Veterinary Immunology and Immunopathology 161 (2014) 240–250

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Modulation of innate immune function and phenotype in bred dairy heifers during the periparturient period induced by feeding an immunostimulant for 60 days prior to delivery

E.L. Nace[a], S.C. Nickerson[a,*], F.M. Kautz[a], S. Breidling[b], D. Wochele[b], L.O. Ely[a], D.J. Hurley[b]

[a] Department of Animal and Dairy Science, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA
[b] Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA

ARTICLE INFO

Article history:
Received 18 February 2014
Received in revised form 21 August 2014
Accepted 25 August 2014

Keywords:
Innate immune function
Dietary supplementation
Dairy heifers
Periparturient period

ABSTRACT

The purpose of this study was to evaluate the effect of a feed additive (OmniGen-AF®, reported to have immune modulating activity) on innate immunity and health events during the periparturient period in dairy heifers when immunity is suppressed. From 60 days prepartum through calving, supplemented heifers ($n = 20$) received OmniGen-AF® daily and were compared with unsupplemented controls ($n = 20$). Blood leukocyte innate immune activity (phenotype markers, phagocytic activity, and reactive oxygen species—ROS production) was measured prior to feeding (60 days prepartum), 30 days later, and on days 1, 7, 14, and 30 postpartum. Adverse health events (udder edema, ketosis, displaced abomasum, and death) and milk production were measured at calving and into early lactation. The fraction of leukocytes with measurable CD62L (L-selectin) on their surface from supplemented heifers tended to be greater during the periparturient period in treated heifers than controls ($p = 0.100$). Likewise, leukocyte phagocytosis of *Escherichia coli* and *Staphylococcus aureus* during this time period tended to be greater in heifers supplemented with OmniGen-AF® ($p = 0.100$). Conversely, ROS production in response to phorbol myristate acetate or when leukocytes were stimulated with killed *S. aureus* lysate tended to be greater among control heifers compared with supplemented animals ($p = 0.100$). Supplemented heifers exhibited fewer incidents of udder edema than controls ($p = 0.030$) and tended to exhibit a lower rate of new cases of mastitis ($p = 0.098$); however, no differences were observed in milk somatic cell counts or level of milk production. Results demonstrate a positive role of OmniGen-AF® in amplifying leukocyte function consistent with antibacterial activity during the periparturient period, and support the continued study of dietary supplementation to enhance mammary gland health in dairy cows.

Published by Elsevier B.V.

1. Introduction

The periparturient period is a critical point in the lactation cycle of the dairy cow because it is a period of depressed immunity, which renders the animal susceptible to diseases such as mastitis (Smith et al., 1985; Burton and Erskine, 2003). Innate immunity is the most important

Abbreviations: FACS, fluorescence activated cell sorter; IMI, intramammary infection; ROS, reactive oxygen species; SCC, somatic cell count.

* Corresponding author. Tel.: +1 706 542 0658/+1 706 340 3367; fax: +1 706 542 2465.
E-mail address: scn@uga.edu (S.C. Nickerson).

http://dx.doi.org/10.1016/j.vetimm.2014.08.013
0165-2427/Published by Elsevier B.V.

defense system of the bovine mammary gland and relies heavily on polymorphonuclear neutrophilic leukocytes (PMN), which migrate from the blood to the mammary gland in response to bacterial infection (Paape et al., 2000). Macrophages in milk, originating from circulating monocytes, are the predominate cells that act as sentinels to invading mastitis-causing pathogens and recruit the PMN (Paape et al., 2000). From about 2–4 weeks prior to parturition through approximately 2 weeks after birth, PMN as well as mononuclear cell function has been shown to be compromised, and changes in phenotype have been documented (Paape et al., 1981; Burton et al., 1995; Nonnecke et al., 2003; Weber et al., 2004). Strategies aimed at enhancing the immune system of the mammary gland during these periods of immunosuppression might greatly impact the ability of the cow to resist infection.

OmniGen-AF®, a general dietary immunostimulant for ruminants, appears to enhance immunity by stimulating PMN function and trafficking protein expression. Although the basic mechanisms of how the supplement exerts its effects have not been fully elucidated, initial studies demonstrated that the main effect of dietary supplementation is to alter the expression of mRNA transcripts. For example, the down-regulation of genes due to OmniGen-AF® was shown to enrich the oxidative phosphorylation pathway, which suggests that PMN with lower oxidative phosphorylation activity may be more efficient in distributing O_2 toward effector functions such as ROS production (Revelo et al., 2013). Another study suggested that restoration of PMN function by feeding OmniGen during periods of immunosuppression may be dependent on MyD88, which is critical for the development of innate and adaptive immunity by the induction of inflammatory cytokines triggered by TLRs (Ortiz-Marty et al., 2013).

In ruminants, Forsberg (Omnigen Research, LLC., Corvallis, Oregon, personal communication) found that PMN harvested from lactating cows receiving OmniGen-AF® for 60 days showed significantly increased phagocytosis of *Streptococcus uberis* compared to PMN of unsupplemented controls. Likewise, Wang et al. (2004) showed that OmniGen-AF® increased L-selectin (a PMN cell surface protein that promotes diapedesis and migration to infection sites) mRNA expression in pathogen-challenged sheep, which suggested that this immunostimulant enhanced the potential capacity of PMN to enter infection sites and kill bacteria. In a subsequent study, Wang et al. (2007) determined that OmniGen-AF® increased the expression of L-selectin and IL-1β mRNA in sheep that had been immune suppressed with dexamethasone. Thus, OmniGen-AF® appeared to ameliorate the immunosuppressive effects similar to those encountered around parturition by enhancing PMN activity and function.

More recently, Ryman et al. (2012, 2013) evaluated dairy heifers on a continuous feeding program with OmniGen-AF® (compared to unsupplemented controls) that were vaccinated against *Staphylococcus aureus* mastitis at 6 months of age followed by boosting every six months. The trial was conducted to determine if daily feeding of OmniGen-AF® would enhance the antibody titer response to this vaccine in these young heifers. They also examined the function and phenotype of leukocytes 30 and 60 days after initiation of continuous feeding of these heifers. OmniGen-AF® induced no improvement in antibody titers during calfhood or throughout the first pregnancy; however, heifers receiving extended continuous feeding of OmniGen-AF® exhibited enhanced PMN phagocytic activity against *Escherichia coli* and *S. aureus*, and greater reactive oxygen species (ROS) production compared to unsupplemented controls 30 and 60 days after supplementation. In addition, PMN L-selectin mRNA expression was significantly increased in heifers receiving continuous feeding with the supplement, and IL-8R mRNA expression was enhanced, but not significantly. These data suggest that supplementing heifer diets continuously with OmniGen-AF® stimulated the innate immune system in an effort to protect against bacterial challenge.

In the present study, we wished to determine if short-term daily supplementation with OmniGen-AF® beginning approximately 60 days prior to calving, would be sufficient to induce the previously observed enhancements in leukocyte function. It was our hypothesis that OmniGen-AF® would enhance blood PMN and monocyte function and provide a phenotype consistent with reduced periparturient immune depression, which may enhance resistance to mastitis during this time.

2. Materials and methods

2.1. Animals used in the study

Control ($n = 20$) and OmniGen-AF® treated ($n = 20$) Holstein heifers were serially selected at 6 months of age based on an even (treated) or odd (control) ear tag identification number for inclusion in the study, commingled by age, placed on pasture, and bred by 15 months of age. As the pregnant heifers reached 60 days prior to expected calving date, they began receiving a supplementation (OmniGen-AF® at the rate of 4 g/45 kg of body weight/day) or a control diet as described by Ryman et al. (2013).

Briefly, heifers were locked-up along a bunk-line feeding pad with head-locks and fed once daily a total mixed ration (TMR) based on wheat or sorghum silage and 2.3 kg of dry cow grain mix. The grain mix contained the following in kg/t: rolled corn (790), soybean meal (100), dicalcium phosphate (7.8), Clarifly® (4.3), salt (3.8), a trace mineral pack (4.4), vitamins A, D, and E (4.4), Zinpro performance minerals® (3.4), and limestone (2.0). At feeding time, treated heifers received the recommended dose of OmniGen-AF® delivered through the dry cow grain mix as a topdress at the rate of 4 g OmniGen-AF®/45 kg of body weight/day. Based on this rate, the supplement contained 10% Omnigen-AF, 10% molasses (to allow for binding), and 80% grain mix. Control heifers received a supplement of only the grain mix. The treatment and control diets were fed directly on the concrete slab in front of the heifers.

The average body weight, which was recorded each month starting 60 days prepartum, determined the amount of OmniGen-AF®/grain mixture that treatment groups received. This average corresponded to a designated amount of OmniGen-AF®/grain mix that was fed once a day. In addition to the grain supplement, heifers were given 22.7 kg/head/day of wheat or sorghum silage depending

on the season. Water and Bermudagrass hay were available to the heifers ad libitum. At approximately 2 to 3 weeks prepartum, heifers were relocated to a close-up pasture, and the TMR was top-dressed with approximately 0.8 kg/head/day of dietary cation anion diet (DCAD) mix, 2.7 kg/head/day of dry cow grain mix, and 0.11 kg/head/day of limestone. OmniGen-AF® continued to be delivered to treated animals through the dry cow grain mix at the rate of 4 g/45 kg of body weight/day.

Use of animals was approved by the University of Georgia Institutional Animal Care and Use Committee. All procedures were carried out according to the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1999).

2.2. Collection of blood samples

A jugular vein blood sample (60 ml) was collected from each heifer on day −60 (prior to feed supplementation) and on day −30 prior to calving as well as on days 1, 7, 14, and 30 postpartum using a 60 ml syringe containing 1.5 ml of 100 mM EDTA anticoagulant (Sigma-Aldrich, St. Louis, MO) fitted with a 14-G 1½-in needle. The blood was then processed for the isolation of PMN and monocytes to determine leukocyte phenotype markers, phagocytic activity, and ROS production.

2.3. Testing of blood samples

2.3.1. Preparation of leukocytes

Buffy coats were prepared by centrifugation at 850 × *g* for 20 min. The buffy layer and the top 20% of the packed RBC layer were collected in a 50 ml tube and washed in phosphate buffered saline (PBS) (Mediatech, Manassas, VA). For enrichment of PMN and monocytes, a 40-ml aliquot of PBS was used to dilute the washed cell pellet from the buffy coat and this was layered over 10 ml of lymphocyte separation medium (LSM 1.077 gm/l) (Mediatech) and centrifuged at 800 × *g* for 30 min. The PBS layer was removed and the mononuclear cell layer was collected from the top of the tube using a 5-ml pipette. All LSM was then removed and PBS was added to suspend the pellet containing RBC and neutrophils. After centrifugation at 450 × *g* for 10 min, PBS was removed and red blood cells were lysed by adding 10 ml of sterile water to the pellet. The sample was vortexed for 30 s and 10 ml of double strength PBS added followed by 30 ml of PBS. After the red blood cell membrane fraction was removed by washing twice with PBS, the remaining PMN population was washed by centrifugation at 450 × *g* for 5 min and suspended in 10 ml of PBS. The mononuclear cells were washed twice as well. A viable cell count (85–95% viability) was performed using Trypan blue (Sigma-Aldrich).

2.3.2. Monoclonal antibody staining of blood leukocytes

Phenotype was determined by indirect staining and flow cytometry. Leukocytes were stained with the antibodies and dilutions shown in Table 1; the dilution represents twice the minimum saturating concentration for each antibody as previously determined. Leukocytes were stained using 96-well round bottom plates by placing 200-μl aliquots of cell suspensions into each well for incubation with each antibody; an additional well was used for incubating cells with detection antibody only as a control. Prior to staining, the cells were washed once in 200 μl of PBS containing 0.5% bovine serum albumin and 0.1% sodium azide (FACS buffer) followed by centrifugation at 200 × *g*. The buffer was removed and the cells suspended by vortexing in the residual volume. To each well, 20 μl of diluted antibody (or FACS buffer for detection antibody control) was added, and the plate was incubated at 4 °C for 60 min in the dark. Following this incubation, the cells were washed three times with 200 μl of FACS buffer by centrifugation. Following suspension of the cells, 100 μl of detection antibody (anti-mouse IgG (Fab fragment)) labeled with FITC prepared in the goat (Sigma-Aldrich) at a 1:100 dilution was added. The plate was incubated for 30 min in the dark at 4 °C. Following this incubation, the cells were washed with 200 μl of FACS buffer three times, then suspended, and 100 μl of FACS buffer with 0.2% formalin was added and fixed overnight at 4 °C. The samples were diluted to 400 μl in FACS buffer in 12 × 75 mm tubes for assessment by flow cytometry as described below. For the assessment of phenotype, 10,000 events were collected in a forward angle peak (height) versus forward angle area (integrated) window that represented the expected size for PMN or mononuclear cells. In the PMN preparations, between 65% and 92% of the events fell into the PMN forward angle and side scatter window. For the mononuclear cells preparation, 55–75% fell in the lymphocyte and 25–45% in the monocyte window.

Table 1
Antibodies used for phenotyping PMN and monocytes.

Antibody marker	Supplier—Catalog#	Dilution
MHC class I	VMRD[a]—H58A	1:100
MHC class II	VMRD—TH14B	1:100
CD11b	AbD Serotec—MCA1425	1:50
CD11c	VMRD—BAQ153A	1:50
CD13	AbD Serotec—MCA2338	1:20
CD43	AbD Serotec—MCA1096	1:50
CD62L	AbD Serotec—MCA1649G	1:100

[a] VMRD, Pullman WA; AbD Serotec, Raleigh, NC.

2.3.3. Flow cytometric assessment of phenotype

Samples were assayed using a BD Accuri C6 cytometer and BD CFlow Plus software (BD-Accuri, Ann Arbor, MI). PMN and monocyte populations were identified in forward angle and side scatter gates as previously described (Hart et al., 2011). Green fluorescent signal was analyzed for the PMN and monocyte populations independently. To calculate the percentage of cells bearing each marker, the raw percentage positive for each antibody was determined, and the percentage positive for detection antibody for that animal was subtracted. The data are reported as percentages of cells positive for each marker.

2.3.4. Assessment of phagocytic function

To measure phagocytic activity, PMN-enriched leukocytes and mononuclear cells were incubated with bodipy-labeled killed *E. coli* (Molecular Probes/Invitrogen, Eugene, OR) and *S. aureus* (Molecular Probes/Invitrogen).

Incubations were performed for 30 min at 37 °C in the dark, and then assay tubes were fixed at 4 °C with 300 μl of 2% paraformaldehyde and held overnight at 4 °C before analysis. Phagocytosis of internalized bacteria (measured in the presence of 0.04% Trypan blue, Sigma-Aldrich) was then quantified (using a C6 flow cytometer, BD-Accuri, Ann Arbor, MI) in a population of leukocytes falling in size (forward scatter) and granularity (side scatter) within the gates containing PMN or monocytes. Percentage fluorescence was measured using BD Accuri C6—CFlow Plus Software according to techniques described by Hart et al. (2011) and Wiggins et al. (2011).

2.3.5. Assessment of ROS production by PMN

ROS production was measured in the PMN population using dihydrorhodamine-123 (DHR-123) (Molecular Probes/Invitrogen) as described by Wiggins et al. (2011). Stimulants of ROS production included PMA (Sigma-Aldrich) at 10^{-7} M, *Salmonella typhimurium* LPS (List Biologicals Laboratories, Inc., Campbell, CA) at 1 μg/ml, *S. aureus* peptidoglycan (PGN, Sigma-Aldrich) at 1 μg/ml, staphylococcal enterotoxin B (SEB; Toxin Technology, Sarasota, FL) at 1 μg/ml, and killed *S. aureus* lysated whole cell antigen at dilutions of 1:100, 1:200, and 1:400 (prepared in the UGA College of Veterinary Medicine Applied Immunology Laboratory). Total ROS production was measured using a fluorescent plate reader (Thermo-Fisher, Pittsburg, PA) in cumulative arbitrary fluorescence units (AFU) at the end of a 2-h incubation period at37 °C. The AFU represented the cumulative conversion of DH-R123 to R-123 over the 2 h of incubation.

2.4. Statistical analysis of blood leukocyte measurements

Mean percentage fluorescence of cell surface markers, mean percentage fluorescence of bacterial phagocytosis, and mean ROS production among PMN and monocytes were compared for treated and control heifers using unpaired *t*-tests with Welch's correction. Similarly, the changes over time from baseline (day −60) were compared for each day within the control and within the treated heifers (e.g., days −30, 0, 7, 14, and 30 of controls were each compared with day −60 control and no intermediate values were assessed) using unpaired *t*-tests with Welch's correction because several animals could not be followed for the full duration of the study. Normality and homogeneity of variance were evaluated using the Kolmogorov–Smirnov test and the *F*-test, respectively. Analyses were performed with Prism version 5.05 (Graphpad Software, La Jolla, CA), a 2-sided alternative hypothesis was assumed. Trends were declared with $p \leq 0.100$ and significance with $p \leq 0.050$ for differences among means.

2.5. Collection of mammary secretion samples, health data, SCC, and milk production

Prior to feeding (day −60) and on day −30 prepartum, mammary secretions were collected and cultured for comparison with milk samples collected and cultured on days 3 and 10 postpartum. The teat orifice was sanitized and mammary secretions were expressed into a sterile 12-ml tube. Once samples were collected, teats were sanitized with a 1% iodine solution (FS-103 X, IBA, Inc., Millbury, MA). Mammary secretions and milk samples were cultured, and any bacterial growth was identified using standard microbiological procedures (NMC, 2004). The somatic cell count (SCC) for mammary secretion and milk samples was also determined using a Direct Cell Counter (DeLaval, Tumba, Sweden). Heights and body weights were also recorded on days −60 and −30 prior to calving to ensure proper growth rate. Height was measured in centimeters at the hip using a height stick and weight was taken in kilograms using a weigh tape measuring around the heart girth.

Heifers calved in maternity paddocks and any adverse health events associated with parturition such as a retained placenta, displaced abomasum, ketosis, udder edema, and death were recorded. Adverse health events were analyzed by treatment for the variable: (1) all heifers having a health event, (2) number of events per heifer, (3) incidence of mammary edema, (4) incidence of retained placenta, (5) incidence of ketosis, (6) incidence of displaced abomasum, (7) incidence of deaths, and for (8) the cumulative adverse event score. The adverse event score, which was accumulative, was calculated by assigning a numerical value to each event such that no event = 0, mammary edema = 1, retained placenta = 2, ketosis = 2, displaced abomasum = 3, and death = 4. Means values for each variable among treated and control heifers were separated using SAS 9.3 Proc Glm for Windows (SAS, 2013).

Within 24 h of calving, cows began twice daily milking in a double-6 herringbone parlor using a DeLaval system equipped with automatic milking unit takeoffs, milk volume meters, and electronic cow identification. Daily milk production was recorded and milk samples from individual mammary quarters were collected on days 3 and 10 postpartum, analyzed bacteriologically to determine the development of new IMI, and processed to determine SCC and somatic cell score (SCS).

Averages for SCC and SCS on day 3 and day 10 postpartum were determined, and means for treated and control heifers were separated using SAS 9.3 (SAS, 2013). To determine new IMI rate, the diagnosis of an infection in a previously uninfected quarter (e.g., a quarter not infected prepartum) on day 3 or day 10 postpartum was considered a new IMI. Means for treated and control heifers were expressed on a per animal basis and a per quarter basis, and were separated using SAS 9.3 (SAS, 2013).

3. Results

3.1. Leukocyte phenotyping

Leukocytes were assessed for phenotypic expression of several markers. The expression of MHC I was used as an internal control and was always between 88 and 100% for both PMN and monocytes and never significantly different between control and treated heifers during the course of the experiment. The expression of MHC II was utilized to confirm the gating of the monocyte population and was always expressed by 85–100% of the cells in the monocyte gate defined by forward angle and side scatter. There was no significant change in MHC II expression among

monocytes between treated and control heifers during the course of the experiment.

The expression of CD11b was used as a quality marker for PMN, and cells in the PMN gate defined by forward angle and side scatter were between 95 and 100% positive, with uniform brightness on PMN samples over the course of the study. A fraction of monocytes expressed CD11b, but with a lower brightness than the PMN, consistently during the study. We also attempted to use an antibody against CD13 to estimate the fraction of less mature PMN in circulation. However, this antibody did not recognize circulating PMN as anticipated, so did not bear useful data.

The percentage of CD11c-positive PMN was not significantly different between control and treated heifers at the initiation of the study (day −60) (Fig. 1A). Control heifers had a significantly higher expression of CD11c on day 1 ($p = 0.003$) and day 7 ($p = 0.006$) relative to treated heifers. However, on day 30, treated heifers expressed a significantly higher percentage of CD11c-positive cells than control heifers ($p = 0.004$). The percentages of PMN positive for CD11c in the controls were significantly increased on day 1 and day 7 relative to the day −60 values ($p = 0.027$ and $p = 0.017$, respectively), then decreased to pretreatment values on days 14 and 30. In contrast, the percentage of PMN bearing CD11c declined significantly on day 1 in treated heifers compared with day −60, but did not vary from pretreatment values over the rest of the trial.

The percentage of CD11c-positive monocytes was not significantly different at the initiation of the study between the treatment groups (Fig. 1B). Treated heifers expressed a higher percentage of positive cells than controls on day 14 ($p = 0.020$) with a trend for significance on day 7 ($p = 0.090$) and day 30 ($p = 0.063$). Expression of CD11c-positive monocytes from control and treated heifers did not change over time except for a significant decline in expression in the control heifers on day 14 relative to day −60 ($p = 0.016$).

The expression of CD62L was similar among PMN at the initiation of the experiment between control and treated heifers (Fig. 1C), but the percentage of CD62L-positive PMN was numerically higher in treated than control heifers on day 1 through day 30, showing a trend toward significance on day 1 ($p = 0.073$) and day 7 ($p = 0.089$). On day 1 after calving, CD62L expression on PMN of the control heifers was significantly lower than on day −60 ($p = 0.043$), but not different from pretrial values thereafter. There were no significant differences in the percentages of PMN expressing CD62L among the treated heifers across days, and the typical reduction in expression at parturition was not observed.

The expression of CD62L was not significantly different among monocytes at the initiation of the experiment between control and treated heifers (Fig. 1D). After calving, monocyte CD62L expression in treated heifers remained numerically higher than the controls until day 30. The percentage of CD62L-positive monocytes tended to be higher than controls on day 1 ($p = 0.071$), was significantly higher than controls on day 7 ($p = 0.034$), and tended to be higher than controls on day 14 ($p = 0.066$). On days 7 and 14 after calving, CD62L expression on monocytes of the control heifers was significantly lower than on day −60 ($p = 0.008$ and 0.039, respectively), but there were no significant differences in the percentages of monocytes expressing CD62L among the treated heifers across days. Thus, as with PMN, the typical reduction in CD62L expression on monocytes at parturition collected from treated animals was not observed.

For both groups of heifers, expression of CD43 remained approximately the same over the course of the experiment; treated and control heifers never had significantly different levels of expression on PMN or monocytes (data not shown). The percentage of CD43-positive PMN remained numerically higher among controls until day 14 postpartum. On day 30, there was a trend toward significance ($p = 0.086$) in the percentage of CD43-positive PMN in the treated over the control heifers.

3.2. Leukocyte phagocytic activity against mastitis-causing bacteria

The percentages of PMN exhibiting phagocytic activity against *E. coli* were similar among control and treated heifers at the initiation of the trial (day −60) (Fig. 2A). On day −30, percentage phagocytosis tended to be greater for treated heifers compared with controls ($p = 0.086$) and remained numerically higher throughout the rest of the trial. Compared with day −60 values, the percentage phagocytosis among control heifers was significantly lower on day 1 ($p = 0.017$), day 7 ($p < 0.001$), day 14 ($p = 0.003$), and day 30 ($p = 0.001$), whereas the percentage phagocytosis among treated heifers was significantly lower than day −60 values only on day 7 ($p = 0.003$) and day 30 ($p < 0.001$).

The percentages of PMN exhibiting phagocytic activity against *S. aureus* were similar among treatments at day −60 (Fig. 2B). On day 7, percentage phagocytosis tended to be greater for treated heifers compared with controls ($p = 0.078$) and remained numerically higher throughout the rest of the trial. Compared with day −60 values, the percentage phagocytosis among PMN from control heifers was significantly lower on day −30 ($p = 0.015$), day 1 ($p < 0.001$), day 7 ($p < 0.001$), day 14 ($p < 0.001$), and day 30 ($p < 0.001$), and the percentage phagocytosis among treated heifers was significantly lower on day 1 ($p = 0.009$), day 7 ($p = 0.019$), day 14 ($p = 0.005$), and day 30 ($p = 0.003$) compared with day −60 values.

The percentage of monocytes exhibiting phagocytic activity against *E. coli* was similar on day −60, then remained numerically higher for treated heifers over controls across all study days except day 1, showing a trend toward significance on day −30 ($p = 0.072$) and day 7 ($p = 0.090$) (Fig. 2C). Compared with day −60 values, the percentage phagocytosis among control heifers was significantly lower on day −30 ($p < 0.001$), day 7 ($p < 0.001$), day 14 ($p = 0.002$), and day 30 ($p < 0.001$), whereas the percentage phagocytosis among treated heifers did not differ over time, suggesting maintenance of phagocytic activity in treated animals.

The percentages of monocytes exhibiting phagocytic activity against *S. aureus* were similar across treatments at day −60, then became significantly higher for treated heifers over controls on day −30 ($p = 0.037$) (Fig. 2D). Compared with day −60 values, the percentage phagocytosis among control heifers was significantly lower on day −30 ($p = 0.008$), day 1 ($p = 0.034$), day 7 ($p = 0.017$), and

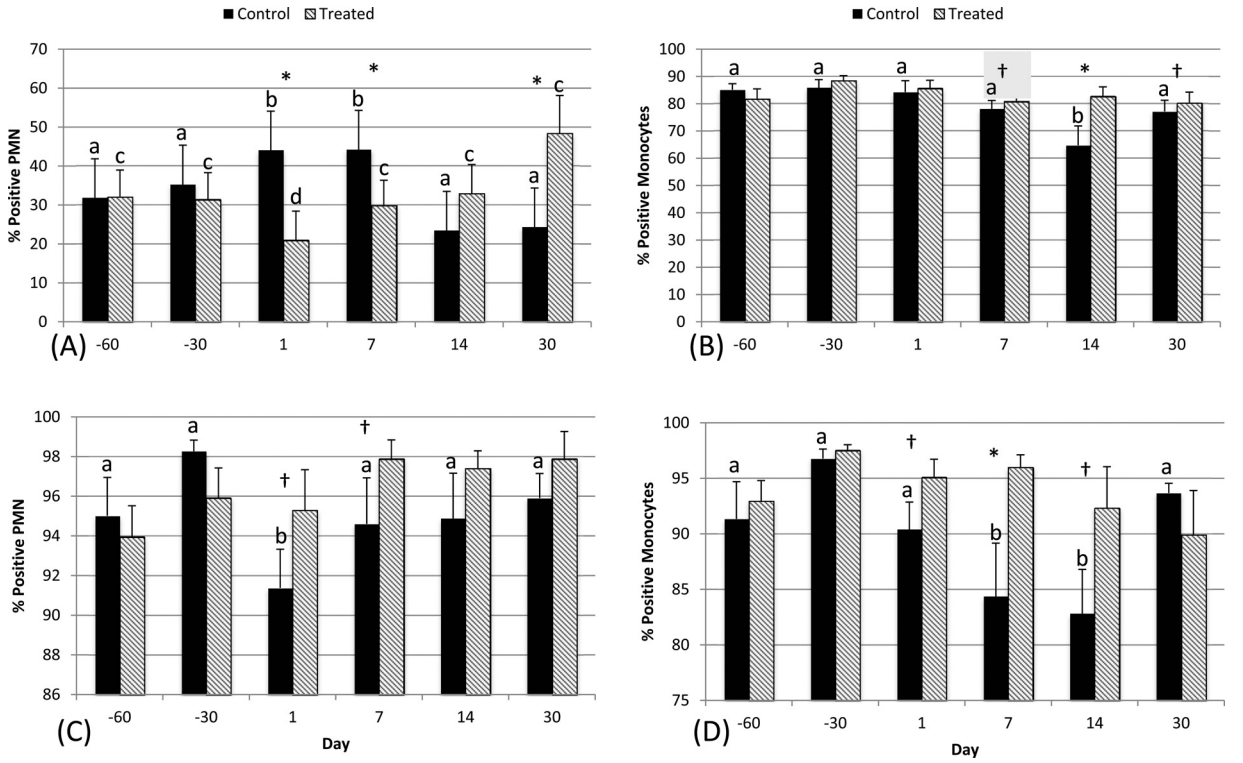

Fig. 1. (A) The percentage of PMN binding CD11c antibody among control and treated heifers from day 60 prepartum to day 30 postpartum. (B) The percentage of monocytes binding CD11c antibody. (C) The percentage of PMN binding CD62L antibody. (D) The percentage of monocytes binding CD62L antibody. Percentages are means ± SEM. * Treatments differ $p < 0.050$. † Treatments differ $p < 0.100$. [a,b] Control values for days without a superscript in common differ ($p < 0.050$). [c,d] Treated values for days without a superscript in common differ ($p < 0.050$).

day 14 ($p = 0.004$), whereas the percentage phagocytosis among treated heifers did not differ over time until day 30 ($p = 0.033$) when it decreased compared with day −60 values.

3.3. ROS production among PMN from control and treated heifers

There were no significant differences in ROS produced by PMN after stimulation with PMA (total capacity) at day −60 prepartum between control and treated heifers (Fig. 3A). At day −30 prepartum and day 1 postpartum, control heifers exhibited a significantly higher total ROS capacity than treated heifers ($p = 0.036$ and $p = 0.024$, respectively). In addition, the total ROS capacity of controls was significantly higher on day −30 than day −60 ($p = 0.050$), but not significantly different thereafter. In contrast, the total ROS capacity for treated heifers was significantly lower on day 1 and day 7 after calving than at day −60 ($p = 0.023$ and 0.016, respectively) then returned to pretreatment levels on days 14 and 30. The two treatment groups demonstrated similar total ROS capacity on day 7 and beyond.

ROS production by PMN after exposure to whole cell *S. aureus* antigen was also tested. The level of ROS production to the 1:400 *S. aureus* dilution was similar for both control and treated heifers on day −60, but after calving, PMN from control heifers exhibited a significantly higher ROS capacity on day 1 ($p = 0.021$) and day 7 ($p = 0.042$) than treated heifers (Fig. 3B). ROS production by PMN for control heifers was significantly lower on day −30 than at day −60 ($p = 0.013$), but not significantly different thereafter. Treated heifers showed no significant differences across times. No differences between treatments or across times were observed for the 1:100 and 1:200 dilutions of *S. aureus*.

ROS production by PMN after exposure to LPS, PGN, and SEB was also measured; however, no significant differences between treatments or across times were observed (data not shown).

3.4. Adverse health events, mastitis, SCC, and milk production

Although 20 treated and 20 control heifers began the trial, animal numbers were reduced to 16 and 14, respectively, due to removal from the study for a variety of reasons including recurring resistance to sampling procedures (bleeding), abortion, displaced abomasum complications, bloat, leg injury, and culling. All data from removed heifers were excluded from the study.

Among the health event variables measured, only the incidence of mammary edema was significantly different between treatments (Table 2). Control heifers exhibited an edema incidence of 75%, whereas in treated heifers, the incidence was appreciably lower at 36% ($p = 0.030$).

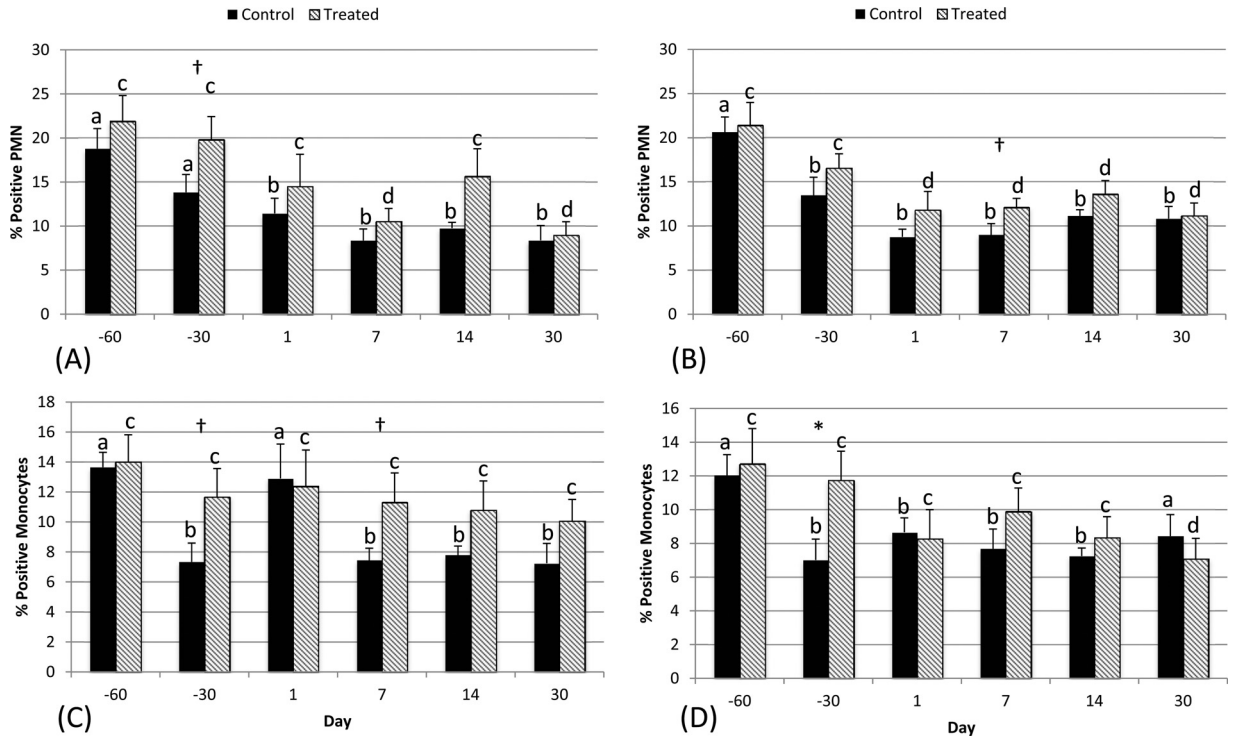

Fig. 2. (A) The percentage of PMN that engulfed *E. coli* among control and treated heifers from day 60 prepartum to day 30 postpartum. (B) The percentage of PMN engulfing *S. aureus* among control and treated heifers. (C) The percentage of monocytes engulfing *E. coli* among control and treated heifers. (D) The percentage of monocytes engulfing *S. aureus* among control and treated heifers. Percentages are means ± SEM. * Treatments differ $p < 0.050$. † Treatments differ $p < 0.100$. [a,b] Control values for days without a superscript in common differ ($p < 0.050$). [c,d] Treated values for days without a superscript in common differ ($p < 0.050$).

Table 2
Adverse health events during early lactation in control and treated heifers.

Variable	Control	Treated	*p*
Event (%)[a]	0.88 (14/16)[e]	0.64 (9/14)[e]	0.143
Event (no.)[b]	1.69	1.07	0.182
Mammary edema[c]	0.75 (12/16)	0.36 (5/14)	0.030
Retained placenta[c]	0.12 (2/16)	0.28 (4/14)	0.288
Ketosis[c]	0.31 (5/16)	0.14 (2/14)	0.289
Displaced abomasum[c]	0.19 (3/16)	0.14 (2/14)	0.754
Death[c]	0.31 (5/16)	0.14 (2/14)	0.289
Adverse event score[d]	3.44	2.21	0.318

[a] Percentage of heifers having an event.
[b] Number of events per heifer.
[c] Incidence (%).
[d] Calculated by assigning a numerical value to each event such that no event = 0, mammary edema = 1, retained placenta = 2, ketosis = 2, displaced abomasum = 3, and death = 4.
[e] Number of heifers affected over total number of animals.

Table 3
New IMI rate, SCC, and milk production during early lactation in control and treated heifers.

Variable	Control	Treated	*p* Value
New IMI among heifers[a]	0.50	0.36	0.464
New IMI among quarters[b]	0.93	0.36	0.098
SCC × 10^3 ml^{-1} on day 3[c]	685	963	0.382
SCC × 10^3 ml^{-1} on day 10[c]	470	471	0.994
SCS on day 3[d]	5.04	5.51	0.469
SCS on day 10[d]	4.66	4.32	0.608
Yield (kg)[e]	28.8	28.8	Not determined

[a] Percentage of heifers with new IMI on days 3 and 10 postpartum.
[b] Average number infected quarters per heifer on days 3 and 10 postpartum.
[c] Average somatic cell count × 10^3 ml^{-1} on day 3 or day 10 postpartum.
[d] Average somatic cell score on day 3 or day 10 postpartum.
[e] Average daily milk yield per heifer over first 5 weeks of lactation.

Although not significant, treated heifers exhibited fewer incidents of ketosis (0.14 vs. 0.31), displaced abomasum (0.14 vs. 0.19), death (0.14 vs. 0.31), and a lower adverse event score (2.21 vs. 3.44) compared with controls. Health event results should be interpreted with caution due to the low number of animals used. The incidence of adverse health events was high and partly attributed to the elevated age at calving (32 months) rather than the preferred 24 months; older animals generally have more health issues at calving. Also, several heifers calved during the summer of 2013, one of the warmest on record, and heat stress may have been an issue.

An analysis of the new IMI rate among heifers postpartum (Table 3) demonstrated that new IMI were numerically more prevalent among control heifers than treated heifers (50% vs. 36%, $p = 0.464$). Similarly, on a mammary quarter basis, there was a tendency for more new IMI in control than treated heifers (0.93 vs. 0.36, $p = 0.098$).

Average SCC from quarter milk samples taken 3 days postpartum demonstrated that counts were numerically lower for control (685×10^3 ml^{-1}) vs. treated (963×10^3 ml^{-1}) heifers ($p = 0.382$); however, by day 10

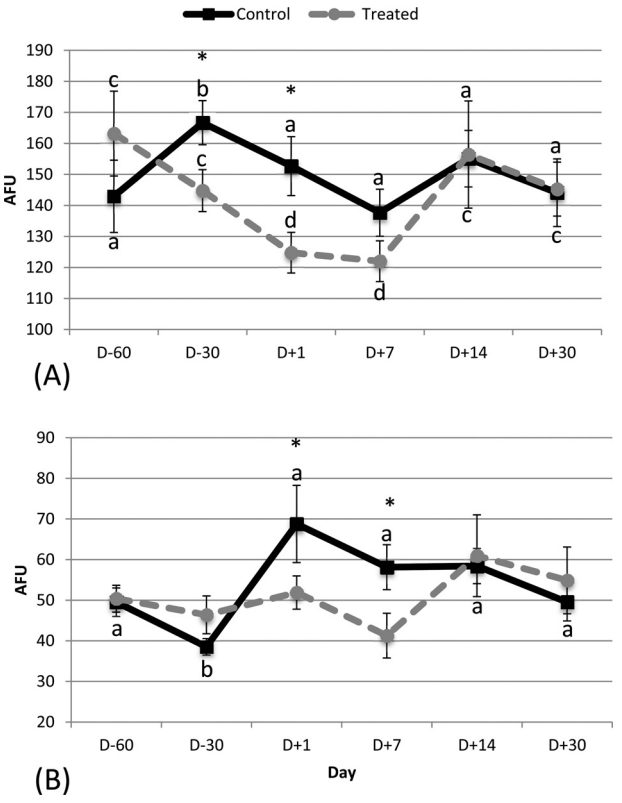

Fig. 3. (A) A comparison of the maximum generating capacity of ROS by PMN reacting to PMA in control and treated heifers from day 60 prepartum to day 30 postpartum. The values are expressed in arbitrary fluorescent units (AFU) (B) Production of ROS by PMN reacting to killed *S. aureus* lysate at a 1:400 dilution in control and treated heifers from 60 days prepartum to day 30 postpartum, measured in AFU. AFU values are means ± SEM. * Treatments differ $p < 0.050$. [a,b] Control values for days without a superscript in common differ ($p < 0.050$). [c,d] Treated values for days without a superscript in common differ ($p < 0.050$).

postpartum, there was no difference between control (470×10^3 ml^{-1}) and treated (471×10^3 ml^{-1}) animals. Likewise, average SCS from quarter milk samples taken 3 days postpartum demonstrated that scores were numerically lower for control (5.04) vs. treated (5.51) heifers ($p = 0.469$); however, by day 10 postpartum, there was no difference between control (4.66) and treated (4.32) animals ($p = 0.608$).

The average milk production from day 7 through day 35 postpartum demonstrated identical yields between control and treated heifers (28.8 kg/day vs. 28.8 kg/day, respectively). Among controls, milk production averaged 21.5 kg on day 7 and increased to 34.2 kg on day 35. Treated heifers' milk production averaged 21.0 kg on day 7 and increased to 33.3 kg by day 35.

Heights and weights measured on days −60 and −30 showed no differences between control and treated heifers and were recorded to ensure proper growth in supplemented animals. Average hip heights for control and treated heifers were 162 cm and 150 cm, and average weights were 598 kg and 589 kg, both which were within the expected growth range for Holstein heifers at 24 to 30 months of age (Hoffman, 1997).

4. Discussion

The dairy cow's diet plays an important role in its ability to resist disease, and the provision of micronutrient supplements is one practical means of enhancing the innate immune defense system of the cow against invading mastitis pathogens. Selenium is probably the best characterized micronutrient with the capacity to modulate immunoregulation in the bovine, and Se deficiencies result in compromised PMN function and increased susceptibility to IMI (Erskine, 1993). If other immunomodulators can be used to augment natural immune function at critical periods of the lactation cycle when the mammary gland becomes immunocompromised (Kehrli et al., 1989a,b), then the losses caused by mastitis should be reduced. Enhancing the natural ability of the host to resist mastitis through dietary supplementation without introducing undesirable drug residues into the food chain is compatible with current consumer demands.

This study evaluated a simple method to address the well-documented depression in innate immune function that occurs during the periparturient period. The product assessed (OmniGen-AF®) is a low cost powder that is simply added to the daily ration as a top dressing. A pilot study by our group (Ryman et al., 2012, 2013) evaluated the long-term (18 months) feeding of this supplement, beginning at six months of age, and generated data that suggested enhanced innate immune function, reduced mastitis, and improved milk production. In the present study, we wished to determine if a shorter period of feeding (60 days prepartum) would provide similar benefits by (1) promoting the expression of certain leukocyte receptors, (2) increasing phagocytic activity against common mastitis pathogens, and (3) regulating ROS production.

It has been established that the concentration, effective adhesion, phagocytosis, and bactericidal properties of circulating PMN highly influence the outcome and severity of mastitis in the dairy cow; hence, these leukocytes are regarded as the first line of defense against pathogenic bacteria (see review by Paape et al., 2002). In order to migrate into infected tissue sites, such as the mammary gland, PMN (as well as monocytes) must first adhere to capillary endothelia, and this is accommodated by the adhesion molecule L-selectin (CD62L) cell-surface receptors that allow leukocytes to adhere to L-selectin present on endothelial cells (Kishimoto et al., 1989; Burvenich et al., 1994).

Unfortunately, L-selectin is shed from surface of PMN at parturition (Lee and Kehrli, 1998; Kimura et al., 1999), remaining low for several days postpartum, which could contribute to the reported reduction in bovine PMN chemotactic activity immediately following calving (Berning et al., 1993). Loss of CD62L is associated with elevated corticoid levels (Weber et al., 2004), which increase as calving approaches, are associated with the stress of calving, and have been shown to dampen the immune activity of the dam and her calf (Chase et al., 2008). Indeed, corticoid administration to cows has been shown to cause CD62L shedding from neutrophils (Burton et al., 1995). Reduced CD62L expression leads to compromised neutrophil immune surveillance and diapedesis into infected

tissues, and this delayed inflammatory response allows bacteria to multiply, produce more toxins, and invade mammary tissues further.

In our study, CD62L expression on PMN from control heifers was reduced to the lowest percentage of cells on day 1 after calving, and expression on monocytes was lowest on day 7 and 14 after calving. However, CD62L expression on PMN and monocytes from treated heifers did not decrease during these times, and expression remained elevated over control values on days 1, 7, and 14 suggesting a greater ability of PMN to adhere to capillary walls for subsequent extravasation into infected tissue sites. Thus, the feeding of OmniGen-AF® appeared to ameliorate the typical reduction in CD62L expression observed during the stress of calving. Likewise, Wang et al. (2007) observed that OmniGen-AF® prevented the decrease in L-selectin expression in sheep that were immunosuppressed with dexamethasone. This is further evidence that corticosteroid induced reduction of CD62L can be reversed. Our results are also in agreement with Ryman et al. (2013) who observed an increase in L-selectin mRNA expression in blood leukocytes from heifers fed OmniGen-AF® over a 15-month period compared with control heifers.

The expression of CD11c on PMN and monocytes is more difficult to interpret during the periparturient period of the present study. CD11c is a transmembrane protein found on leukocytes that induces extravasation, cellular activation, and helps trigger PMN respiratory burst (Adams and Barnum, 2007). The elevation in CD11c expression of control PMN on days 1 and 7 may be a reflection of the stress of parturition, which forces PMN from control heifers to overcompensate for the stress-associated immunosuppression at calving, whereas this stress is ameliorated in treated heifers, thus PMN from treated heifers do not need to overcompensate by increasing expression of CD11c. Changes in CD11c expression have been demonstrated as a result of stress in other studies as well. For example, Heidari et al. (2001) demonstrated that granulocyte-colony stimulating factor, produced in response to inflammatory stress, enhanced the expression of CD11c on bovine PMN in vitro by 87%. Likewise, van Werven et al. (1997) reported that increased expression of CD11c in younger cows during the periparturient period led to milder signs of *E. coli* mastitis, and that CD11c expression increased over the course of infection in all cows, but faster and to a greater degree in the younger animals. In another study, Zerbe et al. (2000) demonstrated an increase in the fraction of CD11c-positive uterine PMN in cows under stress with of fatty liver syndrome, which was associated with a reduction in ROS activity of these PMN. In humans, the expression of CD11c increased significantly with higher doses of lipopolysaccharide, platelet activating factor, and tumor necrosis factor-alpha, all inflammatory stressors, which was correlated with enhanced PMN function and enhanced expression of the whole family of adhesion molecules: CD11b, CD35, and CD62L (Condliffe et al., 1996).

Furthermore, CD11c expression has been shown to increase in response to inflammatory activation and sheer stress in humans, including the inflammatory mechanisms of atherosclerosis and heart disease (Gower et al., 2011). In the present study, as lactation ensued, the stress of parturition was no longer an issue, and CD11c expression increased in treated heifers to a greater degree than controls (e.g., days 14 and 30 postpartum), possibly in response to environmental factors.

The expression of the surface marker CD43 is important for immune function, and is associated with homing and trafficking of leukocytes, including transendothelial migration (Andrew et al., 1998). Expression of CD43 among PMN and monocytes was similar over the course of the present experiment for treated and control heifers, except on day 30 when the percentage of CD43-positive PMN in treated heifers increased over controls. As with CD11c, by day 30, the stress of parturition was no longer an issue, and CD43 expression increased in treated heifers to a greater degree than controls.

Once leukocytes have extravasated into infected tissues, phagocytosis by PMN is the most effective defense against bacterial infection of the mammary gland (Paape et al., 2002); however, the phagocytic and bactericidal activities of these cells are especially diminished during the periparturient period (Paape et al., 1981; Cullor et al., 1990). The results of the present trial showed that although phagocytic activity of PMN against *E. coli* and *S. aureus* was significantly lower during the periparturient period compared to day −60 across treatments, activity was numerically greater in PMN isolated from OmniGen-AF®-supplemented vs. control heifers from day −30 prepartum through 14 of lactation, and trended toward significance on days −30 and 7.

Similarly, monocyte phagocytic activity against *E. coli* and *S. aureus* decreased significantly during the periparturient period, but in cells from the control heifers only; monocytes from treated heifers showed no decrease in activity during this time, and activity was elevated significantly or tended to be elevated over controls between days −30 and 7. The decreased activity observed through day 7 may reflect the inhibitory effect of the stresses associated with calving and may be related to the impact of negative energy and protein balances in early lactation on innate immune function (Nonnecke et al., 2003).

Thus, the feeding of OmniGen-AF® for 60 days prepartum appeared to ameliorate the immunosuppression typically observed during calving. In support of the increased leukocyte phagocytic activity observed, Forsberg (Omnigen Research, LLC., Corvallis, Oregon, personal communication) found that PMN harvested from lactating cows receiving OmniGen-AF® for 60 days showed significantly increased phagocytosis of *S. uberis* compared to PMN collected from unsupplemented controls. Likewise, Ryman et al. (2013) found that heifers supplemented with OmniGen-AF® for 30 or 60 days exhibited greater leukocyte phagocytic activity compared with unsupplemented controls.

Once bacteria are attached to the PMN surface and the phagocytic process has begun, ROS (hydroxyradicals, singlet oxygen, oxygen halides, hydrogen peroxide, nitrogen oxide) are released that kill bacteria as they are ingested (Burvenich et al., 1994; Paape et al., 2002). ROS production, however, is typically decreased during the periparturient period, which may increase susceptibility to mastitis at this time (Dosogne et al., 1999; Mehrzad et al., 2002). In fact,

the severity of coliform mastitis has been reported to be less pronounced in animals with higher pre-infection PMN ROS production (Heyneman et al., 1990).

In the present study, prior to treatment, both groups of heifers exhibited similar levels of ROS production in response to PMA and killed *S. aureus* lysate activation, but during the periparturient period, control heifers produced more ROS than treated heifers, then both groups decreased to day −60 values by days 14 and 30 postpartum. This sequence of events suggests that although the two treatment groups started with no significant differences in ROS production, once the treatment began to take effect, the treated heifers managed the stress associated with parturition better and were producing fewer active inflammatory products, hence lower ROS production. When the effect of treatment began to wear off in early lactation and the animals were recovering from the stress of parturition nearing day 14, the two groups were similar and leveled out in ROS production through day 30. Likewise, Nightingale et al. (2013) found that ROS production was lower in OmniGen-AF® supplemented multiparous cows at or near parturition, and suggested that the supplement improved health status during this period of immunosuppression.

It has been suggested that when ROS is produced adequately but not excessively, collateral cell and tissue damage is minimized, as uncontrolled generation of ROS upon the initiation of phagocytic activity is harmful for many cell systems (Capuco et al., 1986; Mehrzad et al., 2002). For example, Capuco et al. (1986) found that culturing mammary explants with lysed PMN or intact PMN that were allowed to phagocytize opsonized zymosan resulted in morphologic damage to milk-producing cells, and concluded that PMN phagocytic function and release of ROS was responsible for the damage observed. The lower (more controlled) generation of ROS by PMN from treated heifers in the present study may be a safety mechanism to minimize collateral cell and tissue damage during the periparturient period. In contrast, Ryman et al. (2013) observed increased ROS production in PMN from OmniGen supplemented vs. control heifers at 30 and 60 days after OmniGen-AF® supplementation; however, these heifers averaged 6–9 months of age, were not bred yet, and therefore not subject to the stress associated with parturition. Thus, the increase in ROS production in that study (Ryman et al., 2013) could be interpreted as beneficial in potentially killing bacteria without resulting in collateral cell and tissue damage.

No differences were observed between treatments or over time for the ROS stimulants LPS, PGN, or SEB in the present study.

Thus, we observed a trend toward enhancement of immune parameters (increased cell surface receptor expression and phagocytic activity) and stabilization of ROS production in animals supplemented with OmniGen-AF®. This tendency for increased immune capacity may have been associated with the observed reductions in udder edema and new IMI rate during the periparturient period and in early lactation, respectively, in treated heifers compared with controls. Likewise, cytokine treatment with recombinant human granulocyte colony-stimulating factor and recombinant bovine granulocyte-macrophage colony-stimulating factor in the 1st week of lactation to control bovine mastitis enhanced the functional activity of blood PMN and led to fewer new IMI, faster bacterial clearance rates, and reduced clinical severity of mastitis (Kehrli, 1997).

While our pilot study (Ryman et al., 2012, 2013) demonstrated some of the enhancements in innate immunity documented in the present study after feeding OmniGen-AF®, it also demonstrated a lower prevalence of mastitis, reduced SCC, and an enhancement in milk production that we did not observe in the present study. However, we did observe a tendency for a reduction in numbers of new IMI among quarters at calving in heifers fed OmniGen-AF® in the present study. It should be emphasized that heifers in the first study were fed continuously for a period of up to 24 months, whereas in the present trial, heifers were fed for up to 2 months, and the positive effects on milk production in the former may reflect long-term action of the supplement on the treated heifers.

The incidences of adverse health events, deaths, and culling in the present trial were high. This was, in part, attributed to the age at which heifers calved, which averaged 32 months rather than the preferred 24–26 months. Older animals generally have more health issues, especially around the time of calving. Additionally, many heifers calved over the summer months of 2013, which was one of the warmest summers on record; thus, heat stress may have accounted for less than optimal animal health.

5. Conclusions

Results of this study demonstrated that the feeding of OmniGen-AF® for 60 days prepartum (1) enhanced the function bovine blood leukocytes during the periparturient period when the innate immune system is typically immunosuppressed and susceptible to new IMI (Smith et al., 1985), and (2) tended to reduce the new infection rate at calving. Such immunomodulators work best in hosts with suboptimal immune function; thus, the periparturient period is an opportune time to investigate the use of such products as part of a good nutrition program using a diet that meets or exceeds the nutritional recommendations for dairy heifers. Our data suggest that OmniGen-AF® could also be considered a candidate as novel interventions that modulate the mammary gland immune response to mastitis are developed, and that continued study of this feed additive as a potential immunostimulant is warranted. The fact that it is of low cost and introduces no recombinant products or antibiotics to the production system is also worthy of consideration.

Acknowledgements

This work was funded by a gift from Prince Agri, Inc., and USDA Animal Health Formula Funds grant GOEV 0538. The authors wish to thank Ms. Natalie Norton for her assistance with the laboratory studies reported here.

References

Adams, J.E., Barnum, S., 2007. CD11c is critical in modulating cytokine production by activated T cells. J. Immunol. 178, 88–93.

Andrew, D.P., Spellberg, J.P., Takimoto, H., Schmits, R., Mak, T.W., Zukowski, M.M., 1998. Transendothelial migration and trafficking of leukocytes in LFA-1-deficient mice. Eur. J. Immunol. 28, 1959–1969.

Berning, L.M., Paape, M.J., Peters, R.R., 1993. Functional variation in endogenous and exogenous immunoglobulin binding to bovine neutrophils relative to parturition. Am. J. Vet. Res. 54, 1145–1153.

Burton, J.L., Erskine, R.J., 2003. Immunity and mastitis. Some new ideas for an old disease. Vet. Clin. N. Am.: Food Anim. Prac. 19, 1–45.

Burton, J.L., Kehrli Jr., M.E., Kapil, S., Horst, R.L., 1995. Regulation of L-selectin and CD18 on bovine neutrophils by glucocorticoids: effects of cortisol and dexamethasone. J. Leukocyte Biol.V 57, 317–325.

Burvenich, C., Paape, M.J., Hill, A.W., Guidry, A.J., Miller, R.H., Heyneman, R., Kremer, W.D.J., Brand, A., 1994. Role of the neutrophil leukocyte in the local and systemic reactions during experimentally induced *E. coli* mastitis in cows immediately after calving. Vet. Q. 16, 45–50.

Capuco, A.V., Paape, M.J., Nickerson, S.C., 1986. In vitro study of polymorphonuclear leukocyte damage to mammary tissue of lactating cows. Am. J. Vet. Res. 47, 633–668.

Chase, C.C.L., Hurley, D.J., Reber, A.J., 2008. Neonatal immune development in the calf and its impact on vaccine response. Vet. Clin. N. Am.: Food Anim. Prac. 24 (1), 87–104.

Cullor, J.S., Fairley, N., Smith, W.L., Wood, S.L., Dellinger, J.D., Inokuma, M.S., Souza, I.M., 1990. Hemogram changes in lactating dairy cow given human recombinant granulocyte colony-stimulating factor. Vet. Pathobiol. 27, 311.

Condliffe, A.M., Chilvers, E.R., Haslett, C., Dransfield, I., 1996. Priming differentially regulates neutrophil adhesion molecule expression/function. Immunology 89, 105–111.

Dosogne, H., Burvenich, C., Freeman, A.E., Kehrli Jr., M.E., Detilleux, J.C., Sulon, J., Beckers, J.F., Hoeben, D., 1999. Association between pregnancy-associated glycoprotein and decreased polymorphonuclear leukocyte function in early postpartum dairy cows. Vet. Immunol. Immunopathol. 67, 47–54.

Erskine, R.J., 1993. Nutrition and mastitis. Vet. Clin. N. Am.: Food Anim. Prac. 9, 551.

Federation of Animal Sciences Societies (FASS), 1999. Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. Federation of Animal Sciences Societies, Champaign, IL, USA.

Gower, R.M., Wu, H., Foster, G.A., Devaraj, S., Jialal, I., Ballantyne, C.M., Knowlton, A.A., Simon, S.I., 2011. CD11c/CD18 expression is upregulated on blood monocytes during hypertriglyceridemia and enhances adhesion to vascular cell adhesion molecule-1. Arterioscler. Thromb. Vasc. Biol. 31, 160–166.

Hart, K.A., Barton, M.H., Vandenplas, M.L., Hurley, D.J., 2011. Effects of low-dose hydrocortisone therapy on immune function in neonatal horses. Pediatr. Res. 70 (1), 72–77.

Heidari, M., Harp, J.A., Kerli Jr., M.E., 2001. Expression, purification and in vitro biological activities of recombinant bovine granulocyte-colony stimulation factor. Vet. Immunol. Immunopathol. 81, 45–57.

Heyneman, R., Burvenich, C., Vercauteren, R., 1990. Interaction between the respiratory burst activity of neutrophil leukocytes and experimentally induced Escherichia coli mastitis in cows. J. Dairy Sci. 73, 985–994.

Hoffman, P.C., 1997. Optimum body size of Holstein replacement heifers. J. Anim. Sci. 75, 836–845.

Kehrli Jr., M.E., 1997. Efficacy of granulocyte-colony stimulatory factor alone or in combination with granulocyte macrophage-CSF against *Escherichia coli* bovine mastitis. In: Ellis, R.P. (Ed.), 78th Conference Research Workers Animal Disease. Chicago, IL. Blackwell Publishing, Ames, IA, p. 88.

Kehrli Jr., M.E., Nonnecke, B.J., Roth, J.A., 1989a. Alterations in bovine lymphocyte function during the periparturient period. Am. J. Vet. Res. 50, 215–220.

Kehrli Jr., M.E., Nonnecke, B.J., Roth, J.A., 1989b. Alterations in bovine neutrophil function during the periparturient period. Am. J. Vet. Res. 50, 207–214.

Kimura, K., Goff, J.P., Kerhli, M.E., 1999. Effects of the presence of mammary gland on expression of neutrophil adhesion molecules and myeloperoxidase activity in periparturient dairy cows. J. Dairy Sci. 82, 2385–2392.

Kishimoto, T.K., Jutila, M.A., Berg, E.L., Butcher, E.C., 1989. Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. Science 245, 1238–1241.

Lee, E.K., Kehrli Jr., M.E., 1998. Expression of adhesion molecules on neutrophils of periparturient cows and neonatal calves. Am. J. Vet. Res. 59, 37–43.

Mehrzad, J., Duchateau, L., Pyorala, S., Burvenich, C., 2002. Blood and milk neutrophil chemiluminescence and viability in primiparous and pluriparous dairy cows during late pregnancy, around parturition and early lactation. J. Dairy Sci. 85, 3268–3276.

National Mastitis Council (NMC), 2004. Microbial Procedures for the Diagnoses of Bovine Udder Infection and Determination of Milk Quality, fourth ed. National Mastitis Council, Inc., Verona, WI, pp. 9–41.

Nightingale, C.R., Sellers, M.D., Pepper-Yowell, A.R., Chapman, J.D., O'Connor, D.L., Ballou, M.A., 2013. OmniGen-AF® supplementation improves leukocyte responses and hematology of multiparous peripartum cows. J. Dairy Sci. 96 (E-Suppl. 1), 59.

Nonnecke, B.J., Kimura, K., Goff, J.P., Kehrli Jr., M.E., 2003. Effects of the mammary gland on functional capacities of blood mononuclear leukocyte populations from periparturient cows. J. Dairy Sci. 86, 2359–2368.

Ortiz-Marty, R.J., Forsberg, N.E., Chapman, J.D., Mullarky, I.K., 2013. OmniGen-AF® restores GR-1, L-selectin, and RANTES expression by immunosuppressed murine PMN challenged with lipopolysaccharide in a MyD88-dependent manner. J. Dairy Sci. 96 (E-Suppl. 1), 397.

Paape, M., Mehrzad, J., Zhao, X., Detilleux, J., Burvenich, C., 2002. Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. J. Mammary Gland Biol. Neoplasia 7, 109–121.

Paape, M.J., Shafer-Weaver, K., Capuco, A.V., Van Oostveldt, K., Burvenich, C., 2000. Immune surveillance of mammary tissue by phagocytic cells. Adv. Exp. Med. Biol. 480, 259–277.

Paape, M.J., Wergin, W.P., Guidry, A.J., 1981. Phagocytic defense of the ruminant mammary gland. Adv. Exp. Med. Biol. 137, 555.

Revelo, X.S., Davis, J.W., Schnabel, R.D., Kenny, A.L., Barkley, N.M., Waldron, M.R., 2013. Effects of an immunomodulatory dietary supplement on the global gene expression profile of neutrophils from periparturient dairy cows. J. Dairy Sci. 96 (E-Suppl. 1), 485.

Ryman, V.E., Nickerson, S.C., Kautz, F.M., Hurley, D.J., Ely, L.O., Wang, Y.Q., Forsberg, N.E., 2013. Effect of dietary supplementation on the antimicrobial activity of blood leukocytes isolated from Holstein heifers. Res. Vet. Sci. 95, 969–974.

Ryman, V.E., Hurley, D.J., Ely, L.O., Kautz, F.M., Nickerson, S.C., Forsberg, N.E., Wang, Y.Q., Zanzalari, K., Chapman, J., 2012. Pre- and postpartum immunomodulatory effects of a dietary supplement on the immune system of dairy heifers. J. Anim. Sci. Vol. 90 (Suppl. 3/J. Dairy Science. Vol. 95, Suppl. 2. Pg 222. Abstr # 220).

SAS, 2013. Business and Analytical Software. SAS Institute, 100 SAS Campus Drive, Cary, NC, USA.

Smith, K.L., Todhunter, D.A., Schoenberger, P.S., 1985. Environmental mastitis: cause, prevalence, prevention. J. Dairy Sci. 68, 1531–1553.

van Werven, T., Noordhuizen-Stassen, E.N., Daemen, A.J.J.M., Schukken, Y.H., Brand, A., Burvenich, C., 1997. Preinfection in vitro chemotaxis, phagocytosis, oxidative burst, and expression of CD11/CD18 receptors and their predictive capacity on the outcome of mastitis induced in dairy cows with *Escherichia coli*. J. Dairy Sci. 80, 67–74.

Wang, Y.Q., Puntenney, S.B., Burton, J.L., Forsberg, J.L., Forsberg, N.E., 2007. Ability of a commercial feed additive to modulate expression of innate immunity in sheep immunosuppressed with dexamethasone. Animal: Int. J. Anim. Biosci. 1, 945–951.

Wang, Y.Q., Puntenney, S.B., Forsberg, N.E., 2004. Identification of the mechanisms by which OmniGen-AF®, a nutritional supplement, augments immune function in ruminant livestock. Proc., West. Sec., Am. Soc. Anim. Sci. 55, 349–435.

Weber, P.S., Toelboell, T., Chang, L.C., Tirrell, J.D., Saama, P.M., Smith, G.W., Burton, J.L., 2004. Mechanisms of glucocorticoid- induced down-regulation of neutrophil L-selectin in cattle: evidence for effects at the gene-expression level and primarily on blood neutrophils. J. Leukocyte Biol. 75, 815–827.

Wiggins, M.C., Woolums, A.R., Hurley, D.J., Sanchez, S., Ensley, D.T., Donovan, D.C., 2011. The effect of various *Mycoplasma bovis* isolates on bovine leukocyte responses. Comp. Immunol. Microbiol. Infect. Dis. 34, 49–54.

Zerbe, H., Schneider, N., Leibold, W., Wesing, T., Kruip, T.A.M., Schuberth, H.L., 2000. Altered functional and immunophenotypical properties of neutrophilic granulocytes in postpartum cows associated with fatty liver. Theriogenology 54, 771–786.