Reducing gut effects from *Cryptosporidium parvum* infection in dairy calves through prophylactic glucagon-like peptide 2 therapy or feeding of an artificial sweetener

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ABSTRACT

Glucagon-like peptide 2 (GLP-2) therapy was shown previously to reduce inflammation-related gut damage from coccidiosis in dairy calves, and feeding of artificial sweetener stimulates GLP-2 secretion from intestinal L cells. The purpose of this study was to determine whether GLP-2 treatment or artificial sweetener feeding beginning 1 wk before an experimental inoculation with the coccidian parasite *Cryptosporidium parvum* can reduce infection-related intestinal damage in Holstein bull calves. Newborn calves were assigned to 1 of 4 treatment groups of 6 calves each, including noninfected control calves injected s.c. every 12 h with control buffer (CON), infected control calves injected s.c. every 12 h with control buffer (INF), infected calves injected s.c. every 12 h with 50 µg/kg of body weight of GLP-2 (GLP2), and infected calves injected s.c. every 12 h with control buffer and supplemented in the diet with Sucram (Pancosma, Geneva, Switzerland) at 400 mg/kg of dry matter of milk replacer (SUC). Treatments were initiated on d 1, and calves in INF, GLP2, and SUC were orally dosed on d 8 with 12,500 *C. parvum* oocysts. Fecal scores were recorded daily, plasma was collected on d 1, 8, 12, 15, and 18 to evaluate markers of inflammation, and fecal samples were collected on d 1, 8, and every other day thereafter to determine the presence of oocysts. Calves were euthanized on d 18 for collection of intestinal tissues and histological and gene expression analyses. Relative to CON, calves in INF exhibited an increase in diarrhea severity, increased plasma serum amyloid A concentration on d 15 and 18, reduced intestinal villus height, increased villus apoptosis and crypt cell proliferation, and increased intestinal

mRNA expression of *MARVELD2* and *GPX2*. However, calves in SUC and GLP2 had reduced diarrhea severity and fecal *C. parvum* oocyst shedding, reduced plasma serum amyloid A concentration on d 15 and 18, and, depending on the intestinal segment, increased villus height, reduced crypt cell proliferation, and reduced mRNA expression of *MARVELD2*, *GPX2*, and other tight junction proteins relative to INF. Lastly, GLP2 and SUC exhibited increased intestinal mass-to-length ratio and decreased length-to-empty body weight ratio relative to INF. Our findings suggest that GLP-2 and Sucram treatments administered before a low-level *C. parvum* exposure may contribute to fewer effects on intestinal integrity, morphology, and inflammation in response to infection, and shorter, denser intestines.

Key words: coccidiosis, dairy calf, glucagon-like peptide 2, gut health

INTRODUCTION

Over 50% of early-life calf mortality is due to diarrhea (scours), and *Cryptosporidium parvum* is among the primary causative factors of this disease (Cho and Yoon, 2014). Diarrhea also contributes to calf morbidity, nutrient malabsorption, and poor production performance (Lorenz et al., 2011). Depending on the cause and severity of disease, treatment of diarrhea often is focused on palliative care, including replacement of lost fluids and electrolytes, reestablishment of acid-base balance within the digestive tract, and may include administration of anti-inflammatory drugs or antibiotics (Kahn and Line, 2010). In the case of cryptosporidiosis, halofuginone lactate is the only licensed drug available for prevention and treatment of the disease in newborn calves, which has shown limited benefits on reducing infection or animal production (Jarvie et al., 2005; De Waele et al., 2010) and is not licensed for use in the United States. Thus, therapies that minimize the nega-

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tive effects of diarrhea on gut function and improve animal productivity could greatly benefit the profitability of dairy calf production and improve animal well-being.

Recent studies in dairy calves suggest glucagon-like peptide 2 (**GLP-2**) therapy may be beneficial in the treatment of calf scours to reduce intestinal damage, improve barrier function, and increase nutrient absorption based on its various effects on the intestinal mucosa and blood flow (Taylor-Edwards, et al., 2011; Connor et al., 2013; Walker et al., 2015). Furthermore, artificial sweeteners can stimulate natural GLP-2 release from ruminant intestine, which is believed to be through the activation of sweet taste receptors expressed on intestinal L cells (Moran et al., 2014). Therefore, the potential exists to use dietary sweeteners to promote GLP-2-mediated benefits on intestinal health and function and reduce the negative effects of calf scours on dairy production. The purpose of the current study was to determine whether GLP-2 treatment or feeding of artificial sweetener to newborn dairy calves beginning 1 wk before oral inoculation with *C. parvum* oocysts can reduce the severity of diarrhea and negative effects of cryptosporidiosis on the gut, including changes in intestinal villus height, crypt cell proliferation, cellular apoptosis, indicators of inflammation and barrier function, and nitro-oxidative stress.

MATERIALS AND METHODS

All procedures involving animals were approved by the Beltsville Area Animal Care and Use Committee protocol number 14–001.

Animals

Holstein bull calves (n = 24; 6/treatment) were purchased from a local commercial dairy farm or obtained from the Beltsville Agricultural Research Center (**BARC**) dairy herd. Calves were fed colostrum at their first feeding and were transported at 1 to 2 d of age to an environmentally controlled building on the BARC research campus. All calves were housed in individual concrete pens (7.3 m²) bedded with wood shavings. Calves received 750,000 IU of penicillin G benzathine and penicillin G procaine, 2.5 mL of BO-SE (Merck Animal Health, Summit, NJ), 750 mg of vitamin C, 100,000 IU of vitamin A, 10,000 IU of vitamin D, 200 mg of iron dextran, and 35 mL of Quatracon-2 × (antiserum to provide passive immunity to *Arcanobacterium pyogenes*, *Escherichia coli*, *Pasteurella multocida*, and *Salmonella typhimurium*; Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO) at arrival and were confirmed negative for bovine viral diarrhea by a PCR-based blood test conducted by the Cornell University Animal Health Di-

agnostic Center (Ithaca, NY). All calves were provided continuous access to water and were fed a commercial milk replacer (Premium 20/20 Biomos, nonmedicated; Strauss Feeds, LLC, Watertown, WI) according to BW at a rate of 84 mL/kg daily, divided equally between 2 meals fed at approximately 0700 and 1300 h. The milk replacer contained 20.0% CP, 20.0% crude fat, 0.15% crude fiber, 0.75 to 1.25% Ca, 0.70% P, 66,000 IU/kg of vitamin A, 22,000 IU/kg vitamin D₃, and 440 IU/kg vitamin E. On d 1, 8, 12, 15, and 18, calves were weighed before feeding and the volume of milk replacer fed was adjusted at the next (afternoon) feeding. On d 1, mean (±SD) calf age was 8 (±2) d and BW was 44 (±6) kg.

Experimental Design

The experiment was conducted in 4 blocks: the first block consisting of noninfected control calves injected with buffer (**CON**; n = 6), and the remaining 3 blocks consisting of 6 calves each assigned by age and BW to 1 of 3 treatment groups (n = 2 per treatment group in each of 3 blocks) and experimentally infected with *C. parvum*. The 3 groups of infected calves were (1) injected with buffer (**INF**; n = 6); (2) injected with buffer and supplemented with Sucram (Pancosma, Geneva, Switzerland) at 400 mg/kg of DM of milk replacer (**SUC**; n = 6) as recommended by the manufacturer; and (3) injected with GLP-2 (**GLP2**; n = 6).

Plasma Analyses

On d 1, 8, 12, 15, and 18, blood was collected from each calf before feeding by jugular venipuncture using a heparinized Vacutainer tube (Becton Dickinson, Franklin Lakes, NJ). Plasma was harvested from whole blood after centrifugation (1,932 × g, 20 min, 4°C) and stored at −20°C. Serum amyloid A (**SAA**) was measured in duplicate samples using a multispecies SAA ELISA kit (Cat. No. TP-802; Tridel Development Limited, Maynooth, County Kildare, Ireland) according to manufacturer's instructions. Intra- and interassay coefficients of variation for SAA were 4.6 and 10.0%, respectively. Fatty acid binding protein 2 (**FABP-2**) was measured in duplicate samples using the Bovine FABP2, Intestinal ELISA Kit (Cat. No. E11F0030; Blue Gene, Shanghai, China) according to manufacturer's instructions. Intra- and interassay coefficients of variation for FABP-2 were 4.8 and 7.1%, respectively. Plasma tumor necrosis factor α (**TNF-α**) was measured in duplicate 250-μL samples by RIA according to Kenison et al. (1990) using rabbit anti-bovine (recombinant) TNF-α antibody (Kingfisher Biotech, Saint Paul, MN) at a 1:160,000 final dilution. Intra-assay coefficient of variation was

12.1%, minimum assay detection was 28 pg/mL, and the linearity (R^2) of the standard curve was 0.97.

GLP-2 Treatment

On d 1 through 18, calves in the GLP2 group received subcutaneous injections of bovine GLP-2 (AA 1 to 33 of the native bovine sequence, 95% purity; AAPPTec, LLC, Louisville, KY) every 12 h at a dosage of 50 µg/kg of BW (i.e., 100 µg/kg of BW daily) in sterile-filtered 0.01 M sodium carbonate/bicarbonate (pH 8.5) buffer. This dosage was shown to have beneficial effects on gut development and health in cattle (Taylor-Edwards et al., 2011; Connor et al., 2013; Walker et al., 2015). During the same period, calves in the CON, INF, and SUC groups received subcutaneous injections every 12 h with equivalent volumes of 0.01 M sodium carbonate/bicarbonate (pH 8.5) buffer. The volume of GLP-2 administered was adjusted according to BW measured on d 1, 8, 12, and 15.

C. parvum Inoculation and Fecal Analyses

Fecal samples were collected by digital rectal stimulation from all calves at arrival to the BARC campus and on experimental d 1, 8, 10, 12, 14, 16, and 18. Feces were smeared directly onto glass microscope slides and examined for *Cryptosporidium* oocysts by immunofluorescence microscopy using MeriFluor *Cryptosporidium*/*Giardia* reagents (Meridian Bioscience Inc., Cincinnati, OH). The slide was covered with a coverslip and the entire well area was examined by fluorescence microscopy at 400× using a Zeiss Axioskop equipped with epifluorescence and a FITC-Texas Red dual-wavelength filter (Carl Zeiss Microscopy, LLC, Thornwood, NY). Calves in the INF, SUC, and GLP2 groups were orally infected with 12,500 *C. parvum* oocysts [Iowa isolate (Harley Moon) passaged through calves; Waterborne Inc., New Orleans, LA) on d 8 (i.e., 1 wk after treatments began) by spiking the milk replacer fed through a nipples bottle at the afternoon feeding period. As points of reference, *C. parvum* oocyst shedding in feces was reported as high as 132,000 eggs/g among Canadian beef calves sampled up to 70 d of age (McAllister et al., 2005), yet the ID₅₀ (infectious dose 50%) is only 20 oocysts for symptoms of diarrhea and fecal parasite shedding in newborn dairy calves maintained in an experimental (clean) environment (Zambriski et al., 2013). Fecal scores were recorded daily for each calf, where 1 = normal; 2 = stool not formed; 3 = stool loose and runny; 4 = stool watery with visible solids; and 5 = stool watery with no visible solids, or bloody. Diarrhea severity index (DSI) was calculated for each calf as the sum of its daily fecal scores beginning the

day of inoculation through the day of euthanasia; thus, a normal score of 1 every day would equate to a DSI of 11. At slaughter, fecal samples of *C. parvum*-infected calves from experimental block 4 ($n = 2/\text{treatment}$) were processed and oocysts quantified as described by Santín et al. (2004).

Tissue Collection and RNA Extraction

On d 18, calves were administered a bolus intravenous injection of 5-bromo-2'-deoxyuridine (BrdU; MP Biomedicals LLC, Santa Ana, CA; 20 mg/mL in 0.9% sterile saline, pH 8.2) at a dosage of 5 mg/kg of BW through the jugular vein 2 h before slaughter to monitor proliferating intestinal cells. Calves were stunned by a penetrating captive bolt followed by exsanguination. The complete digestive tract was removed and processed as described by Connor et al. (2013). In addition, the epithelial mucosal layer was collected from segments of jejunum and ileum by scraping with a glass slide, stabilized in RNAlater (Life Technologies, Grand Island, NY) according to the manufacturer's instructions, and stored at -80°C until RNA extraction. Tissues were homogenized in Qiazol Lysis Reagent (Qiagen, Valencia, CA) and total RNA was extracted using the RNeasy Mini Kit (Qiagen) with on-column DNase digestion. The 2100 Bioanalyzer with RNA 6000 Nano LabChip Kits (Agilent Technologies, Palo Alto, CA) was used to evaluate RNA quality, and RNA concentration was measured using a ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE). The RNA integrity number of all RNA samples was at least 7.4, with the exception of a single sample from jejunum with a value of 6.6.

Morphometry and Immunostaining of Jejunum and Ileum

Mean villus height was calculated using captured images of hematoxylin-and-eosin-stained tissue sections of well-oriented villi using a BX-40 microscope equipped with a DP-70 digital CCD camera (Olympus America Inc., Center Valley, PA). An average ($\pm$ SD) of 11 ($\pm$ 2) villi per calf was measured in jejunum and 13 ($\pm$ 2) villi were measured in ileum using Image-Pro software (Media Cybernetics Inc., Rockville, MD).

To estimate the number of apoptotic cells in ileum and jejunum, immunolabeling of fragmented DNA formed during the process of apoptosis was performed on formalin-fixed, paraffin-embedded sections by Histoserv Inc. (Germantown, MD) using terminal deoxynucleotidyl transferase end-labeling with digoxigenin-conjugated deoxyuridine triphosphate (TUNEL assay). Labeled and unlabeled cells were manually counted

from captured images (as described above using an Olympus BX-40 microscope fitted with a DP-70 digital CCD camera) of 10 villi and 10 crypts per calf, and the percentage of labeled cells in each intestinal segment was calculated. In ileum, an average ($\pm$ SD) of 256 ($\pm$ 73) cells/crypt and 276 ($\pm$ 121) cells/villus were counted. In jejunum, 240 ($\pm$ 76) cells/crypt and 278 ($\pm$ 127) cells/villus were counted. Due to quality and orientation of histological sections from jejunum, only 11 samples (3 CON, 3 INF, 3 GLP2, and 2 SUC) were suitable for cell counting from the TUNEL assay. Cell proliferation in intestinal crypts was assessed by BrdU staining as described by Connor et al. (2013). For each calf, the numbers of BrdU-labeled and -unlabeled cells were manually counted from captured images obtained as described above of 9 to 13 well-oriented crypts, and the percentage of BrdU-labeled cells was calculated. In jejunum, 547 ($\pm$ 194) cells/crypt were counted, and in ileum, 449 ($\pm$ 125) cells/crypt were counted. All counts were made by a single observer blinded to experimental treatment.

The presence and abundance of nitrated intestinal proteins in ileal mucosa was estimated as an indicator of inflammation-related nitro-oxidative stress using quantitative immunohistochemical procedures for 3'-nitrotyrosine (NT) previously described and validated for similar applications (Elsasser et al., 2007; Connor et al., 2013), with minor modifications described herein. Paraffin-embedded tissue sections on glass slides were deparaffinized by traditional immersion in a triplicate series each of xylene then absolute ethanol baths. Slides were transferred to a solution of methanol containing 3% H₂O₂ for 30 min and then rehydrated into aqueous Tris-saline buffer (pH 7.5) through a series of 5 baths decreasing in ethanol concentration (100, 100, 95, and 70%, then distilled water). Tissues were treated for 10 min with 0.1% Triton-X-100 (Sigma-Aldrich, St. Louis, MO) and rewashed in Tris-saline buffer. Nonspecific blocking was accomplished by incubating the slides for 1 h at 24°C in Tris-saline containing 3.5% normal goat serum (Sigma Biologicals, St. Louis, MO). Slides were further incubated overnight with rabbit anti-NT primary antibody (1:300, Life Technologies, Eugene, OR) at 4°C. After 3 washes with Tris-saline buffer, secondary antibody (goat anti-rabbit IgG, Abcam Laboratories, Cambridge, MA) was applied for 2 h at room temperature. Following rinsing, all slides were incubated simultaneously by immersion into 200 mL of Tris buffer (pH 7.5) containing 150 mg of 3,3'-diaminobenzidine HCl and 50 μ L 30% H₂O₂. A slide containing intestinal tissue known to be positive for NT immunostaining from Connor et al. (2013) was processed along with the present experiment slides and the 3,3'-diaminobenzidine HCl color development monitored with the development of

the brown staining in the correct locations of the positive control. Staining development was quenched by immersion into ice-cold Tris buffer followed by nuclear counterstaining for 1 min with Carazzi's hematoxylin (Bancroft and Stevens, 1982), dehydration through ethanol to xylene, and finished with the addition of glass coverslips. For quantitating cellular NT content as a valid marker for the presence of nitro-oxidative stress across treatments, 5 separate images of each animal's intestinal tissue were captured using an Olympus DP-70 CCD camera mounted on an Olympus BX-40 microscope. In addition, 1 image of a slide containing the known NT-positive control was captured. For each animal, a master composite of the 5 images for that animal plus the 1 image of the positive control was formed. The NT staining for the NT-positive control was used to establish the *.RBG pixel identification algorithm used by the analysis program (Image-Pro Premier Ver. 9.1, Media Cybernetics). For each master image and to maximize continuity across the master images, the total pixels per cell of the positive control was adjusted to differ among all master images by less than 1%. With that optimization established, the positive pixel identification criteria were applied to each of the other 5 images of the master and the analytical data captured as associated with areas of interest particular to the villi. Data consisted of percentage of positive cells, mean number of positive pixels per cell, and pixel cluster size (area) for positive cells.

Quantitative Real-Time PCR

The mRNA expression of the pathogen recognition protein toll-like receptor 2 (*TLR2*), proinflammatory cytokines interleukin 18 (*IL18*) and tumor necrosis factor α (*TNF*), the antioxidant enzyme glutathione peroxidase 2 (*GPX-2*), the cellular tight junction proteins tricellulin (*MARVELD2*), occludin (*OCN*), and tight junction protein 1 (*TJP1*), and the secretory protein trefoil factor 3 (*TFF3*) was quantified in jejunum and ileum epithelium using the CFX96 Touch qPCR System (Bio-Rad Laboratories, Hercules, CA). Reverse transcription using 1.0 μ g of total RNA per 20- μ L reaction was conducted using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories), according to the manufacturer's instructions. Real-time quantitative PCR was performed in triplicate 20- μ L reactions using SsoAdvanced Universal SYBR Green Super Mix (Bio-Rad Laboratories) according to kit instructions and a final concentration of 500 nM for each primer. Cycling conditions were 95°C for 1 min, followed by 40 cycles of 95°C for 10 s, annealing temperature for 10 s, and 72°C for 30 s, then 95°C for 10 s followed by a melt curve analysis. Primer sequences (Table 1) were designed

Table 1. Primer sequences and annealing temperatures used for real-time quantitative PCR

Gene symbol	Gene name	Sense primer (5' → 3')	Antisense primer (5' → 3')	Annealing temperature °C
<i>GPX2</i>	Glutathione peroxidase 2	TCCTGAGCATTCCTGTGCC	CAACCAGAAGGTTGCCGGTA	59.0
<i>IL18</i>	Interleukin 18	ATAAAGATGGCTGCAGAACAACT	GATCTGATTCCAGGTCTTCATCA	59.0
<i>MARVELD2</i>	Tricellulin	CCGATCTGGGTTTCGGAGC	AGTGTCTGTCCCGATTCTCTG	59.0
<i>TFF3</i>	Trefoil factor 3	TTTTCTGAGCAGTCGGGTCC	CCACATACTGCCCGGTTGAA	59.0
<i>TLR2</i>	Toll-like receptor 2	TCCTGTTGCTCCTGCTCAC	GAATCCCGCTCGCTGTAGG	57.0
<i>TNF</i>	Tumor necrosis factor α	CCCCTGAGATAACCTCCCA	CAGACGGGAGACAGGAGAGC	56.0
<i>OCN</i>	Occludin	ACGCAGGAAGTGCCTTTGGTAGC	GCAGCCATGGCCAGCAGGAA	63.0
<i>TJP1</i>	Tight junction protein 1	AATGCATCCTGACCACCAGG	GATGGTGCCGGGTTTGTTC	63.0

using primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/) except those for *OCN* (from Malmuthuge et al., 2013), *TJP1* (from Walker et al., 2015), and *TNF* (from Mookherjee et al., 2006). Primer specificity was validated by direct DNA sequencing (CEQ 8000 GeXP genetic analysis system and DTCS Quick Start Kit, Beckman Coulter Inc., Brea, CA) of gel-purified (Qiagen Gel Extraction Kit) amplicons from a standard end-point PCR reaction. Transcript abundance in samples was based on interpolation from a standard curve evaluated simultaneously with samples as described by Connor et al. (2005). Assay amplification efficiencies ranged from 89.9 to 103.9%, and linearity (R^2) of standard curves ranged from 0.997 to 1.000.

Statistical Analyses

The presence of *Giardia* was detected in fecal samples of 6 calves during the experimental trial ($n = 1$ CON; $n = 1$ INF; $n = 2$ SUC; and $n = 2$ GLP2); thus, *Giardia* infection status (positive or negative) was included as a main effect and an interaction term in all statistical models. Due to the small number of *Giardia*-positive animals, no *Giardia* status-specific interpretations were made when *Giardia* main effects or interactions were significant. For plasma SAA, FABP-2, and TNF- α , treatment means were compared using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC), specifying calf, *Giardia* infection status, treatment, and day as CLASS variables; in the MODEL statement, plasma analyte was the dependent variable and *Giardia* status, treatment, day, and the interactions of treatment by day, *Giardia* by day, *Giardia* by treatment, and *Giardia* by treatment by day were the independent effects. Within-calf correlations among days for FABP-2 and TNF- α were modeled using a heterogeneous compound symmetric covariance structure; no correlations were observed for SAA. For all other variables measured at a single time point, treatment means were compared using the GLIMMIX procedure of SAS to fit generalized linear mixed effects

models (Stroup, 2015), where *Giardia* status, calf, and treatment were the CLASS variables and the MODEL statement included the measurement of interest as the dependent variable and *Giardia* status, treatment, and *Giardia* by treatment as the independent effects. Jejunal and ileal gene counts, ileal nitrotyrosine as pixels per cell, and fecal oocyst counts were modeled using a negative binomial distribution with log link function. The DSI and ileal nitrotyrosine measured as a percentage of positive cells were modeled using a β distribution with logit link function. All other dependent variables were modeled using a normal distribution with identity link function. Significance tests were conducted for the contrasts (1) CON versus INF, (2) INF versus (GLP2 + SUC)/2, and (3) GLP2 versus SUC.

RESULTS

All CON calves tested negative for *Cryptosporidium* by fecal examination throughout the trial, and the experimentally *C. parvum*-infected groups were negative for *Cryptosporidium* until at least 6 d after oral inoculation. As expected, INF, SUC, and GLP2 exhibited mild diarrhea in response to *C. parvum* infection. The DSI of INF was greater ($P < 0.001$) than CON and was reduced ($P = 0.02$) in GLP2 and SUC combined relative to INF (Figure 1A). No difference ($P = 0.66$) in DSI was observed between SUC and GLP2. Fecal oocyst counts at slaughter also were reduced ($P = 0.09$) in GLP2 and SUC combined, relative to INF (Figure 1B), despite a limited sample size of 2 calves per treatment group. We found no difference ($P = 0.78$) between GLP2 and SUC in fecal oocyst counts. Mean ($\pm$ SE) ADG (kg/d) of calves was 0.43 (± 0.03) and gain-to-feed was 0.05 (± 0.00); neither variable differed ($P \geq 0.17$) by experimental treatment.

Plasma Analytes

Figure 2 summarizes plasma concentrations of FABP-2, TNF- α , and SAA throughout the experimental pe-

riod. We noted no treatment by day interaction ($P > 0.32$) on plasma FABP-2 or TNF- α concentrations, but did note an interaction ($P = 0.04$) for SAA concentration. No effects ($P \geq 0.39$) of treatment or day were detected on plasma FABP-2 or TNF- α concentrations. For plasma SAA concentration, we observed no differences ($P \geq 0.29$) among treatments on d 1, 8, or 12, but differences were detected ($P \leq 0.05$) among treat-

ments on d 15 and 18 (Figure 2C). On d 15 and 18, plasma SAA was elevated ($P \leq 0.001$) in INF relative to CON and was reduced ($P < 0.01$) in SUC and GLP2 combined relative to INF. No difference ($P \geq 0.47$) was detected in plasma SAA between GLP2 and SUC.

Intestinal Morphometry

Table 2 summarizes the effects of *C. parvum* infection and GLP-2 or Sucram treatments on intestinal measurements of calves obtained at slaughter. Of all intestinal measures evaluated, only jejunum 1-m section weight was increased ($P = 0.04$) in INF relative to CON. Combined, SUC and GLP2 exhibited increased ($P = 0.04$) mass-to-length ratio and decreased ($P \leq 0.03$) length-to-empty BW ratio in both small and large intestines relative to INF. Ileum 1-m section weight was also greater ($P = 0.09$) in SUC and GLP2 combined versus INF. Total small intestine mass as a percentage of empty BW was reduced ($P < 0.01$) and length-to-empty BW ratio in small intestine was decreased ($P = 0.06$) in SUC relative to GLP2.

Effects of treatment and infection on intestinal villus height are shown in Figure 3. Villus height was reduced in INF relative to CON in both jejunum ($P = 0.09$) and ileum ($P = 0.02$), and was reduced ($P = 0.02$) in ileum of INF relative to SUC and GLP2 combined. We found no difference ($P = 0.37$) in jejunal villus height between INF and SUC and GLP2 combined. Villus height was greater ($P \leq 0.10$) in GLP2 than SUC in both jejunum and ileum.

Intestinal Apoptosis and Crypt Cell Proliferation

The percentage of cells undergoing apoptosis, as assessed by TUNEL assay, in crypts and villi of the small intestine are shown in Figure 4. In the jejunum, we observed no effect ($P = 0.91$) of treatment on apoptosis in crypts, but percentage of cells undergoing apoptosis in jejunal villi was increased ($P = 0.01$) in INF relative to CON. We noted no difference ($P \geq 0.76$) in cellular apoptosis between INF and GLP2 and SUC combined, or between GLP2 and SUC.

In ileum, percentage of apoptotic cells in villi was greater ($P = 0.07$) in INF relative to CON, as well as in GLP2 and SUC combined relative to INF. Apoptosis did not differ ($P = 0.96$) between SUC and GLP2 in ileal villi. In ileal crypts, apoptosis did not differ ($P \geq 0.32$) between INF and CON or between INF and SUC and GLP2 combined. Cellular apoptosis in ileum was reduced ($P = 0.02$) in crypts of SUC relative to GLP2.

Crypt cell proliferation in ileum and jejunum, as measured by positive nuclear staining for BrdU, is sum-

Figure 1. Fecal analyses of noninfected control Holstein bull calves (CON), calves infected with *Cryptosporidium parvum* (INF), calves infected with *C. parvum* and injected subcutaneously every 12 h with 50 μ g/kg of BW of glucagon-like peptide 2 beginning 1 wk before infection (GLP2), and calves infected with *C. parvum* and fed milk replacer supplemented with Sucram (400 mg/kg of DM; Pancosma, Geneva, Switzerland) beginning 1 wk before infection (SUC). (A) Mean diarrhea severity index (DSI) with 95% CI; $n = 6$ /treatment. The DSI is the sum of the daily fecal scores recorded over an 11-d period, where score 1 = normal; 2 = stool not formed; 3 = stool loose and runny; 4 = stool watery with visible solids; and 5 = stool watery with no visible solids, or bloody. DSI = 11 indicates normal; DSI = 55 indicates the worst possible diarrhea. (B) Mean ($\pm$ SE) oocysts per gram of feces on d 18; $n = 2$ /treatment. DSI contrasts: INF vs. CON, $P < 0.001$; INF vs. (GLP2 + SUC)/2, $P = 0.02$; GLP2 vs. SUC, $P = 0.66$; Oocyst shedding contrasts: INF vs. (GLP2 + SUC)/2, $P = 0.09$; GLP2 vs. SUC, $P = 0.78$.

Table 2. Effects of *Cryptosporidium parvum* infection and glucagon-like peptide 2 (GLP-2) or Sucram (Pancosma, Geneva, Switzerland) treatments beginning 1 wk before infection on mean (±SE) intestinal measures of Holstein bull calves obtained at slaughter on d 18

Variable	Treatment ¹ (n = 6/group)				P-value ²
	CON	INF	GLP-2	SUC	
Small intestine					
Mass, % EBW ³	3.35 (±0.17)	3.27 (±0.17)	3.38 (±0.13)	2.83 (±0.13)	0.04
Mass:length, g/m	50.7 (±3.6)	56.7 (±3.6)	66.3 (±2.9)	65.1 (±2.9)	0.01
Length:EBW, m/kg	0.658 (±0.035)	0.580 (±0.035)	0.516 (±0.027)	0.433 (±0.027)	<0.001
Duodenum 1-m section wt, g	48.8 (±3.7)	50.1 (±3.7)	55.4 (±2.9)	57.3 (±2.9)	0.26
Jejunum 1-m section wt, g	37.8 (±4.0)	50.7 (±4.0)	59.1 (±3.2)	52.1 (±3.2)	<0.01
Ileum 1-m section wt, g	91.7 (±11.7)	85.1 (±11.7)	111.0 (±9.3)	108.1 (±9.3)	0.28
Large intestine					
Mass, % EBW	1.51 (±0.09)	1.42 (±0.09)	1.43 (±0.07)	1.28 (±0.07)	0.28
Mass:length, g/m	169.1 (±14.3)	169.8 (±14.3)	209.0 (±11.3)	205.2 (±11.3)	0.07
Length:EBW, m/kg	0.090 (±0.006)	0.084 (±0.006)	0.070 (±0.005)	0.064 (±0.005)	0.02

¹CON = noninfected control calves; INF = calves infected with *Cryptosporidium parvum*; GLP-2 = calves infected with *C. parvum* and injected subcutaneously every 12 h with 50 µg/kg of BW of GLP-2 beginning 1 wk before infection; and SUC = calves infected with *C. parvum* and fed milk replacer supplemented with Sucram (400 mg/kg of DM) beginning 1 wk before infection.

²P-values ≤0.10 are indicated in bold font. Contrast 1 = INF vs. CON, Contrast 2 = INF vs. (GLP-2 + SUC)/2, Contrast 3 = GLP-2 vs. SUC.

³EBW = empty body weight.

Figure 2. Plasma concentrations (mean ± SE) of fatty acid binding protein 2 (FABP-2), tumor necrosis factor α (TNF-α), and serum amyloid A (SAA) in noninfected control Holstein bull calves (CON), calves infected with *Cryptosporidium parvum* (INF), calves infected with *C. parvum* and injected subcutaneously every 12 h with 50 µg/kg of BW of glucagon-like peptide 2 beginning 1 wk before infection (GLP2), and calves infected with *C. parvum* and fed milk replacer supplemented with Sucram (400 mg/kg of DM; Pancosma, Geneva, Switzerland) beginning 1 wk before infection (SUC). All infected calves were orally inoculated with 12,500 *C. parvum* oocysts on d 8. Asterisk (*) represents contrasts: INF vs. CON, $P < 0.001$; INF vs. (GLP2 + SUC)/2, $P = 0.004$; GLP2 vs. SUC, $P = 0.02$; dagger (†) represents contrasts: INF vs. CON, $P = 0.001$; INF vs. (GLP2 + SUC)/2, $P = 0.002$; GLP2 vs. SUC, $P = 0.47$.

marized in Figure 5. In jejunum, crypt cell proliferation was greater ($P = 0.01$) in INF relative to CON, and was greater ($P = 0.10$) in INF relative to SUC and GLP2 combined. Cell proliferation in jejunal crypts was similar ($P = 0.42$) between GLP2 and SUC. In ileum, crypt cell proliferation was greater ($P < 0.01$) in INF compared with CON, but did not differ ($P \geq 0.25$) between INF and GLP2 and SUC combined or between GLP2 and SUC.

Figure 3. Villus height (mean $\pm$ SE) in (A) jejunum and (B) ileum of noninfected control Holstein bull calves (CON), calves infected with *Cryptosporidium parvum* (INF), calves infected with *C. parvum* and injected subcutaneously every 12 h with 50 μ g/kg of BW of glucagon-like peptide 2 beginning 1 wk before infection (GLP2), and calves infected with *C. parvum* and fed milk replacer supplemented with Sucram (400 mg/kg of DM; Pancosma, Geneva, Switzerland) beginning 1 wk before infection (SUC); $n = 6$ /treatment. Jejunum contrasts: INF vs. CON, $P = 0.09$; INF vs. (GLP2 + SUC)/2, $P = 0.37$; GLP2 vs. SUC, $P = 0.06$; Ileum contrasts: INF vs. CON, $P = 0.02$; INF vs. (GLP2 + SUC)/2, $P = 0.02$; GLP2 vs. SUC, $P = 0.10$.

Figure 4. Apoptosis in (A) jejunum and (B) ileum expressed as mean ($\pm$ SE) percentage of cells positive for terminal deoxynucleotidyl transferase end-labeling of non-infected control Holstein bull calves (CON), calves infected with *Cryptosporidium parvum* (INF), calves infected with *C. parvum* and injected subcutaneously every 12 h with 50 μ g/kg of BW of glucagon-like peptide 2 beginning 1 wk before infection (GLP2), and calves infected with *C. parvum* and fed milk replacer supplemented with Sucram (400 mg/kg of DM; Pancosma, Geneva, Switzerland) beginning 1 wk before infection (SUC); $n = 6$ /treatment unless otherwise indicated. Due to quality and orientation of histological sections from jejunum, fewer than 6 calves per treatment were suitable for cell counting from the TUNEL assay. Jejunal villi contrasts: INF vs. CON, $P = 0.01$; INF vs. (GLP2 + SUC)/2, $P = 0.86$; GLP2 vs. SUC, $P = 0.76$; Ileal villi contrasts: INF vs. CON, $P = 0.07$; INF vs. (GLP2 + SUC)/2, $P = 0.07$; GLP2 vs. SUC, $P = 0.96$; Ileal crypts contrasts: INF vs. CON, $P = 0.87$; INF vs. (GLP2 + SUC)/2, $P = 0.32$; GLP2 vs. SUC, $P = 0.02$.

Protein Tyrosine Nitration in Ileum

Results of ileal nitrated protein immunostaining estimated as number of positive pixels per cell, percentage of positive cells, and positive pixel area are summarized in Table 3. Two of the estimates of tissue nitro-oxidative stress, namely positive pixels per cell and positive

pixel area, were reduced ($P \leq 0.09$) in SUC relative to GLP2. No other effects on protein tyrosine nitration were observed.

Intestinal Gene Expression

Table 4 summarizes mRNA expression of 8 genes of interest (*IL18*, *TNF*, *GPX2*, *MARVELD2*, *TFF3*,

OCN, *TLR2*, and *TJP1*) by treatment group in jejunum and ileum of calves evaluated on d 18 of the study. In jejunum, we found no treatment effect ($P = 0.60$) on *TLR2* mRNA expression, but expression of *IL18*, *GPX2*, and *MARVELD2* was increased ($P \leq 0.02$) and *TNF* was decreased ($P = 0.02$) in INF versus CON. Among these genes, only *MARVELD2* mRNA expression in jejunum was reduced ($P = 0.01$) in SUC and GLP2 combined relative to INF. The mRNA expression of *MARVELD2* was no different ($P = 0.97$) in GLP than in SUC, but expression of *GPX2* was reduced ($P = 0.01$) in GLP2 relative to SUC. The mRNA expression of *TFF3*, *OCN*, and *TJP1* was not different ($P \geq 0.25$) in jejunum of INF relative to CON or in GLP2 versus SUC, although expression of these transcripts was reduced ($P \leq 0.08$) in GLP2 and SUC combined compared with INF. As in jejunum, mRNA expression of *GPX2* and *MARVELD2* was increased ($P \leq 0.03$) in INF versus CON. The *GPX2* mRNA expression in ileum was reduced ($P = 0.02$) in GLP2 and SUC combined relative to INF; however, no difference ($P = 0.95$) in *GPX2* mRNA expression was detected between SUC and GLP2 in ileum.

Figure 5. Crypt cell proliferation in (A) jejunum and (B) ileum expressed as mean ($\pm$ SE) percentage of cells positive for 5-bromo-2'-deoxyuridine (BrdU) labeling of noninfected control Holstein bull calves (CON), calves infected with *Cryptosporidium parvum* (INF), calves infected with *C. parvum* and injected subcutaneously every 12 h with 50 μ g/kg of BW of glucagon-like peptide 2 beginning 1 wk before infection (GLP2), and calves infected with *C. parvum* and fed milk replacer supplemented with Sucram (400 mg/kg of DM; Pancosma, Geneva, Switzerland) beginning 1 wk before infection (SUC); $n = 6$ /treatment. Jejunum contrasts: INF vs. CON, $P = 0.01$; INF vs. (GLP2 + SUC)/2, $P = 0.10$; GLP2 vs. SUC, $P = 0.42$; Ileum contrasts: INF vs. CON, $P < 0.01$; INF vs. (GLP2 + SUC)/2, $P = 0.25$; GLP2 vs. SUC, $P = 0.59$.

DISCUSSION

In the United States, *Cryptosporidium* is the most common diarrhea-causing pathogen in newborn calves, wherein the most prevalent form is *C. parvum* (Santín et al., 2004). The prepatent period of *C. parvum* in ruminants is about 4 d, and peak shedding of oocysts typically occurs at approximately 2 wk of age during natural infections (Thompson et al., 2008). Cryptosporidiosis may cause mucous or watery diarrhea lasting 4 to 14 d or be asymptomatic, but pathology associated with infection includes atrophy of intestinal villi, reduced gut barrier function, altered apoptosis rate of mucosal cells, and inflammation (Thompson et al., 2008). In the present study, calves orally infected with 12,500 *C. parvum* oocysts exhibited mild diarrhea and a significant increase in DSI compared with noninfected control calves. However, in general, infected calves treated daily with GLP-2 or Sucram beginning 1 wk before oral inoculation had a reduced DSI compared with nontreated, infected calves, suggesting that both treatments may provide some protection to the damaging effects of *C. parvum* on the gut. Furthermore, fecal analysis of a subset of GLP-2 and Sucram-fed calves suggested these treatments may contribute to reduced number of *Cryptosporidium* oocysts shed per gram of feces on d 10 of infection. Based on results from the 2 observed calves per treatment (mean $\pm$ SE; INF = 25,814 $\pm$ 38,656, GLP2 = 212 $\pm$ 318, and SUC = 400 $\pm$ 599), a post-hoc power analysis indicated 4 or

Table 3. Effects of *Cryptosporidium parvum* infection and glucagon-like peptide 2 (GLP-2) or Sucram (Pancosma, Geneva, Switzerland) treatments beginning 1 wk before infection on mean ($\pm$ SE) protein tyrosine nitration immunostaining in ileum of Holstein bull calves at d 18

Variable	Treatment ¹ (n = 6/group)				P-value ²			
	CON	INF	GLP2	SUC	Treatment	Contrast 1	Contrast 2	Contrast 3
Pixels/cell	68.5 ($\pm$ 17.0)	66.1 ($\pm$ 16.4)	83.4 ($\pm$ 16.4)	50.1 ($\pm$ 10.0)	0.37	0.92	0.94	0.09
% Positive	29.8 ($\pm$ 5.2)	26.0 ($\pm$ 4.9)	33.5 ($\pm$ 4.4)	24.3 ($\pm$ 3.9)	0.45	0.61	0.66	0.14
Positive area	217.6 ($\pm$ 15.9)	227.4 ($\pm$ 15.9)	241.6 ($\pm$ 12.6)	206.4 ($\pm$ 12.6)	0.29	0.67	0.85	0.06

¹CON = noninfected control calves; INF = calves infected with *Cryptosporidium parvum*; GLP-2 = calves infected with *C. parvum* and injected subcutaneously every 12 h with 50 μ g/kg of BW of GLP-2 beginning 1 wk before infection; and SUC = calves infected with *C. parvum* and fed milk replacer supplemented with Sucram (400 mg/kg of DM) beginning 1 wk before infection.

²P-values ≤ 0.10 are indicated in bold font. Contrast 1 = INF vs. CON, Contrast 2 = INF vs. (GLP-2 + SUC)/2, Contrast 3 = GLP-2 vs. SUC.

more calves per treatment would allow the observed difference between INF and GLP2 to be detected as statistically significant. Similarly, 5 or more calves per treatment would allow the observed difference between INF and SUC to be detected as statistically significant. Hence, the 2 calves per treatment means comparisons of INF versus SUC ($P = 0.14$) and GLP2 ($P = 0.11$) suggests potential reduction in fecal oocyst counts with these treatments may have been detected with a larger sample size and are of biological interest.

Because GLP-2 has been shown to affect expression of tight junctions in epithelial cells of the intestine (Yu et al., 2014; Walker et al., 2015), treatment may reduce the ability of *C. parvum* to attach to the microvilli and penetrate the host epithelium. Alternatively, Costa et al. (2011) demonstrated that well-nourished weaned mice challenged with *C. parvum* shed fewer fecal oocysts and for a shorter duration than malnourished infected mice. Thus, effects of GLP-2 and Sucram on DSI may be related to their effects on the calf's nutrient status, as both GLP-2 and Sucram are known to increase intestinal nutrient transport and absorption (Moran et al., 2014; Connor et al., 2015). Clearly, additional study is needed to determine whether GLP-2 or Sucram treatments consistently reduce DSI in cryptosporidiosis or could affect oocyst shedding, as well as their mechanisms of action.

Plasma indicators of impaired intestinal integrity (FABP-2) and the acute phase response (TNF- α) evaluated in infected calves relative to noninfected controls showed no significant changes in plasma concentrations over time or by treatment through d 10 of *C. parvum* infection despite a significant increase in DSI. Circulating concentrations of FABP-2 are elevated in neonatal humans with necrotizing enterocolitis (Schurink et al., 2015) and patients with celiac disease and villus atrophy (Derikx et al., 2009; Adriaanse et al., 2013), as the binding protein is primarily produced by epithelial cells of the small intestine and is released into circulation during gut damage (Bottasso Arias et al., 2015). No studies to date have examined relationships between

intestinal disease or damage and circulating FABP-2 in cattle, although we anticipate similar relationships to those observed in humans. Failure to detect a change in FABP-2 in *C. parvum*-infected calves indicates that plasma FABP-2 may not be a reliable indicator of intestinal damage in cattle or that calves in our study did not have sufficient mucosal injury to significantly increase circulating FABP-2 concentration. Previous work in similarly aged Holstein calves to those in the present study and infected with a much larger infective dose of *C. parvum* (5×10^5 oocysts) showed intestinal permeability more than double when measured 7 d postinfection, which remained elevated through 14 d postinfection (Klein et al., 2008).

Previous studies of humans and mice during experimentally induced cryptosporidiosis reported increased local *TNF* mRNA production within the intestinal mucosa (Lacroix et al., 2001; Robinson et al., 2001; Ehigiator et al., 2005), as well as increased systemic TNF- α in children naturally infected with *Cryptosporidium* (Kirkpatrick et al., 2006) relative to healthy individuals. Incubation of isolated bovine PMNL with *C. parvum* also increased *TNF* gene transcription (Muñoz-Caro et al., 2015), and experimental *C. parvum* inoculation of BALB/C mice with 10^6 oocysts increased serum TNF- α concentration (Codices et al., 2013). In our study, plasma TNF- α levels of calves indicated that the cryptosporidiosis induced by an inoculation with 12,500 oocysts was not sufficient to elicit systemic inflammation and *TNF* mRNA was actually reduced at this time point. Furthermore, we evaluated the local inflammatory response in the jejunum and ileum by examining *TLR2* and *IL18* mRNA expression. In previous studies, *TLR2* mRNA was upregulated in cultured bovine intestinal epithelial cells exposed to *C. parvum*, which is believed to serve as an initial activator of the innate immune response to exposure to the parasite (Yang et al., 2015). Similarly, expression of the proinflammatory response gene *IL18* was increased in human primary intestinal epithelial cells in response to *C. parvum* infection (McDonald et al., 2006), and

Table 4. Effects of *Cryptosporidium parvum* infection and glucagon-like peptide 2 (GLP-2) or Sucram (Pancosma, Geneva, Switzerland) treatments beginning 1 wk before infection on intestinal mRNA expression of 8 genes of interest in Holstein bull calves at d 18.

Gene	Treatment ¹ (n = 6/group)				P-value ²			
	CON	INF	GLP2	SUC	Treatment	Contrast 1	Contrast 2	Contrast 3
Jejunum ³								
<i>IL18</i>	11,577 (±2,275)	23,433 (±4,603)	16,735 (±2,599)	15,714 (±2,440)	0.13	0.02	0.12	0.78
<i>TNF</i>	1,664 (±281)	920 (±156)	1,099 (±147)	1,293 (±173)	0.11	0.02	0.20	0.40
<i>GPX2</i>	46,039 (±8,288)	170,422 (±30,677)	90,534 (±12,884)	159,390 (±22,683)	<0.001	0.0001	0.11	0.01
<i>MARVELD2</i>	2,850 (±366)	5,168 (±662)	3,471 (±352)	3,449 (±350)	0.03	<0.01	0.01	0.97
<i>TFF3</i>	616,669 (±118,074)	762,232 (±145,944)	515,956 (±78,101)	500,214 (±75,718)	0.34	0.45	0.08	0.89
<i>OCLN</i>	61,725 (±10,035)	76,254 (±12,396)	53,999 (±6,940)	47,692 (±6,130)	0.18	0.37	0.04	0.50
<i>TLR2</i>	885 (±223)	1,034 (±260)	680 (±136)	885 (±176)	0.60	0.67	0.33	0.36
<i>TJP1</i>	27,974 (±3,161)	33,788 (±3,818)	26,347 (±2,354)	24,055 (±2,149)	0.17	0.25	0.04	0.48
Ileum								
<i>IL18</i>	19,074 (±1,983)	17,526 (±1,822)	17,404 (±1,431)	14,206 (±1,168)	0.16	0.57	0.38	0.10
<i>TNF</i>	3,786 (±339)	3,058 (±274)	3,602 (±255)	3,142 (±223)	0.23	0.11	0.37	0.19
<i>GPX2</i>	40,159 (±10,239)	218,290 (±55,653)	102,206 (±20,600)	104,147 (±20,992)	<0.01	<0.001	0.02	0.95
<i>MARVELD2</i>	804 (±142)	1,449 (±254)	1,208 (±168)	1,245 (±173)	0.14	0.03	0.42	0.88
<i>TFF3</i>	164,067 (±42,582)	229,693 (±59,614)	195,181 (±40,048)	310,739 (±63,758)	0.26	0.37	0.82	0.13
<i>OCLN</i>	7,588 (±1,584)	10,380 (±2,166)	8,607 (±1,420)	10,335 (±1,705)	0.62	0.30	0.69	0.44
<i>TLR2</i>	1,676 (±230)	1,659 (±228)	1,930 (±209)	1,289 (±140)	0.11	0.96	0.75	0.02
<i>TJP1</i>	9,474 (±1,541)	13,695 (±2,227)	12,571 (±1,616)	11,995 (±1,542)	0.43	0.13	0.57	0.80

¹Values are mean (±SE) numbers of transcripts per 100 ng of total RNA. CON = noninfected control calves; INF = calves infected with *Cryptosporidium parvum*; GLP-2 = calves infected with *C. parvum* and injected subcutaneously every 12 h with 50 µg/kg of BW of GLP-2 beginning 1 wk before infection; and SUC = calves infected with *C. parvum* and fed milk replacer supplemented with Sucram (400 mg/kg of DM) beginning 1 wk before infection.

²P-values ≤0.10 are indicated in bold font. Contrast 1 = INF vs. CON, Contrast 2 = INF vs. (GLP-2 + SUC)/2, Contrast 3 = GLP-2 vs. SUC.

³*IL18* = interleukin 18; *TNF* = tumor necrosis factor α; *GPX2* = glutathione peroxidase 2; *MARVELD2* = triceulinin; *TFF3* = trefoil factor 3; *OCLN* = occludin; *TLR2* = toll-like receptor 2; *TJP1* = tight junction protein 1.

at 3 d postinfection in mice with an impaired TH-1 immune response (Ehigiator et al., 2005). In our calves, we observed no detectable changes in *TLR2* mRNA in response to *C. parvum* measured 10 d postinfection, although this time point may have been too late to detect the tissue response to infection. Sucram-treated calves did exhibit lower *TLR2* mRNA in ileum relative to GLP-2-treated calves, but similar effects were not observed in jejunum. We did observe an increase in *IL18* mRNA in calf jejunum of infected calves, consistent with responses in humans, but treatments with GLP-2 and Sucram showed no significant benefits on intestinal *IL18* transcript abundance even though *IL18* mRNA tended to be reduced in ileum of Sucram-treated calves relative to those receiving GLP-2.

Unlike plasma FABP-2 and TNF- α , we did observe increased abundance of the acute phase plasma marker SAA in *C. parvum*-infected calves versus noninfected controls by d 15 of the study (8 d postinoculation). Serum amyloid A has been shown to increase in animals exhibiting diarrhea caused by *C. parvum* and rotavirus (Enemark et al., 2003). The GLP-2- and Sucram-treated calves, however, exhibited reduced SAA concentrations relative to infected controls on d15 and 18. These findings suggest that the GLP-2 and Sucram treatments may contribute to a shortened duration of intestinal injury in response to *C. parvum* infection.

To evaluate possible means by which GLP-2 and Sucram treatments may be reducing intestinal effects of *C. parvum* infection, we evaluated mRNA expression of tight junction protein genes *MARVELD2*, *OCN*, and *TJP1*, antioxidant gene *GPX2*, as well as *TFF3*, a mucin-associated protein that helps to maintain integrity of the intestinal epithelium and promote healing of damaged cells (Hoffmann et al., 2001). Moran et al. (2012) reported that a human colon cell line treated with GLP-2 reduced cellular permeability, which was associated with increased expression of the tight junction proteins occludin and tight junction protein 1, and diminished the detrimental effects of TNF- α treatment on cellular barrier function. In addition, GLP-2 treatment of porcine jejunal epithelium increased expression of *OCN* and *TJP1* mRNA and proteins (Yu et al., 2014). We previously observed reduced expression of tight junction protein genes in intestinal segments of newborn calves infected with the coccidian parasite *Eimeria bovis*, and restored tight junction gene expression in infected calves treated with GLP-2 for 10 d, beginning after signs of clinical disease (Walker et al., 2015). In the current study, calves were treated with GLP-2 and Sucram for 7 d before oral inoculation with *C. parvum* and each day thereafter for 10 d. Under these conditions, we did not detect any changes in *OCN* or *TJP1* mRNA in ileum with infection or experimental

treatment, but did observe reductions in *OCN* and *TJP1* mRNA jejunum in GLP-2- and Sucram-treated calves relative to infected controls. In addition, we observed an increase in *MARVELD2* mRNA expression in both ileum jejunum of *C. parvum*-infected calves, which was eliminated in jejunum by GLP-2 and Sucram treatments. The *MARVELD2* gene encodes tricellulin, a protein that helps to regulate permeability and barrier function at multicellular junctions of epithelial membranes (Mariano et al., 2011). Increased expression of intestinal epithelial *MARVELD2* mRNA in response to infection may reflect local remodeling and recovery of cellular membranes occurring after disruption of the mucosal layer caused by *C. parvum*, or perhaps a host defense response to reinforce intestinal barrier function against further pathogen invasion. Reduction of *MARVELD2*, *OCN*, and *TJP1* mRNA with GLP-2 and Sucram treatments during *C. parvum* infection suggests that these treatments may help to protect the host from changes in intestinal integrity and local cellular remodeling induced by infection.

Furthermore, we observed increased expression of *GPX2* mRNA in both jejunum and ileum of infected control calves, which was attenuated in ileum by GLP-2 and Sucram treatments and was reduced in jejunum of GLP-2-treated calves relative to those receiving Sucram. Induction of *GPX2* expression has been demonstrated in a mouse model of acute colitis and is believed to contribute to tissue recovery after inflammation (Hiller et al., 2015). Thus, our results indicate some level of intestinal inflammation or injury was induced by *C. parvum* infection, which was mitigated in calves treated with GLP-2 and Sucram before inoculation. Despite responses in tight junction protein mRNA and *GPX2* transcript expression, we observed no treatment effects on *TFF3* mRNA expression in ileum, and only a tendency for reduced *TFF3* expression in jejunum of infected calves receiving GLP-2 and Sucram relative to infected controls. A preliminary study using 2 piglet models of short bowel syndrome indicated that GLP-2 treatment increases expression of *TFF3* mRNA and may assist with tissue repair after injury (Koepke et al., 2015). Thus, changes in *TFF3* mRNA expression did not appear to play a significant role in response to *C. parvum* infection or GLP-2 and Sucram treatments in our study.

Consistent with observed changes in gene expression related to gut injury and integrity as well as plasma SAA results, we detected intestinal disruption and mucosal damage in infected control calves relative to non-infected controls, which were congruent with an established *C. parvum* infection (Gookin et al., 2002). These disruptions included reduced intestinal villus height, increased apoptosis in villi, and increased cell prolifera-

tion in intestinal crypts. Shortening and fusion of villi results from loss of epithelium at the villus surface as the invading parasites replicate and invade neighboring microvilli (Tzipori and Ward, 2002). Stimulation of cell proliferation then follows in the intestinal crypts, which serve as the site of stem cell-mediated villus regeneration (Neal et al., 2011). It is believed that apoptosis is increased by the host in late infection as a protective mechanism to limit replication of the intracellular parasites and to minimize inflammation associated with cell injury or necrosis (Liu et al., 2008). Similar to rodents and swine (Drucker and Yusta, 2014; Deng et al., 2016), treatment of healthy ruminating calves with GLP-2 increases villus height (Taylor-Edwards et al., 2011) and has been shown to reduce cell apoptosis in villi and crypts of newborn pigs receiving total parenteral nutrition (Burrin et al., 2005). In the present study, treatment of infected calves with GLP-2 and Sucram maintained villus height in ileum, although GLP-2 effects on intestinal villus height tended to be greater than the effects of Sucram. Moran et al. (2014) reported that Sucram feeding increased both villus height and crypt depth in duodenum of ruminating calves, but failed to alter either measure in preruminating calves, suggesting the response may be affected by age or developmental stage. Increased apoptosis in villi of small intestine associated with *C. parvum* infection was not reduced by GLP-2 and Sucram treatments, but tended to increase apoptosis in ileal villi. Sucram feeding did reduce apoptosis in ileal crypts relative to GLP-2, suggesting potential reduction in damage to this region with Sucram treatment. It is noteworthy that Liu et al. (2008) reported the capability of intracellular *C. parvum* to inhibit apoptosis within epithelial cells to limit its host's ability to defend against pathogen replication. Thus, the observed increase in apoptosis in ileal villi of Sucram- and GLP-2-treated calves may reflect a reduction of intracellular *C. parvum* in villi of these animals. Furthermore, GLP-2 and Sucram treatments tended to reduce cell proliferation in jejunal crypts induced by infection. This reduction in crypt cell proliferation may reflect a diminished requirement for cell replacement from *C. parvum*-induced mucosal injury.

On a gross tissue level, GLP-2 and Sucram treatments increased the mass-to-length ratio and reduced length-to-empty BW ratio of both the small and large intestines relative to infected controls, indicating the tissues were more dense and shorter as a result of GLP-2 and Sucram treatments. Additionally, Sucram feeding reduced the mass and length of the small intestine (normalized to empty BW) relative to GLP-2 treatment, indicating an overall reduction in the relative size of the small intestine and potential benefits to energetic efficiency in this group, given the high metabolic de-

mands of intestinal tissue (Baldwin et al., 2004). To date, no other studies have evaluated intestinal size in response to Sucram feeding, but ruminating calves treated for the same duration and dose of GLP-2 used in the present study showed no change in length-to-empty BW ratio of either the small or large intestine (Taylor-Edwards et al., 2011; Connor et al., 2013). Thus, the reason for the observed reductions in the present study is unknown. Nonetheless, it is interesting to consider that Allen (1984) proposed that *Eimeria acervulina*-infected chickens exhibiting greater mass of the distal small intestine relative to controls may be expressing a compensatory response of the tissue to its reduced nutrient uptake functions during disease. Because Sucram and GLP-2 treatments increase expression of glucose transporters and increase glucose uptake and mucosal growth (Cheeseman, 1997; Taylor-Edwards et al., 2011; Moran et al., 2014), perhaps a corresponding reduction in overall intestinal size can be afforded by the animal while maintaining sufficient nutrient absorption. Further study is needed regarding the effects of Sucram and GLP-2 treatments on overall intestinal size in cattle, particularly during coccidiosis and potential longer-term gains in growth performance. Lastly, increased 1-m section weights of the jejunum in *C. parvum*-infected control calves relative to non-infected controls likely was related to symptomatic thickening of the folds of the small intestine associated with the disease (Federle and Raman, 2015), and no significant benefits of GLP-2 and Sucram treatments on this measure were detected.

Finally, we observed no main effects of infection or treatment on nitro-oxidative stress in the ileum of calves, measured as nitrated protein abundance, although 2 estimates of nitrated protein abundance tended to be reduced in Sucram-treated calves relative to those administered GLP-2. We previously demonstrated an increase in nitro-oxidative stress in ileocecum of calves infected with *E. bovis*, which was greatly reduced (47 to 69%) with GLP-2 therapy (Connor et al., 2013). Not surprisingly, no benefits of GLP-2 treatment in reducing inflammation-related nitro-oxidative stress among noninfected control calves were found in that study. In the present study, nitrated protein abundance was highly variable among calves within each group, and it appeared that the tissue inflammatory response to infection was too small to elicit a significant increase in nitro-oxidative stress to evaluate mitigation potential by GLP-2 and Sucram treatments. More severe coccidial infection is needed to effectively assess the anti-inflammatory effects of these treatments in cattle.

In conclusion, GLP-2 and Sucram treatment of calves before oral inoculation with 12,500 *C. parvum* oocysts showed some protection relative to untreated infected

calves, including reduced diarrhea, improved intestinal morphology, a reduced plasma indicator of inflammation, and reduced mRNA expression of genes participating in cellular remodeling and injury repair. For the majority of variables examined, no clear difference was evident between the effects of GLP-2 versus Sucram. Additional evaluation of these therapies is needed in calves with more severe cases of coccidiosis to more effectively determine their beneficial effects.

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