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Evaluating effects of zinc hydroxychloride on biomarkers of inflammation and intestinal integrity during feed restriction

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ABSTRACT

Objectives were to evaluate effects of supplemental zinc hydroxychloride (HYD; Micronutrients, Indianapolis, IN) on gut permeability, metabolism, and inflammation during feed restriction (FR). Holstein cows (n = 24; 159 ± 8 d in milk; parity 3 ± 0.2) were enrolled in a 2 × 2 factorial design and randomly assigned to 1 of 4 treatments: (1) ad libitum fed (AL) and control diet (ALCON; 75 mg/kg Zn from zinc sulfate; n = 6); (2) ad libitum fed and HYD diet (ALHYD; 75 mg/kg Zn from HYD; n = 6); (3) 40% of ad libitum feed intake and control diet (FRCON; n = 6); or (4) 40% of ad libitum feed intake and HYD diet (FRHYD; n = 6). Prior to study initiation, cows were fed their respective diets for 21 d. The trial consisted of 2 experimental periods (P) during which cows continued to receive their respective dietary treatments. Period 1 (5 d) served as the baseline for P2 (5 d), during which cows were fed ad libitum or restricted to 40% of P1 feed intake. In vivo total-tract permeability was evaluated on d 4 of P1 and on d 2 and 5 of P2, using the paracellular permeability marker chromium (Cr)-EDTA. All cows were euthanized at the end of P2 to assess intestinal architecture. As anticipated, FR cows lost body weight (~46 kg), entered into calculated negative energy balance (−13.86 Mcal/d), and had decreased milk yield. Circulating glucose, insulin, and glucagon decreased, and nonesterified fatty acids and β-hydroxybutyrate increased in FR relative to AL cows. Relative to AL cows, FR increased lipopolysaccharide-binding protein, serum amyloid A (SAA), and haptoglobin (Hp) concentrations (2-, 4-, and 17-fold, respectively); and peak SAA and Hp concentrations were observed on d 5. Circulating SAA and Hp from FRHYD tended to be decreased (47 and 61%, respectively) on d 5 relative to FRCON. Plasma Cr area under the curve increased (32%) in FR treatments on d 2 and tended to be increased (17%) on d 5 of P2 relative to AL treatments. No effects of diet were observed on Cr appearance. Relative to AL cows, FR increased jejunum villus width and decreased jejunum crypt depth and ileum villus height and crypt depth. Relative to FRCON, ileum villus height tended to increase in FRHYD cows. Feed restriction tended to decrease jejunum and ileum mucosal surface area, but the decrease in the ileum was ameliorated by dietary HYD. In summary, FR induced gut hyperpermeability to Cr-EDTA, and feeding HYD appeared to benefit some key metrics of barrier integrity.

Key words: inflammation, leaky gut, Cr-EDTA

INTRODUCTION

Livestock are often challenged with transient periods of insufficient feed intake, which jeopardize animal performance and profitability. Recent ruminant literature has demonstrated deleterious effects of feed restriction (**FR**) on gut barrier integrity (Gäbel and Aschenbach, 2002; Zhang et al., 2013; Kvidera et al., 2017a,c), a phenomenon which had previously been well characterized in avian (Yamauchi et al., 1996; Gilani et al., 2018) and monogastric species (Rodriguez et al., 1996; Boza et al., 1999; Pearce et al., 2013). Feed restriction negatively affects intestinal architecture as a consequence of decreased epithelial cell proliferation and migration and increased apoptosis (Ferraris and Carey, 2000). Furthermore, physical and chemical defense mechanisms (mucus production, tight junction proteins, and Paneth cell function) are compromised during FR (Thompson and Applegate, 2006; Hodin et al., 2011; Kvidera et al., 2017c). Mechanistically, what triggers the alterations in barrier defense strategies remains poorly understood; however, convincing evidence suggests a neuroendocrine role (Horn et al., 2014; Najafi et al., 2018). Regardless of the mechanism, alterations in physical and chemical defenses contribute to the loss of barrier function, which increases the risk of pathogen entry into portal, lymph, and systemic circulation (Deitch et al., 1990; Kvidera

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et al., 2017a). Leukocyte recognition of the infiltrating pathogens initiates a cascade of events culminating in immune activation and inflammation; a process which is energetically demanding (Johnson, 2012; Kvidera et al., 2017b; Horst et al., 2018, 2019). Fueling the energetic requirement of the immune system markedly disrupts the hierarchy of nutrient partitioning away from profitable functions. Therefore, dietary strategies that may strengthen the epithelial barrier and reduce the risk of immune activation are currently of great interest.

Zinc is an essential trace mineral that plays a role in variety of biological processes, including immune regulation and epithelial barrier defense (Wessels et al., 2017; Ohashi and Fukada, 2019). Effects of zinc on gut integrity are conserved across species and experimental models of intestinal barrier dysfunction (Rodriguez et al., 1996; Sturniolo et al., 2001; Zhang and Guo, 2009). Zinc increases cell proliferation (Shao et al., 2017), turnover, and repair (Cario et al., 2000) and increases tight junction protein complexes (Finamore et al., 2008; Sarkar et al., 2019). Efficacy of dietary zinc is generally more evident when improved sources with better stability and bioavailability relative to zinc sulfate are used (Pearce et al., 2015; Horst et al., 2019; Opgenorth et al., 2021). Enhanced bioavailability with hydroxychlorides is ostensibly explained by reduced ruminal solubility (compared with inorganic sulfates) and, thus, decreased formation of insoluble complexes between zinc and feed constituents, ruminal metabolites, and microorganisms (Shaeffer et al., 2017; Caldera et al., 2019), a scenario that increases zinc delivery throughout the intestinal tract. Therefore, we hypothesized that feeding zinc hydroxychloride would strengthen epithelial barrier integrity in response to FR and reduce biomarkers of inflammation. Thus, experimental objectives were to evaluate the effects of replacing zinc sulfate with zinc hydroxychloride on intestinal architecture, paracellular permeability, and the acute-phase protein response following 5 d of FR in lactating Holstein cows.

MATERIALS AND METHODS

Animals and Experimental Design

All procedures were approved by the Iowa State University Institutional Animal Care and Use Committee (Ames, IA). Twenty-four lactating Holstein cows (685 ± 9 kg; 159 ± 8 DIM; parity 3 ± 0.2) were used in an experiment conducted in 2 replications (12 cows/replicate). Cows were randomly assigned to 1 of 2 dietary treatments: (1) control (**CON**: 75 mg/kg supplemental zinc from zinc sulfate) or (2) zinc hydroxychloride (**HYD**: 75 mg/kg supplemental zinc from Zn HYD; Micronutrients USA LLC, Indianapolis, IN). Zinc level of 75 mg/kg was chosen based on prior research demonstrating a beneficial effect of improved zinc source on gut health during heat stress in calves (Opgenorth et al., 2021). Total diet zinc analyzed 92 mg/kg for both treatments (Table 1), providing approximately 2,392 mg/d, which is well above NRC (2001) recommendations of 1,261 mg/d. Prior to the start of the study (before cows were moved into individual pens), cows were fed their respective diets for 21 d. After the initial feeding phase, cows were moved to sand- and straw-bedded individual box-stalls (4.57 × 4.57 m) at the Iowa State University Dairy Farm (Ames, IA). Cows were allowed 3 d to acclimate to housing conditions, during which time they were implanted with jugular catheters. The trial consisted of 2 experimental periods. Period 1 (**P1**) lasted 5 d and served as the baseline, which yielded data for covariate analysis. Period 2 (**P2**) lasted 5 d, during which cows were assigned to 1 of 2 treatments: (1) 100% ad libitum feed intake (**AL**; n = 12) or (2) 40% ad libitum feed intake (FR; n = 12). Dietary and FR combinations resulted in 4 total treatments: (1) ad libitum fed and control diet (**ALCON**; n = 6); (2) ad libitum fed and HYD diet (**ALHYD**; n = 6); (3) 40% of ad libitum feed intake (42.9% actual) and control diet (**FRCON**; n = 6); or (4) 40% of ad libitum feed intake (37.6% actual) and HYD diet (**FRHYD**; n = 6). One cow from the ALCON treatment was removed from the experiment due to health issues, and her data were not included in the final data set.

Throughout the experiment, all cows were fed a TMR formulated to meet or exceed the predicted requirements for energy, protein, minerals, and vitamins (NRC, 2001). Samples of TMR were obtained daily and composited into weekly samples for nutrient analysis (Dairyland Laboratories Inc., Arcadia, WI; Table 1). Trace mineral analysis was conducted on the same weekly composited TMR samples as well as on the premix and vitamin trace mineral mix at preparation of each batch, using inductively coupled emission spectroscopy (AOAC method no. 985.01, AOAC International, 2000; SDK Laboratories Inc., Hutchinson, KS; Tables 1 and 2). Daily feed intake during P2 was determined as a percentage (40%) of each cow's mean daily ad libitum intake during P1. Feed was provided once daily during P1 for all cows (0800 h), once daily during P2 for AL treatments (0800 h), and divided into 3 equal portions during P2 for FR treatments (0800, 1300, and 1800 h). Multiple meals were provided during FR to minimize metabolic variation due to gorging. Cows in the FR treatment appeared to consume a minimal amount (not quantified) of straw bedding during P2. Drinking water samples were obtained at the beginning of the pre-feeding phase and at the end of P2 for analysis of trace mineral content and hardness (SDK Laboratories

Table 1. Ingredients and nutrient composition of TMR[1]

Item	Control	HYD
Ingredient, % of DM		
Corn silage	30.34	30.34
Alfalfa hay	23.50	23.50
Ground corn	18.80	18.80
Dry corn gluten pellets	10.26	10.26
Premix[2]	6.84	6.84
Soy Plus[3]	4.27	4.27
Whole cottonseed	3.42	3.42
Soybean meal	1.71	1.71
Molasses	0.85	0.85
Chemical analysis, % of DM[4]		
Starch, %	24.6	24.9
CP, %	16.2	16.6
NDFom,[5] %	32.5	32.0
ADF, %	23.4	22.6
NE_L, Mcal/kg	1.61	1.60
Mineral, % of DM[6]		
Calcium, %	1.00 ± 0.44	0.90 ± 0.21
Phosphorus, %	0.39 ± 0.04	0.38 ± 0.04
Potassium, %	1.31 ± 0.10	1.34 ± 0.08
Magnesium, %	0.34 ± 0.02	0.34 ± 0.02
Sodium, %	0.51 ± 0.05	0.56 ± 0.08
Sulfur, %	0.22 ± 0.02	0.21 ± 0.03
Aluminum, mg/kg	293 ± 123	281 ± 82
Cobalt, mg/kg	0.57 ± 0.21	0.52 ± 0.15
Copper, mg/kg	15.64 ± 2.83	16.80 ± 2.56
Iron, mg/kg	252 ± 124	258 ± 52
Manganese, mg/kg	60.90 ± 9.80	63.35 ± 11.68
Molybdenum, mg/kg	1.00 ± 0.32	1.01 ± 0.21
Selenium, mg/kg	0.61 ± 0.05	0.60 ± 0.06
Zinc, mg/kg	92.39 ± 24.98	92.54 ± 16.45

[1]Diet DM = 49.45% for control and 51.59% for HYD. HYD = diet supplemented with zinc hydroxychloride.

[2]See Table 2 for premix ingredients and composition.

[3]Dairy Nutrition Plus, Ralston, IA.

[4]A composite of all weekly samples was sent off for analysis.

[5]NDFom = neutral detergent fiber organic matter.

[6]Mineral composition was analyzed weekly throughout the trial. Analysis presented as the mean ± standard error.

Table 2. Ingredients and analyzed mineral composition of premix

Item[1]	Control	HYD[2]
Soybean meal	27.33	27.33
Bloodmeal	16.00	16.00
Pork meat and bone meal	11.39	11.39
Sodium bicarbonate	10.29	10.29
MagnaPalm	9.14	9.14
Calcium carbonate	9.11	9.11
Salt	5.49	5.49
VTM[3]	2.85	2.85
Urea	2.29	2.29
Magnesium oxide	2.06	2.06
FloMatrix	1.37	1.37
Alimet	0.69	0.69
Diamond V XPC	0.68	0.68
Choice white grease	0.58	0.58
Smartamine M	0.58	0.58
Rumensin 90	0.10	0.10
Biotin 2%	0.05	0.05
Mineral analysis,[4] % of DM		
Calcium, %	5.10 ± 0.06	5.61 ± 0.43
Phosphorus, %	0.72 ± 0.12	0.72 ± 0.09
Potassium, %	0.95 ± 0.05	0.86 ± 0.06
Magnesium, %	1.22 ± 0.03	1.32 ± 0.04
Sodium, %	5.22 ± 0.31	6.21 ± 0.30
Sulfur, %	0.85 ± 0.24	0.63 ± 0.15
Aluminum, mg/kg	476 ± 113	633 ± 142
Cobalt, mg/kg	3.21 ± 0.01	2.31 ± 0.78
Copper, mg/kg	142 ± 46	143 ± 10
Iron, mg/kg	380 ± 153	613 ± 194
Manganese, mg/kg	474 ± 11	552 ± 71
Molybdenum, mg/kg	0.78 ± 0.04	0.92 ± 0.47
Selenium, mg/kg	4.48 ± 1.11	4.18 ± 0.06
Zinc, mg/kg	1,368 ± 855	1,162 ± 499

[1]Magna Palm (Energy Feeds International, Lago Vista, TX), FloMatrix (PMI, Neosho, MO), Ailmet (Novus, Saint Charles, MO), Diamond V (Cedar Rapids, IA), Smartamine (Adisseo, Alpharetta, GA), Rumensin (Elanco, Greenfield, IN).

[2]HYD = diet supplemented with zinc hydroxychloride.

[3]VTM = vitamin trace mineral mix. Analyzed mineral composition was as follows. Control diet: Ca 15.12%, P 0.09%, K 0.21%, Mg 0.12%, Na 0.13%, S 3.07%, Al 1,002 mg/kg, Co 119 mg/kg, Cu 4,336 mg/kg, Fe 763 mg/kg, Mn 18,375 mg/kg, Mo 1.76 mg/kg, Se 103 mg/kg, and Zn 32,549 mg/kg. HYD diet: Ca 15.12%, P 0.04%, K 0.21%, Mg 0.13%, Na 0.06%, S 1.42%, Al 771 mg/kg, Co 106 mg/kg, Cu 4,495 mg/kg, Fe 454 mg/kg, Mn 18,527 mg/kg, Mo 0.77 mg/kg, Se 121 mg/kg, Zn 31,673 mg/kg.

[4]Samples collected each time a new batch of premix was made (twice for the control premix and 4 times for the HYD premix). Analysis presented as mean ± SE.

Inc.; Table 3). Energy balance (**EBAL**) was calculated using the following equations: EBAL = energy intake − energy output, where energy intake = 1.6 Mcal/kg × DMI, and energy output = (NE_M = 0.08 Mcal/kg × $BW^{0.75}$) + [NE_L = milk yield × (0.0929 × fat % + 0.0547 × protein % + 0.0395 × lactose %)]. Body weights and BCS were obtained on d 1 of acclimation and on d 5 of P2 to calculate BW and BCS change.

Cows were milked twice daily (0600 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 & F Control Systems Inc., San Ramon, CA) until analysis by Dairy Lab Services Inc. (Dubuque, IA), using AOAC-approved infrared analysis equipment and procedures (AOAC International, 1995). Rectal temperature and respiration rate were recorded after each milking. Respiration rate was measured as flank movements during a 15-s interval and later transformed to breaths/min. Rectal temperature was measured using a digital thermometer (M700 Digital Thermometer, GLA Agricultural Electronics, San Luis Obispo, CA).

Blood samples for metabolite and inflammatory biomarker analysis were obtained on d 3 and 5 of P1 and d 1, 3, and 5 of P2 following the a.m. milking. Samples were collected from the catheter and divided equally between a tube containing K_2EDTA (Becton, Dickinson and Company, Franklin Lakes, NJ; for plasma collection) and an empty glass tube (for serum collection).

Table 3. Water sample analysis (± SD)

Item	Lactating barn, control pen	Lactating barn, HYD pen	Maternity barn, control + HYD
Number of samples[1]	2	2	2
pH	7.90 ± 1.33	7.75 ± 1.17	8.84 ± 0.20
Chloride, mg/L	21.25 ± 5.30	23.75 ± 1.77	22.50 ± 3.54
Total hardness, mg/L	126.0 ± 4.24	121.5 ± 6.36	121.5 ± 6.36
Nitrate-nitrogen, mg/L	2.16 ± 0.57	2.23 ± 0.78	2.17 ± 0.52
Calcium, mg/L	33.25 ± 0.78	31.8 ± 2.40	31.9 ± 2.55
Magnesium, mg/L	10.40 ± 0.57	10.25 ± 0.21	10.20 ± 0.00
Sodium, mg/L	11.75 ± 0.92	12.25 ± 2.33	13.8 ± 2.69
Sulfate, mg/L	57.25 ± 2.47	56.00 ± 7.07	53.00 ± 8.49
Iron, mg/L	<0.01	<0.01	<0.01
Manganese, mg/L	<0.01	<0.01	<0.01
EC,[2] s/m	34.75 ± 1.77	33.10 ± 2.69	33.35 ± 2.05
TDS,[3] mg/L	246.5 ± 12.02	234.5 ± 19.09	236.5 ± 14.85

[1]Water samples were collected at the beginning and end of the experiment.

[2]EC = electrical conductivity.

[3]TDS = total dissolved solids, calculated.

Samples for glucagon analysis (3 mL) were collected from the catheter into K_2EDTA Vacutainers containing 150 μL of aprotinin (BP2503-10; Thermo Fisher Scientific, Fair Lawn, NJ). Serum samples were allowed to clot at room temperature for 1 h before centrifugation. Plasma and serum were harvested following centrifugation at 1,500 × *g* for 15 min at 4°C, and were subsequently frozen at −20°C until analysis. Samples for complete blood count analysis were collected on d 3 and 5 of P1 and twice daily during P2 following milking. A 3-mL blood sample was collected from the catheter (K_2EDTA; Becton, Dickinson and Co.) and stored at 4°C for approximately 12 h before submitting to the Iowa State University Department of Veterinary Pathology for analysis.

Laboratory Analyses

Serum cortisol and plasma insulin, nonesterified fatty acids (**NEFA**), BHB, BUN, glucose, glucagon, LPS-binding protein (**LBP**), serum amyloid A (**SAA**), haptoglobin (**Hp**), and L-lactate concentrations were determined using commercially available kits according to manufacturers' instructions (cortisol: Enzo Life Sciences, Farmingdale, NY; insulin: Mercodia AB, Uppsala, Sweden; NEFA: Wako Chemicals USA, Richmond, VA; BHB: Pointe Scientific Inc., Canton, MI; BUN: Teco Diagnostics Anaheim, CA; glucose: Wako Chemicals; glucagon: RD Systems Inc., Minneapolis, MN; LBP: Hycult Biotech, Uden, the Netherlands; SAA: Tridelta Development Ltd., Kildare, Ireland; Hp: Life Diagnostics Inc., West Chester, PA; and L-lactate: Biomedical Research Service Center, Buffalo, NY). All assays were analyzed in duplicate, aside from BUN, which was analyzed in triplicate (outliers were dropped when necessary). Samples were reanalyzed when coefficients of variation exceeded 15%. The inter- and intra-assay coefficients of variation for insulin, NEFA, BHB, BUN, glucose, glucagon, cortisol, LBP, SAA, Hp, and L-lactate were, respectively, 2.2 and 6.5%, 5.6 and 3.5%, 4.9 and 12.6%, 6.7 and 5.1%, 4.3 and 4.7%, 8.4 and 6.7%, 14.1 and 6.2%, 6.7 and 6.5%, 10.6 and 4.9%, 8.7 and 6.6%, and 9.3 and 11.6%.

Neutrophil Isolation and Functional Analysis

Blood samples (32 mL) for neutrophil isolation were collected on d 3 of P1 and on d 3 and 5 of P2, into 50-mL conical tubes containing acid citrate dextrose (8 mL). Samples were immediately transported to the laboratory for neutrophil isolation and functional analysis, as previously described (Kimura et al., 2014; Horst et al., 2019). Neutrophil function was assessed by oxidative burst (cytochrome C reduction) and extracellular release of myeloperoxidase (**MPO**). Three cell preparations were used to assess MPO activity: (1) cells were lysed by treatment with cetyltrimethyhylammonium bromide solution as a measure of total MPO; (2) PMN were stimulated with equal parts calcium ionophore A23187 and cytochalasin B in Hank's balanced salt solution (HBSS) to assess release of MPO with stimulation; and (3) PMN were treated with HBSS alone as a measure of unstimulated MPO release. Percentage of MPO released from PMN was determined using the following equation:

$$\text{Exocytosis (\%)} = [(\text{OD of stimulated PMN})/(\text{OD of lysed PMN})] \times 100.$$

In Vivo Total-Tract Barrier Function

Total-tract barrier function was evaluated in vivo using the paracellular permeability marker chromium

(Cr)-EDTA as previously described (Wood et al., 2015). Beginning at 0700 h (after milking, before feeding) on d 4 of P1 and d 2 and 5 of P2, a 180 m*M* solution of Cr-EDTA (1.5 L) was pulse dosed into the rumen using a cattle pump system. Blood samples were collected at 0, 1, 2, 4, 8, 12, 18, and 24 h relative to Cr-EDTA administration, into a tube containing K_2EDTA (Becton, Dickinson and Co., reference no. 368381; plasma for trace element analysis). Plasma was harvested following centrifugation at 1,500 × *g* for 15 min at 4°C and subsequently frozen at −20°C until analysis. Samples were submitted to Brooks Applied Labs (Bothell, WA) for analysis of total chromium, using inductively coupled plasma triple quadrupole (ICP-QQQ)-MS (Agilent 8900, Agilent Technologies, Santa Clara, CA).

Tissue Collection

At the end of P2, all cows were transported to the Iowa State Livestock Infectious Disease Isolation Facility and euthanized with a CASH Special captive bolt gun (Accles & Shelvoke Ltd., Sutton Coldfield, UK) using a large animal charge, followed by exsanguination. Liver and intestinal tissues (duodenum, jejunum, ileum, and colon) were harvested immediately following euthanasia. Liver was obtained from the right lobe and was snap-frozen in liquid nitrogen and stored at −80°C until later analysis. Intestinal samples were obtained as follows: duodenum was collected 20 cm distal to the pyloric sphincter; jejunum was collected 1 m proximal to the ileocecal junction; ileum was collected 18 cm proximal to the ileocecal junction; and colon was collected 50 cm proximal to the rectum. All intestinal samples were flushed with saline in an attempt to remove any intestinal content, and a 4-cm section from the middle of the segment was collected and fixed in 10% neutral buffered formalin for later histological analysis, as previously described (Kvidera et al., 2017a,c).

Liver Analysis

Hepatic triglyceride content was measured as previously described (Morey et al., 2011). A 20-mg (approximate) liver sample was weighed and homogenized with 500 μL of chilled PBS. The homogenate was then centrifuged at 8,000 × *g* for 2 min at 4°C. Free glycerol was immediately determined using 10 μL of supernatant via enzymatic glycerol phosphate oxidase method (Sigma-Aldrich, St. Louis, MO). An additional 300 μL of supernatant was removed and incubated with 75 μL of lipase (MP Biomedicals, Solon, OH) at 37°C for 16 h before determining total glycerol using the same method. Free glycerol (before lipase digestion) was subtracted from total glycerol (after lipase digestion) to determine triglyceride content, and this was expressed as a percentage of wet weight of the original sample. The intra-assay coefficients of variation for free glycerol and total glycerol were 9.4 and 2.0%, respectively.

Histological Analysis

For histological analysis, 10% neutral buffered formalin-fixed duodenum, jejunum, ileum, and colon were submitted to the Iowa State University Veterinary Comparative Pathology Core for sectioning and staining. Hematoxylin and eosin (H&E) staining was used for morphology, periodic acid-Schiff (PAS) for goblet cell area (**GCA**) quantification, and May-Grünwald-Giemsa (MGG) for mast cell quantification. One slide per cow per tissue was generated. Using a microscope (DMI3000 B Inverted Microscope, Leica Microsystems Inc., Bannockburn, IL) with an attached camera (12-bit QICAM Fast 1394, Teledyne QImaging, Surrey, BC, Canada), 5 images per intestinal section were obtained at 5× magnification for the H&E and PAS stains and at 40× magnification for the MGG stain. All image processing and quantification was done using ImageJ version 1.48 (National Institutes of Health, Rockville, MD). Goblet cell area, villus morphology, and mucosal surface area were quantified using previously described methods (Kvidera et al., 2017a,c). Mast cells were counted in a grid fashion using ImageJ software. Cells falling within a single grid (20,000 μm^2) on each image were counted and later summed. The cell number was then divided by the area to obtain cells per micrometer squared and multiplied by 1×10^6 to express values as cells per millimeter squared of tissue area.

Statistical Analyses

Sample size calculation was determined using PROC Power in SAS version 9.4 (SAS Institute Inc., Cary, NC) and was based on the effects of FR on inflammation using 80% power and a 95% confidence level. The sample size of 12 cows per group was based on a detection of difference of 363 ± 299 μg/mL (mean ± SD) in plasma SAA (Kvidera et al., 2017c). Data were analyzed using SAS version 9.4. For production and postmortem parameters (DMI, EBAL, BW, BCS, milk yield, ECM, feed efficiency, milk composition, rectal temperature, respiration rate, intestinal histology) effects of group (AL or FR), diet (CON or HYD), and their interaction were assessed using PROC MIXED, and cow was included as a random effect. For blood parameters, a repeated-measures analysis, with an autoregressive covariance structure and time as the repeated effect, was

Table 4. Effects of group (ad libitum intake or feed restriction) and diet (zinc sulfate or zinc hydroxychloride) on production parameters during P2[1]

	AL		FR			*P*-value		
Parameter	CON	HYD	CON	HYD	SEM	Group	Diet	Group × Diet
Number of animals (n = 23)								
DMI, kg/d	25.9	27.1	11.1	10.2	0.9	<0.01	0.85	0.24
Milk yield, kg/d	38.8	39.1	27.6	24.3	1.3	<0.01	0.37	0.22
ECM, kg/d	44.3	43.0	31.8	28.6	1.1	<0.01	0.11	0.49
Milk composition								
Fat, %	4.36	4.15	4.86	4.64	0.09	<0.01	0.05	0.97
Fat, kg	1.70	1.62	1.27	1.18	0.04	<0.01	0.12	0.92
Lactose, %	4.61[z]	4.57[y]	4.50[x]	4.53[x]	0.02	<0.01	0.59	0.12
Protein, %	3.23	3.29	3.08	3.13	0.04	<0.01	0.23	0.94
Protein, kg	1.26	1.27	0.86	0.75	0.04	<0.01	0.32	0.19
MUN, mg/dL	14.45	14.52	15.29	15.16	0.20	<0.01	0.90	0.63
SCS	1.84	2.25	2.85	2.79	0.41	0.08	0.70	0.57
FE,[2] ECM:DMI	1.72	1.59	2.94	2.89	0.08	<0.01	0.37	0.65
BWΔ,[3] kg	2.05	10.61	−39.39	−53.41	9.52	<0.01	0.78	0.25
BCSΔ[4]	−0.10	−0.04	−0.25	−0.25	0.12	0.16	0.80	0.80
EBAL,[5] Mcal/d	1.40	3.94	−14.30	−13.42	0.82	<0.01	0.05	0.31
Rectal temperature, °C	37.9[ab]	38.0[b]	38.0[b]	37.8[a]	0.1	0.22	0.73	0.02
Respiration rate, breaths/min	45	42	37	35	1	<0.01	0.11	0.73
Liver TG,[6] %	1.08	0.90	2.02	1.94	0.40	0.04	0.56	0.69

[1]During period 2 (P2), cows were either fed ad libitum (AL) or feed restricted (FR) and fed a diet with supplemental zinc from zinc sulfate (CON) or zinc hydroxychloride (HYD).

[2]Feed efficiency.

[3]Final BW − initial BW.

[4]Final BCS − initial BCS.

[5]EBAL = energy balance.

[6]TG = triglycerides.

used to determine effects of time (d or h), group, diet, and their interactions. The P1 value of each specific variable was used as a covariate. Data are reported as least squares means and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.15$.

RESULTS

Production

By design, DMI was decreased (60%) in FR relative to AL-fed cows during P2 ($P < 0.01$; Table 4), and no effect of diet was observed ($P > 0.85$). As anticipated, FR cows lost BW (~46 kg) and entered into calculated negative EBAL (−13.86 Mcal/d) during P2 ($P < 0.01$; Table 4). Dietary HYD improved EBAL similarly in both groups (AL and FR) compared with CON cows ($P = 0.05$; Table 4). No effects of group or diet were observed for BCS (Table 4). Regardless of zinc source, FR decreased milk yield (33%) during P2 relative to AL-fed cows ($P < 0.01$; Table 4). No main effects of diet were observed on milk yield ($P > 0.37$). Similarly, FR decreased ECM (31%) during P2, compared with AL-fed cows ($P < 0.01$). Regardless of group, HYD supplementation tended to decrease ECM (6%) relative to CON ($P = 0.11$; Table 4). Feed efficiency increased (76%) in FR relative to AL-fed cows ($P < 0.01$), although no dietary effect was detected ($P > 0.37$; Table 4).

Overall, milk lactose content decreased (2%) in FR relative to AL-fed cows ($P < 0.01$; Table 4). Milk lactose tended to decrease (1%) in ALHYD relative to ALCON, whereas it was similar between FRCON and FRHYD cows ($P = 0.12$; Table 4). Milk fat percentage increased (12%) and yield decreased (26%) in FR relative to AL-fed cows ($P < 0.01$). Regardless of feed intake, HYD supplementation decreased milk fat percentage (5%; $P = 0.05$) and tended to decrease milk fat yield (6%; $P = 0.12$; Table 4). Relative to AL cows, FR decreased milk protein content and protein yield during P2 (5 and 37%, respectively; $P < 0.01$; Table 4), but no main effects of diet were observed ($P \geq 0.23$). Overall, MUN increased (5%) in FR relative to AL-fed cows ($P < 0.01$), but no dietary effect was observed ($P > 0.90$; Table 4). Feed restriction tended to increase SCS relative to AL cows during P2 (38%; $P = 0.08$; Table 4). No main effects of diet were observed for these parameters ($P > 0.42$). Rectal temperature was similar across diets in AL-fed groups; however, during FR it decreased (0.1°C) in HYD-supplemented cows relative to CON ($P = 0.02$; Table 4). Respiration rate decreased (8 breaths/min) in FR cows relative to AL-fed cows (P

< 0.01; Table 4). Regardless of feed intake (AL or FR), HYD supplementation tended to decrease respiration rate (3 breaths/min; $P = 0.11$).

Metabolism

Glucose and insulin concentrations from FR cows were consistently decreased (8 and 57%, respectively) throughout P2 relative to AL-fed cows ($P \leq 0.05$; Figure 1A and B). Feed restriction decreased circulating glucagon (31%; $P < 0.01$; Figure 1C) and markedly increased NEFA relative to AL-fed cows ($P = 0.03$; Figure 1D). Circulating BHB from FR cows increased (37%) on d 3 and 5 of P2, whereas in AL-fed cows it did not change from P1 ($P = 0.09$; Figure 1E). Blood urea nitrogen concentrations initially increased (19% on d 1) and then steadily declined throughout P2 in FR relative to AL-fed cows ($P < 0.01$; Figure 1F). No main effects of diet or interactions between group and diet were observed in any of the aforementioned blood metabolites and hormones ($P \geq 0.24$).

Inflammation

Although circulating insulin was decreased with FR, no differences were observed in the insulin-to-DMI ratio ($P > 0.85$; Table S1, https://doi.org/10.3168/jds.2020-18860). Overall, circulating LBP was increased in FR relative to AL-fed cows (63%; $P = 0.02$) and tended to be increased in HYD relative to CON cows (38%; $P = 0.10$) throughout P2. Circulating LBP increased (94%) in FRHYD cows on d 1 and 3 of P2, whereas concentrations from all other treatments remained similar ($P = 0.14$; Figure 2A). Serum amyloid A concentrations from FR cows increased progressively throughout P2 ($P = 0.07$) and were increased 4-fold overall in FR compared with AL cows ($P = 0.04$). We detected a tendency for a diet × day interaction for SAA, such that SAA increased in CON cows from d 3 to 5 of P2 and decreased in HYD cows ($P = 0.14$); this effect was more pronounced in FR cows than in AL (Figure 2B). Overall, Hp concentrations tended to increase 17-fold in FR relative to AL cows ($P = 0.08$), and the increase was largely driven by differences on d 5 of P2 (Figure 2C). Dietary HYD tended to decrease Hp concentrations in FR cows on d 5 of P2 relative to CON; however, no dietary effects were observed in AL-fed cows at the end of P2 ($P = 0.10$; Figure 2C). Regardless of group, circulating L-lactate tended to decrease (18%) in HYD-supplemented cows ($P = 0.12$; Figure 2D). No effects of group or diet were observed for circulating cortisol during P2 ($P > 0.43$; Figure 2E). Feed restriction increased liver triglyceride content 2-fold at the end of P2 relative to AL feeding ($P = 0.04$; Table 4); however, neither main effects of diet ($P > 0.56$) nor an interaction were observed.

White blood cell counts tended to increase throughout P2 in HYD-supplemented cows, regardless of group, compared with CON (6%; $P = 0.10$; Table S1, https://doi.org/10.3168/jds.2020-18860). Circulating neutrophil counts from FRCON cows decreased (25%) relative to ALCON, but counts did not differ between AL and FR groups fed dietary HYD ($P = 0.05$; Table S1, https://doi.org/10.3168/jds.2020-18860). No effects of group, diet, or their interaction were observed on neutrophil oxidative burst ($P \geq 0.27$; Figure 3A) or neutrophil total MPO activity ($P \geq 0.23$; Figure 3B). However, oxidative burst tended to be decreased in FRCON versus ALCON cows on d 5 (16%; $P = 0.15$) and was numerically increased (19%) in FRHYD relative to FRCON cows on d 5 ($P > 0.17$). Myeloperoxidase activity following in vitro neutrophil stimulation did not differ across dietary treatments under AL-fed conditions; however, during FR, HYD supplementation increased stimulated MPO release (24%; $P = 0.01$; Figure 3C). On d 5, stimulated MPO release tended to decrease in FRCON relative to ALCON cows (27%; $P = 0.07$). Myeloperoxidase exocytosis tended to decrease in HYD-supplemented cows regardless of group ($P = 0.15$; Figure 3D).

Relative to AL cows, circulating platelets decreased (14%) in both dietary treatments during FR ($P = 0.03$; Table S1, https://doi.org/10.3168/jds.2020-18860). No effects of group or diet were detected for circulating monocytes ($P \geq 0.39$; Table S1, https://doi.org/10.3168/jds.2020-18860). Lymphocytes were similar in ALCON and FRCON cows but increased (7%) in FRHYD relative to ALHYD cows ($P = 0.02$; Table S1, https://doi.org/10.3168/jds.2020-18860). We detected a tendency for eosinophil counts to decrease (21%) in FRHYD relative to FRCON cows, but no dietary treatment differences were detectable in AL-fed cows ($P = 0.11$; Table S1, https://doi.org/10.3168/jds.2020-18860). Overall, HYD supplementation tended to increase basophil counts (17%) during P2 ($P = 0.10$), but no differences were detected across groups ($P > 0.67$; Table S1, https://doi.org/10.3168/jds.2020-18860). Feed restriction and HYD supplementation increased hemoglobin and hematocrit throughout P2 ($P \leq 0.01$; Table S1, https://doi.org/10.3168/jds.2020-18860), and the greatest differences were detected from d 1.5 to 5 ($P \leq 0.01$).

Gastrointestinal Integrity

Plasma Cr area under the curve (**AUC**) increased (32%) in FR treatments on d 2 ($P = 0.01$; Figure 4A) and tended to be increased (17%) on d 5 of P2 relative

to AL-fed treatments ($P = 0.09$; Figure 4B). No effects of diet were observed on Cr AUC. No effects of group or diet were observed on duodenum villus height, width, crypt depth, or villus height-to-crypt depth ratio at the end of P2 ($P > 0.32$; Figure 5A). Feed restriction increased jejunum villus width and decreased crypt depth ($P = 0.02$; Figure 5B), but no differences were detected for jejunum villus height or the villus height-

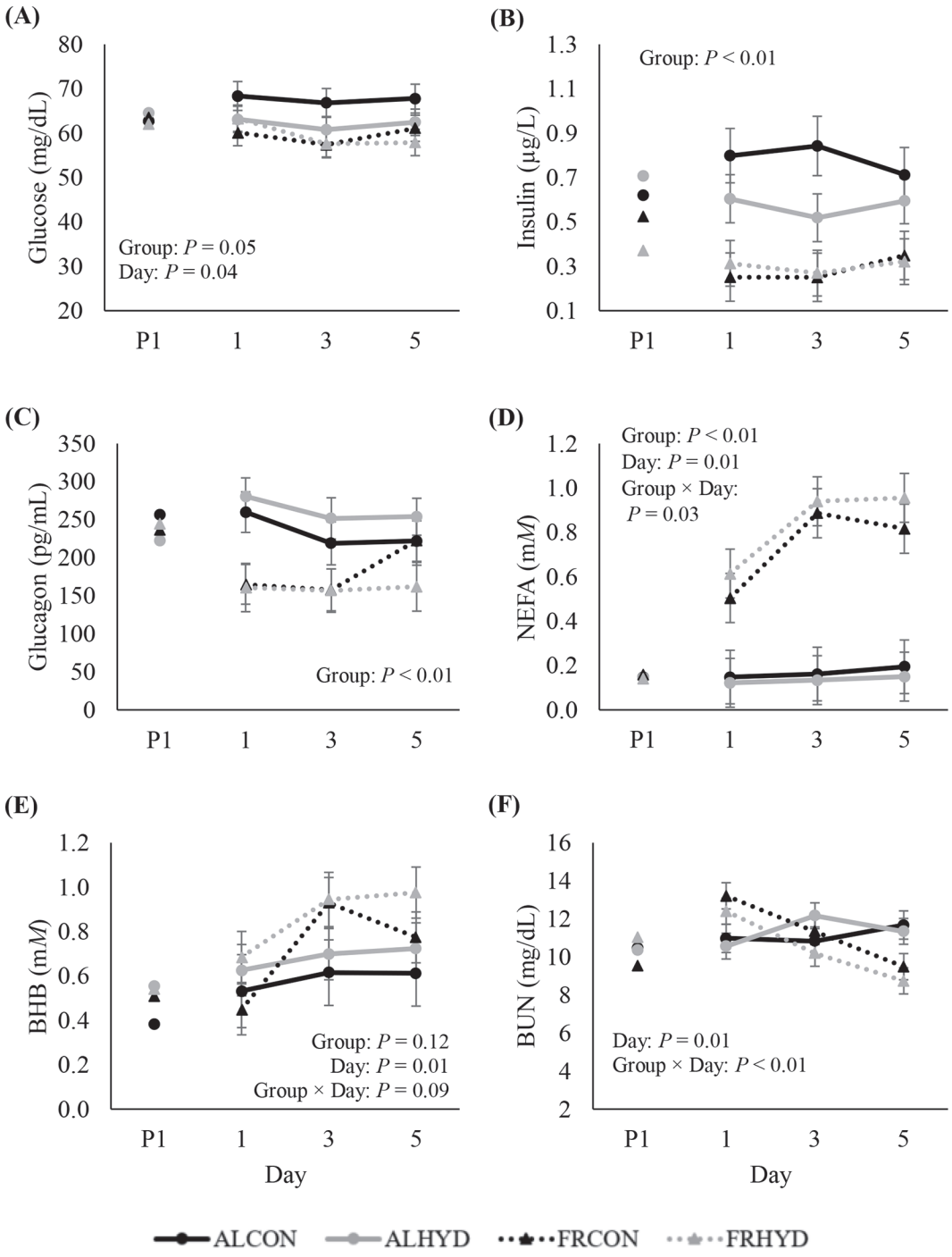

Figure 1. Effects of group [ad libitum intake (AL) or feed restricted (FR)] and diet [zinc sulfate (CON) or zinc hydroxychloride (HYD)] on circulating (A) glucose, (B) insulin, (C) glucagon, (D) nonesterified fatty acids (NEFA), (E) BHB, and (F) BUN during period 2 (P2). Data were analyzed using PROC MIXED (SAS version 9.4; SAS Institute Inc., Cary, NC) and included fixed effects of group, diet, day, and their interactions. P1 represents an average of measurements obtained during the 5 d of period 1 and was used as a covariate. Data are represented as LSM ± SEM and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.15$. Only P-values ≤0.15 are reported.

to-crypt depth ratio ($P \geq 0.32$). Feed restriction tended to decrease ileum villus height and crypt depth relative to AL feeding ($P \leq 0.14$; Figure 5C). Ileum villus height was similar across diets during AL conditions but tended to be increased in FRHYD cows relative to FRCON ($P = 0.09$). No treatment differences were observed for colon crypt depth ($P > 0.34$; data not shown). No effects of group or diet were observed on

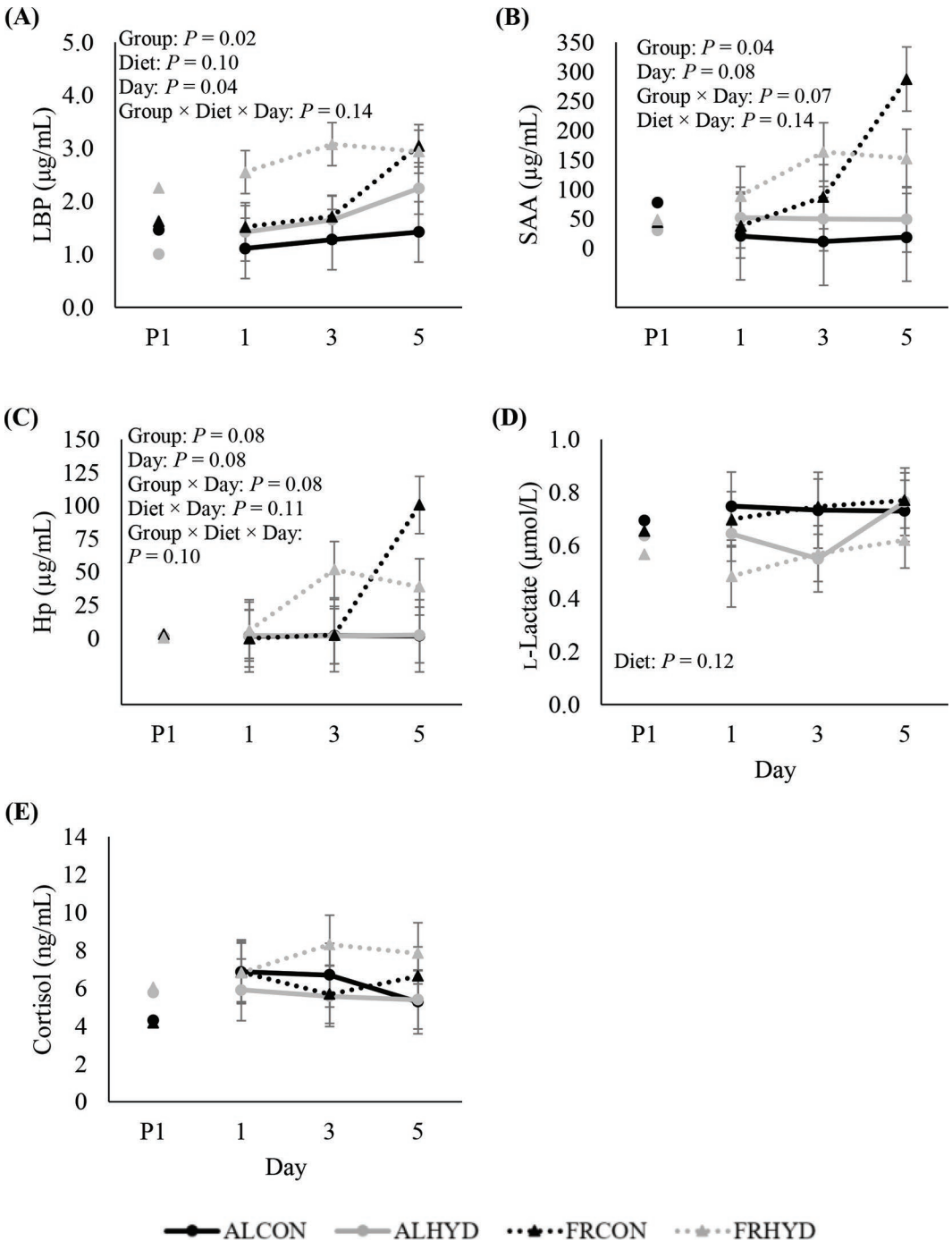

Figure 2. Effects of group [ad libitum intake (AL) or feed restricted (FR)] and diet [zinc sulfate (CON) or zinc hydroxychloride (HYD)] on circulating (A) LPS-binding protein (LBP), (B) serum amyloid A (SAA), (C) haptoglobin (Hp), (D) L-lactate, and (E) cortisol during period 2 (P2). Data were analyzed using PROC MIXED (SAS version 9.4; SAS Institute Inc., Cary, NC) and included fixed effects of group, diet, day, and their interactions. P1 represents an average of measurements obtained during the 5 d of period 1 and was used as a covariate. Data are represented as LSM ± SEM and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.15$. Only P-values ≤0.15 are reported.

duodenum mucosal surface area ($P > 0.50$; Figure 5D). Overall, the jejunum mucosal surface area tended to be decreased in FR relative to AL cows ($P = 0.15$; Figure 5E). Ileum mucosal surface area tended to decrease in FRCON relative to ALCON cows, but was similar in ALHYD and FRHYD treatments ($P = 0.12$; Figure 5F). Duodenum GCA was similar in ALCON and FRCON cows; however, in HYD-supplemented animals, FR increased GCA ($P = 0.03$; Table 5). No effects of group or diet were observed for jejunum or ileum GCA ($P \geq 0.17$; Table 5). Feed restriction decreased colonic GCA (40%) at the end of P2 ($P = 0.02$; Table 5). Feed restriction increased mast cell number similarly across diets within the duodenum ($P = 0.02$). No effects of group or diet were observed for the quantity of mast cells in the jejunum, ileum, or colon (Table 5).

DISCUSSION

Weaning (Moeser et al., 2007), heat stress (Baumgard and Rhoads, 2013; Pearce et al., 2015), rumen acidosis (Emmanuel et al., 2007; Khafipour et al., 2009),

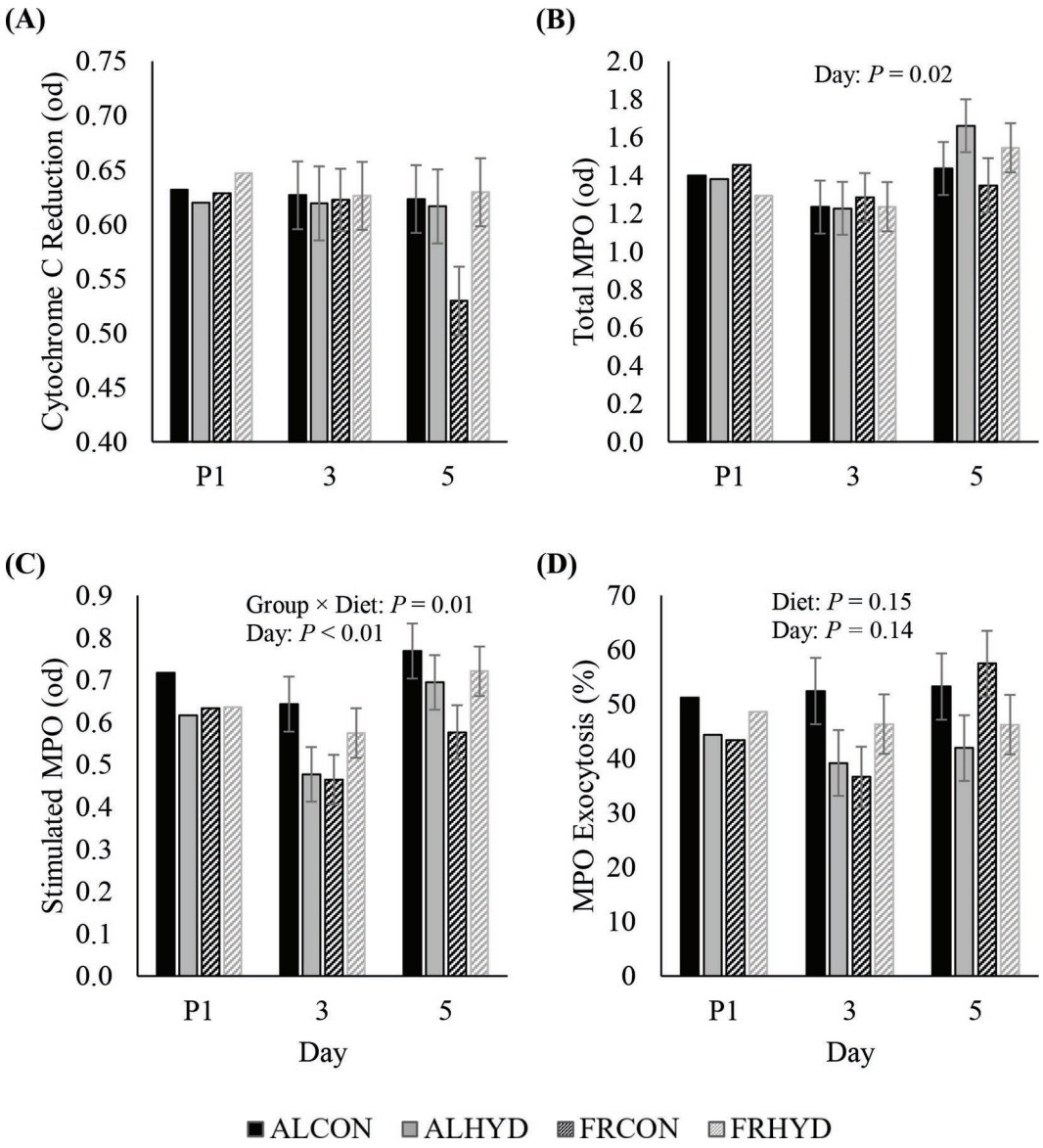

Figure 3. Effects of group [ad libitum intake (AL) or feed restricted (FR)] and diet [zinc sulfate (CON) or zinc hydroxychloride (HYD)] on (A) neutrophil oxidative burst, (B) total myeloperoxidase (MPO) release, (C) stimulated MPO release, and (D) MPO exocytosis. Data were analyzed using PROC MIXED (SAS version 9.4; SAS Institute Inc., Cary, NC) and included fixed effects of group, diet, day, and their interactions. P1 represents an average of measurements obtained d 3 of period 1 and was used as a covariate. Data are represented as LSM ± SEM and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.15$. Only P-values ≤0.15 are reported. od = optical density.

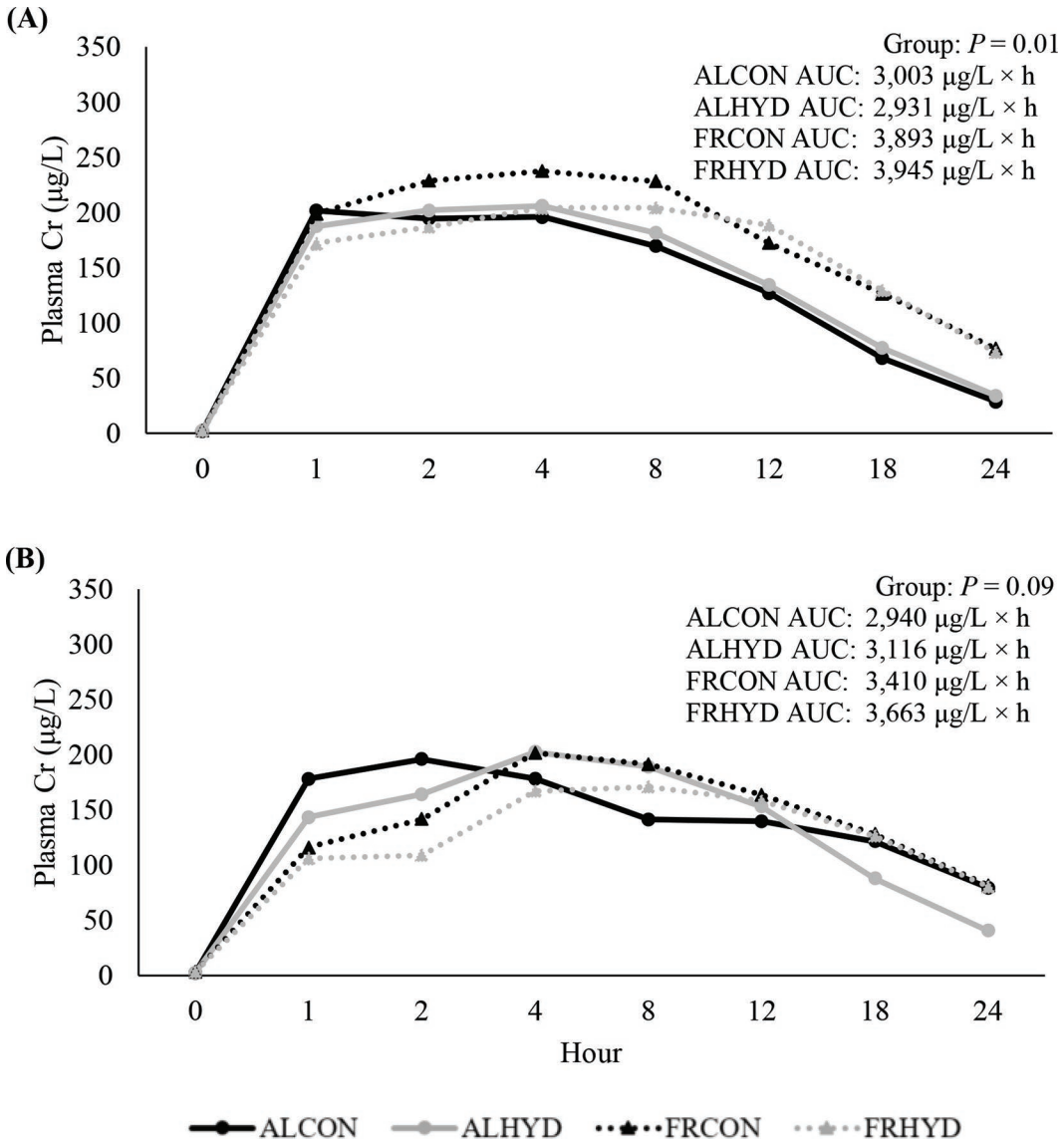

Figure 4. Effects of group [ad libitum intake (AL) or feed restricted (FR)] and diet [zinc sulfate (CON) or zinc hydroxychloride (HYD)] on circulating chromium (Cr) concentrations on (A) d 2 and (B) d 5 of period 2 (P2). Data were analyzed using PROC MIXED (SAS version 9.4; SAS Institute Inc., Cary, NC) and included fixed effects of group, diet, and their interaction. P1 represents an average of measurements obtained during the 5 d of period 1 and was used as a covariate. Data are represented as LSM ± SEM and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.15$. Only P-values ≤0.15 are reported.

and the periparturient period (Bertoni et al., 2008; Trevisi et al., 2012; Abuajamieh et al., 2016) are just a few of the stressors production animals frequently encounter that are characterized by gut barrier dysfunction, inflammation, hypophagia, and compromised productivity. The short-term FR that accompanies these situations independently affects gastrointestinal permeability and pathogen translocation (Zhang et al., 2013; Pearce et al., 2015; Kvidera et al., 2017a,c). Feed restriction decreases intestinal cell number, proliferation, and migration rates, simultaneously increasing cell loss and apoptosis (Bayer et al., 1981; Goodlad et al., 1988; Ferraris and Carey, 2000). Consequently, villus height and mucosal surface area are reduced, and, ultimately, intestinal barrier function is compromised (Mayhew, 1990; Ferraris and Carey, 2000). Loss of barrier integrity permits infiltration of pathogens (as well as other noxious substances) into circulation and consequently initiates a systemic immune response (Pearce et al., 2013; Kvidera et al., 2017a). When activated, most leukocytes switch their metabolism from oxidative phosphorylation to aerobic glycolysis and begin utilizing copious amounts of glucose (Palsson-McDermott and O'Neill, 2013). As a means of supporting the

increased glucose demand, several marked alterations in nutrient partitioning are employed, which ultimately deemphasizes productive processes (i.e., milk and meat synthesis; Waldron et al., 2003; Kvidera et al., 2017b; Horst et al., 2018, 2019). Dietary mitigation strategies aimed at enhancing barrier integrity have the potential to improve animal performance.

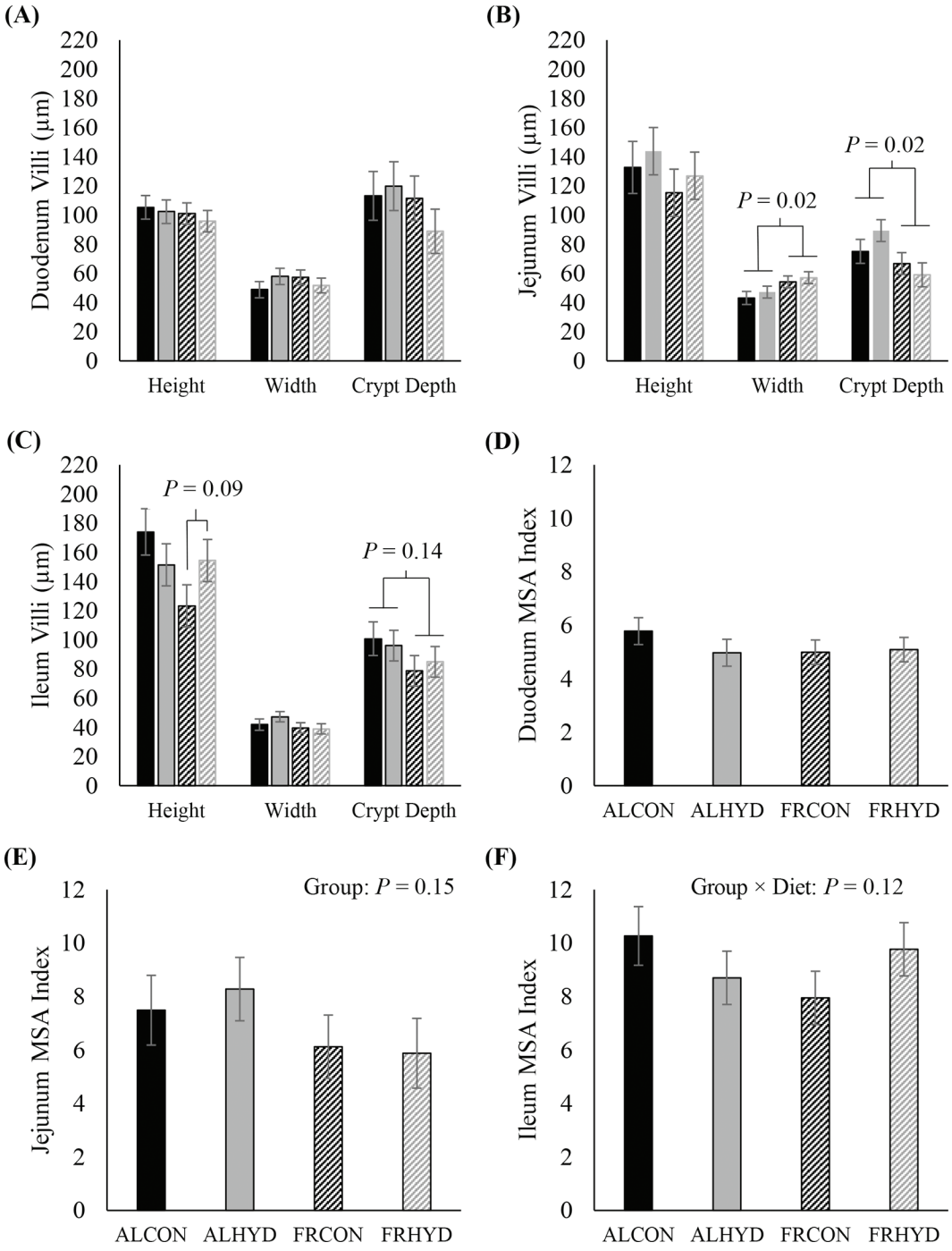

Figure 5. Effects of group [ad libitum intake (AL) or feed restricted (FR)] and diet [zinc sulfate (CON) or zinc hydroxychloride (HYD)] on (A) duodenum villus morphology, (B) jejunum villus morphology, (C) ileum villus morphology, (D) duodenum mucosal surface area (MSA), (E) jejunum MSA, and (F) ileum MSA. Data were analyzed using PROC MIXED (SAS version 9.4; SAS Institute Inc., Cary, NC) and included fixed effects of group, diet, and their interactions. Data are represented as LSM ± SEM and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.15$. Only P-values ≤0.15 are reported.

Table 5. Effects of group (ad libitum intake or feed restriction) and diet (zinc sulfate or zinc hydroxychloride) on intestinal goblet cell area (GCA) and mast cell number at the end of P2[1]

	AL		FR			*P*-value		
Parameters	CON	HYD	CON	HYD	SEM	Group	Diet	Group × Diet
Number of animals (n = 23)								
Duodenum								
GCA, %	6.75[ab]	4.89[a]	5.55[ab]	7.72[b]	0.85	0.35	0.86	0.03
Mast cells, cells/mm^2	288.1	253.3	383.3	396.7	46.9	0.02	0.82	0.62
Jejunum								
GCA, %	3.58	3.56	3.41	3.14	0.51	0.57	0.78	0.81
Mast cells, cells/mm^2	370.5	383.3	370.0	450.0	50.4	0.52	0.37	0.52
Ileum								
GCA, %	3.96	5.01	3.56	5.30	0.98	0.96	0.17	0.73
Mast cells, cells/mm^2	353.0	381.2	375.0	388.3	39.1	0.72	0.60	0.85
Colon								
GCA, %	15.27	17.00	10.27	9.06	2.45	0.02	0.92	0.56
Mast cells, cells/mm^2	161.7	168.3	218.3	198.3	41.3	0.31	0.87	0.75

[a,b]Values within a row with differing superscript letters indicate $P \leq 0.05$.

[1]During period 2 (P2), cows were either fed ad libitum (AL) or feed restricted (FR) and fed a diet with supplemental zinc from zinc sulfate (CON) or zinc hydroxychloride (HYD).

Zinc is an essential micronutrient crucial for maintaining mammary (Weng et al., 2018), lung (Bao and Knoell, 2006), and intestinal epithelial integrity (Sanz-Fernandez et al., 2014; Miyoshi et al., 2016; Opgenorth et al., 2021). Zinc ameliorates gut permeability in several leaky-gut models (Rodriguez et al., 1996; Lambert et al., 2003; Weng et al., 2018) and plays an essential role in cellular processes such as metabolism, inflammation, and oxidative stress (as reviewed by Olechnowicz et al., 2018). The efficacy of zinc is improved when more bioavailable sources are provided, such as amino acid complexes and hydroxychlorides (Sanz-Fernandez et al., 2014; Pearce et al., 2015; Horst et al., 2019; Opgenorth et al., 2021). Therefore, we hypothesized that replacing zinc sulfate with HYD would alleviate the influence of FR on gastrointestinal permeability and modify the inflammatory response in lactating Holstein cows.

In the current experiment, FR cows lost significant BW (~46 kg), entered into calculated negative EBAL (−13.86 Mcal/d), and had reduced milk and component yields, indicating successful implementation of our experimental protocol. The decrease in production metrics was consistent with our previous report executing the same magnitude and duration of FR (Kvidera et al., 2017a) as well as others utilizing a comparable approach (Velez and Donkin, 2005; Carlson et al., 2006; Ferraretto et al., 2014). Dietary zinc source had no effect on DMI or milk yield in our study, which agrees with past studies evaluating improved zinc sources (Weng et al., 2018; Horst et al., 2019) but contradicts others (Kellogg et al., 2004; Cope et al., 2009; Nayeri et al., 2014; Osorio et al., 2016). Regardless, production metrics were not the primary objective of our experiment.

Feed restriction induced well characterized changes in metabolism, including decreased circulating glucose and insulin, and increased NEFA and BHB. These changes are consistent with previous FR studies in ruminants (Carlson et al., 2006; Ferraretto et al., 2014; Lérias et al., 2015; Kvidera et al., 2017a) and demonstrate the coordinated changes in nutrient partitioning that animals implement to survive insufficient feed intake (McCue, 2010). Interestingly, despite the catabolic state, we observed decreased circulating glucagon (a potent stimulator of hepatic glucose output; Faulkner and Pollock, 1990) in FR relative to AL groups. Hypoglucagonemia in response to FR has been reported in sheep (Carruthers et al., 1974), whereas others have observed no FR-induced change in glucagon concentrations (de Boer et al., 1985; Drackley et al., 1989, 1991). Unlike monogastrics, glucagon concentrations increase in response to a meal in ruminants, as a means of stimulating hepatic glucose production from ruminally derived substrates (Mineo et al., 1994). Thus, the decrease in glucagon concentrations observed herein is likely a reflection of a fasted versus a fed state and of species differences in how gluconeogenesis is controlled.

Although the negative consequences of FR on production and metabolism have been well described in ruminant literature, its effect on gut barrier function has only recently received attention (Zhang et al., 2013; Kvidera et al., 2017a,c). In addition to regulating digestion and nutrient uptake, the intestinal epithelium serves as a crucial barrier against bacteria (commensal and pathogenic) and toxins residing within the gut

lumen. Two physical strategies imperative to gut barrier integrity are the mucus layer, produced by Paneth and goblet cells, and tight junction complexes (as reviewed by Steele et al., 2016). The mucus layer serves as a physical barrier to prevent pathogen penetration (Barreau and Hugot, 2014), whereas tight junction complexes allow for selective permeability (Mani et al., 2012). Interestingly, intestinal mucus production is decreased during insufficient feed intake (Nuñez et al., 1996; Thompson and Applegate, 2006; Kvidera et al., 2017c), thereby permitting pathogen colonization at the epithelium. In accordance, we detected decreased colonic GCA in response to FR, but no changes were detected in the duodenum, jejunum, or ileum. Reasons for not observing differences in small intestinal GCA are not clear and contradict our previous report implementing the same magnitude and duration of FR (Kvidera et al., 2017a). Although we did not detect an overall effect of FR on duodenum GCA, duodenum GCA was increased in HYD cows during FR. Additionally, we observed a numerical increase in ileum GCA from HYD-supplemented cows independent of feed intake. Increased GCA agrees with a previous report on feeding high levels of zinc oxide in weaned pigs (Hedemann et al., 2006). The mode of action by which zinc (especially zinc source) may alter GCA and mucus production remains incompletely understood and warrants further investigation, as it likely has multiple implications for animal agriculture.

In agreement with changes in GCA, FR negatively influenced villus morphology. These architectural changes were characterized particularly by decreased jejunum and ileum crypt depth and decreased ileum villus height, changes which corroborate our previous study in cows (Kvidera et al., 2017a) as well as reports on other species (Holt et al., 1986; Thompson and Applegate, 2006; Pearce et al., 2013). The decrease in villus height and crypt depth likely reflects a reduction in cellular proliferation, as previously described (Kvidera et al., 2017a). Alterations in villus morphology do not directly indicate changes in permeability; therefore, we used Cr-EDTA as an indicator of total-tract paracellular leakiness, a technique adapted from Zhang et al. (2013). Feed restriction increased total-tract permeability to Cr-EDTA acutely and chronically, as indicated by increased plasma Cr AUC; however, effects were more pronounced on d 2 compared with d 5 of FR, as will be further discussed.

In agreement with past reports (Pearce et al., 2015; Li et al., 2019; Opgenorth et al., 2021), improved zinc source ameliorated the negative effects of FR on ileum villus architecture and mucosal surface area. A plausible explanation for positive effects of improved zinc sources is superior bioavailability, particularly during times of stress when DMI (and thus mineral intake) is low. In the current study, animals were consuming approximately 2,392 mg of Zn/d during ad libitum conditions (92 mg/kg dietary Zn × 26 kg of DMI). When the FR treatment is applied, daily zinc intake decreased to roughly 1,012 mg of Zn/d (92 mg/kg dietary Zn × 11 kg of DMI), which is approximately 80% of NRC recommendations. Thus, a more bioavailable source should be able to supply more absorbable zinc, to maintain proper immune and mucosal function. In addition to improved bioavailability, source-mediated differences in gut parameters may be explained by altered release of free metal along the tract. Sulfate sources of trace minerals dissociate quickly in an aqueous environment, which can lead to a high metal load in the proximal intestine and less extractable mineral in the distal intestine (Klasing and Naziripour, 2010). In vitro, high levels of zinc salts (including zinc sulfate) caused rapid accumulation of Zn^{2+} in porcine jejunum epithelial cells, leading to cell damage and oxidative stress compared with zinc glycinate, a more stable and nonreactive form (Chen et al., 2020). However, high zinc concentrations were used in these studies, making interpretation in a practical dairy setting difficult. Improved morphology was not reflected by a corresponding change in total-tract permeability to Cr-EDTA. Reasons for the discrepancy in our gut integrity metrics are not fully understood but may be explained by evaluation at different time points or by differences in sensitivity or variation of the different techniques. In addition, the Cr-EDTA technique measures total-tract permeability, and therefore it is difficult to isolate where the greatest degree of "leakiness" occurred. The reticulorumen and omasum consist of 4 distinct strata (basale, spinosum, granulosum, and corneum), which together make up an approximately 85-μm-thick epithelial layer, whereas the lower gut is composed of a single layer of columnar epithelium (Steele et al., 2016). Because of these structural differences, we presume the risk of permeability is greater in the lower gut.

Although measuring circulating Cr levels in response to ruminal Cr-EDTA administration appears to be a promising technique to evaluate total-tract permeability, some limitations require consideration. First, it is not entirely clear how altered passage rate (which presumably occurred during FR) influences Cr-EDTA appearance along the gastrointestinal tract. However, because the temporal pattern of circulating Cr following Cr-EDTA administration was similar and Cr concentrations were comparable at 24 h across challenges, it is likely that passage rate had only a marginal affect. Second, it is not clear whether FR influenced renal and milk Cr clearance rate. However, Zhang et al. (2013) previously reported increased urinary Cr recovery in

FR relative to AL-fed heifers, suggesting that the increased plasma appearance is not simply a reflection of decreased renal removal from circulation. Additionally, it is reasonable to assume that effects of FR on clearance rate were similar on d 2 and 5, yet we observed larger Cr AUC on d 2, suggesting that increased blood Cr reflected increased gut leakiness. Regardless, further validation of this technique is warranted.

Fasting-induced gastrointestinal damage occurs rapidly, with several reports in pigs and chickens observing detrimental consequences within 24 h of FR (Thompson and Applegate, 2006; Horn et al., 2014; Gilani et al., 2018). Interestingly, herein we observed increased permeability (as indicated by increased Cr AUC) on d 2 compared with d 5 of FR. Rapid effects of FR on gut permeability suggest that a mechanism other than decreased luminal nutrient content is responsible, and some reports point to a neuroendocrine role (Horn et al., 2014; Najafi et al., 2018). In response to stress, the hypothalamic pituitary axis is activated, which stimulates nervous system and peripheral tissue production of corticotropin-releasing factor (**CRF**). Administering CRF induces intestinal barrier dysfunction in rodents (Santos et al., 1999; Teitelbaum et al., 2008) and initiates systemic inflammation in ruminants (Cooke and Bohnert, 2011; Cooke et al., 2012). Consequences of CRF are mediated by stimulation of intestinal mast cell degranulation and release of pre-formed and de novo mediators, such as histamine, proteases, and cytokines, which negatively affect intestinal barrier function (Abraham and St. John, 2010; Overman et al., 2012). Stress-mediated effects on the gut barrier may explain why so many seemingly unrelated situations (heat stress, weaning, acidosis) share a common consequence of leaky gut and systemic inflammation (Mayorga et al., 2020).

In the current study, we observed no effect of FR on circulating cortisol concentrations, suggesting that the hypothalamic pituitary axis may not have been activated. Reasons for not detecting increased cortisol are likely explained by insufficient sample collection during the acute phase of FR, as several reports demonstrate resolution of the cortisol response within 24 h of an insult (Chouzouris et al., 2018; Marques et al., 2019). In agreement, we detected no treatment differences in mast cell abundance in the jejunum, ileum, or colon, although FR increased duodenum mast cell number (5 d following FR initiation). To the authors' knowledge, no reports in ruminants exist demonstrating effects of stress on mast cell activation. Additionally, it is of interest to evaluate the influence of zinc on the stress response, as previous reports have demonstrated attenuated hypercortisolemia when improved zinc sources were provided (Lippolis et al., 2017; Xu et al., 2018; Horst et al., 2019). Furthermore, zinc may act as a mast cell stabilizer within the intestinal tract (Penissi et al., 2003). Further investigation is needed to determine effects of zinc source on gut hyperpermeability and whether potential changes can be explained by alterations in mast cell activation.

Our results indicate that 5 d of FR increased gut permeability to Cr-EDTA, and this ostensibly permitted pathogen infiltration into systemic circulation. In corroboration, we observed increased acute-phase proteins (LBP, SAA, and Hp) and altered neutrophil function during FR. Despite detecting increased permeability on d 2 of FR, meaningful increases in SAA and Hp were not observed until d 5 of FR, which agrees with our previous report (Kvidera et al., 2017a). The delayed increase in SAA and Hp concentrations was expected, based on patterns previously observed in response to intravenous LPS administration (Horst et al., 2018, 2019) and CRF infusion (Cooke et al., 2012). Herein we observed a tendency for decreased SAA and Hp concentrations on d 5 of FR in HYD-supplemented cows. Both increased (Pearce et al., 2015; Jarosz et al., 2019; Horst et al., 2019) and decreased (Mayorga et al., 2018) inflammatory mediators have been reported in previous studies feeding improved zinc sources. Based on the HYD-mediated changes in intestinal morphology, decreased acute-phase proteins presumably reflect improved barrier integrity and reduced passage of luminal antigens across the intestinal barrier. However, zinc's inhibitory effect on the nuclear factor kappa-light-chain-enhancer of activated B-cells pathway (Prasad, 2008; Foster and Samman, 2012), which is crucial in regulating the immune response, may be another explanation. In addition to stimulating an acute-phase protein response, FR negatively influenced neutrophil oxidative burst and stimulated MPO activity. Haptoglobin directly inhibits leukocyte activity by binding to receptor-ligand sites (Oh et al., 1990; Arredouani et al., 2005). Haptoglobin's potent anti-inflammatory actions are crucial for the development of immune tolerance and preventing an over-exaggerated inflammatory response (Raju et al., 2019). Interestingly, no differences were observed in neutrophil function in FRHYD relative to AL-fed groups, which is likely explained by the decreased Hp concentrations also observed in this treatment. The immunosuppressive actions of Hp warrant further investigation, especially considering its relevance to the dysregulated immune response that occurs around parturition.

CONCLUSIONS

Corroborating our previous report, we found that 5 d of FR negatively influences intestinal morphology and

initiates a systemic inflammatory response. Furthermore, FR affects total-tract permeability both acutely and chronically, and the effects appear to be more pronounced after 2 d of FR. The temporal intestinal barrier response to inadequate nutrient intake has practical implications to multiple scenarios in a dairy cow's life-cycle. Replacing zinc sulfate with Zn HYD improves barrier morphology and modulates acute-phase protein response and neutrophil function.

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