollment to 28 DIM, which was used to provide the assigned feeding treatment. The prepartum diet was mixed and delivered once daily at 1030 h. The postpartum diet was mixed once a day at 0830 h and delivered in 2 parts, 60% at 0900 h and 40% at 1530 h. The amount of feed offered was adjusted daily to allow 8% orts. Represen-tative feed samples were collected each week right after morning feeding for analyses of DM, and composited monthly for chemical composition. Feed ingredients and chemical composition of pre- and postpartum diets, including confirmation of TM concentrations, are described in a companion study (Mion et al., 2022). Lactating cows were milked twice daily in a rotary milking parlor (DeLaval) at 0530 and 1700 h, and milk fat and protein content were measured once a month by DHIA (Lactanet, Guelph, ON, Canada). After each milking, BW was recorded using a walk-through scale, which was also used to record BW of prepartum cows once a week. Body condition score was evaluated at enrollment and on d −21, 0, and 21 relative to calving using a 1 to 5 scale (Ferguson et al., 1994). Energy balance (EBAL) of prepartum and postpartum cows were calculated according to NRC (2001) and detailed in a companion study (Mion et al., 2022). Daily records of DMI, BW, milk yield, ECM, and EBAL were summarized weekly for statistical analyses.

Two days before the expected calving date or when demonstrating signs of calving, cows were moved from the prepartum pens to individual box stalls with chopped wheat straw bedding and remained there until 7 DIM, when they were moved to postpartum freestall pens. The individual stalls were also used for cows diagnosed with a clinical disease that required treatment. For the individual stalls, diets were delivered using a feed cart equipped with an electronic scale (Super Data Ranger, American Calan) and refusals were weighed daily. Daily feed intake in the individual stalls was calculated as the weight difference between offered feed and refusals.

**Neutrophil Function Assays**

Blood samples from a subgroup of 131 cows were obtained 7 ± 3 d before the expected calving date by puncture of the coccygeal vessels into vacutainer tubes containing acid citrate dextrose solution A for assessment of neutrophil phagocytic activity. Similarly, blood samples from a subgroup of 133 lactating cows were obtained from the coccygeal vessels at 7 ± 3 DIM to perform the same neutrophil function assay. Of all cows sampled, 86 had both pre- and postpartum assays and were used to investigate differences between prepartum and postpartum times. All cows sampled were clinically healthy on the day of blood collection. Blood samples were transported on ice to the laboratory and processed within 2 h of collection. The subgroups of cows were selected based on logistics of the research personnel and their availability to run in vitro assays.

The protocol for neutrophil isolation and function assays were previously described by Miltenburg et al. (2018). Briefly, 20 mL of 1 × PBS-EDTA was added to 8 mL of whole blood, inverted multiple times to mix, and gently overlaid on top of 8 mL of Ficoll Paque PLUS (General Electric Healthcare Bio-Sciences AB) in a 50 mL tube. Tubes were centrifuged at 420 × $g$ (1,500 rpm; Sorvall ST 16R, ThermoScientific) for 30 min at 23°C to separate red blood cells and granulocytes from the rest of the blood components. The plasma and buffy coat were discarded, leaving 5 mL of blood. Red blood cells were lysed by adding 30 mL of Milli-Q water and inverting the tubes for 45 s before osmolarity was restored with 15 mL of 3 × PBS-EDTA.
Samples were centrifuged again at 270 × g (1,200 rpm) for 10 min at 4°C, and the procedure of erythroylysis was repeated. After centrifugation, the supernatant was discarded and cells in the pellet were resuspended with 1 mL of 1 × PBS-EDTA before an additional 9 mL of 1 × PBS-EDTA was added. A subsample of 500 µL of this suspension was aliquoted into a 1.5 mL tube for hemocytometry, from which 10 µL was mixed with 10 µL of Trypan Blue stain (0.4%) and loaded onto a hemocytometer to determine the concentration of live cells. The remaining 9.5 mL suspension was centrifuged at 270 × g for 10 min at 4°C. Supernatant was removed and the pellet was resuspended in 1 × PBS with 10% fetal bovine serum according to the cell count, aiming for a final concentration of 50 × 10⁶ live cells/mL. Aliquots of 200 µL of the isolated neutrophils was removed and the pellet was resuspended in 1 × PBS with 10% fetal bovine serum according to the cell count, aiming for a final concentration of 50 × 10⁶ live cells/mL. Aliquots of 200 µL of the isolated neutrophils suspension containing 1 × 10⁶ live cells were distributed into two 5-mL polystyrene round-bottom tubes for the phagocytic activity assay (1 negative control and 1 test sample).

To perform the phagocytosis assay, 50 µL of zymosan-activated serum was added to both negative control and test sample tubes containing the isolated neutrophils. The zymosan-activated serum was prepared by adding 100 mg of zymosan A from Saccharomyces cerevisiae (Sigma-Aldrich) into 10 mL of pooled bovine serum of healthy mid-lactation cows. In addition, 1 µL of 1.0 µm labeled TransFluoSpheres Carboxylate-Modified Microspheres (488/560 nm, excitation/emission, 2% solids; Thermo Fisher Scientific) beads were added to test sample tubes to be phagocytized by the neutrophils. Both test sample and negative control tubes were incubated in a covered shaking water bath for 30 min at 37°C. Two hundred microliters of flow cytometer buffer was added to each tube and kept on ice, protected from light until flow cytometry analysis.

Flow Cytometry Analyses

A total of 10,000 cells/tube were evaluated in the area of interest using the BD FACSCanto flow cytometer (BD Biosciences) and BD FACSDiva software (version 8). Using the FlowJo software (version 10.5.3; Tree Star), neutrophils were gated in a side scatter by front scatter plot and converted into histograms. The fluorescence of beads was evaluated at 585 ± 42 nm. The percentage of cells that successfully performed phagocytosis of beads (pPhago) was defined as the percent of fluorescing cells in the test sample tube. Phagocytosis intensity (iPhago) was defined as the median fluorescence intensity in the test sample tube minus the background median fluorescence intensity in the negative control tube. Change in pPhago (pPhagoCh) and iPhago (iPhagoCh) between pre-partum and postpartum assays were calculated in cows that had both assays by subtracting the prepartum value from the respective postpartum value.

Immunoglobulin G Responses to Ovalbumin Challenge

On d −45 ± 3, −21 ± 3 relative to expected date of parturition, and on d 3 after parturition, subcutaneous injections of 1 mL of a solution containing 0.5 mg of OVA from chicken egg white (Sigma-Aldrich), diluted in Quil-a adjuvant (Sigma-Aldrich) in PBS (1 mg of Quil-a/mL of PBS), were administered in the shoulder region of the first 189 cows enrolled in the study. Blood was collected via coccygeal venipuncture on d −45 ± 3, −21 ± 3, 3, 7, and 23 ± 3 to measure the concentration of anti-OVA IgG in serum using an indirect ELISA assay described by Silva et al. (2015). Briefly, 96-well plates were coated with OVA in carbonate-bicarbonate buffer (1.4 mg of OVA/mL) and incubated for 48 h at 4°C. Plates were washed with PBS containing 0.05% Tween 20 (Sigma-Aldrich), and blocked with PBS containing 4% BSA during 2 h incubation. A second wash was then performed, and serum samples diluted 1:200 in PBS were plated in duplicate and allowed to incubate for 2 h. Following a third wash, 1:1,500 dilution of alkaline phosphatase conjugated antibody (Sigma-Aldrich) in Tris-buffered saline was added to the plate and incubated for 1 h. Plates were washed, and a p-nitrophenyl phosphate substrate was added and incubated for 30 min before reading at 410 and 540 nm (Cytation5, BioTek). The variable of interest reported for anti-OVA IgG was the optical density value. If the optical density reading exceeded 4.0, then samples were further diluted to 1:400, 1:800, or 1:1,600, as needed. The final optical density value for diluted samples was calculated by multiplying the optical density of the diluted sample by the dilution factor. Negative (unstimulated cows) and positive (d −21 samples) controls were included in each plate, and the intra- and interassay coefficients of variation for these controls were 3.77 and 7.94%, respectively. The coefficient of variation between duplicate test samples was 3.14%. Measurement of anti-OVA on d −45 was considered the basal level, and measurements on d −21, 3, 7, and 21 were considered as responses to OVA injections. Average and maximum values in the last 4 measurements were considered as additional dependent variables for statistical analyses.

Clinical Diseases

Cows were observed daily through 21 DIM by the research team and farm personnel for abnormal atti-
tude and visible clinical signs of disease, and classified according to Carvalho et al. (2019). Retained placenta was defined as visible fetal membranes 24 h after calving, and metritis was characterized by watery, bloody fetid vaginal discharge examined by Metricheck (Simcro, Datamars Inc.) on at 5, 7, 10, and 14 DIM, regardless of body temperature. Incidence of clinical mastitis was evaluated before every milking and characterized by the presence of abnormal milk. Locomotion was scored biweekly, and cows that stood and walked with arched back and had short strides in one or more legs were classified as clinically lame. Digestive problems were characterized by displacement of the abomasum or diarrhea. Respiratory problems were characterized by increased respiration rate associated with abnormal lung sounds at auscultation. Cows with positive diagnosis of at least 1 of the health problems above were classified as having clinical disease in the first 21 DIM (ClinD21). The information of ClinD21 was used in this study to investigate the association between immune function and disease. The effect of TM treatments on incidence of clinical disease was evaluated in the full set of 273 cows in a separate study.

**Blood Metabolites**

Blood samples for serum were collected by puncture of the coccygeal blood vessels into vacutainer tubes without an anticoagulant, and allowed to clot at room temperature. Samples for plasma were collected in the same manner into vacutainer tubes containing sodium heparin and were refrigerated until centrifugation. All samples were centrifuged at 2,000 × g for 15 min at 4°C for serum and plasma separation. Serum and plasma samples were harvested and frozen at −20°C or −80°C until analyses. Concentration of TM in serum were evaluated on d −45, −21, −7, 0, 7, and 21 by inductively coupled plasma mass spectrometry (Agilent 7900 ICP-MS, Agilent) at the Animal Health Laboratory (University of Guelph, Guelph, ON). The metabolic profile of serum samples collected on d −45, −21, −10, −3, 0, 3, 7, 21 was also assessed at the Animal Health Laboratory using an automated analyzer (Cobas 6000 c 501, Roche Diagnostics). The data on blood metabolites were used here to investigate the association between immune function and changes in metabolism during the periparturient period. The effects of TM treatments on concentrations of blood metabolites were described in a companion paper (Mion et al., 2022).

**Statistical Power and Analyses**

The minimum sample size for neutrophil function assays and IgG responses were calculated based on expected mean differences that would be biologically meaningful, and on the expected variability of the responses, which were extrapolated from studies that used the same assays used in this study (Silva et al., 2015 for IgG responses; and Couto Serrenho et al., 2020 for neutrophil phagocytosis). To allow 80% probability of detecting, at 5% statistical significance, a 7 percentage point difference with a standard deviation of 12.5 in percentage of neutrophils performing phagocytosis, a sample size of 102 (51 per treatment) was required. To allow 80% probability of detecting, at 5% statistical significance, 1,000 units difference in phagocytosis intensity with a standard deviation of 2,000, a sample size of 126 was required. For anti-OVA IgG responses, a minimum sample size of 168 was required for detection of 1 unit difference in average anti-OVA IgG responses, considering a standard deviation of 2.3, 80% power, and 5% statistical significance level. All power calculations were performed in the WinPepi program, version 11.65 (Abramson, 2011).

Data were analyzed by ANOVA using the GLIMMIX procedure of SAS (version 9.3; SAS Institute Inc.). To test the effects of treatment on neutrophil function responses (pPhago, iPhago, pPhagoCh, iPhagoCh) and on average and maximum anti-OVA IgG responses on d −21, 3, 7, and 21, statistical models included the fixed effects of treatment, parity (primiparous vs. multiparous), interaction between treatment and parity, and season. For every model, the distribution of residuals was tested for normality and data were transformed (square root or log10) if needed. Concentrations of anti-OVA IgG on d −21, 3, 7, and 21 were analyzed as repeated measures, and the statistical model included the fixed effects of treatment, day, parity, interactions between treatment and day, treatment and parity, and season, and the continuous values of IgG concentration on d −45 as a baseline covariate, and the random effect of cow nested within treatment. To investigate the association of immune responses with the incidence of ClinD21, the same models were used, but the fixed effects of treatment were replaced by the fixed effect of ClinD21 (0 or 1). Treatment was included as a covariable in these models when significant, and a backward stepwise elimination (when $P > 0.10$) was used for variable selection.

To evaluate the associations between immune responses and metabolic variables of cows between 21 d prepartum and 21 d postpartum, cows were ranked from lowest to highest for each immune response and classified as below or above the median. Ranking of cows was performed within parity and season when these variables significantly affected the immune response in question, which was tested previously. The statistical models included the fixed effects of immune response
category (below or above the median), time (day or week of assessment), parity, season, and the interactions between immune response category and time, and immune response category and parity. Within day differences in blood metabolites were explored by examining the pairwise comparison of least squares means within day of sampling when an interaction of category and time was observed. Pearson correlation coefficients and probability values between immune responses were evaluated using the CORR procedure of SAS.

For all analyses, group differences with $P \leq 0.05$ were considered significant and those with $0.05 < P \leq 0.10$ were declared tendencies.

**RESULTS**

Descriptive characteristics of the sample population used in each analysis (treatment, parity, incidence of ClinD21) are presented in Table 1.

### Effects of Feeding Treatment on Immune Responses

#### Phagocytic Activity of Neutrophils.

Data from 2 prepartum samples (1 STM and 1 OTM) and 5 postpartum samples (3 STM and 2 OTM) were excluded because of errors in the protocol that led to similar small values in phagocytic intensity in both the negative and positive tubes. The remaining 129 prepartum assays and 128 postpartum assays were used for statistical analyses. Of those, 82 cows had both prepartum and postpartum values for the evaluation of changes in phagocytic activity during transition into lactation.

Supplementary trace mineral source did not affect pPhago (Table 2). Prepartum pPhago, postpartum pPhago, and pPhagoCh were similar between STM and OTM groups (Table 2). Nonetheless, cows in the OTM group tended ($P = 0.08$) to have lesser prepartum iPhago but greater ($P = 0.05$) postpartum iPhago than the STM group (Table 2). Moreover, iPhagoCh differed ($P = 0.03$) between dietary treatment groups. On average, cows in OTM increased 892 units of median fluorescence intensity from prepartum to postpartum, whereas cows in the STM group reduced 317 units, resulting in a 1,209 unit difference between treatment groups (Table 2). No interactions between treatment and parity were observed for any of the responses above.

**Anti-OVA IgG Responses.** One hundred and eighty-nine cows received all 3 injections of OVA and had all 5 measurements of anti-OVA IgG. Cows responded to injections of OVA and gradually increased ($P < 0.01$) the concentration of anti-OVA IgG on d $-21$, $3$, $7$, and $21$. Compared with basal readings on d $-45$ (before first injection), IgG concentrations increased on average 4.6, 8.6, 11.1, and 15.6-fold on d $-21$, $3$, $7$, and $21$, respectively. Supplementary trace mineral source had no effect on the concentrations of anti-OVA IgG on d $-21$, $3$, $7$, and $21$ (Figure 1A), and did not affect the average or maximum concentrations across sampling days (Figure 1C). No interaction between treatment and parity was observed. However, we found a significant interaction ($P < 0.01$) between parity and time, as the pattern of increase in concentration of anti-OVA IgG over time differed between primiparous and multiparous cows, especially during the postpartum period (Figure 1B).

### Associations Between Immune Responses and Incidence of Clinical Disease

The incidence of ClinD21 in the entire sample of the study was 24.9% (68 out of 273; 38 STM and 30 OTM) and 28.7% (37 out of 129; 23 STM and 15 OTM) for the subgroup of cows with prepartum neutrophil function data, 26.6% (34 out of 128; 19 STM and 15 OTM) for the subgroup of cows with postpartum neutrophil function data, 25.6% (21 out of 82; 11 STM and 10 OTM) for the subgroup of cows with both prepartum and postpartum neutrophil function data, and 23.8% (45 out of 189; 27 STM and 14 OTM) for the subgroup of cows with anti-OVA IgG data.
no interactions between ClinD21 and time or between pre- and postpartum periods (Figure 2D). We found was not associated with anti-OVA IgG responses during 2C).

iPhagoCh and pPhagoCh (r = −0.31; < 0.01; Figure P be partially explained by a negative correlation between 2B). The opposite directions of these associations can points in cows that developed ClinD21, resulting in a 1,181 unit difference between groups (Figure 2A). In contrast, pPhagoCh had an average reduction of 2.9 percentage points in cows that did not have ClinD21, compared with an reduction of 1,331 units of median intensity between groups (Figure 2B). The opposite directions of these associations can be partially explained by a negative correlation between iPhagoCh and pPhagoCh (r = −0.31; P < 0.01; Figure 2C).

Anti-OVA IgG Responses. Incidence of ClinD21 was not associated with anti-OVA IgG responses during pre- and postpartum periods (Figure 2D). We found no interactions between ClinD21 and time or between ClinD21 and parity. The average (no ClinD21 = 2.88 ± 0.20 vs. ClinD21 = 2.93 ± 0.35; P = 0.85) and maximum (no ClinD21 = 5.42 ± 0.52 vs. ClinD21 = 5.31 ± 0.62; P = 0.53) IgG values were also similar between disease groups.

| Item | Treatment1 | Inorganic | Organic | P-value |
|------|------------|-----------|---------|---------|
| Percentage of cells with phagocytic activity2 | | | |
| Prepartum (square root scale) | 4.73 ± 0.13 | 4.81 ± 0.12 | 0.67 |
| Postpartum (square root scale) | 4.75 ± 0.15 | 4.45 ± 0.14 | 0.14 |
| Peripartum change (original scale) | 2.55 ± 2.33 | −2.18 ± 2.27 | 0.15 |
| Phagocytic activity intensity2 | | | |
| Prepartum (square root scale) | 87.3 ± 1.50 | 83.6 ± 1.46 | 0.08 |
| Postpartum (square root scale) | 85.7 ± 1.46 | 89.7 ± 1.40 | 0.05 |
| Peripartum change (original scale) | −316.7 ± 412.6 | 891.7 ± 400.4 | 0.03 |

1At 45 d before the expected calving date, cows were blocked by parity and BCS, and were randomly assigned to receive inorganic or organic sources of supplementary trace minerals in both prepartum and postpartum diets. Cows assigned to the inorganic group received supplementation of Co, Cu, Mn, and Zn selenates and Na selenite. Cows assigned to the organic group received supplementation with Co, Cu, Mn, and Zn proteinates (Bioplex, Alltech) and selenized yeast (Sel-Plex, Alltech). Data were transformed when needed to reach normality of statistical model residuals. Data are presented as LSM ± SEM.

2Neutrophils were isolated from peripheral blood approximately on d −7 and +7 relative to calving, and phagocytic activity was evaluated in vitro using fluorescence-labeled beads and flow cytometry. The percentage of cells that successfully performed phagocytosis of beads was defined as the percent of fluorescing cells in the test sample minus the percent of fluorescing cells in the negative control sample (no beads). Phagocytosis intensity was defined as the median fluorescence intensity in the test sample minus the median fluorescence intensity in the negative control. Peripartum changes were calculated by subtracting the prepartum value from the respective postpartum value. Two prepartum and 5 postpartum samples were excluded because of operator errors in the assays, and the remaining 128 and 129 assays were used for statistics of prepartum and postpartum phagocytic activity. Only 82 cows had both prepartum and postpartum assays for calculation of peripartum changes.

Associations Between Immune Function and Metabolic Parameters

Neutrophil Function. Out of the 6 variables evaluated, we selected the 2 variables associated with incidence of ClinD21 (iPhagoCh and pPhagoCh) to further investigate their association with markers of various aspects of metabolism during transition into lactation. For each measure of phagocytosis by neutrophils, cows were ranked from lowest to highest and classified as below or above the median.

For iPhagoCh, cows below the median had an average reduction of 1,331 units of median intensity fluorescence, whereas cows above the median had an average increase of 1,889 units, resulting in a 3,220 unit difference between groups (Figure 2A). In contrast, pPhagoCh had an average reduction of 2.9 percentage points in cows that did not have ClinD21, compared with an increase of 626 units in cows that developed ClinD21, resulting in a 3,220 unit difference between groups (Figure 2B). The opposite directions of these associations can be partially explained by a negative correlation between iPhagoCh and pPhagoCh (r = −0.31; P < 0.01; Figure 2C).
in cows above the median than in cows below the median (64.1 ± 0.76 vs. 61.9 ± 0.79 g/L; \( P < 0.01 \)). For concentration of albumin in serum, a tendency \(( P = 0.07 \)) for interaction between category of iPhagoCh and time was observed (Table 3; Figure 3C), and significant differences between groups were observed on d 0 (below median = 35.8 ± 0.34 vs. above median = 37.0 ± 0.32 g/L; \( P < 0.01 \)). In addition, cows above the median of iPhagoCh had greater \(( P = 0.03 \)) concentration of Mn and tended to have \(( P = 0.08 \)) greater concentration of Co in serum from d −21 to d 21, compared with cows below the median (Table 3; Figure 3D–E). Other metabolic markers were not associated with category of iPhagoCh (Table 3).

For pPhagoCh, cows below the median had an average reduction of 11.6 percentage points, whereas cows above the median had an average increase of 9.5 percentage points, resulting in a 21.2 percentage point difference between groups (Figure 4A). Cows with above-median pPhagoCh had lesser serum concentrations of Ca \(( P = 0.02 \)), K \(( P = 0.01 \)), and Se \(( P = 0.01 \)) between d −21 to 21, lesser DMI in wk 1 to 3 \(( P < 0.05 \)), and lesser milk production \(( P < 0.03 \)) in wk 2 and 3 compared with cows below median of pPhagoCh (Table 3; Figure 4B–F). Other metabolic variables were not associated with category of pPhagoCh (Table 3).

**Anti-OVA IgG Responses.** To investigate the association of variability in anti-OVA IgG responses and metabolism of transition cows, cows were ranked from lowest to highest average concentration of anti-OVA IgG (average of all samples from d −21 to 21) and classified as below or above the median. Cows above the median had concentrations of anti-OVA IgG 2.6, 2.7, 3.3, and 4.6 times greater than cows below the median (48x51).
median on d −21, 3, 7, and 21, respectively (Figure 5A). Because basal readings values on d −45 were different (below = 0.20 ± 0.04 vs. above = 0.33 ± 0.04; \( P = 0.04 \); Figure 5B), anti-OVA IgG on d −45 was used as a covariate in the statistical models. By design, the difference in average (below = 1.33 ± 0.18 vs. above = 4.47 ± 0.18; \( P < 0.01 \)) and maximum (below = 2.30 ± 0.41 vs. above = 8.22 ± 0.40; \( P < 0.01 \)) concentrations were highly different between groups (Figure 5B).

When category of anti-OVA IgG (below vs. above median) was used as an independent variable to explain the variability of metabolic parameters in transition cows, multiple dependent variables were found to be significantly associated with category of anti-OVA IgG concentration or by the interaction of category of anti-OVA IgG concentration and time, or both (Table 4). Cows with below-median anti-OVA IgG concentration were heavier (\( P < 0.01 \)) and had a lesser DMI as percentage of BW (\( P = 0.02 \)) than cows above the median (Table 4; Figure 5C–D). In addition, cows below the median had greater concentrations of nonesterified fatty acids (NEFA; \( P = 0.04 \)) and aspartate aminotransferase (AST; \( P = 0.04 \)) in serum, tended to have lesser concentrations of gamma-glutamyl transpeptidase (GGT; \( P = 0.06 \)) and Cu (\( P = 0.08 \)) in serum, and tended to have lesser EBAL (\( P = 0.08 \)) than cows above the median (Figure 5E–I). Differences in EBAL were significant at wk 1 (below median = −8.1 ± 0.65 vs. above median = −6.3 ± 0.65 Mcal of NEL/d; \( P = 0.049 \); Figure 5E), and differences in Cu were significant on d −21 (below = 0.77 ± 0.01 vs. above = 0.81 ± 0.01 Mcal of NEL/d; \( P = 0.049 \); Figure 5I).

Interactions between category of anti-OVA IgG concentration and time were observed for serum concentrations of NEFA, albumin, Na, and P (Table 4). For cows with above-median anti-OVA IgG concentration, NEFA

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**Figure 3.** Associations between changes in phagocytic intensity of neutrophils and metabolic parameters through the transition period. The 6 panels show the average change in phagocytic median fluorescence intensity (panel A) and serum concentrations of total protein (panel B), albumin (panel C), Mn (panel D), and Co (panel E) through the transition period, according to category of change in phagocytic intensity from d −7 to d 7 relative to calving (iPhagoCh), classified as below or above the median. Data points and error bars represent the LSM and SEM, respectively.
concentration declined after calving, whereas for cows below the median, it continued to increase up to d 3 (Figure 5F). Serum concentrations of albumin differed on d 10 \((P = 0.05)\) and 21 \((P = 0.01)\), Na on d 10 \((P = 0.03)\) and 14 \((P < 0.01)\), and P on d −21 \((P = 0.029)\) and −3 \((P = 0.027)\).
However, we detected no differences between OTM and STM in percentage of cells performing phagocytic activity, or in IgG responses to OVA injections.

The observed increase in intensity of neutrophil phagocytic activity suggests that individual neutrophils, harvested from cows supplemented with OTM, had greater capacity to perform phagocytosis of multiple foreign particles than those isolated from cows supplemented with STM. This difference in neutrophil function could be a result of differences in TM or energy statuses during the periparturient period, or both. In a companion study (Mion et al., 2022), we reported greater concentrations of Se in serum throughout the periparturient period, greater DMI, and lower concentrations of NEFA in serum of cows supplemented with OTM. Thatcher et al. (2010) reported that supplementation with selenized yeast from 23 ± 8 d prepartum to 80 d postpartum increased Se concentration in serum and postpartum phagocytosis and killing activity of neutrophils compared with supplementation of Na selenite. Hammon et al. (2006) reported that myeloperoxidase activity of blood neutrophils was impaired in cows with lesser DMI and greater concentrations of NEFA in serum during the peripartum period. Partial replacement of STM (Co, Cu, Mn, and Zn) by OTM fed as boluses to transition cows enhanced postpartum DMI and percentage of neutrophils with phagocytic activity on d 30 postpartum, which was not observed on d −15 and 10 relative to calving (Osorio et al., 2016). Unfortunately, Thatcher et al. (2010) and Osorio et al. (2016) did not report intensity of phagocytosis, and Hammon et al. (2006) did not evaluate phagocytic activity of neutrophils, only oxidative burst activity. A recent study investigating the use of immune and metabolic measures of periparturient cows to predict the incidence of retained placenta and metritis observed that intensity of phagocytosis, and not percentage of phagocytosis or oxidative burst activities, was predictive of uterine health (Chebel, 2021), which supports the importance of efforts to improve immune response during the peripartum period.

Figure 4. Associations between changes in percentage of phagocytosis and metabolic parameters through the transition period. The 6 panels show the average change in percentage of phagocytosis (panel A), DMI (panel B), milk yield (panel C), and in serum concentrations of Ca (panel D), K (panel E), and Se (panel F) through the transition period, according to category of change in phagocytic percentage from d −7 to d 7 relative to calving (pPhagoCh), classified as below or above the median. Data points and error bars represent the LSM and SEM, respectively.
Figure 5. Associations between anti-ovalbumin IgG responses and metabolic parameters through the transition period. Cows were ranked according to average concentration of anti-OVA IgG on d −21, 3, 7, and 21 and classified as below or above the median. By design, concentration of anti-OVA IgG on d −21, 3, 7, and 21 (panel A), basal signal on d −45 and average and maximum concentration from d −21 to 21 (panel B) were all greater in cows above the median than in cows below the median. The following panels show changes in BW (panel C), DMI (panel D), energy balance (panel E), and serum concentration of nonesterified fatty acids (NEFA; panel F), aspartate aminotransferase (AST; panel G), gamma-glutamyl transpeptidase (GGT; panel H), Cu (panel I), albumin (panel J), Na (panel K), and P (panel L) according to category of anti-OVA IgG response. Data points and error bars represent the LSM and SEM, respectively.
of intensity of phagocytosis to the health of transition cows. Nonetheless, another study that compared Na selenite versus selenized yeast supplementation during the transition period, with consequent differences in Se concentration in serum, did not observe any differences in percentage of neutrophils performing phagocytosis or in the number of bacteria phagocytized per neutrophil that contained at least 1 bacterium, which should correspond to our measure of intensity of phagocytosis (Weiss and Hogan, 2005). In their study, neutrophil function assays were performed at 28 DIM, and no differences in DMI or EBAL were observed. Nemec et al. (2012) also did not observe any differences in neutrophil function of cows supplemented Cu, Mn, and Zn from organic versus inorganic forms in mid-lactation cows. Thus, it is possible that source of supplementary TM has an effect on neutrophil function, only when overall DMI or the energy status of the animal is reduced (i.e., during the peripartum period, clinical disease, or stress response) and potentially improved by the use of OTM (Mion et al., 2022). In our study, cows supplemented with OTM not only had greater intensity of postpartum phagocytic activity, but also a positive change in this measure of neutrophil function between prepartum and postpartum evaluations, which was different from the small decrease in cows supplemented with STM. This measure of neutrophil function was also associated with incidence of ClinD21 regardless of TM feeding treatments. Cows that did not develop ClinD21 had increased intensity of phagocytic activity from prepartum to postpartum evaluations, versus decreased intensity in cows that had ClinD21. This result indicates that enhancing phagocytic activity intensity during the periparturient period could improve the cow’s ability to fight postpartum infections, and that complete replacement of STM by OTM in both prepartum and postpartum diets could help to arrive at such an outcome.

Table 4. Selected metabolic markers of transition cows according to categories of anti-ovalbumin IgG responses

| Item1 | Above median | Below median | Group | Group × time |
|-------|--------------|--------------|-------|--------------|
| BCS, 1–5 scale | 3.49 ± 0.02 | 3.52 ± 0.02 | 0.27 | 0.33 |
| BW, kg | 719.9 ± 6.8 | 752.1 ± 6.9 | <0.01 | 0.05 |
| DMI, kg/d | 14.1 ± 0.20 | 13.9 ± 0.20 | 0.56 | 0.77 |
| DMI, % of BW | 1.97 ± 0.03 | 1.88 ± 0.03 | 0.02 | 0.55 |
| EBAL, Mcal of NE3/d | −0.28 ± 0.31 | −1.03 ± 0.31 | 0.08 | 0.67 |
| ECM, kg/d | 34.5 ± 0.72 | 34.8 ± 0.72 | 0.83 | 0.85 |
| Milk, kg/d | 30.5 ± 0.58 | 30.6 ± 0.58 | 0.89 | 0.86 |
| Glucose, mmol/L | 3.77 ± 0.03 | 3.71 ± 0.03 | 0.14 | 0.11 |
| NEFA, mmol/L | 0.45 ± 0.02 | 0.49 ± 0.02 | 0.05 | 0.04 |
| BHB, µmol/L | 586.7 ± 20.98 | 597.8 ± 20.71 | 0.70 | 0.71 |
| Total protein, g/L | 66.17 ± 0.41 | 65.99 ± 0.41 | 0.76 | 0.08 |
| Albumin, g/L | 36.00 ± 0.20 | 35.79 ± 0.21 | 0.45 | 0.05 |
| Haptoglobin, g/L | 0.32 ± 0.02 | 0.34 ± 0.02 | 0.55 | 0.58 |
| AST, U/L | 66.03 ± 1.76 | 71.11 ± 1.74 | 0.04 | 0.15 |
| GGT, U/L | 22.23 ± 1.05 | 19.46 ± 1.04 | 0.06 | 0.72 |
| Ca, mmol/L | 2.30 ± 0.01 | 2.29 ± 0.01 | 0.17 | 0.92 |
| Mg, mmol/L | 0.98 ± 0.01 | 0.97 ± 0.01 | 0.92 | 0.63 |
| Na, mmol/L | 143.5 ± 0.24 | 143.0 ± 0.24 | 0.32 | 0.24 |
| K, mmol/L | 4.69 ± 0.02 | 4.65 ± 0.02 | 0.17 | 0.99 |
| P, mmol/L | 1.78 ± 0.02 | 1.78 ± 0.02 | 0.92 | 0.04 |
| Co, ng/mL | 0.31 ± 0.01 | 0.32 ± 0.01 | 0.42 | 0.53 |
| Cu, mg/mL | 0.88 ± 0.01 | 0.85 ± 0.01 | 0.08 | 0.62 |
| Mn, ng/mL | 1.63 ± 0.02 | 1.61 ± 0.02 | 0.46 | 0.80 |
| Se, µg/mL | 0.078 ± 0.001 | 0.078 ± 0.001 | 0.87 | 0.84 |

1BCS was evaluated on d −45, −21, 0, and 21. Daily BW, DMI, and energy balance (EBAL) were summarized weekly and evaluated from wk −5 to 3. Daily ECM and milk yield were summarized weekly and evaluated from wk 1 to 3. Concentration of trace minerals were evaluated on d −45, −21, −7, 0, 7, and 21. Blood metabolites were evaluated on d −21, −10, −3, 0, 3, 7, 10, 14, and 21. NEFA = nonesterified fatty acid; AST = aspartate aminotransferase; GGT = gamma-glutamyl transpeptidase.

2Subcutaneous injections of 0.5 mg of ovalbumin (OVA) were administered on d −45 ± 3, −21 ± 3, and 3 relative to (expected) date of calving in 189 cows. Peripheral blood was collected on d −45 ± 3, −21 ± 3, 3, 7, and 21 ± 3 to measured concentration of anti-OVA IgG in serum using an indirect ELISA assay. Cows were ranked from lowest to highest average concentration of anti-OVA IgG on d −21, 3, 7, and 21 and classified as below or above the median. By design, concentrations of anti-OVA IgG were on average 3.4 times higher in cows above the median compared with cows below the median. Probability values include the effects of anti-OVA IgG category (group) and its interaction with time (group × time). Data are presented as LSM ± SEM.
Considering that changes in intensity of phagocytosis during the periparturient period are relevant to postpartum health, then understanding what factors are associated with it is also relevant. We observed that cows above the median had greater concentrations of albumin and total protein on the day of calving, and greater concentration of Mn and Co through the periparturient period. Concentrations of circulating proteins and AA affect immune cell function and might become limited around the time of parturition (Meijer et al., 1995; Garcia et al., 2016; Coleman et al., 2020). Thus, reducing the magnitude of the decrease of circulating serum proteins close to time parturition is likely important for immune cell function and health of transition cows. Concentrations of Co and Mn contribute to oxidative balance, especially during the transition period, which, in turn, is critical for immune cell function and the health of cows (Paterson and MacPherson, 1990; Spears and Weiss, 2008; Sordillo and Aitken, 2009).

Change in intensity of phagocytic activity was negatively correlated with change in percentage of phagocytic activity which, as opposed to the former, was positively associated with incidence of ClinD21. Cows that had ClinD21 had an increase in percentage of phagocytic activity from pre- to postpartum evaluations, which was different from the average reduction observed in those that did not have ClinD21. Interestingly, the pattern of periparturient change in percentage of phagocytosis was associated with several differences in the indicators of metabolism. Cows classified as above the median had lesser DMI and milk production in the first 21 DIM, and lesser concentrations of Ca, K, and Se in serum. These differences suggest a less successful transition to lactation in cows with above-median peripartum change in percentage of phagocytosis, with effects on energy, macro- and micronutrient statuses, all of which have been previously reported to be important to immune function (Hammon et al., 2006; Kimura et al., 2006; Spears and Weiss, 2008; Martinez et al., 2012).

A limitation of our study was the fact that we did not sample postpartum cows for neutrophil function when they were clinically ill, which might have created a sample selection bias. This criterion was put in place to avoid changes in neutrophil function caused by an active systemic immune response. Thus, the reported association between peripartum changes in neutrophil function and diseases refer to clinical cases that occurred before or after the postpartum blood sampling. Our results could still be confounded with potential long-term effects of disease on neutrophil function; however, ClinD21 was not associated with individual prepartum or postpartum neutrophil function measures and was only associated with the changes in function during peripartum (postpartum minus prepartum values). Thus, the possible effects of disease on neutrophil function were less likely to explain the observed association between peripartum changes in phagocytic activity and incidence of ClinD21. Moreover, concentration of haptoglobin in serum was not associated with any of the neutrophil functions evaluated, which further supports the hypothesis that the reported associations between peripartum changes in phagocytic activity and ClinD21 were not a consequence of disease. Nevertheless, we acknowledge that our data cannot identify cause and effect in this association.

We did not find any association between anti-OVA IgG responses during the transition period and incidence of ClinD21, suggesting that the cow’s ability to mount a humoral response to a generic antigen might not be important for susceptibility to common postpartum infections. This finding agrees with Chebel (2021), who did not find a strong connection between anti-OVA IgG responses during the transition period and development of uterine diseases. The lack of association between periparturient IgG responses and ClinD21 does not imply that variability in acquired-immunity responses during transition to lactation is not important, because it is for vaccine responses, colostrogenesis, and health beyond common ClinD21. Thus, understanding the factors associated with variability in IgG responses during transition is relevant. In the present study, we calculated the average anti-OVA IgG response for each cow, ranked from lowest to highest, and classified cows as below and above the median. By design, the difference in average response between these 2 groups was remarkable, with cows above the median averaging a response 3.4 times greater than those below the median. Metabolic differences between these 2 groups were also noteworthy. Cows that responded better to the antigen in vivo challenge were lighter, had greater postpartum DMI (as % of BW) and EBAL, lesser postpartum concentrations of NEFA, and greater postpartum concentrations of albumin and Na in serum. The latter 2 blood metabolites were likely a result of the differences in DMI relative to BW. Our findings are supported by Ropstad et al. (1989) who explored the effects of estimated EBAL on response to various mitogens, and observed a positive relationship between mitogenic responses by lymphocytes and EBAL, with cows having a less negative EBAL exhibiting stronger responses. High levels of NEFA have been associated with impaired lymphocyte proliferation and secretion of IgM and IFN-γ (Lacetera et al., 2004, 2005). It is noteworthy that the immune activation caused by the OVA injections could directly affect the circulating concentration of metabolites. Moreover, it would be
expected that cows with a greater IgG response would use more energy and nutrients to build the humoral response than cows with a smaller IgG response. Serum Cu was reduced in cows classified as below-median anti-OVA IgG response. In calves, Cu deficiency has been shown to impair superoxide dismutase activity and antibody production in response to porcine erythrocytes (Gengelbach and Spears, 1998). Additional studies have also linked Cu levels to variability in immunity in multiple species (Lukasewycz and Prohaska, 1990; Scuderi, 1990; Prohaska and Failla, 1993). Aspartate aminotransferase is a marker for liver and cellular damage, and is involved on cytoplasmic transamination of aspartate into oxaloacetate, and on the formation of glutamate from α-ketoglutarate. Cows classified as below-median anti-OVA IgG response exhibited greater levels of AST during transition into lactation, which might reflect a higher degree of cellular damage, or even a metabolic adjustment for the observed reduced EBAL and potential differences in gluconeogenesis. Different from AST, GGT was increased in the serum of cows that responded above the median to OVA. Gamma-glutamyl transpeptidase is also commonly used as biomarker of liver damage, but also serves as catalyst for glutathione hydrolysis as part of the γ-glutamyl cycle. This latter function of GGT is very important for glutathione recycling, becoming highly expressed on the surface of activated T cells and performing an important protective feature against oxidative stress (Carlisle et al., 2003). Moreover, GGT knockout mice showed a 30% reduction of glutathione content and a 50 to 60% reduction of cellularity and organ weight of lymphoid organs, thymus, and spleen (Paige Lawrence et al., 2000). Nonetheless, no differences in antibody responses against OVA were observed in the GGT knockout mice model.

CONCLUSIONS

Total replacement of inorganic sources of TM with organic sources in both prepartum and postpartum diets enhanced postpartum intensity of phagocytic activity of neutrophils, but did not alter percentage of neutrophils performing phagocytosis. Cows supplemented with OTM had an increase in the intensity of phagocytosis by neutrophils from d −7 to 7 relative to calving, a pattern that was also observed in cows that did not develop ClinD21, regardless of feeding treatment. Cows that had postpartum clinical disease had a reduction in intensity of neutrophil phagocytosis, but an increase in percentage of neutrophil phagocytosis from d −7 to 7, and these 2 responses were negatively correlated. The changes in neutrophil function associated with incidence of postpartum clinical disease were also associated with changes in concentrations of Ca, K, Se, Mn, and total protein in serum from d −21 to 21, as well as DMI and milk yield in the first 3 wk postpartum. Anti-OVA IgG responses to in vivo immunizations were not affected by TM treatments and were not associated with the incidence of ClinD21. Nonetheless, anti-OVA IgG responses were associated with changes in BW, DMI, and EBAL, and serum concentration of NEFA, AST, GGT, albumin, Na, P, and Cu during the transition into lactation.

ACKNOWLEDGMENTS

Financial support for this study was provided by the Natural Sciences and Engineering Research Council of Canada (Ottawa, ON, Canada; NSERC Collaborative Research and Development Grant #503732), the Ontario Centres of Excellence (Toronto, ON, Canada; Alberta-Ontario Innovation Program Grant #26426), and Alltech Animal Nutrition (Lexington, KY). The authors thank the staff of the Elora Dairy Research and Innovation Centre (Elora, ON) for all of their support, and the Ontario Ministry of Agriculture, Food and Rural Affairs (Guelph, ON, Canada) for providing access to animals and research facilities and for providing kind support. The authors also gratefully acknowledge the assistance from Dorothee Bienzle and Rita Couto Serrenho (University of Guelph, Guelph, ON) with the nutrient function assays. The authors have not stated any conflicts of interest.

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