Effect of heat-treated canola meal and glycerol inclusion on performance and gastrointestinal development of Holstein calves

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ABSTRACT

The objectives of this study were to assess the effect of using heat-treated canola meal (CM) and glycerol inclusion in starter mixtures on starter intake, growth, and gastrointestinal tract development in Holstein bull calves. In the first study, a protocol for the heat treatment of CM was evaluated by comparing commercial CM that was exposed to 0, 100, 110, or 120°C of heat treatment for 10 min. Following heat treatment, *in situ* crude protein (CP) ruminal degradability and estimated intestinal CP digestibility were assessed. It was observed that the degradable fractions of dry matter and CP in CM decreased linearly with increasing temperature of heat treatment. The estimated intestinal CP digestibility was greatest when CM was heated to 110°C. In the second study, 28 bull calves were used in a randomized complete block design. Calves were fed pelleted starters containing CM or CM that was heat-treated to 110°C for 10 min. Diets also contained 0 or 5% glycerol on a dry matter basis. The study lasted 51 d, ending on the first day of weaning. Starter intake, average daily gain (ADG), ruminal short-chain fatty acid concentrations, morphology of the rumen and small intestine, gene expression (*MCT1*, *GPR41*, *GPR43*, *UTB*, *AQP3*, *PEPT1*, *PEPT2*, *ATB0+*, and *EAAC1*) in the ruminal, jejunal, and ileal epithelium, and brush border enzyme activities in the duodenum, jejunum, and ileum were investigated. Few interactions between heat-treated CM and glycerol inclusion were observed. Feeding heat-treated CM did not affect starter intake. However, feeding heat-treated CM to calves tended to reduce ADG and decreased the weight of ruminal and jejunal tissue. Heat treatment did not affect gene expression or brush border enzyme activi-

ties in the small intestine. Glycerol inclusion tended to increase cumulative starter intake and increased cumulative body weight gain. Use of glycerol reduced ruminal pH and increased the concentration of ruminal short-chain fatty acids. Additionally, glycerol inclusion increased abomasal, duodenal, jejunal, and cecal digesta weights and tended to increase the weight of the jejunal tissue. Glycerol supplementation tended to downregulate the expression of *MCT1* in the ruminal epithelium, and upregulated the expression of *MCT1* in the epithelium of proximal jejunum. In conclusion, heat treatment of CM may negatively affect calf growth and gastrointestinal tract development. Glycerol inclusion may increase starter intake, ADG, ruminal fermentation, and intestinal development in calves when CM is used as a main source of protein in pelleted starter mixture.

Key words: brush border enzymes, gene expression, intestine, rumen

INTRODUCTION

The weaning transition represents a critical phase for calves (Weary et al., 2008), with marked nutritional changes occurring as the provision of milk is reduced and calves start to rely on solid feed intake. The shift from milk to solid feed both stimulates and relies on gastrointestinal tract (GIT) development (Roth et al., 2009; Khan et al., 2016). Though considerable focus has been placed on stimulating ruminal digestion around weaning as a strategy to promote ruminal development, Górka et al. (2011) reported that development of the small intestine may also influence ruminal development. As such, dietary strategies that provide nutrients to promote ruminal fermentation while ensuring substrate availability in the small intestine may provide benefit for calves at weaning.

Canola meal (CM) is a common protein source used in diets for lactating cows and has been reported to increase milk yield when used as a replacement for soybean meal or wheat dried distiller grains (Huhtanen et al., 2011; Martineau et al., 2013). However, CM is

Received December 28, 2019.

Accepted April 21, 2020.

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not commonly used in diets for calves around weaning due to concerns over palatability (Miller-Cushon et al., 2014a,b) and digestibility (Fiems et al., 1985; Khorasani et al., 1990). In contrast, some studies have suggested that CM inclusion has limited effects on starter intake and growth, at least when inclusion is limited to a portion of the soybean meal (Claypool et al., 1985; Hadam et al., 2016). However, mildly heat-treated CM might be beneficial because it may increase the RUP fraction (McKinnon et al., 1991) and deliver a greater supply of AA to calves, and thus potentially hasten small intestine development, starter intake, and consequently, whole GIT development in calves before weaning. Nevertheless, the effect of heat-treated sources of protein, likely to increase rumen bypass protein content in starter feed, for calves at weaning has not been extensively evaluated.

Glycerol is a byproduct of bio-diesel production (Gergen, 2005) that is rapidly fermented in the rumen (Rémond et al., 1993) and shifts fermentation to increase the proportion of butyrate and propionate in ruminal fluid (Rémond et al., 1993; Paiva et al., 2016). Butyrate is known to stimulate development of the ruminal epithelium (Sander et al., 1959; Mentschel et al., 2001), and thus glycerol may be a valuable feed ingredient in diets for calves during the weaning transition. Additionally, due to its sweet taste (Quispe et al., 2013), glycerol may improve palatability of feed, which may be an important contributing factor when CM is used as a source of protein in calf starters.

We hypothesized that the use of heat-treated CM in a starter mixture will promote the development of the small intestine, likely through greater supply of AA to the small intestine, which will have a positive effect on starter intake and intestinal and ruminal development. Finally, we hypothesized that glycerol inclusion in a starter mixture containing CM would increase the proportion of butyrate in ruminal fluid, and thus further stimulate ruminal development and starter intake. The objectives were to evaluate the optimal heat-treatment temperature of CM in regard to ruminal and estimated intestinal digestibility of nutrients, and to determine the effect of CM heat treatment, glycerol supplementation, and their interaction on starter intake, calf growth, and the development of the GIT of dairy calves at weaning.

MATERIALS AND METHODS

Experimental procedures were pre-approved by the University of Saskatchewan Animal Research Ethics Board (protocol no. 20100021) in accordance with the guidelines of the Canadian Council on Animal Care (Ottawa, ON, Canada).

Study 1: Optimal Heat-Treatment Temperature of CM

Heat Treatment of CM. Four subsamples of commercial solvent-extracted CM (100 kg each) from a single source were used to evaluate the effect of heat-treated CM on in situ ruminal and estimated intestinal CP digestibility. For each treatment, a 25-kg portion from each subsample was subjected to 1 of 4 treatments: (1) no additional heat (**CON**), or (2) additional heat treatment to achieve a temperature of 100°C (**H-100**), (3) 110°C (**H-110**), or (4) 120°C (**H-120**). All treatment temperatures were held at the designated temperature for 10 min (McKinnon et al., 1991, 1995). Heat treatment was conducted in a tumble dryer (POS Bioscience, Saskatoon, SK, Canada) and the temperature was steadily increased from ambient temperature to the treatment temperature. Following 10 min of heat exposure at the specific temperature, the CM was cooled to 50°C. A total of (mean $\pm$ SEM) 26 $\pm$ 2.1 min for H-100, 43 $\pm$ 4.4 min for H-110, and 67 $\pm$ 6.7 min for H-120 were required to achieve target temperatures. The durations required for cooling were 21 $\pm$ 2.3 min for H-100, 24 $\pm$ 4.1 min for H-110, and 30 $\pm$ 0.3 min for H-120. All temperature changes occurred linearly.

In Situ Ruminal Degradability. Four ruminally cannulated (internal diameter of 10 cm, Bar Diamond Inc., Parma, ID) Hereford-cross heifers (616 $\pm$ 26.0 kg, initial BW $\pm$ SD) were used for in situ incubations. Heifers were housed in individual pens (3 $\times$ 3 m) at the Livestock Research Building (University of Saskatchewan, Saskatoon, SK, Canada) and were fed a TMR twice a day at 0900 and 1700 h. The diet consisted of barley silage (25% DM), rolled barley grain (47% DM), grass hay (20% DM), and a mineral and vitamin pellet (8% DM) with the amount of TMR provided at 2.5% of BW. Feed refusals were weighed and collected daily at 0830 h. Barley grain and the mineral and vitamin pellet samples were collected once per week and forage samples were collected twice per week. Feed and refusal samples were analyzed for DM content by drying at 55°C in a forced-air oven until achieving a constant weight. The DM content of the ingredients was used to adjust their proportions in the diet on an as-fed basis, as necessary. Feed samples were ground to pass through a 1-mm sieve (Christy and Norris, Christy Turner Ltd., Chelmsford, UK) and were pooled by sampling date and analyzed for chemical composition as described below.

Empty polyester bags (53 $\pm$ 10 μ m pore size; #BG510, Bar Diamond Inc.) were dried to 55°C for 1 h, placed in a desiccator for 15 min, and weighed. Approximately 7 g of CM was weighed into each bag and then sealed. The sequential-in all-out procedure,

as recommended by the NRC (2001), was used for in situ incubations. Incubation times for the nylon bags were 0, 2, 4, 8, 12, 16, 24, and 48 h. Incubations were completed in 2 separate, consecutive, randomized runs with treatments and times balanced among heifers. Nylon bag insertion into the rumen was initiated at 0900 h on d 1 and all bags were removed at 0900 h on d 3. Bags for the 0 h of incubation were soaked in warm (37 to 39°C) distilled water for 30 min. As the in situ incubation approach used a sequential-in all-out method, the number of nylon bags inserted into a single heifer did not exceed 44 bags. In addition, 4 bags for each treatment replicate were incubated for 12 h to allow for a 3-step in vitro procedure to measure estimated intestinal digestibility (Calsamiglia and Stern, 1995).

Upon removal from the rumen, bags were immediately placed in cold water (4°C) and washed 5 times. For each wash, 60 bags were placed in 15 L of cold water and manually agitated for 1 min. After washing, bags were placed on a flat pan and frozen at -20°C for 24 h. Following this step, bags were removed from the freezer, placed in cold water, and rinsed 1 additional time to reduce microbial contamination (Kamel et al., 1995). The bags were then dried at 55°C until achieving a constant weight, placed in desiccators for 15 min, and weighed. The residual feed from each time point within a treatment replicate were pooled for chemical analysis.

Estimated Intestinal Digestibility. The 3-step estimated intestinal digestibility procedure was conducted as described by Calsamiglia and Stern (1995). The residuals remaining after 12 h of ruminal incubation were pooled for each treatment and subjected to digestion in pepsin (1 g/L; No. P-7012, Sigma, St. Louis, MO) and HCl (0.1 N) for 1 h at 38°C in a shaking water bath. Samples were then neutralized using 1 N sodium hydroxide and subsequently incubated in the presence of pancreatin (3 g/L; No. P-7545, Sigma; in 0.5 M KH₂PO₄ buffer) for 24 h. During incubation, samples were placed in a shaking water bath (38°C). At the end of incubation, enzymatic activity was stopped by addition of trichloroacetic acid (100% wt/vol, T6399, Sigma). Samples were then stored at 4°C until further analysis of CP content.

Chemical Analysis of Feed and In Situ Samples. Analytical DM content was determined by drying samples at 135°C for 2 h. Crude protein concentration was measured using the Kjeldahl method (984.13, AOAC International, 1994). Ash was determined according to method 942.05 (AOAC International, 1994) and was used to calculate OM concentration. The NDF and ADF concentrations were analyzed independently using Ankom F57 filter bags in the Ankom 200 Fiber Analyzer (ANKOM Technology Corp., Fairport, NY) according to the method of van Soest et al. (1991); NDF

samples were treated with α -amylase and sodium sulfite. Ether extract analysis was carried out in Goldfish Fat Extraction Apparatus (Model 35001, Goldfish, ExpoTechUSA, Houston, TX) according to method 920.39 (AOAC International, 1994). Amino acid concentrations of the CM samples were analyzed by Evonik Nutrition and Care GmbH (Essen, Germany).

Study 2: Effect of Heat-Treated CM and Glycerol on Calves

Animals, Housing, and Feeding Regimen.

Twenty-eight newborn bull calves were sourced from a single commercial herd near Saskatoon (SK, Canada) for this study. All calves were separated from their dam immediately after birth and provided a commercial colostrum replacer (Headstart bovine dried colostrum; The Saskatoon Colostrum Co. Ltd., Saskatoon, SK, Canada) to target total supply of 180 g of IgG within first 12 h of life. Two bags of colostrum replacer (120 g of IgG) were fed within 6 h of birth, and a third bag (60 g of IgG) was fed within 12 h of birth. Colostrum replacer was fed from a bottle with a nipple. Following colostrum, all calves received a commercial milk replacer (Grober Nutrition, Cambridge, ON, Canada) containing dried skim milk powder (Grober Energy Supplement 983681), dried whey powder, vegetable oil, Grober rearing premix (950261), autolyzed yeast, soy lecithin, calcium chloride, DL-methionine, calcium carbonate L-lysine, flavor (983030; Grober Nutrition), viable microbial product (982931; Grober Nutrition), polyethylene glycol, mono and di-oleates, and selenium-enriched yeast (982026; Grober Nutrition) with a mixing rate of 150 g (DM basis) of milk replacer powder in 1 L of water. (Table 1). Until 7 d of age, all calves were fed 4 L/d of milk replacer in 2 equal feedings. Within 1 wk of birth (3.6 ± 2.1 d of age), calves were transported to the Livestock Research Building (University of Saskatchewan, Saskatoon, SK, Canada). Upon arrival, calves were placed in individual pens (1.2×2.4 m) with wood shavings as bedding. Bedding was kept at a minimum and replaced as needed.

At 8 d of age calves were weighed (mean 43.2 ± 3.9 kg) and BW was recorded weekly thereafter. The amount of milk replacer provided was adjusted to deliver 10% BW on d 8 and 9, 11.5% BW on d 10 and 11, 13% BW on d 12 and 13, and 15% BW from d 14 until d 28 of age with the milk replacer fed in 3 equal feedings at 0800, 1200, and 1600h. Calves were exposed to a gradual weaning protocol by reducing the amount of milk replacer offered to 10% of BW starting on d 29, 5% BW starting on d 36 (both fed in 2 equal feedings at 0800 and 1600 h), and 2.5% BW from d 43 (fed in 1 feeding at 0800 h) with no further milk replacer offered

after d 50. The amount of milk replacer offered and refused were recorded at each feeding and summarized to reflect each feeding level and the cumulative amount fed over the course of the study. Representative samples of milk replacer were collected from each 25-kg bag and used for DM analysis to monitor and ensure the mixing rate was static at 150 g of DM/L. Samples of milk replacer were also composited monthly and used for chemical analysis (described below).

Calves were blocked by birth date and within block randomly assigned to 1 of 4 treatments, using BW at 8 d of age as a secondary blocking factor. The treatments differed in composition of offered pelleted starter mixtures that contained: (1) CM without glycerol (**NH-NG**), (2) CM with glycerol (**NH-G**), (3) heat-treated CM without glycerol (**H-NG**), or (4) heat-treated CM with glycerol (**H-G**) in a 2×2 factorial arrangement (Table 1). Starters were formulated to be isonitrogenous and isoenergetic. To balance the nitrogen content between glycerol supplemented and nonsupplemented diets, urea was included in the glycerol starters. Based on results of study 1 (see Results section), the heat-treated CM was heated in a tumble dryer (POS, Saskatoon) at 110°C for 10 min before mixing and pelleting. The crude glycerol made from soybean (minimum

of 80% purity and maximum of 2.30% sodium) was sourced from Canadian Feed Research Centre (North Battleford, SK, Canada) and was included at 5% of the pellet DM. Glycerol was included in the starters in exchange for barley grain. Starter mixtures were pelleted at the Canadian Feed Research Centre. First, the starter mash was prepared for pelleting with UAS-Muyang pellet conditioner (model MUTZ350-J Triple layered twin; Muyang, Yangzhou, China) by addition of 2.5% moisture at atmospheric pressure. Next, the mash was pelleted using a 3.5-mm die at 65°C for 20 to 25 s in the UAS-Muyang pellet mill (model MUZL350II). The compression ratio of the die was 14.0 as calculated by dividing the effective length of the die hole (50 mm) by the diameter of the die hole (3.57 mm). Pellets were then cooled to 5°C above ambient temperature in a UAS-Muyang counter-flow cooler (model SLNF14X14A). Pelleting added 2.5% moisture, which was removed by the cooling process.

Starter was offered from d 8 of age at a rate of 400 g/d and the amount was adjusted daily to ensure ad libitum intake by increasing the amount offered by 500 g when refusals dropped below 250 g initially, and when refusals were less than 500 g for the rest of the study. Refusals of the starter were removed and

Table 1. Ingredient and nutrient composition of pelleted starter mixtures containing canola meal either nonheated (NH) or heated to 110°C for 10 min (H), without (NG) or with (G) inclusion of 5% DM of glycerol, and nutrient composition of milk replacer

Item	Treatment				Milk replacer ¹
	NH-NG	NH-G	H-NG	H-G	
Ingredient composition, % of DM					
Nonheated canola meal	34.00	34.00	—	—	
Heated canola meal	—	—	34.00	34.00	
Barley grain	32.00	26.82	32.00	26.82	
Corn grain	29.00	29.00	29.00	29.00	
Whey permeate (deproteinized)	2.00	2.00	2.00	2.00	
Mineral supplement ²	3.00	3.00	3.00	3.00	
Urea	—	0.18	—	0.18	
Glycerol	—	5.00	—	5.00	
Chemical composition, ³ % of DM					
DM, %	91.5 ± 0.6	91.5 ± 0.8	92.6 ± 0.2	92.7 ± 0.8	93.2 ± 0.9
CP	20.4 ± 0.5	20.6 ± 0.5	21.2 ± 0.7	21.2 ± 0.4	27.3 ± 0.7
ADF	10.2 ± 0.2	10.1 ± 0.4	10.3 ± 0.2	10.7 ± 0.3	NA ⁴
NDF	16.1 ± 0.2	17.1 ± 0.8	17.0 ± 0.4	17.0 ± 0.2	NA
Starch	39.2 ± 0.2	35.9 ± 0.9	38.1 ± 0.8	33.9 ± 0.8	NA
NSC	41.1 ± 0.1	44.7 ± 1.4	42.5 ± 1.0	46.0 ± 0.8	NA
Ether extract	2.99 ± 0.2	3.00 ± 0.1	3.23 ± 0.2	3.00 ± 0.0	17.7 ± 0.1
OM	91.6 ± 0.1	91.9 ± 0.3	91.9 ± 0.2	91.5 ± 0.3	92.4 ± 0.1
Ca	1.31 ± 0.0	1.19 ± 0.0	1.24 ± 0.0	1.32 ± 0.0	1.1 ± 0.0
P	0.96 ± 0.0	0.94 ± 0.0	0.97 ± 0.0	1.01 ± 0.0	0.9 ± 0.0

¹Grober Nutrition (Cambridge, ON, Canada). The milk replacer contained dried skim milk powder (Grober energy supplement 983681), dried whey powder, vegetable oil, Grober rearing premix (950261), autolyzed yeast, soy lecithin, calcium chloride, DL-methionine, calcium carbonate L-lysine, flavor (983030), viable microbial product (982931), polyethylene glycol, mono- and di-oleates, and selenium yeast (982026).

²Composition: Ca 1.2%; P 0.6%; Mg 0.4%; K 1.2%; S 0.3%; Na 0.4%; Cl 0.7%; Se 0.5%; Fe 230 mg/kg; Mn 120 mg/kg; Zn 220 mg/kg; Cu 50 mg/kg; vitamin A 10,000 IU; vitamin D 1,600 IU; vitamin E 50 mg.

³Mean ± SD.

⁴NA = not analyzed.

recorded daily before the morning milk replacer feeding, and fresh starter offered after provision of milk replacer. Furthermore, refusals from each calf were collected and pooled on a weekly basis and analyzed for DM as previously described. In addition, samples of starters were collected weekly for DM determination and chemical analysis. Samples were then composited to yield a monthly sample for each treatment and were then ground to pass through a 1-mm sieve (ZM 200 Retsch, Haan, Germany). Data on starter intake were summarized to indicate starter intake from d 8 to 28, 29 to 35, 36 to 42, and 43 to 49 to represent periods of time where milk replacer was provided at 15% of BW, 10% of BW, 5% of BW, and 2.5% of BW, respectively. In addition, cumulative starter intake over the duration of the study was calculated.

Samples of the starter pellets and milk replacer were sent to Cumberland Valley Analytical Services (Hagerstown, MD) for analysis of DM, CP, ether extract, OM, ADF, NDF, starch, ethanol soluble carbohydrates, Ca, and P. Dry matter was analyzed using method 930.15 by drying the sample at 135°C (AOAC International, 2000). Crude protein was analyzed using method 990.03 (AOAC International, 2000) using Leco FP-528 Nitrogen Combustion Analyzer (Leco, St. Joseph, MI). Acid detergent fiber was analyzed using method 973.18 (AOAC International, 2000) with modifications; we used Whatman 934-AH glass microfiber filters with 1.5- μ m particle retention. Neutral detergent fiber was analyzed as described by van Soest et al. (1991) using sodium sulfate and α -amylase with the same microfiber filters as used for ADF. Starch was analyzed according to Hall (2009). Ethanol soluble carbohydrates were analyzed according to DuBois et al. (1956). Ether extract was analyzed using method 2003.05 (AOAC International, 2006) using Tecator Soxtec System HT 1043 Extraction unit (Tecator, Foss, Eden Prairie, MN). To calculate OM, ash was analyzed with method 942.05 (AOAC International, 2000) using a 1.5-g sample with a 4-h ashing duration. Mineral analysis was conducted using method 985.01 (AOAC International, 2000) and Perkin Elmer 5300 DV ICP (Perkin Elmer, Shelton, CT).

Blood Sample Collection and Analysis. Blood samples were collected on d 22 (15% BW of milk replacer provision), d 43 (5% BW of milk replacer provision) and on d 51 (1 d after weaning) from the jugular vein at 1000 h (2 h postfeeding). Two samples (10 mL each) were collected at each time point with blood placed in a tube containing Na-heparin (plasma separation; 158 IU, Becton Dickinson, Franklin Lakes, NJ) and a tube without anticoagulant (serum separation; Becton Dickinson). Blood collected into the tube with Na-heparin was immediately placed on ice until centrifugation for 15 min at $2,600 \times g$ at 4°C (Sorvall ST 16R, Thermo

Scientific, Waltham, MA), whereas blood collected into the tube without an anticoagulant was allowed to clot for 1 h before centrifugation at the same conditions as for plasma separation. The supernatant from both samples was then transferred into separate vials and frozen at -20°C until analysis of glucose, insulin, BHB, nonesterified fatty acids (NEFA), and urea.

Plasma glucose concentration was analyzed by enzymatic reaction of glucose with glucose oxidase/peroxidase (No. P7119 Sigma) and dianisidine dihydrochloride (No. D3252 Sigma) in a 96-well plate and the absorbance was measured at 450 nm using a plate reader (Epoch 2, BioTek Instruments Inc., Winooski, VT). Plasma insulin concentration was analyzed using a bovine-specific ELISA kit (no. 10-120-01, Mercodia, Uppsala, Sweden). The BHB concentration in serum was measured based on the method described by Williamson et al. (1962). Plasma NEFA concentration was analyzed using a commercial kit (HR Series NEFA-HR(2); Wako Diagnostics, Mountain View, CA) using 2 solvents (no. 995-34791 and 993-35191) and 2 color reagents (no. 999-34691 and 991-34891). Plasma urea concentration was analyzed using the method described by Fawcett and Scott (1960).

Postslaughter Data and Sample Collection.

One day after weaning (d 51), all calves were killed via captive bolt stunning followed by exsanguination at 1400 h, or at 1330 h and 1430 h if 2 calves were killed on the same day. The whole GIT was dissected to determine tissue and digesta weights and to obtain tissue and digesta samples. The weights, both with and without digesta, were recorded for the reticulo-rumen, omasum, abomasum, duodenum, jejunum, ileum, cecum, and colon. The reticulo-rumen was cut open along the dorsal sac and digesta was transferred into a clean container. Ruminant tissue samples were collected from the cranial ventral sac of the rumen. For intestinal tissues, length measurements were conducted for each region. Then, tissue samples were taken from the mid-point of the duodenum (the distal portion of the duodenum was determined at the ligament of Treitz), 3 regions of jejunum including the proximal (25% of the total length starting at the ligament of Treitz), middle (50% of the length), and distal regions (75% of the length) with the region adjacent to the ileocecal fold used to denote the end of the jejunum and start of the ileum. The mid-point of the ileum was also collected using the ileocecal fold and ileocecal junction to define the ileum. All tissue samples were gently washed in ice-cold sterile phosphate buffered saline (Sigma) to remove digesta before any further processing.

Ruminal Fermentation Characteristics. A representative sample of ruminal digesta was strained through 2 layers of cheesecloth and pH was measured

using a portable pH meter (Accumet AP110, Fischer Scientific, Ottawa, ON, Canada). Ten milliliters of strained ruminal fluid was mixed with 2 mL of metaphosphoric acid (25% wt/vol). The sample was stored at -20°C until analysis of short-chain fatty acid (SCFA) concentration with gas chromatography (Agilent Technologies Inc., Santa Clara, CA) as described by Khorasani et al. (1996). A second sample of strained ruminal fluid (10 mL) was collected and mixed with 2 mL of 1% sulfuric acid and frozen at -20°C until analysis of ammonia concentration based on the method described by Fawcett and Scott (1960).

Tissue Collection for Gene Expression. Representative tissues from the cranial sac of the rumen (CRA), proximal jejunum (PROX), and ileum (ILE) were used to obtain epithelia and to measure the mRNA expression of selected target genes (Table 2). The genes of interest were monocarboxylate transporter 1 (*MCT1*) analyzed in CRA, PROX, and ILE; G-protein-coupled receptors (*GPR41*, *GPR43*) analyzed in CRA, PROX, and ILE; urea transporters (*UTB* and *AQP3*) analyzed in CRA; peptide transporters (*PEPT1* and *PEPT2*) and amino acid transporters (*EAAC1* and *ATB0+*) analyzed in PROX and ILE; as well as reference genes (*GAPDH*, *RPLP0*, and *HPRT1*) analyzed in CRA, PROX, and ILE. The ruminal epithelium was manually peeled from the underlying muscle layer and cut into small pieces, the samples of the intestinal tissues were cut open through the mesenteric line, and the mucosa was scraped off using a glass slide. All tissues were collected using sterile equipment, rinsed in sterile ice-cold phosphate buffered saline, and transferred to 2-mL test tubes with 1.8 mL of RNeasy lysis solution (Applied Biosystems, Foster City, CA). Tubes were stored for 24 h at 4°C and then frozen at -20°C .

Quantitative Real-Time PCR. Samples of epithelia were thawed, removed from RNeasy lysis solution, and ground with a mortar and pestle while purged with liquid nitrogen to keep the tissue frozen. Approximately 100 mg of tissue was transferred into a 2-mL sterile microcentrifuge tube. We extracted RNA through the phenol-chloroform extraction process based on the Chomczynski and Sacchi (1987) and Trizol (Thermo Fisher Scientific) protocols, with an additional isopropanol precipitation step.

The nucleic acid concentration and the 260:280 nm absorbance ratio were measured using a Nanodrop 2000c spectrophotometer (Thermo Scientific). Samples were then evaluated for RNA integrity on a 1.2% agarose denaturing gel. The RNA was considered good quality if there was distinct separation of the 18S and 28S ribosomal RNA bands. Four samples were suspected to contain genomic DNA contamination. As such, they were DNase-treated using the TURBO DNA-free

Kit (Thermo Fisher Scientific) and re-evaluated on the agarose gel to ensure lack of contamination.

For each sample, 2 μg of RNA was reverse transcribed to produce cDNA (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems). Primer (Table 2) efficiency was determined (Ramakers et al., 2003) using a composite sample of cDNA from all calves in the study. The melting curve of the primers was analyzed to ensure their specificity. The primer efficiency values ranged from 98 to 110%, with an average of 103.6%. Quantitative real-time PCR was carried out using the CFX96 Real-Time PCR system and SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA). We ran the reaction in duplicate with different gastrointestinal regions for the same gene analyzed on consecutive 96-well plates (Bio-Rad).

The expression (C_T , threshold cycle) of reference genes (*GAPDH*, *RPLP0*, *HPRT1*) in all analyzed regions of GIT were used to determine whether there were differences between the treatments. All 3 reference genes were deemed to have stable C_T as treatment effects were not present ($P \geq 0.15$, data not shown). The expression of target genes was analyzed by calculating the difference between the C_T for the gene of interest and the geometric mean of C_T for the reference genes (ΔC_T). With gene expression calculated in this manner, greater ΔC_T values indicate lesser mRNA expression, and lesser ΔC_T values indicate greater expression.

Brush Border Enzyme Activity Assays. The epithelium from the duodenum, the proximal, middle, and distal jejunum, and the ileum were scraped using sterile glass slides on a clean surface on ice. Epithelia samples were placed into 2-mL cryo-vials, snap frozen in liquid nitrogen, and then stored at -80°C until analysis of lactase (EC 3.2.1.23), maltase (EC 3.2.1.20), dipeptidase IV (EC 3.4.14.5), aminopeptidase A (EC 3.4.11.7), and N (EC 3.4.11.2) enzyme activity (Maroux et al., 1973; Nagatsu et al., 1976; Dahlqvist, 1984). Briefly, small intestine mucosa samples were thawed on ice and 1 g of tissue was weighed and transferred into a 10-mL vial. Five milliliters of reverse osmosis water was added per 1 g of tissue and samples were then homogenized for 30 s, and following a 10 s break, for another 30 s, while kept on ice at all times. The homogenate was centrifuged (SORVALL ST 16R, Thermo Scientific) at $1,000 \times g$, 4°C for 5 min and the supernatant was transferred into multiple 1.5-mL tubes and frozen at -80°C .

Protein content was determined for each supernatant of tissue homogenate using the bicinchoninic acid method described by Smith et al. (1985). Lactase and maltase were analyzed according to Dahlqvist (1984) with minor modifications. The released glucose was measured according to the same procedure as plasma glucose described above. The L-glutamyl-*p*-nitroanilide

Table 2. Target gene name, accession number, primer sequences, and function of the genes of interest

Target gene name abbreviation ¹	Accession number	Forward (F) and reverse (R) primers (3'-5')	Efficiency, %	Product length, bp
Reference genes				
<i>GAPDH</i> ²	NM_001034034	F: TCTGGCAAAAGTGACATCGT R: ATGACGAGCTTCCCGTTCTC	101.3	134
<i>RPLP0</i> ³	NM_001012682	F: TTGTGGAGACAGACAACTG R: GCCGGGTGTTTCCAGATG	102.2	136
<i>HPRT1</i> ³	NM_001034035	F: AGTTGCGAGCTTGCTGAT R: AGGCAATATCCACAAACAA	103.3	103
Short-chain fatty acid receptors and transporters				
<i>MCT1</i> ³ (<i>SLC16A1</i>)	NM_001037319	F: CTGATGGACCTTGTGGGAC R: CGGTAATGATGCCATGCC	98.5	206
<i>GPR41</i> ³ (<i>FFAR3</i>)	FJ562214.1	F: CCACATCTATCTACGTCCC R: GGGAGAGTCCCGGAGAT	99.6	192
<i>GPR43</i> ⁴ (<i>FFAR2</i>)	FJ562212.1	F: TCATGGGTTTCGGCTTCTAC R: GGGAAAGAAAGAGGAGGA	107.0	323
Urea transporters				
<i>UTB</i> ⁵ (<i>SLC14A1</i>)	NM_001008666	F: TATGTCCATGACGTGTCCAGTCT R: GCAGGTCCCATTTGCTCAGC	104.9	65
<i>AQP3</i> ⁵	NM_001079794	F: CGCGAGCCCTGGATCA R: CCCAGATCGCATCGTAATACAA	103.3	103
Peptide and AA transporters				
<i>PEPT1</i> ⁶ (<i>SLC15A1</i>)	NM_001099378	F: AGCAAAGGCTTTCGAAAGACA R: ACAGCCATCCTGCTTGAAGT	107.1	170
<i>PEPT2</i> ³ (<i>SLC15A2</i>)	NM_001079582	F: TGCTGACTCATGGTTGGAA R: TTGGAAGCAAGAGGCTAGAA	107.7	110
<i>ATB0+</i> ⁷ (<i>SLC6A14</i>)	NM_001098461	F: AGTCGGAGCAGCATATTAAAGGAA R: ACCAGGATGAGTAGGACACATAGG	103.3	93
<i>EAAC1</i> ³ (<i>SLC1A1</i>)	NM_174599	F: TGTGCTACATGCCGATTGGT R: GATTGCAAGCCACTCAGGA	110.4	122

¹*RPLP0* = 60S acidic ribosomal protein P0; *HPRT1* = hypoxanthine phosphoribosyltransferase 1; *MCT1* = monocarboxylic acid transporter 1; *GPR41* = G protein-coupled receptor 41; *GPR43* = G protein-coupled receptor 43; *UTB* = urea transporter B; *AQP3* = aquaporin 3; *PEPT1* = peptide transporter 1; *PEPT2* = peptide transporter 2; *ATB0+* = amino acid transporter B0+; *EAAC1* = excitatory amino acid carrier 1.

²Verdugo (2016).

³Designed using NCBI Primer-BLAST (NCBI, 2018).

⁴Wang et al. (2009).

⁵Røjen et al. (2011).

⁶Connor et al. (2010).

⁷Liao et al. (2009).

and L-leucyl-*p*-nitroanilide (Bachem, Budendorf, Sweden) were, respectively, used as substrates for analysis of aminopeptidase A and N activities (Maroux et al., 1973) and L-glycyl-*p*-nitroanilide (Bachem) was the substrate for dipeptidase IV activity (Nagatsu et al., 1976). The reaction was carried out in glass cuvettes and absorbance was measured using spectrophotometer at 410 nm for 10 min (Beckman Coulter, Brea, CA) fitted with a connection to a circulating water bath (1130-1S, VWR International, Mississauga, ON) to ensure stable reaction temperature. Brush border enzyme activities were calculated based on the amount of released product over the reaction period, either glucose for lactase and maltase or *p*-nitroanilidine for aminopeptidase A and N, and for dipeptidase IV. Enzyme activity was normalized per unit of protein in the tissue homogenate and 1 min of reaction.

Statistical Analysis

Optimal Heat-Treatment Temperature. Degradation rates were initially analyzed using the PROC NLIN of SAS (ver. 9.4, SAS Institute, Cary, NC) following Ørskov's model (Ørskov and McDonald, 1979) with the equation

$$R(t) = U + D \times e^{-K_d \times (t - T_0)},$$

where $R(t)$ is the percentage of residue at a given incubation time point (t), U is the undegradable fraction (%), D is the potentially degradable fraction (%), e is Euler's number, K_d is the degradation rate of D (%/h), and T_0 is lag time (h). Effective degradability was calculated following the equation

$$ED = S + \frac{(D \times K_d)}{K_d + K_p},$$

where ED is the effective degradability, S is the soluble fraction, which was washed out from 0-h incubation bags, and K_p is the rate of passage (assumed $K_p = 5\%/h$).

The effect of CM heat treatment on degradation rates for DM and CP, the degradable fractions, the effective degradability, and the estimated intestinal digestibility were analyzed using the mixed model in SAS version 9.4, considering heat treatment as the fixed effect with polynomial contrasts used to determine the linear or quadratic effect of heat treatment. Significance was declared when $P \leq 0.05$ and tendencies when $0.05 < P \leq 0.10$.

Effect of Heat-Treated CM and Glycerol on Calves. Data were analyzed as a randomized complete block design with a 2×2 factorial design using the mixed model of SAS. The model included the fixed effects of CM heat treatment (untreated and heated), glycerol inclusion (0 and 5%), and the 2-way interaction. Block was considered as a random effect. Data were tested to verify whether the data and residuals were normally distributed and homoscedastic. Data that were collected over time (BW, ADG, starter intake, and milk replacer intake) were also analyzed using repeated measures with time (week or day) as a fixed effect and the relevant 2- and 3-way interactions. Covariance error structures were tested and the error structure for each variable with the least Akaike's and Bayesian Information Criterion was chosen. In case of a significant interaction between fixed effects, the Bonferroni mean separation was used to determine if treatment means differed. Differences were declared significant when $P \leq 0.05$, and tendencies were declared when $P \leq 0.10$. As there were no interactions (2- or 3-way) of treatment with time, only the main treatment effects and the interaction among the heat treatment and glycerol inclusion are reported.

RESULTS

Study 1: Optimal Heat-Treatment Temperature for CM

Increasing the temperature for the heat treatment of CM increased DM concentration at a decreasing rate (quadratic, $P = 0.001$; Table 3), decreased OM concentration at a decreasing rate (quadratic, $P = 0.002$), increased CP concentration at a decreasing rate (quadratic, $P = 0.002$), increased NDF and ADF concentrations at an increasing rate (quadratic, $P < 0.001$), and increased ether extract concentration linearly ($P = 0.006$). The increases in CP, NDF, ADF, and ether extract were offset by a quadratic reduction in NFC concentration with the rate of reduction increasing as the temperature of heat treatment for CM increased ($P < 0.001$).

The rate of DM disappearance from the rumen decreased at a decreasing rate with the increase of heat-treatment temperature for CM (quadratic, $P = 0.017$; Table 3). The soluble fraction of DM decreased at a decreasing rate (quadratic, $P = 0.024$). The degradable fraction of DM decreased at an increasing rate with the increase of temperature (quadratic, $P < 0.001$), with values of 60.0% for CON and 34.1% for H-120. The undegraded fraction of DM increased at an increasing rate (quadratic, $P < 0.001$) with values of 14.1% for CON

Table 3. Chemical composition and digestion characteristics of the nonheated (CON) and heat-treated canola meal when heated to achieve temperatures of 100 (H-100), 110 (H-110), or 120°C (H-120) for 10 min (n = 3)

Item	Treatment				SEM	P-value	
	CON	H-100	H-110	H-120		Linear	Quadratic
Chemical composition							
DM, %	89.7	92.7	94.4	95.6	0.3	<0.001	0.001
OM, % of DM	93.6	93.3	93.1	93.0	0.0	<0.001	0.002
CP, % of DM	32.7	34.7	35.5	36.3	0.2	<0.001	0.002
NDF, % of DM	25.5	30.9	36.8	44.2	0.7	<0.001	<0.001
ADF, % of DM	19.3	22.4	25.2	33.4	0.4	<0.001	<0.001
NFC, ¹ % of DM	31.9	23.9	17.0	8.8	0.8	<0.001	<0.001
Ether extract, % of DM	3.53	3.65	3.87	3.93	0.08	0.006	0.073
In situ DM degradation							
K_d , ² %/h	4.50	4.20	3.14	3.06	0.21	0.002	0.017
Soluble, %	25.8	21.9	19.6	18.8	0.5	<0.001	0.024
Degradable, %	60.0	57.1	52.7	34.2	1.9	<0.001	<0.001
Undegradable, %	14.1	21.0	27.7	47.0	2.1	<0.001	<0.001
EDDM, ³ %	54.2	46.8	39.1	31.7	0.8	<0.001	<0.001
In situ CP degradation							
K_d , %/h	4.89	4.59	3.54	7.77	0.59	0.28	0.005
Soluble, %	16.4	11.8	8.6	12.1	2.9	0.14	0.73
Degradable, %	79.0	70.0	59.0	22.3	3.5	<0.001	<0.001
Undegradable, %	4.6	18.2	32.3	65.7	3.9	<0.001	<0.001
EDCP, ³ %	55.5	45.3	32.7	25.4	2.7	<0.001	0.003
Intestinal CP digestibility ⁴ , %	45.9	46.1	51.0	37.2	2.6	0.48	0.034

¹NFC calculated based on NRC (2001): 100 – (NDF, % + CP, % + ether extract, % + ash, %).

² K_d = the degradation rate of degradable fraction.

³EDDM or EDCP = effectively degradable DM or CP assuming a 5%/h passage rate.

⁴Estimated CP intestinal digestibility was based on a 3-step procedure described by Calsamiglia and Stern (1995).

and 47.0% for H-120. The effectively degradable DM, assuming a constant passage rate of 5%/h, decreased at an increasing rate with increasing temperature of treatment (quadratic, $P < 0.001$) from 54.2 for CON to 31.7% for H-120. The rate of CP disappearance from the rumen decreased from CON (4.89%/h) to H-110 (3.54%/h) and increased for H-120 (7.77%/h; quadratic, $P = 0.005$). The soluble fraction of CP was not affected by heat treatment ($P \geq 0.14$). The degradable fraction of CP decreased at an increasing rate when exposed to greater treatment temperatures (quadratic, $P < 0.001$) with values ranging from 79.0 and 22.3% for CON and H-120 treatments, respectively, and the CP undegraded fraction increased at an increasing rate (quadratic, $P < 0.001$). The effectively degradable CP decreased at an increasing rate after exposure to increasing heat-treatment temperatures (quadratic, $P = 0.003$) from 55.5% for CON to 25.4% for H-120. The estimated intestinal digestibility responded quadratically to the increase of heat-treatment temperature ($P = 0.034$), with digestibility increasing from CON (45.9%) to H-110 (51.0%) and decreasing for H-120 (37.2%).

With increased heat-treatment temperature of CM, linear decreases in the concentration of Arg ($P = 0.002$; Table 4), His ($P = 0.003$), Met ($P = 0.048$), Asp ($P = 0.026$), Cys ($P = 0.001$), Glu ($P = 0.035$), Pro ($P = 0.043$), and Ser ($P = 0.049$) were detected. In addition,

Lys ($P = 0.059$), Phe ($P = 0.058$), Thr ($P = 0.099$), and Val ($P = 0.088$) concentrations tended to decrease linearly. Isoleucine, Leu, Ala, and Gly concentrations were not affected by the heat treatment ($P \geq 0.101$).

Study 2: Effect of Heat-Treated CM and Glycerol on Calves

Even though our treatment diets were designed to be isonitrogenous and could not be statistically evaluated, we observed numerically greater CP content in the heat-treated treatment diets (Table 1). Treatments that contained glycerol had numerically less starch and greater NSC concentration. There were no 2- or 3-way interactions of treatments with time for BW, ADG, starter intake, and milk replacer intake ($P \geq 0.21$, data not shown). The aforementioned variables increased with time ($P \leq 0.001$, data not shown); however, these observations were expected for growing calves.

Initial and average BW of calves ($P \geq 0.47$; Table 5) were not affected by CM heat treatment. Heat treatment of CM decreased final BW ($P = 0.041$) and tended to reduce ADG ($P = 0.079$) and total BW gain ($P = 0.097$). When comparing starters without glycerol to those with glycerol, glycerol inclusion tended to increase ADG ($P = 0.088$) and increased total BW gain ($P = 0.003$). Heat treatment of CM in the starter

Table 4. Amino acid composition of nonheated (CON) and heat-treated canola meal when heated to achieve temperatures of 100 (H-100), 110 (H-110), or 120°C (H-120) for 10 min (n = 3)

AA	Treatment				SEM	P-value	
	CON	H-100	H-110	H-120		Linear	Quadratic
EAA, % of CP							
Arg	6.19	5.89	5.74	5.58	0.09	0.002	0.11
His	2.72	2.62	2.52	2.52	0.04	0.003	0.27
Ile	4.17	4.07	3.63	3.97	0.14	0.11	0.88
Leu	7.30	7.12	6.95	6.78	0.17	0.101	0.72
Lys	5.44	4.95	4.97	4.36	0.30	0.059	0.28
Met	2.06	2.00	1.92	1.94	0.04	0.048	0.58
Phe	4.25	4.11	3.94	4.02	0.09	0.058	0.73
Thr	4.56	4.46	4.33	4.24	0.11	0.099	0.63
Val	5.36	5.18	5.00	5.09	0.12	0.088	0.83
NEAA, % of CP							
Ala	4.66	4.55	4.34	4.43	0.11	0.104	0.69
Asp	7.31	7.10	6.78	6.83	0.14	0.026	0.37
Cys	2.45	2.32	2.25	2.25	0.03	0.001	0.40
Gly	5.30	5.17	4.94	5.05	0.13	0.12	0.73
Glu	18.06	17.50	16.73	17.03	0.35	0.035	0.61
Pro	6.57	6.33	6.10	6.14	0.14	0.043	0.56
Ser	4.41	4.28	4.08	4.15	0.09	0.049	0.57

did not affect starter intake, with the exception of a tendency for reduced starter intake ($P = 0.090$) from d 43 to 49—a time when milk replacer provision was limited to 2.5% of BW. Inclusion of glycerol tended to increase starter intake from d 29 to 35 ($P = 0.056$), d 36 to 42 ($P = 0.087$), d 43 to 49 ($P = 0.087$), and the cumulative starter intake throughout the study ($P = 0.069$). Milk replacer intake did not differ among treatments ($P \geq 0.14$).

We observed a tendency for a heat treatment $\times$ glycerol interaction for ruminal pH ($P = 0.070$; Table 5) with the numerically greatest pH for the H-NG treatment, intermediate for the NH-NG and NH-G treatments, and the least for the H-G treatment. Heat treatment of CM did not affect ruminal SCFA concentration ($P = 0.11$), but glycerol inclusion increased SCFA concentration ($P = 0.005$). There was a tendency for an interaction between heat treatment of CM and glycerol inclusion for the molar proportions of acetate and propionate ($P = 0.075$ and $P = 0.069$, respectively). In general, the greatest molar proportion of acetate was observed for the NH-NG treatment, intermediate for the H-G treatment, and the least for the H-NG and NH-G treatments. The molar proportion of propionate was the greatest for the NH-G treatment, intermediate for the H-NG treatment, and H-G treatment, and the least for the NH-NG treatment. Glycerol inclusion tended to decrease the molar proportions of iso-butyrate ($P = 0.092$); however, the molar proportion of iso-butyrate was not affected by heat treatment ($P = 0.43$). The molar proportion of butyrate was not affected by the glycerol supplementation ($P = 0.62$), but tended to be greater when heat-treated canola was included in

the starters ($P = 0.100$). The molar proportion of isovalerate tended to be decreased by the glycerol supplementation ($P = 0.076$). A heat treatment $\times$ glycerol interaction was observed for valerate ($P = 0.041$), but means that differed could not be detected with the Bonferroni posthoc analysis. The molar proportion of caproate and the ammonia concentration were not affected by the experimental treatments ($P \geq 0.25$).

The weight of the ruminal tissue and ruminal digesta were reduced ($P \leq 0.011$; Table 6) for calves fed heat-treated CM compared with those fed CM without additional heat treatment. Glycerol inclusion in the starter did not affect the weight of the ruminal tissue or digesta ($P \geq 0.18$). Omasal tissue and digesta, and abomasal tissue weights were not affected by the experimental treatments ($P \geq 0.14$). Abomasal digesta weight tended to be greater with glycerol supplementation ($P = 0.080$). Duodenal tissue weight and length were not affected by experimental treatments ($P \geq 0.15$), and digesta weight tended to be greater with glycerol supplementation ($P = 0.087$). Jejunal tissue weight was less with heat treatment ($P = 0.024$) and tended to be greater with glycerol supplementation ($P = 0.079$). Glycerol supplementation also increased jejunal digesta weight ($P = 0.001$). Heat treatment of CM tended to decrease the jejunal length ($P = 0.075$). Ileal tissue weight and length were not affected by the experimental treatments ($P \geq 0.23$). Heat treatment of CM decreased ileal digesta weight ($P = 0.004$). Cecal tissue weight and length were not affected by the experimental treatments ($P \geq 0.26$). The CM heat treatment decreased cecal digesta weight ($P = 0.018$) and glycerol supplementation increased it ($P = 0.009$). A treatment

Table 5. Body weight, BW gain, feed intake for Holstein bull calves (n = 7/treatment) between 8 and 51 d of age, and ruminal fermentation characteristics on 51 d of age (1 d postweaning) when fed pelleted starter mixtures containing either nonheated (NH) or heat-treated (H) canola meal with (G) or without (NG) glycerol supplementation (5% DM)

Item	Treatment					P-value ¹		
	NH-NG	NH-G	H-NG	H-G	SEM	HEAT	GLY	HEAT × GLY
Average BW, kg	55.0	55.6	53.7	55.7	1.8	0.75	0.47	0.69
Initial BW, kg	43.9	43.1	42.5	43.1	1.6	0.66	0.95	0.69
Final BW, kg	65.4	69.1	61.3	64.1	2.4	0.041	0.12	0.82
ADG, g/d	511.3	615.1	443.3	508.6	54.4	0.079	0.088	0.69
BW gain ² , kg	21.5	26.0	18.7	23.9	1.6	0.097	0.003	0.82
Starter intake ³ , kg of DM/d	0.363	0.355	0.359	0.355	0.027	0.63	0.23	0.69
d 8 to 28, kg of DM/d	0.026	0.049	0.020	0.030	0.008	0.16	0.056	0.54
d 29 to 35, kg of DM/d	0.116	0.174	0.113	0.103	0.045	0.39	0.58	0.43
d 36 to 42, kg of DM/d	0.329	0.508	0.305	0.416	0.093	0.48	0.087	0.68
d 43 to 49, kg of DM/d	0.753	0.959	0.602	0.766	0.117	0.090	0.069	0.83
Cumulative, d 8 to 50, kg of DM	9.98	13.87	8.51	10.56	1.93	0.14	0.075	0.57
Milk replacer intake, kg of DM/d	0.700	0.715	0.754	0.746	0.028	0.14	0.90	0.69
d 8 to 28, kg of DM/d	0.921	0.952	0.909	0.929	0.045	0.65	0.51	0.92
d 29 to 35, kg of DM/d	0.856	0.866	0.824	0.859	0.036	0.53	0.46	0.68
d 36 to 42, kg of DM/d	0.456	0.463	0.430	0.449	0.015	0.19	0.37	0.70
d 43 to 49, kg of DM/d	0.233	0.243	0.223	0.232	0.007	0.17	0.18	0.96
Cumulative, d 8 to 50, kg of DM	32.2	33.2	31.5	32.8	1.2	0.59	0.37	0.87
Ruminal fermentation								
pH	5.15	5.12	5.46	4.98	0.13	0.47	0.040	0.070
SCFA ⁴ concentration, mM	130.1	149.7	117.0	140.4	9.7	0.11	0.005	0.78
Acetate, mol/100 mol	59.7	48.5	49.7	53.2	4.3	0.50	0.34	0.075
Propionate, mol/100 mol	23.5	35.9	28.2	26.3	4.2	0.51	0.18	0.069
Iso-butyrate, mol/100 mol	0.280	0.159	0.346	0.210	0.076	0.43	0.092	0.92
Butyrate, mol/100 mol	11.4	11.1	17.9	15.2	3.1	0.100	0.62	0.70
Iso-valerate, mol/100 mol	0.283	0.144	0.321	0.283	0.077	0.45	0.076	0.85
Valerate, mol/100 mol	3.69	3.56	2.86	4.44	0.40	0.96	0.083	0.041 ⁵
Caproate, mol/100 mol	1.11	0.61	0.67	0.57	0.30	0.36	0.25	0.45
Ammonia, mg/dL	15.2	14.8	11.9	14.4	2.3	0.30	0.54	0.41

¹HEAT = effect of canola meal heat treatment; GLY = effect of glycerol inclusion; HEAT × GLY = canola meal heat treatment by glycerol supplementation interaction.²BW gain = final BW – initial BW.³d 8 = provision of starter to the calves begun; d 28 = provision of milk replacer was gradually decreased over time to facilitate weaning; d 50 = first day with no milk provision.⁴SCFA = short-chain fatty acid.⁵No mean separation was detected with the Bonferroni post hoc analysis.

interaction was observed for the colonic length ($P = 0.038$) and tended to be observed for colonic tissue weight ($P = 0.099$). Colonic length was the greatest for NH-NG treatment, intermediate for NH-G and H-G, and shortest for H-NG treatment. Colonic tissue weight was numerically the greatest for the H-G treatment, intermediate for NH-G and NH-NG treatments, and the least for H-NG treatment. Colonic tissue weight also tended to be increased by the glycerol supplementation ($P = 0.072$), and colonic length was decreased by CM heat treatment ($P = 0.010$). Colonic digesta was not affected by the experimental treatments ($P \geq 0.13$).

Heat treatment did not affect the gene expression of any of the analyzed genes of interest ($P \geq 0.22$; Table 7). A heat treatment $\times$ glycerol interaction was observed for *AQP3* ($P = 0.021$) and tended to be observed for the expression of *MCT1* ($P = 0.076$) in ruminal epithelium from the cranial sac. For the expression of *AQP3*, the Bonferroni mean separation test did not identify means that differed, but numerically the greatest expression of *AQP3* was for the H-NG treatment, followed by NH-G and NH-NG treatments, and the least for the H-G treatment. The expression of *MCT1* was numerically greatest for the H-NG treatment, intermediate for the NH-NG and NH-G treatments, and the least for the H-G treatment. Expression of *UTB*, *GPR41*, and *GPR43* in the ruminal epithelium was not affected by the glycerol inclusion ($P \geq 0.26$). Glycerol inclusion resulted in greater expression of *MCT1* ($P = 0.017$) in the epithelium of the PROX. A tendency for a heat treatment $\times$ glycerol supplementation interaction was observed for the expression of *GPR41* in the PROX ($P = 0.089$). Glycerol did not affect any of the genes of interest in the ileal epithelium ($P \geq 0.46$).

We did not detect any treatment interactions nor effect of heat treatment of CM on brush border enzyme activities ($P \geq 0.16$; Table 8). Inclusion of glycerol did not affect activity of aminopeptidase A, aminopeptidase N, lactase, or maltase ($P \geq 0.19$), but tended to increase dipeptidase IV activity in the middle jejunum ($P = 0.059$).

Heat treatment of CM did not affect plasma glucose concentration ($P \geq 0.18$, Table 9). Glycerol supplementation tended to decrease plasma glucose concentration on d 43 of age ($P = 0.076$), but had no effect on glucose concentration on d 22 and 51 of age ($P \geq 0.45$). Plasma insulin concentration did not differ between the heat-treated and nonheated CM ($P \geq 0.27$). On d 22 and 43 of age, plasma insulin was decreased by the glycerol supplementation ($P = 0.016$ and $P = 0.003$, respectively). We observed a tendency for a heat treatment by glycerol supplementation interaction for plasma insulin concentration on d 51 of age ($P = 0.099$), with the numerically greatest concentration for

the both NH-G and H-NG treatments relative to NH-NG and H-G. Serum BHB concentration on d 22 of age tended to be reduced with heat treatment ($P = 0.096$), yet on d 43, BHB concentration was increased by heat treatment ($P = 0.022$). On d 51 of age, serum BHB tended to be decreased by glycerol supplementation ($P = 0.061$). Plasma NEFA concentration was not affected by the experimental treatments on d 22 and 51 of age ($P \geq 0.23$). On d 43, plasma NEFA concentration tended to be less for calves fed heat-treated CM ($P = 0.072$) and tended to be less for glycerol inclusion ($P = 0.092$). A heat treatment $\times$ glycerol supplementation interaction was observed for plasma urea-N at 43 d of age ($P = 0.035$). Mean separation for this variable was not achieved; however, the greatest plasma urea-N concentration was observed for the NH-NG and H-G treatments, intermediate for H-NG and the least for the NH-G treatment. The main effects of heat treatment and glycerol inclusion did not affect plasma urea-N concentration on any of the 3 d of sample collection ($P \geq 0.13$).

DISCUSSION

Optimal Temperature for Heat Treatment of CM

As CM is a byproduct from the canola crushing industry, processing conditions imposed within and among processing plants affect composition, especially for RDP and RUP proportions (Bell and Keith, 1991). The CON CM in the present study had similar proportions of the undegradable protein fraction as those reported by Huang et al. (2015) and Maxin et al. (2013). Moreover, we observed that heat treating CM increased the proportion of RUP, an observation reported previously (McKinnon et al., 1991; Dakowski et al., 1996). Though the intent of the heat treatment in the present study was to increase the rumen undegradable fraction, the heat-treating process also increased the NDF and ADF content, as observed in previous research (McKinnon et al., 1995). The increase in NDF and RUP was likely partially due to the Maillard reaction (van Soest and Mason, 1991), which is when soluble carbohydrate components react with AA to form indigestible compounds that are insoluble in neutral detergent solution. The latter statement is supported by the general reduction in AA concentrations observed with increasing heat treatment. Similar observations for decreased essential AA concentrations due to the Maillard reaction have been made by Ajandouz and Puigserver (1999); although, their treatments occurred in aqueous conditions. Past studies have also reported no effect of heat treatment on the CP content of CM (McKinnon et al., 1991, 1995; Dakowski et al., 1996). Conversely, we ob-

Table 6. Gastrointestinal tract morphometry of Holstein bull calves (n = 7/treatment) at 51 d of age (1 d postweaning) fed pelleted starter mixtures containing either nonheated (NH) or heat-treated (H) canola meal and with (G) or without (NG) glycerol supplementation (5% DM)

Item	Treatment					P-value ¹		
	NH-NG	NH-G	H-NG	H-G	SEM	HEAT	GLY	HEAT × GLY
Rumen								
Tissue, kg	1.16	1.26	0.884	1.03	0.11	0.011	0.18	0.78
Digesta, kg	4.37	3.82	2.68	2.97	0.29	<0.001	0.61	0.12
Onasum								
Tissue, kg	0.179	0.233	0.205	0.221	0.025	0.77	0.14	0.42
Digesta, g	96.7	120.6	135.6	127.4	34.4	0.40	0.77	0.56
Abomasum								
Tissue, kg	0.343	0.361	0.343	0.355	0.019	0.86	0.41	0.87
Digesta, kg	0.534	0.550	0.327	0.632	0.089	0.48	0.080	0.12
Duodenum								
Tissue, g	84.3	90.0	74.5	84.9	6.2	0.23	0.20	0.71
Digesta, g	18.8	19.7	13.8	21.5	2.4	0.50	0.087	0.17
Jejunum								
Length, m	0.650	0.657	0.590	0.633	0.028	0.15	0.38	0.52
Tissue, kg	1.33	1.55	1.14	1.28	0.10	0.024	0.079	0.68
Digesta, kg	0.776	1.278	0.768	0.992	0.103	0.14	0.001	0.17
Length, m	18.2	18.1	15.8	17.9	0.8	0.075	0.18	0.16
Ileum								
Tissue, kg	0.166	0.187	0.135	0.167	0.022	0.25	0.24	0.79
Digesta, g	69.7	63.9	19.6	27.1	13.8	0.004	0.95	0.63
Cecum								
Length, m	1.029	1.000	0.831	0.943	0.120	0.23	0.69	0.50
Tissue, g	80.1	73.9	66.7	76.6	7.0	0.38	0.85	0.26
Digesta, kg	0.288	0.228	0.236	0.156	0.030	0.018	0.009	0.69
Length, m	0.300	0.279	0.274	0.284	0.018	0.42	0.95	0.26
Colon								
Tissue, kg	0.355	0.359	0.301	0.374	0.023	0.33	0.072	0.099
Digesta, kg	0.330	0.426	0.267	0.327	0.055	0.13	0.14	0.73
Length, m	2.79 ^a	2.59 ^{ab}	2.39 ^b	2.54 ^{ab}	0.10	0.01	0.78	0.038

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).¹HEAT = effect of canola meal heat treatment; GLY = effect of glycerol inclusion; HEAT × GLY = canola meal heat treatment by glycerol supplementation interaction.

Table 7. Gene expression in the cranial sac of the rumen (CRA), proximal jejunum (PROX), and ileum (ILE) of Holstein bull calves (n = 7/treatment) at 51 d of age (1 d postweaning) as influenced by feeding pelleted starter mixtures containing either nonheated (NH) or heat-treated (H) canola meal and with (G) or without (NG) glycerol supplementation (5% DM)

Gene of interest ¹		Treatment				SEM	P-value ²		
		NH-NG	NH-G	H-NG	H-G		HEAT	GLY	H × G
CRA	<i>MCT1</i>	1.79 ³	1.84	1.70	2.18	0.13	0.30	0.034	0.076
	<i>UTB</i>	5.18	5.25	5.34	5.29	0.17	0.44	0.94	0.63
	<i>AQP3</i>	0.492	0.303	0.237	0.753	0.164	0.49	0.26	0.021 ⁴
	<i>GPR41</i>	15.2	15.3	15.0	14.5	0.4	0.29	0.64	0.50
	<i>GPR43</i>	14.1	14.5	14.0	13.7	0.4	0.22	0.95	0.33
PROX	<i>MCT1</i>	3.57	3.15	3.83	3.33	0.18	0.22	0.017	0.83
	<i>PEPT1</i>	4.75	5.31	5.15	5.01	0.24	0.83	0.38	0.16
	<i>PEPT2</i>	13.2	13.0	12.6	13.1	0.3	0.41	0.57	0.23
	<i>EAAC1</i>	7.73	8.39	8.33	7.92	0.38	0.85	0.73	0.15
	<i>ATB0+</i>	4.15	4.03	3.72	4.18	0.24	0.48	0.39	0.16
	<i>GPR41</i>	9.33	9.55	9.52	9.29	0.12	0.81	0.95	0.089
	<i>GPR43</i>	9.30	9.18	9.00	8.98	0.23	0.28	0.76	0.83
	<i>MCT1</i>	6.66	6.57	6.57	6.77	0.21	0.63	0.63	0.22
ILE	<i>PEPT1</i>	6.02	5.96	5.83	6.11	0.32	0.93	0.73	0.59
	<i>PEPT2</i>	10.5	10.4	10.5	10.7	0.2	0.36	0.92	0.30
	<i>EAAC1</i>	6.12	6.24	5.96	5.65	0.48	0.41	0.83	0.63
	<i>ATB0+</i>	5.94	5.76	5.40	6.06	0.38	0.74	0.54	0.27
	<i>GPR41</i>	7.59	7.43	7.66	7.53	0.21	0.66	0.46	0.93
	<i>GPR43</i>	10.2	10.1	10.3	10.7	0.4	0.41	0.71	0.51

¹*MCT1* = monocarboxylic acid transporter 1; *GPR41* = G protein-coupled receptor 41; *GPR43* = G-protein-coupled receptor 43; *UTB* = urea transporter B; *AQP3* = aquaporin 3; *PEPT1* = peptide transporter 1; *PEPT2* = peptide transporter 2; *ATB0+* = amino acid transporter B0+; *EAAC1* = excitatory amino acid carrier 1.

²HEAT = effect of canola meal heat treatment; GLY = effect of glycerol inclusion; H × G = canola meal heat treatment by glycerol supplementation interaction.

³Values of gene expression are presented as ΔC_T = threshold cycle (C_T) for the gene of interest – C_T for the reference genes; greater ΔC_T values represent lesser mRNA expression.

⁴No mean separation was detected with the Bonferroni post hoc analysis.

served that CP concentration increased at a decreasing rate with increasing heat-treatment temperature. It is unclear why CP concentration was altered with heat treatment.

As expected, heat treatment decreased the proportion of potentially degradable CP in the rumen, reduced the degradation rate of the degradable fraction (K_d) relative to untreated CM, and quadratically decreased the estimated intestinal digestibility of CP. Heat treatment also decreased the concentration of Glu, a response that was opposite to that previously reported (Newkirk et al., 2003). It is unclear why results in the present study and that of Newkirk et al. (2003) differ, but may be related to the heat-treatment protocol and the inherent composition of the CM. The increase in the undegradable fraction and stimulatory effect of heat treatment on intestinal CP digestibility indicate that we achieved an increase the RUP without having marked reductions in the intestinal digestibility with our approach. Although we measured in situ degradation using heifers, rather than calves, Vazquez-Anon et al. (1993) suggested that measurements made with cows may reasonably approximate in situ digestion in calves within 4 wk of weaning. Other studies have also suggested that ruminal fermentation at the time of weaning is not dif-

ferent from that occurring several weeks after weaning (Quigley et al., 1985; Lallès and Poncet, 1990).

Interactions Between CM Heat Treatment and Glycerol Supplementation

When designing the current study, we anticipated interactions among heat-treated CM and glycerol inclusion in starters because glycerol inclusion could increase the concentration of ruminal butyrate (Rémond et al., 1993; Paiva et al., 2016) and potentially stimulate ruminal development (Sander et al., 1959; Mentschel et al., 2001), whereas heat-treated CM could increase the supply of AA to the intestine, potentially promoting intestinal growth and functions. Altogether, feed intake and calf performance could be enhanced. However, only a few interactions were observed and the detected interactions were primarily only tendencies. It is unclear why the interactions did not occur, but a possible explanation is that heat treatment of CM may have reduced apparent total-tract CP digestibility, thereby limiting one of the potential synergistic effects. This previous explanation is partially supported by reduced BW gain with heat-treated CM; however, we did not measure apparent total-tract digestibility and cannot

Table 8. Brush border enzyme activities¹ in duodenum (DUO), proximal (PROX), middle (MID) and distal (DIST) jejunum, and ileum (ILE) of Holstein bull calves (n = 7/treatment) at 51 d of age (1 d postweaning) as influenced by feeding pelleted starter mixtures containing either nonheated (NH) or heat-treated (H) canola meal and with (G) or without (NG) glycerol supplementation (5% DM)

Item	Treatment				P-value ²			
	NH-NG	NH-G	H-NG	H-G	SEM	HEAT	GLY	HEAT × GLY
Aminopeptidase A	2.94	2.37	2.31	2.52	0.29	0.42	0.54	0.19
	35.3	36.2	24.6	30.5	6.9	0.25	0.63	0.73
	37.9	24.1	22.4	22.9	6.0	0.16	0.26	0.23
Aminopeptidase N	8.41	10.91	9.52	8.67	2.29	0.80	0.72	0.47
	16.6	14.4	23.5	17.7	4.7	0.28	0.39	0.70
	32.3	20.4	23.7	23.0	5.0	0.55	0.22	0.27
Dipeptidase IV	3.37	5.31	3.57	4.16	0.64	0.47	0.059	0.30
	5.65	4.54	4.66	4.37	0.75	0.41	0.33	0.56
	105.6	122.9	113.8	97.2	15.6	0.52	0.98	0.22
Lactase	144.5	128.0	119.5	112.7	20.9	0.32	0.56	0.81
	59.7	68.2	66.3	74.3	11.4	0.54	0.43	0.98
	20.6	21.6	19.9	20.2	1.6	0.47	0.65	0.80
Maltase	19.2	18.2	19.9	19.3	1.1	0.35	0.45	0.85
	19.8	23.2	20.4	20.2	1.8	0.35	0.21	0.18
	32.0	27.8	33.9	26.5	4.8	0.94	0.19	0.71
DIST	27.9	32.8	27.0	27.8	3.6	0.43	0.29	0.61
	36.7	34.3	34.6	41.2	5.6	0.61	0.66	0.34
	20.7	18.6	18.3	20.5	2.8	0.92	0.98	0.37

¹Data are presented as enzymatic units per milligram of protein, which describes the number of micromoles of glucose or *p*-nitroanilide released per minute of reaction at 37°C.

²HEAT = effect of canola meal heat treatment; GLY = effect of glycerol inclusion; HEAT × GLY = canola meal heat treatment by glycerol supplementation interaction.

Table 9. Concentration of plasma glucose, insulin, nonesterified fatty acid (NEFA), and urea, and serum BHB of Holstein bull calves (n = 7/treatment) 2 h after solid feed presentation as influenced by feeding of pelleted starter mixtures containing either nonheated (NH) or heat-treated (H) canola meal and with (G) or without (NG) glycerol supplementation (5% DM)

Variable	Day of age ¹	Treatment				P-value ²		
		NH-NG	NH-G	H-NG	H-G	SEM	HEAT	GLY
Glucose, mg/dL	22	94.7	92.2	91.3	96.8	5.4	0.34	0.45
	43	95.5	84.4	91.7	88.2	4.7	0.99	0.076
	51	66.5	69.9	64.6	62.1	3.6	0.18	0.91
Insulin, µg/L	22	0.671	0.463	0.704	0.271	0.121	0.51	0.016
	43	0.426	0.206	0.534	0.259	0.102	0.27	0.003
	51	0.110	0.126	0.126	0.107	0.010	0.89	0.89
BHB, mmol/L	22	0.454	0.447	0.325	0.402	0.036	0.096	0.33
	43	0.365	0.400	0.465	0.474	0.036	0.022	0.53
	51	0.492	0.463	0.611	0.456	0.049	0.26	0.061
NEFA, mEq/L	22	147.1	144.9	141.7	133.7	8.7	0.35	0.56
	43	199.3	146.6	144.5	142.7	17.8	0.072	0.092
	51	196.6	155.1	151.9	161.6	20.6	0.36	0.45
Urea-N, mg/dL	22	7.90	8.29	9.06	8.13	0.61	0.42	0.65
	43	9.36	7.93	8.44	9.78	0.86	0.45	0.95
	51	9.01	9.61	10.22	10.42	0.68	0.13	0.53

¹Day of age: 22 = during 15% BW milk replacer provision; 43 = at 5% BW milk replacer provision; 51 = a day after weaning.

²HEAT = effect of canola meal heat treatment; GLY = effect of glycerol inclusion; HEAT × GLY = canola meal heat treatment by glycerol supplementation interaction.

³No mean separation was detected with the Bonferroni post hoc analysis.

confirm the digestibility response. Others have also reported that heat-treatment of protein sources (soybean meal) for calves did not alter the intestinal supply of protein from microbial protein or RUP, despite the reduction of effective ruminal degradability measured in situ in a steer from heat treatment (Obitsu et al., 1995). Additionally, glycerol did not increase ruminal butyrate concentration in the present study, and hence the secondary proposed mode of action for beneficial effects of heat-treated CM and glycerol inclusion was not observed. As such, the main effects of heat-treated CM and glycerol will be discussed separately and the most important interactions will be mentioned and discussed.

Effect of Heat-Treating CM on Calf Performance and Development of the GIT

Studies have evaluated the use of CM in starter mixtures for calves, suggesting that use of CM may decrease starter intake (Stake et al., 1973; Miller-Cushon et al., 2014b), CP digestibility (Schingoeth et al., 1974; Fiems et al., 1985; Khorasani et al., 1990), and ADG (Miller-Cushon et al., 2014b; Hadam et al., 2016) relative to soybean meal; however, in some studies starter intake (Ingalls and Seale, 1971; Fisher, 1980), CP digestibility (Stake et al., 1973; Fisher, 1980), and ADG (Stake et al., 1973; Fisher, 1980; Fiems et al., 1985) were not affected by protein source. In the present study, we did not compare different protein sources, but compared untreated and heat-treated CM to evaluate the effect of altering the RUP supply.

In the current study, we observed decreased ADG and, as a result, a lighter final BW when calves were fed starters containing heat-treated CM. Calves fed heat-treated CM tended to have less cumulative starter intake from d 8 to 50; of which the most notable response was from d 43 to 49. In the 2 studies reported by Obitsu et al. (1995), feeding calves heat-treated soybean meal did not affect DMI when compared with soybean meal that was not heated, but calves in those studies received 30 or 40% of their diet as hay, which may have masked treatment effects. In the present study, calves that ate diets containing heat-treated CM also had lighter reticulo-ruminal and jejunal weights. Given the importance of the reticulo-rumen for fermentation of feed and SCFA absorption, and the importance of the jejunum to facilitate protein digestion and absorption, the reduction in tissue weights could indicate decreased digestive capacity. Additionally, ruminal, ileal, and cecal digesta weights were lighter for the heat-treated CM treatment, likely reflecting a smaller gastrointestinal tract size and the tendency for lesser DMI. However, the effect appears to be influenced primarily by the

changes in tissue weight as gene expression and brush border enzyme activities were, in general, not affected by heat treatment.

A critical assumption in this study was that a large portion of the RUP from heat-treated CM could be digested in the small intestine, as indicated by the *in vitro* measurement approach employed. However, the lack of a positive effect of heat treatment may be indicative of reduced CP digestibility with heat treatment, thereby eliminating potential increases for the total metabolizable protein supply, or that provision of RUP may not provide added benefit for calves around weaning. Unfortunately, we did not confirm digestibility of the starters *in vivo*, and therefore cannot determine the cause for the lack of effect. Although we conclude that heat treatment of CM to increase the RUP was not effective, part of this response is attributed to the reduction in starter intake. Using simple predictions from the *in situ* degradability of CM in study 1 combined with starter intake and the composition of the starter in study 2, the RUP supply from CM can be estimated. As expected, until 35 d of age, CM provision in the starter was estimated to supply up to 5% of the total RUP. The low contribution of the CM in the starter toward the RUP supply were mostly due to the relatively small quantities of starter intake, large quantities of MR intake, and the assumption that the CP in milk replacer was 100% RUP (data not shown). Comparatively, during the step-down weaning phase, the estimated contribution of CM to the supply of RUP ranged from 42.90 to 50.11%, resulting in the predicted supply of 48 to 64 g/d. However, between 43 and 49 d of age, heat treatment of CM may have only marginally increased the supply of RUP from 54 g/d for CM that was not heated to 57 g/d for CM that was heated, again mostly due to the negative effect of heated CM on starter intake. Importantly, the predicted RUP contribution from CM in the starter increased the metabolizable protein supply from milk replacer by 75 to 100%, showing that during the step-down phase of the weaning process, solid feed contributes substantially to the potential RUP supply. Even though these calculations are essential to approximate the contribution of the treatments to the RUP supply, they do not account for differences in the digestibility of the RUP fraction and rely on the use of *in situ* degradation measurements conducted in heifers to predict the response. Moreover, these calculations do not account for potential differences in the RUP supply with pelleting of the treatments (Huang et al., 2015) or other ingredients within the pellet, and rely on the assumption that capacity for ruminal digestion of CP in CM was similar to that for mature cattle. The latter suggestion is supported by previous research where ruminal SCFA concentrations

and the contribution of bacterial N to the omasal N supply were not different during the week of weaning relative to that measured postweaning (Quigley et al., 1985). Additionally, the lining of the intestinal tissue can be renewed every 2 to 3.5 d, depending on species (Darwich et al., 2014), which suggests that even short exposure to differing ration RUP fraction might have potential to influence the development of the intestine. With that said, there is a paucity of research evaluating the digestive capability of calves around weaning and how RUP affects metabolizable protein supply (Hill et al., 2013), especially when originating from heat treatment of a protein source (Vazquez-Anon et al., 1993).

Given the relatively high AA requirement for growing calves (NRC 2001), the dietary changes occurring during weaning, and that ruminal fermentation may not provide sufficient quantities microbial protein to meet metabolizable protein requirements, we hypothesized that providing additional RUP may enhance the growth of Holstein calves. However, use of plant-based proteins in milk replacer reduced digestibility (Guiloteau et al., 2011) and altered the intestinal epithelium (Górka et al., 2011); furthermore, cooking soy protein at 50°C overnight further reduced digestibility (Dawson et al., 1988). As such, we speculated that despite the limited effects of heat treatment on the estimated intestinal digestibility determined *in vitro*, heat treatment of CM may have decreased total-tract CP digestibility, thereby limiting AA supply to promote growth of the GIT and BW growth. The potential reduction in digestibility is further supported by Montagne et al. (2003), as they suggested that the digestion of plant-based protein might be limited at oligopeptide digestion and AA absorption steps in calves. On the contrary, Obitsu et al. (1995) reported that there were no differences in bacterial N flow to the intestine when calves were fed soybean meal or heated soybean meal, suggesting that rather than a reduction in digestibility of CP, heat treatment may not have increased the flow of RUP. This latter suggestion is supported by the empirical calculations previously described. Given that we did not observe any changes in proteolytic brush border enzyme activity or gene expression for AA transporters, it may be postulated that intestinal digestibility was not improved or that intestinal supply of microbial N and the flow of RUP were not altered. Nevertheless, it is clear that heat treatment of CM does not stimulate growth or intestinal development in calves around weaning. Alternatively, it cannot be excluded that heat treatment of CM had a suppressive effect on rumen development, particularly ruminal fermentation development; however, ruminal pH and SCFA concentrations in calves were not affected by feeding them heat-treated CM, challenging such a hypothesis. Moreover, heat-treated

CM did not affect rumen ammonia-N, suggesting that the RDP supply was adequate.

Heat treatment of CM had only small effects on the blood metabolites evaluated by decreasing BHB on d 22 and increasing BHB in d 43, as well as NEFA concentration on d 43. The calves consuming heat-treated CM overall consumed less starter between d 8 and 28, which could result in the lesser BHB concentration on d 22. On the other hand, heat treatment of CM tended to result in increased ruminal butyrate proportion postweaning, which could result in the increased BHB concentration in the serum. The reason why heat treatment of CM increased ruminal butyrate is not clear.

Effect of Glycerol on Calf Performance and Development of the GIT

Calves that consumed starters supplemented with glycerol cumulatively consumed more starter during the whole study and especially during the step-down period. A possible reason for the increase in starter intake is that glycerol has been suggested to have a sweet taste (Quispe et al., 2013) and, therefore, may increase palatability of starter feed. However, a recent review has suggested that glycerol has relatively inconsistent effects on DMI, and the response is likely due to purity of the glycerol source and dose (Kholif, 2019). The glycerol used in the present study had greater than 80% purity and was included at a relatively low rate, supporting that glycerol may enhance DMI at low inclusions (Ariko et al., 2015).

As a result of greater starter intake, both ADG and total BW gain were greater in glycerol supplemented calves. In a study conducted on beef calves (309.3 ± 14.0 kg), ADG increased when glycerol was included at 10% of the DM and decreased when included at 20% (Ramos and Kerley, 2012); however, a following study within the same publication did not yield any changes in ADG. Pantophlet et al. (2016) also reported no effect of glycerol on growth of calves. Although calves used for the previous studies were older than the calves in the present study, there is evidence that, even with a low inclusion rate, glycerol might have positive effects on growth of young ruminants.

As expected, with rapid glycerol fermentation in the rumen (Rémond et al., 1993), ruminal SCFA concentration increased in calves fed glycerol supplemented starters, and as a consequence, a decrease in ruminal pH was noted. Similar effects have been previously observed in dairy cows (DeFraen et al., 2004; Mach et al., 2009); however, others have reported no changes for ruminal SCFA concentrations in calves (Ramos and Kerley, 2012) or cows (Paiva et al., 2016) when glycerol was included in the diet. According to Hall

et al. (2015), SCFA concentration might not be the most reliable measure of fermentation, as it does not account for the ruminal volume, ruminal dilution, or absorption rates. The ruminal dilution rate may be particularly important in young calves (Yohe et al., 2018) considering that we observed increased digesta weight in the abomasum, duodenum, jejunum, and cecum, and increased tissue mass of the jejunum and colon when calves were supplemented with glycerol without differences in ruminal tissue weight. We suspect that glycerol might promote faster digesta passage through the GIT because glycerol increased starter intake, did not affect ruminal digesta weight, and increased digesta weight in regions of the GIT distal to the rumen. As a consequence, more nutrients were likely digested postruminally, as indicated by greater dipeptidase IV activity in jejunum, especially when glycerol was combined in the starter with heat-treated CM. Similar observations of increased dipeptidase IV activity in the distal jejunum were made when supplying microencapsulated sodium butyrate, another energy substrate, to calves (Górka et al., 2014). Along this line of thought, Werner Omazic et al. (2015) estimated that 25% of glycerol is fermented, 45% is absorbed, and 30% flows into the omasum of dairy cows; however, such data are not available for calves. Given the potential for ruminal fermentation in the present study and outflow from the reticulo-rumen, it is likely that some of the glycerol was fermented in the intestine, possibly promoting intestinal growth. Further support for this suggestion includes that the expression of *MCT1* was upregulated in the jejunum, implying greater potential for SCFA absorption from the intestine for calves supplemented glycerol. Unfortunately, in the present study, we did not measure SCFA concentration in the small or large intestines, and thus cannot determine whether SCFA concentrations were altered. Nevertheless, SCFA released postruminally or delivered with digesta to lower regions of the GIT may have an important effect on GIT development in calves (Górka et al., 2018).

Werner Omazic et al. (2015) noted, as mentioned previously, that in addition to being fermented in the rumen and passing to the omasum, glycerol can be transported through the epithelium. Apparent transport of glycerol into portal circulation was observed both in mature dairy cows (Werner Omazic et al., 2015) and in calves (Werner Omazic et al., 2013), following a large pulse-dose of glycerol into the rumen. In the current study, we did not observe differences in the expression of *AQP3* in the CRA due to glycerol, although we did observe a heat treatment by glycerol inclusion interaction. Aquaporin 3 is a water channel within the ruminal epithelium and is within a subgroup classified as aquaglyceroporins, as they are able to

transport small particles such as urea (Rojek et al., 2008; Walpole et al., 2015) or glycerol (Wu and Beitz, 2007). It is possible that the dose of glycerol in the present study was not sufficient to stimulate a response for *AQP3* expression or that the transport was mediated by other aquaglyceroporins such as *AQP7*, *AQP9*, or *AQP10* (Rojek et al., 2008) that were not analyzed in the present study.

Glycerol can modulate ruminal fermentation and has been reported to increase the molar proportion of propionate (Rémond et al., 1993; DeFrain et al., 2004; Ramos and Kerley, 2012) and butyrate (Rémond et al., 1993; DeFrain et al., 2004; Paiva et al., 2016) and decrease acetate (Rémond et al., 1993; Ramos and Kerley, 2012; Paiva et al., 2016). In the present study, we did not observe changes in the molar proportions of acetate, propionate, or butyrate with glycerol inclusion. Reasons for the discrepancy between the present study and past studies may be due to the single spot-sampling approach used in the present study compared with more frequent measurements in the previous studies, and that calves in the present study were transitioning to solid feed. Differences could also arise from a lower dose of glycerol in the present study (5% DM). For example, Paiva et al. (2016) used glycerol inclusions ranging from 7 to 21% of DM, Ramos and Kerley (2012) used 0 to 20% DM, and Rémond et al. (1993) dosed ruminally cannulated cows at 20 or 40% of DM. However, DeFrain et al. (2004) used glycerol doses within a similar range as the current study with only very minor changes in SCFA concentration. Thus, in the current study, the positive effect of glycerol inclusion in starter mixture was likely not a result of its effect on ruminal butyrate, and thus stimulation of rumen epithelium development, which was originally hypothesized.

Glycerol decreased both plasma glucose and insulin concentrations on d 43—a time point corresponding to when calves were exposed to the step-down weaning process. The reduction in glucose and insulin might be due to the decrease in the starch supply in the glycerol treatments due to glycerol introduction into the feed. Similar observations for reduced glucose have been reported in mature ruminants when corn was replaced by glycerol (Carvalho et al., 2011). Surprisingly, glycerol also resulted in decreased NEFA concentration on d 43, further suggesting that the reduction in glucose and insulin were not driven by reduced nutrient intake. However, we observed lesser BHB concentration on d 51 for calves fed glycerol, suggesting that the potentially added nutrient supply was likely not mediated through butyrate as was observed by Carvalho et al. (2011). That said, glycerol increased both the total SCFA concentration in the rumen and starter intake during the step-down period and would be expected

to increase, rather than decrease BHB (Quigley et al., 1991). There was also no effect of glycerol on tissue mass in the proximal parts of GIT, and therefore the decreased of BHB concentration is difficult to explain.

In the present study, calves fed starters with heat-treated CM with glycerol had the greatest gene expression of *MCT1* in the CRA and the least when fed heat-treated CM without glycerol. The gene *MCT1* is a monocarboxylate transporter responsible for the removal of SCFA and ketones out of the rumen (Halestrap and Price, 1999). Nakamura et al. (2018) showed that *MCT1* was upregulated in postweaned calves, although *MCT1* was not affected by changes in SCFA profiles. The increased *MCT1* observed in the present study may be related to greater starter intake between d 29 and 50 of age, as calves supplemented with glycerol consumed more starter than calves not supplemented with glycerol. However, heat treatment increased ruminal butyrate, and butyrate is known to increase *MCT1* expression in ruminal epithelium (Dengler et al., 2015).

CONCLUSIONS

Increasing heat-treatment temperature decreased the ruminally degradable DM and CP fractions in CM with minimal effects on estimated intestinal digestibility in cannulated heifers when heated at 110°C for 10 min. Heat-treated CM and glycerol inclusion do not interact to promote development of the GIT in calves around weaning. Feeding heat-treated CM to calves before and during weaning transition may reduce ADG and GIT development. Inclusion of glycerol at 5% DM may positively affect ADG. Furthermore, glycerol may increase ruminal fermentation and *MCT1* expression in the small intestine, suggesting stimulation of GIT development.

ACKNOWLEDGMENTS

The authors acknowledge the technical assistance of G. Gratton, K. Hare, F. Joy, R. Kanafany-Guzman, A. Laarman, R. Pederzoli, C. Rosser, A. Verdugo, D. Watanabe, and K. Wood (Department of Animal and Poultry Science, University of Saskatchewan, Saskatoon, SK, Canada). The authors acknowledge P. Yu and Z. Niu (Department of Animal and Poultry Science, University of Saskatchewan) for their assistance with estimated intestinal digestibility assay. The authors also acknowledge Evonik Nutrition and Care GmbH (Essen, Germany) for their support with amino acid analysis. The authors thank D. Michel and B. Bandy from the Health Sciences Department at the University of Saskatchewan for providing access to their laboratory equipment. Funding for this project was provided by Saskatchewan Canola Development

Commission (Saskatoon, SK, Canada), Saskatchewan Agriculture Development Fund (Regina, SK, Canada), Western Grains Research Foundation (Saskatoon, SK, Canada), and the Alberta Crop Industry Development Fund (Lacombe, AB, Canada). The authors have not stated any conflicts of interest.

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