Effects of dietary zinc source on the metabolic and immunological response to lipopolysaccharide in lactating Holstein dairy cows

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ABSTRACT

The objectives of this study were to evaluate the effects of replacing 40 mg/kg of Zn from Zn sulfate (control; CON) with Zn AA complex (AvZn) on metabolism and immunological responses following an intravenous lipopolysaccharide (LPS) challenge in lactating cows. Cows were randomly assigned to 1 of 4 treatments: (1) pair-fed (PF) control (PF-CON; 5 mL of saline; n = 5), (2) PF AvZn (PF-AvZn; 5 mL of saline; n = 5), (3) LPS euglycemic clamp control (LPS-CON; 0.375 µg of LPS/kg of BW; n = 5), and (4) LPS euglycemic clamp AvZn (LPS-AvZn; 0.375 µg of LPS/kg of BW; n = 5). Cows were enrolled in 3 experimental periods (P). During period 1 (3 d), cows received their respective dietary treatments and baseline data were obtained. During period 2 (P2; 2 d), a 12-h LPS euglycemic clamp was conducted or cows were PF to their respective dietary counterparts. During period 3 (P3; 3 d), cows received their dietary treatment and consumed feed ad libitum. Mild hyperthermia (1°C) was observed in LPS cows at 3 h postbolus. Throughout P2, the rectal temperature of LPS-AvZn cows was decreased (0.3°C) relative to LPS-CON cows. Administering LPS decreased dry matter intake (47%) during P2, and by experimental design the pattern was similar in PF cohorts. During P3, dry matter intake from LPS cows remained decreased (15%) relative to PF cows. Milk yield from LPS cows decreased (54%) during P2 relative to PF cows, but it was similar during P3. During P2, somatic cell count increased 3-fold in LPS cows relative to PF controls. Dietary AvZn tended to decrease somatic cell count (70%) during P3 relative to LPS-CON cows. Insulin increased 7-fold in LPS cows at 12 h postbolus and remained increased (4-fold) for the duration of P2. Circulating glucagon from LPS cows increased (65%) during P2, and supplementing AvZn

blunted the increase (30% relative to LPS-CON). During P2, circulating cortisol increased 7-fold post-LPS infusion relative to PF cows, and supplementing AvZn decreased cortisol (58%) from 6 to 48 h postbolus relative to LPS-CON cows. Administering LPS increased circulating LPS-binding protein and serum amyloid A (3- and 9-fold, respectively) relative to PF cows. Compared with LPS-CON, LPS-AvZn cows had increased circulating serum amyloid A (38%) 24 h postbolus. The 12-h total glucose deficit was 36 and 1,606 g for the PF and LPS treatments, respectively, but was not influenced by Zn source. In summary, replacing a portion of the Zn sulfate with Zn AA complex appeared to reduce the inflammatory response but had no effect on the glucose deficit.

Key words: leukocyte, cortisol, somatic cell count

INTRODUCTION

Uterine, mammary, and intestinal epithelial integrity can become compromised due to a variety of stressors (Khafipour et al., 2009; Kvidera et al., 2017c; Koch et al., 2019), and hyperpermeability of these barriers permits pathogen infiltration and, ultimately, immune stimulation (van Miert and Frens, 1968). Regardless of the pathogen's origin, a robust and transient immune response is imperative to successful pathogen clearance, resolution of inflammation, and return to optimal production (i.e., milk production, growth, and reproduction; Bertoni et al., 2008; Bradford et al., 2015; Trevisi and Minuti, 2018). Mounting a successful immune response is dependent on effective leukocyte communication (cytokine and chemokine production), extravasation, cellular function (i.e., intracellular killing, reactive oxygen species production, cytokine production), and the elimination of cell debris and harmful byproducts. The energetic costs of initiating an immune response are substantial (Johnson, 2012), and this is particularly true for glucose (Kvidera et al., 2016, 2017a,b; Horst et al., 2018). Therefore, dietary strategies capable of improving barrier integrity and immune responses are

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of practical and economic interest, particularly in lactating dairy cows because glucose is critical for copious milk synthesis (Baumgard et al., 2017).

Zinc plays a pivotal role as a structural, catalytic, and signaling component. Although it has multiple responsibilities, its immunomodulatory effects have received considerable attention (Osorio et al., 2016; Wessels et al., 2017). Zinc is required for proper epithelial barrier function and regulates damaged epithelium regeneration (Alam et al., 1994). Furthermore, leukocyte formation, maturation and differentiation, activation, cellular function, and apoptotic rate (among others) are Zn dependent and are largely influenced by changes in Zn homeostasis (Haase and Rink, 2014; Wessels and Cousins, 2015). Thus, providing dietary sources of Zn (with optimal bioavailability) may benefit leukocyte function and communication and consequently could influence the overall energetic cost of immunoactivation. Research suggests that organic trace minerals improve bioavailability and mineral retention compared with inorganic forms (Spears, 1989). Further, altered cytokine and acute phase protein (APP) production have been observed with supplemental complexed Zn in ruminants and monogastrics compared with inorganic forms (Abuajamieh et al., 2016; Mayorga et al., 2018).

We hypothesized that feeding complexed Zn would improve the immune response to an LPS challenge and alter leukocyte glucose consumption. Therefore, the objectives of this study were to evaluate the effects of supplemental Zn AA complex on productivity, metabolism, glucose consumption, and circulating leukocyte dynamics and function in an acute and intensely activated immune system via the LPS euglycemic clamp technique.

MATERIALS AND METHODS

Animals and Experimental Design

All procedures were approved by the Iowa State University Institutional Animal Care and Use Committee. Twenty nonpregnant lactating Holstein cows (715 ± 19 kg of BW; 284 ± 29 DIM; parity 2 ± 0.2) were used in an experiment conducted in 2 replications (10 cows/replicate). Cows were randomly assigned to 1 of 2 dietary treatments: (1) control (CON; 64 mg/kg of supplemental Zn from ZnSO_4) or (2) replacement of 40 ppm of ZnSO_4 with Zn AA complex (AvZn; Availa-Zn, Zinpro Corporation, Eden Prairie, MN). Actual dietary Zn concentrations (Dairyland Laboratories Inc., Arcadia, WI) were 111 and 112 mg/kg for the CON and AvZn diets, respectively. Before the start of the study (i.e., before cows were moved into individual pens), cows were fed their respective diets for 42 d at the

Iowa State University Dairy Farm (Ames). After the initial feeding phase, cows were moved to individual box stalls (4.57×4.57 m) at the Iowa State University Dairy. Cows were allowed 4 d to acclimate to housing conditions; during this time they were implanted with bilateral jugular catheters as previously described (Baumgard et al., 2011). The trial consisted of 3 experimental periods. Period 1 (P1) lasted 3 d and served as the baseline (data generated for covariate analysis) for period 2 (P2) and period 3 (P3). At the initiation of P2, which lasted 48 h, animals either experienced a 12-h LPS euglycemic clamp as previously described (Kvidera et al., 2016, 2017a; Horst et al., 2018) or were pair-fed (PF) to their respective dietary counterparts for the entire 48 h to eliminate the confounding effects of dissimilar nutrient intake. Dietary and challenged combinations resulted in 4 treatments: (1) PF control diet (PF-CON; 5 mL of sterile saline with CON diet; $n = 5$), (2) PF Availa-Zn diet (PF-AvZn; 5 mL of sterile saline with AvZn diet; $n = 5$), (3) LPS euglycemic clamp control diet (LPS-CON; $0.375 \mu\text{g}$ of LPS/kg of BW with CON diet; $n = 5$), and (4) LPS euglycemic clamp Availa-Zn diet (LPS-AvZn; $0.375 \mu\text{g}$ of LPS/kg of BW with AvZn diet; $n = 5$). During P2, cows receiving LPS were allowed to eat ad libitum, but the saline-infused cows were pair-fed to their dietary counterparts to eliminate the confounding effects of dissimilar feed intake. For pair-feeding calculations, feed intake was averaged for each cow and used as a baseline. During P2, the decrease in feed intake in LPS-CON and LPS-AvZn cows was calculated every day as a percentage of feed intake reduction during P1. The percentage of feed intake reduction was averaged for all cows in the LPS-CON and LPS-AvZn treatments on the 2 d of P2 and applied individually to the baseline of each cow in the PF-CON and PF-AvZn treatments. During P3 (3 d), all animals continued to receive their dietary treatment but were allowed to consume feed ad libitum.

All cows were fed a diet formulated to meet or exceed the predicted requirements of energy, protein, minerals, and vitamins (NRC, 2001; Tables 1 and 2). Reduced feed intake in LPS-treated cows during P2 was determined as a percentage of their mean daily ad libitum intake during P1. Throughout the experiment, PF cows lagged 13 d behind LPS-infused cows to allow for pair-feeding calculations as we have previously described (Baumgard et al., 2011). To more accurately mirror the altered feed intake pattern observed in LPS-administered cows, the amount of feed offered to the PF cows was separated into 3 portions: 10% at 1200 h, 30% at 1800 h, and 60% at 0000 h on d 1 of P2. On d 2 of P2 the feed was provided as 2 equal meals at 0600 and 1800 h. Cows were milked 4 times daily (0000, 0600, 1200, and 1800 h), and yield was recorded. A

sample for composition analysis was obtained at each milking. Samples were stored at 4°C with a preservative (bronopol tablet; D and F Control System, San Ramon, CA) until analysis by Dairy Lab Services (Dubuque, IA) using AOAC-approved infrared analysis equipment and procedures (AOAC International, 1995). Rectal temperature (**Tr**) and respiration rate (**RR**) were recorded after each milking. Rectal temperature was measured using a digital thermometer (GLA M700 digital thermometer, GLA Agricultural Electronics, San Luis Obispo, CA). Respiration rate was measured as flank movements during a 15-s interval and later transformed to breaths per minute.

Table 1. Ingredient and analyzed chemical composition (% of DM unless noted) of the diet¹

Item	Value
Ingredient	
Corn silage	26.5
Ground corn	25.7
Alfalfa hay	19.8
Corn gluten feed	8.8
Expeller soybean ²	4.5
Soybean meal	4.5
Mineral mix	4.2
Whole cottonseed	3.5
Molasses supplement ³	1.7
Bypass fat ⁴	0.8
Chemical analysis	
DM (%)	47.5
Starch	25.5
CP	16.7
NDF	32.3
ADF	21.8
Ether extract	4.83
Ca	0.96
P	0.43
S	0.23
K	1.41
Na	0.48
Cl	0.66
NE _L (Mcal/kg of DM)	1.62
Vitamin A (IU/kg of DM)	6,733.3
Vitamin D (IU/kg of DM)	636.3
Vitamin E (IU/kg of DM)	18.7

¹Values represent an average of ration nutrient summary reports collected throughout the trial. Average values between both diets containing dietary treatments of 64 mg/kg Zn from ZnSO₄ (control diet) or 40 mg/kg Zn from Availa-Zn (Zinpro Corporation, Eden Prairie, MN) plus 24 mg/kg Zn from ZnSO₄ (AvZn diet). All diets contained 40 mg/kg Mn from Mn sulfate, 6.5 mg/kg Mn from Availa-Mn (manganese AA complex; Zinpro Corporation), 10.1 mg/kg Cu from copper sulfate, 4.1 ppm of Cu from Availa-Cu (copper AA complex; Zinpro Corporation), 0.79 mg/kg Co from Copro (cobalt glucoheptonate; Zinpro Corporation), 0.8 mg/kg I from ethylenediamine dihydroiodide, and 0.3 mg/kg Se, with 50% from sodium selenite and 50% from selenium yeast. Analyzed Zn concentrations were 111 and 112 mg/kg for the control and AvZn diets, respectively.

²Mechanically processed soybean meal (Dairy Nutrition Plus, Ralston, IA).

³Quality Liquid Feeds (Dodgeville, WI).

⁴MagnaPalm (Energy Feeds International, Lago Vista, TX).

Table 2. Ingredients and composition (% of DM) of the vitamin and mineral mix for the diets¹

Ingredient	Diet ²	
	Control	AvZn
Calcium carbonate	28.78	28.70
Sodium carbonate	20.50	20.50
Blood meal	20.00	20.00
Salt	9.59	9.59
Magnesium oxide	4.53	4.53
Urea	4.01	4.01
Dried distillers grains with solubles	3.05	2.57
Potassium chloride	2.40	2.40
Yeast culture ³	1.61	1.61
Liquid methionine hydroxy analog	1.19	1.19
Rumen-protected methionine ⁴	1.02	1.02
Mineral oil	1.00	1.00
Availa-Zn 120 ⁵	0.00	0.83
Zinc sulfate	0.45	0.17
Manganese sulfate	0.31	0.31
Selenium premix 0.16%	0.26	0.26
Selenium yeast 2000	0.21	0.21
Availa-Mn 80 ⁵	0.20	0.20
Vitamin E 50%	0.19	0.19
Rumensin 90.7 ⁶	0.18	0.18
Availa-Cu 100 ⁵	0.10	0.10
Dairy trace mineral mix	0.10	0.10
Copper sulfate	0.10	0.10
Biotin 2%	0.09	0.09
Copro 25 ⁵	0.08	0.08
Vitamin A 650 kIU	0.05	0.05
Vitamin D ₃ 500 kIU	0.01	0.01
Ethylenediamine dihydroiodide-99%	0.002	0.002

¹Average nutrient levels were as follows. Control diet: 30.6% CP, 11.33% Ca, 0.06% P, 2.62% Mg, 1.35% K, 0.50% S, 19.6 mg/kg Co from Copro 25 (Zinpro Corporation, Eden Prairie, MN), 19.6 mg/kg Co total, 101 mg/kg Cu from Availa-Cu 100 (Zinpro Corporation), 351 mg/kg Cu total, 19.6 mg/kg I total, 160.7 mg/kg Mn from Availa-Mn 80 (Zinpro Corporation), 1,152 mg/kg Mn total, 4.22 mg/kg Se from selenium yeast, 8.43 mg/kg Se total, 0 mg/kg Zn from Availa-Zn (Zinpro Corporation), 1,588.68 mg/kg Zn total, 17.71 mg/kg Biotin (DSM Nutritional Products, Ames, IA), 33,634 kIU/kg vitamin A, 31.79 kIU/kg vitamin D₃, 937 IU/kg vitamin E, and 0.321 g/kg monensin. AvZn diet: 30.7% CP, 11.33% Ca, 0.06% P, 2.62% Mg, 1.35% K, 0.47% S, 19.6 mg/kg Co from Copro 25, 19.6 mg/kg Co total, 101 mg/kg Cu from Availa-Cu 100, 351 mg/kg Cu total, 19.6 mg/kg I total, 160.7 mg/kg Mn from Availa-Mn, 1,152 mg/kg Mn total, 4.22 mg/kg Se from selenium yeast, 8.43 mg/kg Se total, 1,000 mg/kg Zn from Availa-Zn, 1,588.68 mg/kg Zn total, 17.71 mg/kg Biotin, 33,634 kIU/kg vitamin A, 31.79 kIU/kg vitamin D₃, 937 IU/kg vitamin E, and 0.321 g/kg monensin.

²Control = 64 mg/kg Zn from ZnSO₄. AvZn = 40 mg/kg Zn from Availa-Zn plus 24 mg/kg Zn from ZnSO₄.

³NutriTek (Diamond V, Cedar Rapids, IA).

⁴Smartamine-M (Adisseo, Alpharetta, GA).

⁵Availa-Zn, Availa-Mn, and Availa-Cu = Zn, Mn, and Cu AA complexes, respectively; Copro = cobalt glucoheptonate.

⁶Rumensin-90 (Elanco, Greenfield, IN).

To estimate the glucose requirements of an intensely activated immune system, we employed the LPS euglycemic clamp technique as recently described (Kvidera et al., 2016, 2017a; Horst et al., 2018). Selecting the LPS dose was influenced by the magnitude of hypoglycemia observed in earlier reports (Giri et al., 1990;

Waldron et al., 2003; Waggoner et al., 2009; Kvidera et al., 2017a). Lipopolysaccharide (*Escherichia coli* O55:B5; Sigma-Aldrich, St. Louis, MO) was dissolved in sterile saline at a concentration of 66.6 µg/mL and passed through a 0.2-µm sterile syringe filter (Thermo Scientific, Waltham, MA). The total volume of LPS solution administered was approximately 4 mL and was infused within approximately 30 s. Maintaining the euglycemic clamp involved infusing 50% glucose (as dextrose; VetOne, Boise, ID) intravenously at a known and adjustable rate using a modular pump (Deltec 3000, Deltec Inc., St. Paul, MN) to maintain the pre-LPS administration blood glucose concentrations.

Blood samples were obtained at −30, −20, and 0 min relative to LPS or saline bolus administration to establish baseline glucose levels. Each respective treatment bolus was administered immediately following the 0-min blood sample collection. For cows receiving LPS, postbolus blood samples (1 mL) were collected every 10 min and immediately analyzed for glucose concentration (Truebalance glucometer; McKesson, San Francisco, CA). Glucose infusion began when blood glucose content decreased below baseline levels, and infusion rates were adjusted as necessary to maintain euglycemia ($\pm 5\%$ of baseline). The rate of 50% glucose infusion (mL/h) was transformed to rate of glucose infusion (ROGI; g/h). The total glucose infused for each cow was calculated using the rate of glucose infusion for each 10-min interval (72 intervals in total) according to the following equation:

$$\sum_{i=0}^{72} \text{ROGI(g/h)}_i \times \frac{1 \text{ h}}{60 \text{ min}} \times 10 \text{ min.}$$

Blood glucose was measured every hour in PF cows. Vital measurements (Tr and RR) were obtained at −0.5 and 0 h relative to LPS administration every 3 h for the first 12 h postbolus and every 6 h thereafter. Additional blood samples were collected daily at 1800 h during P1 from the catheter and divided equally between a tube containing K₂EDTA (BD, Franklin Lakes, NJ; for plasma collection) and an empty glass tube (for serum collection). Additional plasma and serum samples (~10 mL each) were collected from all treatments at −0.5, −0.33, 0, 3, 6, 9, 12, 18, 24, 30, 36, 42, and 48 h relative to bolus administration. Serum samples were allowed to clot at room temperature for 1 h before centrifugation. Plasma and serum were harvested following centrifugation at $1,500 \times g$ for 15 min at 4°C and were subsequently frozen at −20°C until analysis.

Serum cortisol and plasma insulin, glucagon, non-esterified fatty acids (NEFA), BHB, LPS-binding protein (LBP), serum amyloid A (SAA), BUN, glucose,

and lactose concentrations were determined using commercially available kits according to the manufacturers' instructions (cortisol: Enzo Life Sciences Inc., Farmingdale, NY; insulin: Mercodia AB, Uppsala, Sweden; glucagon: RD Systems Inc., Minneapolis, MN; NEFA: Wako Chemicals USA, Richmond, VA; BHB: Pointe Scientific Inc., Canton, MI; LBP: Hycult Biotech, Uden, the Netherlands; SAA: Tridelta Development Ltd., Kildare, Ireland; BUN: Teco Diagnostics, Anaheim, CA; glucose: Wako Chemicals USA; lactose: BioVision Inc., Milpitas, CA). The inter- and intra-assay coefficients of variation for cortisol, insulin, glucagon, NEFA, BHB, BUN, glucose, LBP, SAA, and lactose were 7.6 and 9.2%, 6.8 and 6.0%, 7.7 and 9.8%, 6.4 and 2.7%, 7.0 and 6.2%, 4.2 and 4.5%, 3.3 and 2.0%, 16.1 and 6.4%, 12.9 and 6.8%, and 7.9 and 4.4%, respectively.

Samples for complete blood count analysis were collected at −0.5, −0.33, 0, 3, 6, 9, 12, 18, 24, 30, 36, 42, and 48 h relative to bolus administration. A 3-mL blood sample was obtained from the catheter and stored at 4°C for approximately 12 h before submission to the Iowa State University's Department of Veterinary Pathology for analysis. Samples for evaluating blood physiology were measured using an iSTAT hand-held machine and cartridge (CG8+; Abbott Point of Care, Princeton, NJ) and were obtained at 0, 12, 24, and 48 h relative to bolus administration.

Neutrophil Isolation and Functional Assays

Neutrophil isolation and functional assay techniques were performed on P1 d 5 and at 12, 24, and 48 h relative to LPS administration using previously described methods (Kimura et al., 2014). In brief, blood samples (32 mL) for neutrophil isolation were collected into 50-mL conical tubes containing acid citrate dextrose (8 mL) and immediately transported to the laboratory for neutrophil isolation. Samples were centrifuged at $1,000 \times g$ for 20 min at room temperature and the plasma, buffy coat, and upper portion of packed red blood cells were removed. The remaining red blood cells were lysed twice and neutrophils were pelleted following centrifugation at $300 \times g$ for 5 min. Neutrophils were resuspended in phosphate-buffered saline and standardized to 5.0×10^7 cells/mL. Neutrophil function was assessed by extracellular release of myeloperoxidase (MPO) and oxidative burst (cytochrome C reduction).

To assess oxidative burst, 2.5×10^6 polymorphonuclear leukocytes (PMN) were treated with either Hanks' balanced salt solution (HBSS) or HBSS plus phorbol myristate acetate followed by treatment with cytochrome C. Cells were incubated at 39°C for 15 min and immediately read at 2 wavelengths (550 and 650 nm) using a spectrophotometer.

Three cell preparations were used to assess MPO activity: (1) cells were lysed by treatment with cetyltrimethylammonium bromide solution as a measure of total MPO, (2) PMN were stimulated with equal parts calcium ionophore A23187 and cytochalasin B in HBSS to assess the release of MPO with stimulation, and (3) PMN treated with HBSS alone as a measure of unstimulated MPO release. Each preparation was loaded with 1.25×10^6 cells in a microtiter plate and incubated at 39°C for 20 min. Following incubation, 50 μ L of 3,3',5,5'-tetramethylbenzidine dihydrochloride (3.25 mM) was added per well followed promptly with 50 μ L of H₂O₂ per well. The reaction was stopped after a 2-min incubation with the addition of 50 μ L of 2 N H₂SO₄ per well. Plates were centrifuged for 1 min at $600 \times g$ and the supernatant was transferred to a second plate. Optical density (OD) was determined at 405 nm using a spectrophotometer. Percentage of MPO released from PMN was determined using the following equation:

$$\text{exocytosis (\%)} = \frac{(\text{OD of stimulated PMN})}{(\text{OD of lysed PMN})} \times 100.$$

Calculations and Statistical Analysis

Administering LPS reduces milk yield and therefore decreases glucose utilized for milk synthesis. The decrease in milk yield allows us to estimate the amount of glucose conserved (milk glucose deficit) due to reduced feed intake alone (PF-CON and PF-AvZn cows) and decreased feed intake coupled with ostensible glucose utilization by the immune system (LPS-CON and LPS-AvZn cows). The amount of glucose utilized for milk synthesis was calculated for each milking based on Kronfeld's (1982) estimation of 72 g of glucose required to synthesize 1 kg of milk. Milk glucose output before the challenge was averaged to establish a baseline. Milk glucose utilization at both 6 and 12 h was subtracted from the baseline to calculate the milk glucose deficit. For PF-CON and PF-AvZn cows, milk glucose deficit was solely used to calculate total glucose deficit. For LPS-CON and LPS-AvZn cows, milk glucose deficit plus the amount of glucose infused to maintain euglycemia were combined to obtain the total glucose deficit. After cessation of the euglycemic clamp (12 h postbolus), milk glucose deficit continued to be calculated every 6 h until 48 h postbolus.

Sample size determination (5 animals/treatment) was based on previous reports and logistical constraints (Kvidera et al., 2017a; Horst et al., 2018). Each animal's respective parameter was analyzed using repeated measures with an autoregressive covariance

structure for milk yield and composition and DMI and a spatial power law for Tr and RR, iSTAT, complete blood count, neutrophil assays, and blood parameters. The repeated effect was time relative to LPS administration. There were no treatment differences due to diet in any parameter during P1; thus, each specific variable's prebolus values (i.e., P1 average, when available) served as a covariate for analysis of P2 and P3 (each period was statistically analyzed separately). Effects of treatment, time (hours or days relative to bolus), and treatment $\times$ time interactions were assessed as a completely randomized design using PROC MIXED (SAS Institute Inc., Cary, NC). A separate analysis was used to make statistical comparisons with baseline in which the average P1 value for each parameter was included as an additional time point. Preplanned contrasts were used to estimate differences between CON- and AvZn-supplemented cows, PF- and LPS-administered cows, and LPS-CON and LPS-AvZn cows, and these multiple comparisons were not mathematically adjusted for (Rothman, 1990). Data are represented as least squares means $\pm$ standard error of the mean and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$.

RESULTS

Mild hyperthermia (1°C, relative to PF cows; $P = 0.02$) was observed in LPS-administered cows at 3 h postbolus. Throughout P2, Tr of LPS-AvZn cows was decreased (0.3°C) relative to that of LPS-CON cows ($P = 0.01$; Figure 1A). No treatment differences were observed in Tr during P3. No treatment differences were observed in RR during P2 (data not shown). However, during P3, RR of AvZn-supplemented cows (LPS and PF) tended to be increased (5 breaths/min; $P = 0.06$) from 60 to 72 h postbolus, whereas RR of CON-supplemented cows (LPS and PF) remained the same (data not shown).

Lipopolysaccharide administration decreased DMI (47%; $P \leq 0.05$) relative to baseline, and the pattern and extent of reduced DMI were similar for cows in the PF treatments by experimental design ($P > 0.82$; Figure 1B). Dry matter intake increased similarly from P2 d 1 to P2 d 2 for all treatments ($P < 0.01$). During P3, DMI of LPS-administered cows remained decreased (12%; $P < 0.01$) relative to PF controls. Zinc source did not have a detectable effect on DMI.

Despite similar DMI, milk yield from LPS-administered cows decreased (54%; $P < 0.01$; Figure 1C) throughout P2 and was decreased 69 and 38% on d 1 and 2 of P2 relative to PF controls, respectively ($P < 0.01$). No dietary effect was observed on daily milk yield. When evaluated on an hourly basis during P3,

Figure 1. Effects of an LPS bolus or pair-feeding in dairy cows fed a control diet or a diet supplemented with a Zn AA complex on (A) rectal temperature, (B) DMI, (C) milk yield, and (D) milk SCC. Cows were randomly assigned to 1 of 2 dietary treatments: (1) control (CON; 64 mg/kg supplemental Zn from ZnSO_4) or (2) replacement of 40 mg/kg ZnSO_4 with Zn AA complex (AvZn; Availa-Zn, Zinpro Corporation, Eden Prairie, MN). At the initiation of period 2 (P2), which lasted 48 h, animals either experienced a 12-h LPS euglycemic clamp as previously described (Kvidera et al., 2016, 2017a; Horst et al., 2018) or were pair-fed (PF) to their respective dietary counterparts for the entire 48 h to eliminate the confounding effects of dissimilar nutrient intake. Dietary and challenged combinations resulted in 4 treatments: (1) PF control diet (PF-CON; 5 mL of sterile saline with CON diet; $n = 5$), (2) PF Availa-Zn diet (PF-AvZn; 5 mL of sterile saline with AvZn diet; $n = 5$), (3) LPS euglycemic clamp control diet (LPS-CON; 0.375 μg of LPS/kg of BW with CON diet; $n = 5$), and (4) LPS euglycemic clamp Availa-Zn diet (LPS-AvZn; 0.375 μg of LPS/kg of BW with AvZn diet; $n = 5$). Time 0 represents an average of measurements obtained during the baseline and used as a covariate for P2 and period 3 (P3) analysis. The solid line separates P2 from P3 and represents 2 separate statistical analyses. Fixed effects of treatment (Trt), time, and treatment $\times$ time were assessed as a completely randomized design using PROC MIXED of SAS (SAS Institute Inc., Cary, NC). Data are represented as LSM $\pm$ SEM and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. When < 0.10 , the P -value for the fixed effects and the preplanned contrasts are listed on the figure.

milk yield from LPS-administered cows remained decreased (8%; $P = 0.01$; data not shown) relative to PF cows. When P3 milk yield was condensed to daily averages, no treatment differences were observed ($P > 0.48$), although milk yield increased in all treatments with time ($P < 0.01$). During P2 and P3, the rate of milk yield recovery (as measured by slope) was increased in LPS-administered cows relative to PF controls ($P < 0.01$; Table 3). The slope of milk yield recovery following LPS administration (for 72 h) increased (49%) in LPS-AvZn cows relative to LPS-CON cows ($P = 0.05$; Table 3).

Milk urea nitrogen in LPS-infused cows gradually increased between 12 and 30 h postbolus, whereas it decreased in PF controls ($P = 0.05$; Table 3). Milk urea nitrogen of LPS-AvZn cows tended to be increased (11%) relative to LPS-CON cows ($P = 0.09$; Table 3). During P3 there was a treatment $\times$ time interaction for LPS-CON cows, such that MUN content remained constant from 54 to 78 h postbolus, whereas in all other treatments it gradually decreased ($P < 0.01$; Table 3). Throughout P3, MUN from LPS-AvZn cows tended to be decreased (10%) relative to LPS-CON cows ($P = 0.06$; Table 3). During P2, LPS administration decreased milk lactose content (17%; $P < 0.01$; Table 3) and increased milk fat and protein concentrations (32 and 19%, respectively; $P < 0.01$; Table 3) relative to PF cows. Milk lactose content of all treatments increased throughout P3 ($P = 0.01$; Table 3). Overall during P3, milk fat content from LPS-administered cows remained increased (11%) relative to PF controls ($P = 0.04$; Table 3). Milk fat and protein content of all treatments gradually returned to baseline with time ($P < 0.01$). Other than slightly increasing MUN in LPS-AvZn cows, AvZn supplementation had little to no effect on milk composition during P2 (Table 3).

Milk SCC from LPS-administered cows tended to increase with time, whereas in PF cows it remained unchanged ($P = 0.10$; Figure 1D). During P3, no treatment differences were observed in SCC; however, post hoc analysis revealed that SCC of LPS-AvZn cows tended to be decreased (70%) from 54 to 78 h postbolus relative to LPS-CON cows ($P = 0.10$; Figure 1D).

Hyperglycemia occurred in LPS-infused cows for approximately 200 min postbolus, after which continuous glucose infusion was necessary to maintain euglycemia (Figure 2A). Rate of glucose infusion did not differ due to dietary Zn source, but did increase with time ($P < 0.01$; Figure 2A). For the first 70 min of the hyperglycemic phase, LPS-AvZn cows tended to have decreased (19%) circulating glucose relative to LPS-CON cows ($P = 0.09$; Figure 2A). Relative to PF cows, the 48-h milk glucose deficit was increased (75%) in LPS-administered cows throughout P2 ($P < 0.01$; data not shown).

Similarly, the total glucose deficit was 44-fold more in LPS-administered cows relative to PF cows. Total glucose deficit accumulated over the 12 h was 5, 67, 1,500, and 1,712 g for PF-CON, PF-AvZn, LPS-CON, and LPS-AvZn cows, respectively (Figure 2B). Dietary Zn source did not affect the glucose deficit metrics.

For the duration of glucose infusion, circulating insulin in LPS-administered cows was 7-fold more than that in PF controls ($P = 0.05$), and it remained increased 5-fold after the 12 h-infusion period ($P < 0.01$; Figure 3A). Although not significant ($P > 0.41$), insulin concentrations were numerically decreased (51%) in LPS-AvZn cows from 3 to 12 h relative to LPS-CON cows (Figure 3A). Circulating glucagon in LPS-administered cows increased (65%) throughout P2 relative to PF controls ($P < 0.01$; Figure 3B). Supplementing AvZn decreased glucagon concentrations (30%) following LPS administration relative to LPS-CON cows ($P = 0.02$; Figure 3B). From 3 to 12 h postbolus, circulating NEFA in LPS-AvZn cows remained similar to baseline levels, whereas in all other treatments it gradually increased ($P = 0.02$; Figure 3C). Throughout P2, circulating NEFA from LPS-infused cows decreased (39%) relative to PF controls ($P = 0.05$; Figure 3C). Circulating BHB in LPS-administered cows decreased relative to the PF controls during P2 ($P < 0.01$; Figure 3D). Blood urea nitrogen concentrations in LPS-infused cows increased (29%) during P2 relative to PF controls ($P < 0.01$). From 12 to 24 h postbolus, circulating BUN from LPS treatments continued to increase, whereas in PF cows it gradually decreased ($P < 0.01$; Table 4). During P2, plasma lactose concentrations from LPS cows increased (26%) relative to PF cows ($P = 0.05$; Table 4). Following LPS administration, ionized Ca markedly decreased (33% at 12 h postbolus) and it gradually returned to baseline, whereas in PF controls ionized Ca levels remained similar to baseline concentrations ($P < 0.01$; Figure 3E). Interestingly, ionized Ca from LPS-AvZn cows tended to be increased (19%) relative to that from LPS-CON cows at 12 h postbolus ($P = 0.10$).

Circulating LBP from LPS-administered cows increased 3-fold ($P < 0.01$; Figure 4A) throughout P2 relative to PF controls. Similarly, circulating SAA increased in LPS-challenged cows with time, whereas in PF controls it remained similar to baseline (9-fold increase relative to PF; $P < 0.01$; Figure 4B). At 24 h postbolus, circulating SAA from LPS-AvZn cows was increased (38%) relative to LPS-CON cows ($P = 0.03$). Post hoc analysis revealed increased SAA from 12 to 24 h postbolus (43%; $P = 0.05$) in LPS-AvZn cows relative to LPS-CON cows (Figure 4B). Administering LPS increased circulating cortisol 7-fold throughout P2 relative to PF cows ($P < 0.01$; Figure 4C), and supplementing AvZn decreased cortisol (37%) from 6

Table 3. Milk yield recovery and composition from cows fed a control diet or a diet supplemented with a Zn AA complex following administration of saline or LPS

Parameter	Treatment ¹				P-value			Contrast			
	PF-CON	PF-AvZn	LPS-CON	LPS-AvZn	SEM	Treatment	Time	Treatment × time	CON vs. AvZn	LPS vs. PF	LPS-CON vs. LPS-AvZn
Milk yield <i>m</i> ² (kg/d)											
d 1–2	−0.77 ^a	−0.97 ^a	5.31 ^b	8.75 ^b	1.93	0.01			0.41	<0.01	0.21
d 1–3	0.61 ^a	0.92 ^a	6.49 ^b	9.66 ^c	1.06	<0.01			0.12	<0.01	0.05
d 1–4	1.15 ^a	1.45 ^a	5.94 ^b	7.56 ^b	0.89	<0.01			0.30	<0.01	0.20
d 1–5	1.03 ^a	1.73 ^a	5.19 ^b	6.04 ^b	0.82	<0.01			0.36	<0.01	0.46
d 2–4	1.97 ^a	2.43 ^a	5.97 ^b	6.47 ^b	1.26	0.05			0.71	0.01	0.78
d 2–5	1.39 ^a	2.37 ^a	4.83 ^b	4.73 ^b	1.03	0.08			0.67	0.01	0.95
P ² milk parameters											
Fat (%)	3.95 ^a	4.13 ^a	5.22 ^b	5.40 ^b	0.31	<0.01	<0.01	0.12	0.57	<0.01	0.68
Lactose (%)	4.75 ^b	4.76 ^b	3.94 ^a	3.99 ^a	0.11	<0.01	0.01	0.05	0.76	<0.01	0.73
Protein (%)	3.21 ^a	3.32 ^a	4.01 ^b	3.75 ^b	0.14	<0.01	<0.01	<0.01	0.61	<0.01	0.19
MUN (mg/dL)	12.62	12.95	12.89	14.32	0.69	0.14	<0.01	0.05	0.22	0.41	0.09
P ³ milk parameters											
Fat (%)	3.80	3.79	4.32	4.05	0.17	0.14	<0.01	0.98	0.43	0.04	0.28
Lactose (%)	4.72	4.77	4.56	4.69	0.07	0.19	0.01	0.43	0.17	0.13	0.16
Protein (%)	3.23	3.30	3.39	3.19	0.14	0.76	<0.01	0.22	0.64	0.87	0.32
MUN (mg/dL)	10.59	10.13	10.70	9.62	0.49	0.15	<0.01	0.01	0.11	0.75	0.06

^{a-c}Values within a row with different superscripts are different ($P < 0.05$) between treatments.

¹Cows were randomly assigned to 1 of 2 dietary treatments: (1) control (CON; 64 mg/kg supplemental Zn from ZnSO₄) or (2) replacement of 40 mg/kg ZnSO₄ with Zn AA complex (AvZn; Availa-Zn, Zinpro Corporation, Eden Prairie, MN). At the initiation of period 2, which lasted 48 h, animals either experienced a 12-h LPS euglycemic clamp as previously described (Kvidera et al., 2016, 2017a; Horst et al., 2018) or were pair-fed (PF) to their respective dietary counterparts for the entire 48 h to eliminate the confounding effects of dissimilar nutrient intake. Dietary and challenged combinations resulted in 4 treatments: (1) PF control diet (PF-CON; 5 mL of sterile saline with CON diet; n = 5), (2) PF Availa-Zn diet (PF-AvZn; 5 mL of sterile saline with AvZn diet; n = 5), (3) LPS euglycemic clamp control diet (LPS-CON; 0.375 µg of LPS/kg of BW with CON diet; n = 5), and (4) LPS euglycemic clamp Availa-Zn diet (LPS-AvZn; 0.375 µg of LPS/kg of BW with AvZn diet; n = 5).

²Milk yield slope after LPS or saline administration.

³During period 2 (P2), cows either were pair-fed or experienced a 12-h LPS euglycemic clamp.

⁴During period 3 (P3), cows were fed ad libitum.

Figure 2. Effects of an LPS bolus or pair-feeding in dairy cows fed a control diet or a diet supplemented with a Zn AA complex on (A) circulating glucose and rate of glucose infusion (ROGI) and (B) milk or total glucose deficit (TGD) from 0 to 6 h, from 6 to 12 h, and accumulated over 12 h. Cows were randomly assigned to 1 of 2 dietary treatments: (1) control (CON; 64 mg/kg supplemental Zn from ZnSO_4) or (2) replacement of 40 mg/kg ZnSO_4 with Zn AA complex (AvZn; Availa-Zn, Zinpro Corporation, Eden Prairie, MN). At the initiation of period 2, which lasted 48 h, animals either experienced a 12-h LPS euglycemic clamp as previously described (Kvidera et al., 2016, 2017a; Horst et al., 2018) or were pair-fed (PF) to their respective dietary counterparts for the entire 48 h to eliminate the confounding effects of dissimilar nutrient intake. Dietary and challenged combinations resulted in 4 treatments: (1) PF control diet (PF-CON; 5 mL of sterile saline with CON diet; $n = 5$), (2) PF Availa-Zn diet (PF-AvZn; 5 mL of sterile saline with AvZn diet; $n = 5$), (3) LPS euglycemic clamp control diet (LPS-CON; 0.375 μg of LPS/kg of BW with CON diet; $n = 5$), and (4) LPS euglycemic clamp Availa-Zn diet (LPS-AvZn; 0.375 μg of LPS/kg of BW with AvZn diet; $n = 5$). Fixed effects of treatment (Trt), time, and treatment $\times$ time were assessed as a completely randomized design using PROC MIXED of SAS (SAS Institute Inc., Cary, NC). Data are represented as LSM $\pm$ SEM and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. When < 0.10 , the P -value for the fixed effects and the preplanned contrasts are listed on the figure.

to 48 h postbolus relative to LPS-CON cows ($P = 0.04$; Figure 4C).

Circulating white blood cells from LPS-infused cows were initially decreased between 3 and 12 h postbolus (51%) relative to PF controls ($P < 0.01$; Table 5), after which cows from the LPS treatments developed leukocytosis (36% increase relative to PF; $P < 0.01$).

Relative to PF-CON cows, circulating white blood cell counts of PF-AvZn cows tended to be increased (31%) at 30 h postbolus ($P = 0.09$; Table 5). Circulating neutrophils reflected a similar biphasic response ($P < 0.01$; Figure 5A). Patterns of circulating monocytes and lymphocytes in LPS-infused cows were comparable with white blood cell counts, although the leukopenia

Figure 3. Effects of an LPS bolus or pair-feeding in dairy cows fed a control diet or a diet supplemented with a Zn AA complex on circulating (A) insulin, (B) glucagon, (C) nonesterified fatty acids (NEFA), (D) BHB, and (E) ionized calcium. Cows were randomly assigned to 1 of 2 dietary treatments: (1) control (CON; 64 mg/kg supplemental Zn from ZnSO_4) or (2) replacement of 40 mg/kg ZnSO_4 with Zn AA complex (AvZn; Availa-Zn, Zinpro Corporation, Eden Prairie, MN). At the initiation of period 2 (P2), which lasted 48 h, animals either experienced a 12-h LPS euglycemic clamp as previously described (Kvidera et al., 2016, 2017a; Horst et al., 2018) or were pair-fed (PF) to their respective dietary counterparts for the entire 48 h to eliminate the confounding effects of dissimilar nutrient intake. Dietary and challenged combinations resulted in 4 treatments: (1) PF control diet (PF-CON; 5 mL of sterile saline with CON diet; $n = 5$), (2) PF Availa-Zn diet (PF-AvZn; 5 mL of sterile saline with AvZn diet; $n = 5$), (3) LPS euglycemic clamp control diet (LPS-CON; 0.375 μg of LPS/kg of BW with CON diet; $n = 5$), and (4) LPS euglycemic clamp Availa-Zn diet (LPS-AvZn; 0.375 μg of LPS/kg of BW with AvZn diet; $n = 5$). Time 0 represents an average of measurements obtained during the baseline and used as a covariate for P2 and period 3 analysis. Fixed effects of treatment (Trt), time, and treatment $\times$ time were assessed as a completely randomized design using PROC MIXED of SAS (SAS Institute Inc., Cary, NC). Data are represented as LSM $\pm$ SEM and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. When < 0.10 , the P -value for the fixed effects and the preplanned contrasts are listed on the figure.

Table 4. Metabolic parameters of cows fed a control diet or a diet supplemented with a Zn AA complex following administration of saline or LPS during period 2¹

Parameter	Treatment ²				P-value		Contrast	
	PF-CON	PF-AvZn	LPS-CON	LPS-AvZn	SEM	Treatment × time	CON vs. AvZn	LPS vs. PF

BUN (mg/dL)	10.0 ^a	9.0 ^a	12.2 ^b	12.5 ^b	0.6	<0.01	0.58	<0.01
Lactose (μM)	26.0	26.2	30.6	32.6	2.6	0.01	0.68	0.05

^{a,b}Values within a row with different superscripts are different ($P < 0.05$) between treatments.

¹During period 2, cows either were pair-fed or experienced a 12-h LPS euglycemic clamp.

²Cows were randomly assigned to 1 of 2 dietary treatments: (1) control (CON; 64 mg/kg supplemental Zn from ZnSO₄) or (2) replacement of 40 mg/kg ZnSO₄ with Zn AA complex (AvZn; Availa-Zn, Zinpro Corporation, Eden Prairie, MN). At the initiation of period 2, which lasted 48 h, animals either experienced a 12-h LPS euglycemic clamp as previously described (Kvidera et al., 2016, 2017a; Horst et al., 2018) or were pair-fed (PF) to their respective dietary counterparts for the entire 48 h to eliminate the confounding effects of dissimilar nutrient intake. Dietary and challenged combinations resulted in 4 treatments: (1) PF control diet (PF-CON; 5 mL of sterile saline with CON diet; n = 5), (2) PF Availa-Zn diet (PF-AvZn; 5 mL of sterile saline with AvZn diet; n = 5), (3) LPS euglycemic clamp control diet (LPS-CON; 0.375 μg of LPS/kg of BW with CON diet; n = 5), and (4) LPS euglycemic clamp Availa-Zn diet (LPS-AvZn; 0.375 μg of LPS/kg of BW with AvZn diet; n = 5).

phase was prolonged (61 and 45% decrease from 3 to 36 h postbolus relative to PF cows; $P < 0.01$; Table 5). Circulating basophils in LPS cows tended to be decreased from 3 to 6 h and increased from 30 to 48 h postbolus, whereas in PF controls they did not change ($P = 0.06$; Table 5). During P2, LPS administration decreased circulating platelets (48%) relative to PF cows ($P < 0.01$; Table 5).

During P2, no treatment differences were observed in neutrophil oxidative burst (data not shown). However, relative to baseline, oxidative burst from LPS-AvZn cows increased (44%) throughout P2 ($P \leq 0.05$; data not shown). Oxidative burst of LPS-CON cows tended to be increased (31%) 48 h post-LPS administration relative to baseline ($P = 0.07$; data not shown). Stimulated MPO activity increased similarly in all treatments during P2 (Figure 5B). In LPS-infused cows, the MPO released increased for the first 12 h postbolus (38% relative to baseline; $P \leq 0.05$) and then returned to baseline ($P < 0.01$; Figure 5B), whereas in PF cows it remained increased throughout all of P2 ($P \leq 0.05$). Dietary Zn source had no effect on stimulated MPO release. Total MPO from LPS-infused cows (determined by cell lysis) decreased (23% relative to PF cows) from 24 to 48 h postbolus, whereas in PF controls it increased ($P < 0.01$; Figure 5C). Relative to baseline, total MPO of LPS-AvZn cows was decreased (10%; $P = 0.02$) at 24 h postbolus, whereas in LPS-CON cows it remained decreased (11%) from 24 to 48 h postbolus ($P \leq 0.02$). Total MPO in PF treatments increased (11%) relative to baseline from 24 to 48 h postbolus ($P \leq 0.03$). Throughout P2, LPS-administered cows had increased (18%) MPO exocytosis relative to PF controls ($P < 0.01$; Figure 5D). Relative to baseline, the percentage of MPO released after in vitro stimulation increased (41%) in all treatments at 12 h postbolus ($P < 0.01$; Figure 5D). Myeloperoxidase exocytosis was or tended to be increased throughout P2 in LPS-CON and LPS-AvZn treatments relative to baseline (37 and 22%, respectively; $P < 0.01$ and $P \leq 0.09$; Figure 5D).

DISCUSSION

Maintenance of epithelial barrier function is crucial for preventing noxious substances and pathogen infiltration. When the intestinal barrier is compromised, antigens can infiltrate into portal and systemic circulation and initiate a cascade of events culminating in immunoactivation and inflammation. In dairy cows, there are myriad possible routes for pathogen entry, including the mammary gland, uterus, lungs, and gastrointestinal tract (Bradford et al., 2015). Although insult etiology may differ, animal welfare and economic profitability are almost always compromised by epithelial hyperper-

Figure 4. Effects of an LPS bolus or pair-feeding in dairy cows fed a control diet or a diet supplemented with a Zn AA complex on circulating (A) LPS-binding protein (LBP), (B) serum amyloid A (SAA), and (C) cortisol. Cows were randomly assigned to 1 of 2 dietary treatments: (1) control (CON; 64 mg/kg supplemental Zn from ZnSO_4) or (2) replacement of 40 mg/kg ZnSO_4 with Zn AA complex (AvZn; Availa-Zn, Zinpro Corporation, Eden Prairie, MN). At the initiation of period 2 (P2), which lasted 48 h, animals either experienced a 12-h LPS euglycemic clamp as previously described (Kvidera et al., 2016, 2017a; Horst et al., 2018) or were pair-fed (PF) to their respective dietary counterparts for the entire 48 h to eliminate the confounding effects of dissimilar nutrient intake. Dietary and challenged combinations resulted in 4 treatments: (1) PF control diet (PF-CON; 5 mL of sterile saline with CON diet; $n = 5$), (2) PF Availa-Zn diet (PF-AvZn; 5 mL of sterile saline with AvZn diet; $n = 5$), (3) LPS euglycemic clamp control diet (LPS-CON; 0.375 μg of LPS/kg of BW with CON diet; $n = 5$), and (4) LPS euglycemic clamp Availa-Zn diet (LPS-AvZn; 0.375 μg of LPS/kg of BW with AvZn diet; $n = 5$). Time 0 represents an average of measurements obtained during the baseline and used as a covariate for P2 and period 3 analysis. Fixed effects of treatment (Trt), time, and treatment $\times$ time were assessed as a completely randomized design using PROC MIXED of SAS (SAS Institute Inc., Cary, NC). Data are represented as LSM $\pm$ SEM and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. When <0.10 , the P -value for the fixed effects and the preplanned contrasts are listed on the figure.

meability, due in large part to high energetic demands of activated leukocytes (Kvidera et al., 2017a,b). Successful immunoactivation relies on effective leukocyte communication, extravasation, cellular function, elimination of cell debris and harmful byproducts, and return

to homeostasis (Kennedy, 2010). Identifying mitigation strategies aimed at improving barrier integrity (thus reducing pathogen entry) and immunocompetence have the potential to improve productivity over a range of physiological stressors. Zinc is involved in more than

Table 5. Complete blood cell count of cows fed a control diet or a diet supplemented with a Zn AA complex following administration of saline or LPS during period 2¹

Parameter ($\times 10^3/\mu\text{L}$)	Treatment ²				P-value			Contrast		
	PF-CON	PF-AvZn	LPS-CON	LPS-AvZn	SEM	Treatment	Time	Treatment $\times$ time	CON vs. AvZn	LPS vs. LPS-AvZn

White blood cells	7.60	8.66	9.53	9.12	0.71	0.31	<0.01	<0.01	0.65	0.11
Platelets	305.8 ^b	340.3 ^b	174.2 ^a	160.4 ^a	16.6	<0.01	<0.01	0.21	0.54	<0.01
Monocytes	0.41 ^b	0.41 ^b	0.22 ^a	0.20 ^a	0.03	<0.01	<0.01	<0.01	0.78	<0.01
Lymph	3.94 ^b	4.13 ^b	2.34 ^a	2.48 ^a	0.20	<0.01	<0.01	<0.01	0.39	<0.01
Basophils	0.05	0.06	0.06	0.06	0.01	0.48	<0.01	0.06	0.40	0.26

^{a,b}Values within a row with differing superscripts denote differences ($P < 0.05$) between treatments.

¹During period 2, cows either were pair-fed or experienced a 12-h LPS euglycemic clamp.

²Cows were randomly assigned to 1 of 2 dietary treatments: (1) control (CON; 64 mg/kg supplemental Zn from ZnSO₄) or (2) replacement of 40 mg/kg ZnSO₄ with Zn AA complex (AvZn; Availa-Zn, Zinpro Corporation, Eden Prairie, MN). At the initiation of period 2, which lasted 48 h, animals either experienced a 12-h LPS euglycemic clamp as previously described (Kvidera et al., 2016, 2017a; Horst et al., 2018) or were pair-fed (PF) to their respective dietary counterparts for the entire 48 h to eliminate the confounding effects of dissimilar nutrient intake. Dietary and challenged combinations resulted in 4 treatments: (1) PF control diet (PF-CON; 5 mL of sterile saline with CON diet; n = 5), (2) PF Availa-Zn diet (PF-AvZn; 5 mL of sterile saline with AvZn diet; n = 5), (3) LPS euglycemic clamp control diet (LPS-CON; 0.375 μg of LPS/kg of BW with CON diet; n = 5), and (4) LPS euglycemic clamp Availa-Zn diet (LPS-AvZn; 0.375 μg of LPS/kg of BW with AvZn diet; n = 5).

300 enzymes and plays a pivotal role in multiple systems (Prasad, 2012; Kambe et al., 2015). Furthermore, Zn improves epithelial integrity characteristics (mammary and intestinal; Sanz Fernandez et al., 2014; Weng et al., 2018) and immune function (cell adherence, clearance of *E. coli*, cytokine production; Kidd et al., 1994a; Finamore et al., 2008). Therefore, we hypothesized that supplemental complexed Zn would modify the inflammatory response to an intravenous LPS challenge and in turn alter the leukocyte energetic requirement.

Successful immunoactivation was induced herein as indicated by mild hyperthermia, increased circulating inflammatory biomarkers, altered leukocyte dynamics, and reduced productivity. Cows administered LPS experienced a mild and transient hyperthermia, and the extent and pattern agree with previous reports (Waldron et al., 2003; Moyes et al., 2014b; Horst et al., 2018). Interestingly, relative to LPS-CON cows, feeding complexed Zn reduced the febrile response to LPS. Both an increased (Roberts et al., 2002; Sanz Fernandez et al., 2014) and decreased (Chirase et al., 1991; Pearce et al., 2015; Abuajamieh et al., 2016) Tr response has been observed with complexed Zn following immunoactivation, and the variability is presumably cytokine driven. Circulating cytokines (e.g., tumor necrosis factor- α or IL-1 β) have pyrogenic properties and are altered by Zn in pigs (Klosterhalfen et al., 1996; Paulk et al., 2014; Mayorga et al., 2018), humans (Bao et al., 2010), chickens (Jarosz et al., 2017a), and cattle (Batistel et al., 2016). Although not always consistent (Driessen et al., 1994; Klosterhalfen et al., 1996; Batistel et al., 2016), Zn supplementation decreases circulating cytokines during inflammation via Zn-finger protein inhibition of the nuclear factor kappa light-chain enhancer of activated B cells pathway (Prasad, 2008; Foster and Samman, 2012; Jarosz et al., 2017b). Therefore, inconsistencies in the febrile response may be explained by the transient and variable pattern of circulating cytokines, the type of inflammatory insult, or stage of infection.

In response to immunogenic stimuli and cytokines, circulating APP (i.e., LBP and SAA) concentrations are markedly increased (Waldron et al., 2003; Kvidera et al., 2017a; Horst et al., 2018), which corroborates our results. During infection, circulating Zn is rapidly redistributed into cells and tissues (particularly the liver), where it alters APP production (Liuzzi et al., 2005; Liu et al., 2014). In the present study, feeding complexed Zn increased circulating SAA post-LPS infusion. Similarly, feeding complexed Zn increased APP production in heat-stressed calves and pigs (Pearce et al., 2015; Abuajamieh et al., 2016), chickens (Jarosz et al., 2019), and transitioning dairy cows (Batistel et al., 2016). Interestingly, Liu et al. (2014) demonstrated that Zn deficiency enhances APP production via activating the

JAK-STAT3 pathway in a murine model. Regardless of the aforementioned inconsistencies, it is clear that Zn can influence the APP response.

Cytokines and APP produced in response to LPS direct cell traffic, activate circulating immune cells, and facilitate “killing” capacity, which markedly alters

temporal leukocyte patterns. In the current study, leukopenia occurred immediately following LPS infusion, likely due to immune cell infiltration (into presumed infection sites) or increased leukocyte appearance in the marginated pool (Hoedemaker et al., 1992; Lang et al., 1992). After approximately 12 h, leukocytosis

Figure 5. Effects of an LPS bolus or pair-feeding in dairy cows fed a control diet or a diet supplemented with a Zn AA complex on (A) circulating neutrophils, (B) neutrophil-stimulated myeloperoxidase (MPO) release, where od = optical density, (C) total MPO release, and (D) MPO exocytosis. Cows were randomly assigned to 1 of 2 dietary treatments: (1) control (CON; 64 mg/kg supplemental Zn from ZnSO_4) or (2) replacement of 40 mg/kg ZnSO_4 with Zn AA complex (AvZn; Availa-Zn, Zinpro Corporation, Eden Prairie, MN). At the initiation of period 2 (P2), which lasted 48 h, animals either experienced a 12-h LPS euglycemic clamp as previously described (Kvidera et al., 2016, 2017a; Horst et al., 2018) or were pair-fed (PF) to their respective dietary counterparts for the entire 48 h to eliminate the confounding effects of dissimilar nutrient intake. Dietary and challenged combinations resulted in 4 treatments: (1) PF control diet (PF-CON; 5 mL of sterile saline with CON diet; $n = 5$), (2) PF Availa-Zn diet (PF-AvZn; 5 mL of sterile saline with AvZn diet; $n = 5$), (3) LPS euglycemic clamp control diet (LPS-CON; 0.375 μg of LPS/kg of BW with CON diet; $n = 5$), and (4) LPS euglycemic clamp Availa-Zn diet (LPS-AvZn; 0.375 μg of LPS/kg of BW with AvZn diet; $n = 5$). Time 0 represents an average of measurements obtained during the baseline and used as a covariate for P2 and period 3 analysis. Fixed effects of treatment (Trt), time, and treatment $\times$ time were assessed as a completely randomized design using PROC MIXED of SAS (SAS Institute Inc., Cary, NC). Data are represented as LSM $\pm$ SEM and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. When < 0.10 , the P -value for the fixed effects and the preplanned contrasts are listed on the figure.

developed and was most pronounced in the neutrophil population. Neutrophilia may be due to increased neutrophil production, demargination from pulmonary circulation, or decreased endothelial adherence (Jagels and Hugli, 1994; Opdenakker et al., 1998). These fluctuating temporal patterns are consistent with LPS infusion in lactating cows (Bannerman et al., 2008; Horst et al., 2018).

Depending on the severity of infection, populations of immature or banded neutrophils are increased during neutrophilia, and the immature population's function is not fully operational (Van Eeden et al., 1995; Paape et al., 2003). Reduced ROS production, increased rates of apoptosis, and reduced adhesion molecule expression (Mehrzad et al., 2001; Yagi et al., 2002) have been observed in circulating neutrophils isolated from LPS-infused cows. However, improved function of bovine PMN has also been reported following LPS infusion or incubation (Sohn et al., 2007; Moyes et al., 2014a,b; Moraes et al., 2017). We detected no differences in respiratory burst or release of MPO in LPS-infused cows relative to their PF counterparts. Lipopolysaccharide administration decreased total MPO content and increased MPO exocytosis. Although LPS depleted MPO stores, it did not appear to affect the neutrophils' capacity to release MPO, suggesting an improved efficiency of neutrophils isolated from LPS-administered cows. Improved PMN activity in LPS cows may be explained by the maintenance of euglycemia following LPS administration. Infusing LPS markedly alters metabolic status (i.e., hypoglycemia, hyperinsulinemia, hypertriglyceridemia, hypergluconemia), and this metabolic milieu may contribute to immunosuppression (Moyes et al., 2010); thus, maintenance of euglycemia may have masked LPS-induced hypoglycemia effects on neutrophil function. No differences were observed due to Zn source on the temporal pattern of circulating leukocytes, which agrees with previous studies (Kidd et al., 1994a,b). Moreover, Zn source did not alter neutrophil oxidative burst or MPO production. The effect of dietary Zn on cellular and humoral immunity is extremely and unexplainably variable across species (Kidd et al., 1994a,b; Kincaid et al., 1997; Nemec et al., 2012; Dietz et al., 2017; Kloubert et al., 2018). The aforementioned inconsistencies may be due to cell type measured, methods for assessing neutrophil function, or pre-existing Zn status.

Although the mechanisms underlying immunosuppression in immature leukocyte populations remain unclear, one hypothesis is an increase in immunosuppressive glucocorticoids, such as cortisol (Burton et al., 1995, 2005). Surprisingly, despite hypercortisolemia, we observed no effect or even improved PMN function (i.e., increased MPO exocytosis) following LPS

infusion. Additionally, LPS-AvZn cows had decreased circulating cortisol relative to LPS-CON cows, which agrees with previous studies (Dang et al., 2013; Lipopolis et al., 2017). Explanations for why AvZn-induced decreased cortisol did not affect PMN function are not clear; however, cortisol concentrations had almost returned to baseline values before PMN isolation, and it is not clear how long neutrophils need to be exposed to corticosteroids before becoming less functional. Additionally, leukocytes can develop steroid intolerance to immunosuppressive glucocorticoids (Briggs et al., 1983; Snavelly et al., 1985). Even though the decrease in cortisol did not coincide with changes in neutrophil activity, it may suggest that the LPS insult was less severe with complexed Zn supplementation, and this agrees with the reduced febrile response. The biological significance of Zn-mediated decreased cortisol warrants further investigation because it has relevance to multiple on-farm situations (e.g., the transition period, heat stress, and transport stress).

Lipopolysaccharide intravenous infusion reduces the integrity of mammary (Wellnitz et al., 2016) and intestinal epithelial barriers (Klunker et al., 2013). In the current study, we observed increased SCC in LPS-infused cows relative to PF controls during P2, which agrees with previous work (Shuster et al., 1991; Kvidera et al., 2017a). Additionally, plasma lactose (a biomarker of reduced mammary barrier function; Shennan and Peaker, 2000) concentrations increased post-LPS infusion. Interestingly, during P3, SCC of LPS-AvZn cows was decreased relative to their LPS-CON counterparts. Reduced milk SCC has been observed repeatedly with dietary Zn supplementation (Kellogg et al., 2004; Machado et al., 2013; Nayeri et al., 2014), and Weng et al. (2018) recently observed reduced circulating lactose with Zn supplementation in heat-stressed cows. Zinc is required for normal epithelial barrier function and has been shown to improve integrity characteristics of mammary (Weng et al., 2018), lung (Bao and Knoell, 2006), and intestinal epithelial barriers (Sanz Fernandez et al., 2014; Pearce et al., 2015; Abuajamieh et al., 2016). Therefore, Zn appears to be a promising mitigation strategy for strengthening the integrity of multiple epithelial barriers, and it is of interest to evaluate whether dietary Zn can improve uterine epithelial integrity.

In addition to markedly initiating an inflammatory response and compromising mammary epithelial integrity, several well-characterized metabolic adjustments (hyperglycemia or hypoglycemia, depending on the stage of infection; hyperglucagonemia and hyperinsulinemia; increased markers of proteolysis; and hypertriglyceridemia) occur post-LPS infusion (Lohuis et al., 1988; Lanza-Jacoby et al., 1998; McGuinness, 2005). These changes, along with reduced insulin sensitiv-

ity in skeletal muscle and adipose tissue coupled with decreased milk synthesis, reflect coordinated strategies that facilitate glucose “sparing” for immune cells. Most leukocytes become obligate glucose utilizers when activated (Palsson-McDermott and O'Neill, 2013) and require copious amounts of glucose. In the present study, the glucose requirement of the immune system was approximately 0.96 g/kg of BW^{0.75} per hour, which is remarkably comparable with our previous studies in monogastrics and ruminants (Kvidera et al., 2016, 2017a,b; Horst et al., 2018). However, despite numerous indications of an altered inflammatory response (i.e., reduced pyrexia, increased SAA, and reduced cortisol), dietary Zn source did not appear to affect overall glucose use by the immune system. However, a clear limitation to our model is the inability to account for the liver's contribution (i.e., glycogenolysis and gluconeogenesis) to the circulating glucose pool. Both glycogenolytic and gluconeogenic rates increase following LPS administration (Spitzer et al., 1985; McGuinness, 1994; Waldron et al., 2003), and we are unable to quantify the liver's glucose output, a scenario that causes the total glucose deficit to be underestimated. Additionally, we observed increased circulating glucagon in LPS-infused cows, and glucagon is a potent stimulator of both glycogenolysis and gluconeogenesis (Faulkner and Pollock, 1990). Interestingly, feeding complexed Zn decreased the acute hyperglycemic response to LPS and blunted the post-LPS glucagon surge. Both of the aforementioned changes may suggest that there was less of a glucose demand in complexed Zn-fed cows, and this is corroborated by altered inflammation parameters. Further investigation into how dietary Zn modifies the immune system and immunometabolism is needed.

Many of the well-characterized glucose-sparing mechanisms mentioned previously occurred post-LPS administration in our study. Marked hyperinsulinemia developed immediately postbolus and was sustained throughout the sampling period. Increased insulin presumably supports insulin-mediated glucose uptake and function of activated leukocytes (Calder et al., 2007). Although not significant, we observed a numerical decrease in circulating insulin in LPS-AvZn cows relative to their LPS-CON counterparts during the first 12 h postbolus. Zinc plays a pivotal role in processing, storage, secretion, and action of insulin (Myers et al., 2012); furthermore, Zn has been shown to attenuate glucose-stimulated insulin secretion (Slepchenko et al., 2013) via inhibiting tyrosine phosphatases (Haase and Maret, 2005). Literature demonstrating a beneficial effect of organic versus inorganic forms of Zn on circulating insulin is limited and warrants further investigation. Regardless, the combined effects of complexed Zn on post-LPS circulating insulin and glucagon suggest

a coordinated response reflective of a reduced glucose requirement during the acute stage of infection.

Nonesterified fatty acids decreased following LPS administration and then gradually increased with time. Interestingly, circulating NEFA from LPS-AvZn cows decreased relative to LPS-CON cows. Two possible mechanisms for blunted lipolysis may be (1) increased insulin antilipolytic sensitivity or (2) antilipolytic effects of increased circulating lactate. Although Zn has been shown to alter insulin sensitivity (Slepchenko et al., 2013), the results should be interpreted with caution because pre-existing Zn status (i.e., deficiency) may influence this response. Amplified leukocyte proliferation and activity increases lactate efflux, and this can cause infection-induced hyperlactatemia (Haji-Michael et al., 1999). Although not observed in the neutrophil parameters we measured, if Zn increased cellular activity of another white blood cell type, hyperlactatemia may have developed and altered lipolysis. Regardless of the mechanism, the biological significance of reduced lipolysis remains unclear and warrants further investigation. Despite observing an altered NEFA pattern, circulating BHB concentrations did not differ in LPS-CON and LPS-AvZn cows. Circulating BHB remained decreased during P2, possibly due to increased utilization by peripheral tissues (Zarrin et al., 2014). However, decreased BHB production from the liver (Takeyama et al., 1990; Gitomer et al., 1995) and rumen epithelium may also contribute to the LPS-induced hypoketonemia. Further investigation into the LPS-induced hypoketonemia is warranted, especially considering its relevance to the transition cow's bioenergetics. Skeletal muscle mobilization, as measured by circulating BUN, increased throughout P2. Proteolysis provides substrate for gluconeogenesis and AA for APP synthesis. However, BUN can be difficult to interpret in ruminant models because muscle proteolysis is not the sole source of urea. Regardless, these metabolic adjustments are consistent with previous literature (Waldron et al., 2003; Graugnard et al., 2013; Zarrin et al., 2014) and discussed further in our recent publications (Kvidera et al., 2016, 2017a,b; Horst et al., 2018).

Hypocalcemia develops in infection models across a range of species, including calves (Tennant et al., 1973; Elsasser et al., 1996), dogs (Holowaychuk et al., 2012), horses (Toribio et al., 2005), pigs (Carlstedt et al., 2000), sheep (Naylor and Kronfeld, 1986), and cows (Griel et al., 1975; Waldron et al., 2003; Kvidera et al., 2017a). The biological significance of infection-induced hypocalcemia is not fully understood, but past literature has demonstrated a relationship between Ca and LPS detoxification (Skarnes and Chedid, 1964); this was recently reviewed by Eckel and Ametaj (2016). Interestingly, complexed Zn supplementation attenuated

LPS-induced hypocalcemia at 12 h postbolus (Figure 3E). This may suggest that the LPS load was dampened in LPS-AvZn cows at 12 h postbolus compared with LPS-CON, perhaps due to improved integrity of mammary and intestinal epithelial barriers or improved LPS detoxification methods. A reduced LPS load is fitting with the other observations described previously (i.e., reduced fever, increased SAA, reduced cortisol, reduced SCC, decreased insulin and glucagon). The biological significance of ameliorated hypocalcemia due to Zn source warrants further investigation because it has practical relevance to periparturient metabolic disorders.

Determining differences in production outcomes was not the primary objective, as the experiment was knowingly underpowered to detect treatment differences in such variable measurements. Nonetheless, analyzing and presenting production variables allows for the primary aims (i.e., immune response, glucose deficit, and metabolism) to be put into context. Administering LPS markedly decreased DMI, and by experimental design the PF controls mirrored this pattern and magnitude of reduced DMI during P2. The total glucose deficit of PF treatments was accounted for exclusively by the decrease in milk yield because the cows did not become hypoglycemic, whereas the glucose deficit from the LPS treatments included the decrease in milk yield in addition to infused glucose. Although cows remained on the same nutritional plane, the milk glucose deficit from LPS treatments was increased 75% relative to the PF controls. In agreement with our previous studies (Kvidera et al., 2017a; Horst et al., 2018), maintaining euglycemia did not rescue milk yield in LPS-administered cows, confirming that hypoglycemia is not responsible for reduced milk synthesis during infection. Reduced milk production represents an important glucose-sparing mechanism during infection and may occur either directly via LPS binding to mammary epithelial Toll-like receptor 4 or indirectly by increased proinflammatory cytokines (Verheijden et al., 1983; Ibeagha-Awemu et al., 2008). During P3, cows in all treatments were allowed to consume feed ad libitum; however, DMI and milk yield remained decreased in cows previously administered LPS relative to PF controls, demonstrating the prolonged consequences of inflammation. Interestingly, although overall DMI and milk yield were not affected by diet during P2 and P3, LPS-AvZn cows had an increased rate (49%) of recovery in milk yield from d 1 to 3 relative to their LPS-CON counterparts. Although not observed as frequently for DMI (Osorio et al., 2016), complexed Zn has been shown to improve milk yield in lactating dairy cows (Kellogg et al., 2004; Nayeri et al., 2014; Osorio et al., 2016). Reasons for not detecting more Zn differences in production parameters

may be explained by small sample size, severity of the immune insult, and length of study.

CONCLUSIONS

Our results indicate that immunoactivation requires copious amounts of glucose, but Zn source did not appear to affect the immune system's fuel consumption. However, replacing a portion of Zn sulfate with Zn AA complex improved some inflammatory, metabolic, and production responses to LPS. The reduced hyperglycemic phase coupled with decreased circulating glucagon and insulin concentrations demonstrate a dampened metabolic requirement following immunoactivation with complexed Zn supplementation. Further, multiple changes in the inflammatory response coincided with altered energetics, including reduced fever, circulating cortisol, and SCC; increased SAA concentrations; and attenuated hypocalcemia. Our results demonstrate well-coordinated alterations in metabolic and inflammatory parameters with complexed Zn supplementation during immunoactivation.

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