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Validation of an in vivo dual permeability marker technique to characterize regional gastrointestinal tract permeability in mid-lactation Holstein cows during short-term feed restriction

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ABSTRACT

This study evaluated the effects of short-term feed restriction in lactating dairy cows on regional permeability of the gastrointestinal tract (GIT), and the recovery of DMI, ruminal pH, and milk yield. In addition, sampling methods for a novel dual marker technique to characterize total GIT and post-ruminal permeability were validated. Six ruminally cannulated lactating Holstein cows were blocked by parity (3 primiparous, 3 multiparous; 189 DIM ± 25.2) and enrolled in a crossover design. Experimental periods included a 5-d baseline phase, 5-d challenge phase (CHAL), and 2 wk of recovery (REC1 and REC2). During CHAL, cows received either 100% ad libitum feed intake (AL) or 40% of ad libitum feed intake (FR). To assess total-tract and post-ruminal permeability, equimolar doses of Cr-EDTA and Co-EDTA were infused on d 3 of CHAL into the rumen and abomasum (0.369 mmol/kg BW). Following infusions, total urine and feces were collected every 8 h over 96 h, and blood samples were collected at h 0, 1, 2, 3, 4, 6, 8, 12, 16, 20, 24, 32, 40, 48, and 64. The plasma area under the curve (AUC) for Cr and Co were calculated. By design, DMI for FR was reduced by 60% during CHAL and remained 19% lower than AL during REC1 but was not different from AL in REC2. Mean ruminal pH for FR was greatest during CHAL and least during REC1, with no differences detected between AL and FR in REC2. The duration that pH was <5.8 was least for FR during CHAL and greatest during REC1, which were different from AL and were no longer different between treatments in REC2. Milk yield was the least for FR during CHAL and REC1 and no longer different from AL in REC2. Feed restriction reduced milk fat, protein, and lactose yields by 26%, 31%, and 31%, respectively. Plasma Cr AUC was 34% greater and Co AUC tended to be 35% greater for FR than AL on d 3 of CHAL. Urinary Cr recovery after 48 h was not affected by treatment; however, urinary Co recovery was 36% greater for FR than AL. Positive correlations between plasma AUC and urinary recovery for Cr and Co were detected. It was determined that blood samples collected at h 2, 8, 20, 40, and 48 could predict the total plasma Cr and Co AUC within 1.9% and 6.2%, respectively. In summary, short-term FR in lactating dairy cows increases permeability of the total GIT and may increase permeability of the post-ruminal regions, with more than 60% of the permeability occurring post-ruminally. After FR, cows experienced low ruminal pH and a sustained reduction in milk yield. When using Cr- and Co-EDTA to evaluate regional GIT permeability, plasma AUC can be used as an alternative to urinary Cr and Co excretion. In addition, blood samples collected at h 2, 8, 20, 40, and 48 result in adequate prediction accuracy, at least when comparing GIT permeability for lactating dairy cows exposed to AL and FR.

Key words: barrier function, Cr-EDTA, Co-EDTA, low feed intake

INTRODUCTION

Dairy cattle face a multitude of physiological, metabolic, nutritional, and environmental stressors that can lead to periods of low feed intake. Challenges that are commonly associated with low feed intake include parturition (Horst et al., 2021); disease (mastitis, metritis, pneumonia; Brown and Bradford, 2021); limited access to feed due to timing of feeding, overcrowding, or social stress (Proudfoot et al., 2018); heat stress (Baumgard and Rhoads, 2013); weaning (Pohl et al., 2017); and transportation (Hutcheson and Cole, 1986). Although these challenges may appear independent of one another, they share a common outcome of hypophagia, which may lead to hyperpermeability of the gastrointestinal tract (**GIT**; Aschenbach et al., 2019; Horst et al., 2021). Increasing

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evidence suggests that GIT barrier dysfunction and the ensuing immune activation are partly responsible for the loss of milk and milk component yields during off-feed events (Kvidera et al., 2017). However, ruminant GIT physiology is complex, and the regional diversity in GIT structure and function create obstacles for measurement of permeability responses occurring in the pre-gastric (reticulorumen and omasum), gastric, and post-gastric regions. It has been reported that, when measured ex vivo, the intestinal regions are more permeable than the pre-gastric regions (Penner et al., 2014) and that intestinal regions, based on morphology, may be compromised to a greater extent than the reticulorumen during periods of short-term feed restriction (Kvidera et al., 2017; Horst et al., 2020). Differentiating regional permeability responses becomes necessary when developing and evaluating targeted release for potential GIT modulating nutritional treatment strategies. However, in vivo methodologies to characterize regional GIT permeability responses in ruminants are limited.

Oral administration of indigestible and nonmetabolizable markers is a minimally invasive in vivo technique used to assess GIT permeability in animal and human research (Bjarnason et al., 1995; Zhang et al., 2013a; von Martels et al., 2019). A combination of synthetic sugars—for example, lactulose and D-mannitol—have been simultaneously dosed orally in humans (Bischoff et al., 2014), preruminant calves (Wilms et al., 2019; Mellors et al., 2023), and sheep (Minuti et al., 2013) to differentiate intestinal and colonic permeability, respectively. Although these synthetic sugars are not enzymatically degraded by the mammalian digestive system, they are susceptible to microbial fermentation in the rumen, distal regions of the small intestine, and large intestine (Bjarnason et al., 1995; Bischoff et al., 2014). Alternatively, both EDTA chelates of Co and Cr are considered inert salts that have been used for decades as liquid digesta markers in ruminants to estimate nutrient digestibility and passage rate (Udén et al., 1980), and more recently Cr-EDTA has been identified as a suitable total GIT permeability marker (Zhang et al., 2013a; Horst et al., 2020). Both Cr-EDTA and Co-EDTA are similar in molecular size and weight (Zubkowski et al., 1995; García-Lafuente et al., 2001) and therefore have the potential to perform as analogous permeability markers to characterize regional permeability in ruminants when delivered into different regions of the GIT.

Following oral or ruminal administration of Cr-EDTA, timely and repeated blood sampling to enable calculation of area under the curve (**AUC**; Horst et al., 2020), and total urine collection (Zhang et al., 2013a; Ellett et al., 2024) have been performed to determine marker translocation. Although total urine collection has been considered the "gold standard," as it allows for a measurement of marker recovery, risk for incomplete collection, discomfort from catheters, and risk for urinary tract infection pose challenges. Alternatively, measuring the concentration of markers in plasma or serum may be more convenient than total urinary collection and may enable more flexibility in housing and management conditions. However, the relationship between total urine collection and plasma AUC has not been determined. In addition, the timing of sample collection and duration of complete marker recovery using the least invasive approach has not been identified. Moreover, it is not clear whether timing of marker appearance in blood and urine differs between healthy cattle and those exposed to a challenge expected to induce hyperpermeability of the GIT, such as an off-feed event.

Therefore, the objectives of this study were as follows: (1) to evaluate the effects of short-term feed restriction in lactating dairy cows on regional permeability of the GIT and the recovery of DMI, ruminal pH, and milk yield; and (2) to validate the sampling collection method (total urine collection vs. timely blood samples) and the timing of sample collection for a dual marker technique that uses a bolus dose of Cr-EDTA infused into the reticulorumen and a concurrent bolus dose of Co-EDTA infused into the abomasum to characterize total GIT and post-ruminal permeability, respectively, in lactating dairy cows consuming ad libitum feed intake or 40% of ad libitum feed intake.

MATERIALS AND METHODS

All procedures used in this study were preapproved by the University of Saskatchewan Animal Research Ethics Board (protocol number 20230005), and cows were handled according to the guidelines of the Canadian Council of Animal Care (Ottawa, ON, Canada).

Animals and Experimental Design

Six ruminally cannulated lactating Holstein cows were used in this study and housed in tiestalls at the Rayner Dairy Research and Teaching Facility at the University of Saskatchewan (Saskatoon, SK, Canada). Cows were blocked into 2 groups by parity, including 3 primiparous cows (167 ± 4.7 DIM; 618 ± 34.1 kg) and 3 multiparous cows (212 ± 5.3 DIM; 690 ± 33.3 kg). The experimental design was a 2 × 2 crossover, where each experimental period (Figure 1) consisted of a 5-d baseline phase (**BASE**), a 5-d challenge phase (**CHAL**), and 2 consecutive weeks of recovery (**REC1** and **REC2**). The recovery phases served as a washout period between experimental periods. Cows within each parity were randomly assigned to a treatment sequence to either receive 100% ad libitum intake (**AL**) in period 1 and 40% of their ad

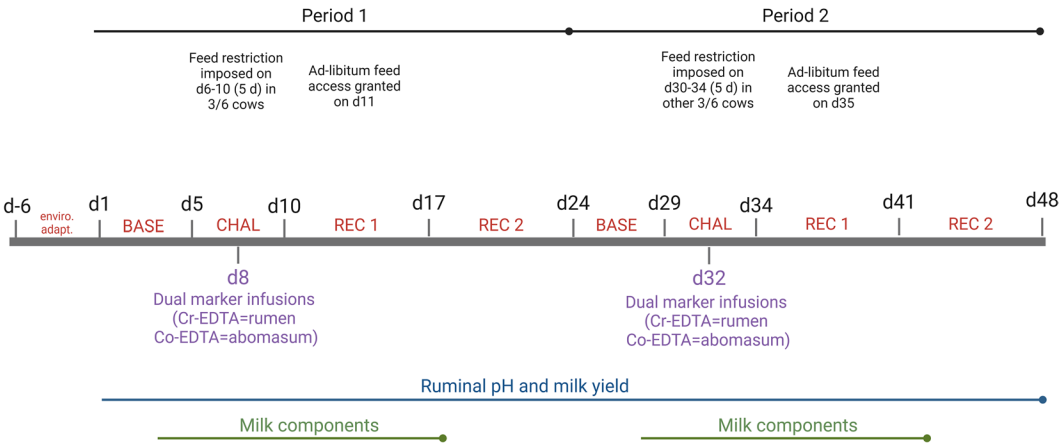

Figure 1. Illustration of the experimental timeline (created by BioRender.com). Enviro. adapt. = environmental adaptation period.

libitum feed intake (60% feed restriction; **FR**) in period 2 during CHAL, or vice versa, ensuring that treatment sequence was balanced within a period.

Diets were formulated to meet or exceed nutrient requirements for a 685-kg mid-lactation Holstein cow consuming 26 kg of DM per day and producing 37 kg of milk per day with 4.1% fat and 3.3% true protein using the Nutritional Dynamic System (NDS, Reggio Emilia, Italy; Table 1). Feed refusals were collected at 0800 h each day, and daily feed allowance was divided into 2 equal portions and fed at 0830 h and 1200 h. The weights of feed offered and refused were measured and recorded daily. The amount of feed provided during BASE was targeted to achieve 10% refusals, on an as fed basis, to ensure ad libitum feed intake (actual achieved across periods was 12.5% refusals as fed). Dry feed ingredients and fermented forages within the TMR were sampled weekly and twice weekly, respectively, throughout the study and dried for 96 h in a forced-air oven at 55°C for determination of DM. Ingredient DM coefficients were adjusted weekly to ensure diets were mixed accurately on an as-fed basis. During the 5-d BASE phase, daily TMR and refusals samples were collected and immediately dried, in duplicate, using a Koster tester (Koster Moisture Tester Inc., Medina, OH), and the average TMR and refusal DM for each cow were used to calculate the amount of DM to be offered for the cows on the FR treatment.

Daily feed ingredient samples were also collected during the BASE and CHAL phases and composited on an equal-weight basis. Thereafter, a subsample of each ingredient (500 g for dry ingredients and 1,000 g for fermented forages) was dried for 96 h in a forced-air oven at 55°C, with samples mixed daily, to determine DM before being ground using a hammer mill (Christie-Norris Laboratory Mill, Christie-Norris Ltd., Chelmsford, UK) to pass through a 2.5-mm screen. Ground samples were then sent to Cumberland Valley Analytical Services (Waynesboro, PA) for determination of nutrient composition (DM, OM, CP, aNDFom (NDF determined using a-amylase, sodium sulfite and ash-corrected), ADF, starch, ether extract, NE_L, Ca, Mg, P, Na, Cl, S, K). During the

Table 1. Ingredient and chemical composition (mean ± SD) of the TMR (% of DM) fed to mid-lactation Holstein dairy cows offered 100% ad libitum feed intake or 40% of ad libitum feed intake for 5d (CHAL) and 2 consecutive weeks of recovery (REC1 and REC2)

Item	Inclusion, % DM
Ingredient	
Barley silage	24.5
Rye silage	12.2
Straw	1.8
Concentrate mix[1]	51.6
Pellet mix[2]	9.9
Chemical analysis, % of DM	
DM	55.9 ± 0.74
CP	17.3 ± 0.16
ADF	18.9 ± 0.36
aNDFom[3]	32.0 ± 0.42
Starch	21.2 ± 0.14
Ether extract	5.4 ± 0.13
OM	92.1 ± 0.52
Ca	0.93 ± 0.063
P	0.46 ± 0.011
Mg	0.34 ± 0.016
K	1.44 ± 0.041
S	0.30 ± 0.003
Na	0.28 ± 0.023
Cl	0.65 ± 0.043
NE_L, Mcal/kg of DM	1.64 ± 0.002

[1]Consisted of 64.7% ground barley (59.0% starch); 14.1% canola meal (13% ether extract); 13.4% protein blend (28% soymeal meal solvent and 72% canola meal); 5.7% macro and micro mineral premix; 1.98% Energizer RP10 (IFFCO, Pasir Gudang, Malaysia).

[2]Consisted of 95.9% pelleted oat hay; 4.1% pelleted beet pulp.

[3]aNDFom = NDF determined using a-amylase, sodium sulfite and ash-corrected.

BASE and CHAL phases, individual cow refusal samples were composited proportionally to the daily amount refused, and a 1,000 g subsample from each phase was collected and dried for determination of DM. However, during the CHAL phase, the cows on FR did not have any refusals.

Cows were milked twice daily at 0630 h and 1800 h in a double-6 DeLaval HDHB herringbone parlor (DeLaval, Tumba, Botkyrka, Sweden). Milk yield and milk samples were recorded and collected during BASE, CHAL, and REC1, to be used for statistical analysis. Milk samples within a day were composited proportionally based on milk yield to create one 40-mL daily milk sample, which was stored at 4°C and preserved with potassium dichromate (Kroger, 1985). Milk samples were then analyzed for determination of fat, true protein, lactose, SCC, and MUN concentrations at the Central Milk Testing Laboratory (Edmonton, AB, Canada). Body weight was measured using an AWS100 DeLaval weigh scale (DeLaval, Tumba, Botkyrka, Sweden) on 2 consecutive days at the start of BASE, d 3 of CHAL (for dosing of permeability markers, described below), and the first day of REC1.

Ruminal pH

Ruminal pH was measured continuously throughout the experiment using indwelling pH measurement systems (Penner et al., 2006). Ruminal pH systems were standardized before placement in the ventral sac of the rumen through the ruminal cannula at 1900 h. For standardization, the millivolt (mV) readings when the pH systems were placed in pH 4 and 7 buffer solutions (Fisher Chemical, Fair Lawn, NJ) were recorded. The location of the pH system (ventral sac) was maintained using two 900-g weights attached to the bottom of the electrode shroud (Penner et al., 2006). Ruminal pH was recorded every 5 min, and every 5 d the pH systems were removed from the rumen, the data were downloaded, and the pH systems were re-standardized and immediately re-inserted into the rumen. Re-standardization occurred on the last day of BASE, CHAL, REC1, and REC2 and data from these days were not used for statistical analysis, to ensure all days had 24 h of complete measurements. The minimum, mean, and maximum pH values along with duration (min/d) and area (pH × min/d) that pH remained below 5.8 were calculated as described by Penner et al. (2007), and were summarized by day for each cow (0700 to 0659 h the following day).

Total and Post-Ruminal Gastrointestinal Tract Barrier Function

The approach to measure total GIT and post-ruminal permeability was based on that described by Zhang et al. (2013a), with a modification that an equimolar abomasal infusion of Co-EDTA was added to enable measurement of post-ruminal permeability. The goal was to deliver the Co-EDTA into the abomasum indicating post-omasal permeability; however, it is possible for some of the Co-EDTA to backflow into the omasum, rendering "post-ruminal permeability" a more appropriate term.

Chromium-EDTA Preparation. Cr-EDTA was prepared according to Binnerts et al. (1968) to achieve a target concentration of 180 m*M*. Briefly, 66.7 g of disodium EDTA dihydrate (Na_2-EDTA·2 H_20; cat. no. E4884; Sigma-Aldrich, St. Louis, MO) was added to a 4-L beaker with 0.5 L of double-distilled water (**ddH_2O**). Heat was applied, and a magnetic stir bar mixed the solution until disodium EDTA dihydrate was completely dissolved. Then 66.7 g of chromium (III) trichloride hexahydrate ($CrCl_3·6H_2O$; cat. no. 27096; Sigma-Aldrich, St. Louis, MO) was added to the first solution. The beaker was covered with a watch glass to avoid evaporation, and the temperature of the solution was increased until a gentle boil was achieved. The solution was boiled for 1 h. Thereafter, the heat was turned off and the mixture was stirred gently for approximately 12 h. The following day, once the mixture had reached room temperature (20°C), 1.937 g of calcium dichloride dihydrate ($CaCl_2·2H_2O$; cat. no. 223506; Sigma-Aldrich, St. Louis, MO) was added to precipitate unbound Cr. The pH was then adjusted to 6.0 using dry sodium hydroxide pellets (NaOH; cat. no. 567530, Sigma-Aldrich, St. Louis, MO). After the desired pH was achieved, the solution was brought to a final volume of 1 L. This process was repeated until the amount required for all cows was achieved, and then all batches were mixed in a 50-L plastic carboy. A 25-mL subsample was collected and analyzed for Cr concentration using inductively coupled plasma-mass spectroscopy (**ICP-MS**) before infusion.

The Cr-EDTA stock solution subsamples (0.2 mL) were prepared for ICP-MS by adding 8 mL of ddH_20 and 1.8 mL of 0.5% HNO_3, followed by mixing. Thereafter the samples were analyzed in duplicates using an iCAP RQ plus ICP-MS (Thermo-Fisher Scientific, Bremen, Germany) using Ga, Y, and In as internal standards. The Cr was identified using the molecular mass (51.996 g/mol), and samples were run in the kinetic energy discrimination (KED) mode on the ICP-MS using an electron multiplier (EM) detector. All reported concentrations were corrected for the dilution performed in the preparation of samples before ICP-MS. The limit of quantification and limit of detection for Cr were 0.09 and 0.009 µg/kg, respectively.

Cobalt-EDTA Preparation. Purified Co-EDTA crystals were prepared as described by Udén et al. (1980) and mixed with ddH_2O to reach a target concentration of 360 m*M*. In a 4-L beaker, 300 g of Co (II) acetate tetrahydrate

($C_4H_6CoO_4 \cdot 4H_2O$; cat. no. 208396; Sigma-Aldrich, St. Louis, MO) and 350 g of EDTA (cat. no. EDS; Sigma-Aldrich, St. Louis, MO) were added to 1 L of ddH_2O while mixing using a magnetic stir bar at a temperature of 100°C. Once the mixture was dissolved, 48 g of NaOH pellets (Sigma-Aldrich, St. Louis, MO) and an additional 1 L of ddH_2O were added. The liquid level was recorded on the beaker, and the beaker was covered with a watch glass. The mixture was allowed to stir while heated for 4 to 5 h. Thereafter, the heat was turned off, and ddH_2O was added to achieve the original liquid volume. Then, the mixture was allowed to stir for another 12 h without heat. The following day, the beaker was placed on ice with continued stirring, and 240 mL of 30% hydrogen peroxide (H_2O_2; cat. no. H1009; Sigma-Aldrich, St. Louis, MO) was slowly added. This reaction activated the transformation of Co (II)-EDTA to Co (III)-EDTA, which greatly increases the stability of the complex between Co and EDTA (Van Soest and Hall, 2020). This transformation was confirmed by a color change from pink to dark purple. After the addition of H_2O_2, the solution was stirred for another 5 to 6 h. Subsequently, the solution was divided into 2 equal parts, no longer stirring, 1.8 L of ethanol (95%–100% purity) was added to both beakers, and the resulting solution was left at room temperature for 2 h. The arising Co-EDTA crystals were allowed to precipitate out of solution at 4°C for 12 h. Thereafter, the Co-EDTA crystals were slowly filtered using a Buchner funnel and No. 1 Whatman filter paper (Sigma-Aldrich, St. Louis, MO) attached to a vacuum and rinsed with ethanol. The filtered crystals were oven-dried at 60°C for 12 h. To prepare Co-EDTA (approximate molecular weight = 370 g/mol) solution for infusions, 66.6 g of Co-EDTA crystals were dissolved in 0.5 L of ddH_2O to target a concentration of 360 m*M*. Batches of 0.5 L were made and then combined until enough volume was made for all cows. A subsample (25 mL) of Co-EDTA was collected and analyzed for Co (58.933 g/mol) concentration by ICP-MS, as described above for Cr-EDTA before infusion. All reported concentrations were corrected for the dilution performed in the preparation of samples for ICP-MS. The limit of quantification and limit of detection for Co by ICP-MS were 0.001 and 0.0008 µg/kg, respectively.

The ICP-MS technique detects the concentrations of Co and Cr ions when not bound to EDTA. A potential analytical measure to determine presence of the chelated salts is through spectrophotometry; however, Cr- and Co-EDTA have similar peak wavelengths of 541 and 535 nm, respectively (Van Soest and Hall, 2020). The authors performed preliminary tests (unpublished) combining Cr- and Co-EDTA in urine and were unable to differentiate the 2 markers, when used in combination, via spectrophotometry. In addition, it was observed that other components within urine may interfere with the ability to detect the markers using spectrophotometry. Therefore, ICP-MS was deemed the most appropriate analytical approach for measuring the concentrations of Cr and Co in urine or plasma following the dual permeability marker technique, and future research is needed to evaluate strategies to confirm they are bound to EDTA.

Dual Marker Infusions. The actual Co and Cr concentrations obtained from ICP-MS were then used to ensure that equimolar concentrations of Cr-EDTA and Co-EDTA were delivered into the rumen and abomasum, respectively, at a dose of 0.369 mmol/kg BW for each marker for each cow. Cows were fitted with jugular and urinary catheters on d 2 of CHAL allowing cows 24 h to acclimatize before infusions. For urinary catheterization, the vulva was surgically scrubbed 3 times with 10% povidone-iodine solution (Betadine, Purdue Pharmaceuticals, Stamford, CT) using a BD B-Z scrub brush 4% cholorhexidine gluconate (BD, West Mississauga, ON, Canada), and rinsed with 70% alcohol. Thereafter, 10 mL of sterile lubricating jelly and 3 mL of topical lidocaine (2%) were placed on the Foley urine catheter (24 Fr 5 mL Bardex Lubricath Catheter, C. R. Bard Inc., Covington, GA), which was aseptically inserted into the bladder and inflated with 80 mL of sterile saline solution. Preparation of the jugular vein area for catheterization began with a surgical clip using 10-mm clipper blades and cleaning procedures similar to those described for urinary catheters above. A 12-gauge stainless steel hypodermic needle (Zalkovic et al., 2001) was used to puncture the skin and jugular vein, and 11 cm of medical-grade Tygon tubing (i.d. 1.02 mm, o.d. 1.78 mm; Cole-Parmer, Quebec City, QC, Canada) was passed through the needle with approximately 30 cm of tubing remaining external to the cow. The needle was then removed, and the tubing was secured in place using a butterfly suture and skin bond glue, and further protected by wrapping the neck with an adhesive bandage (Tensoplast, Oakville, ON, Canada) and 10.2-cm Vetrap (3M, St. Paul, MN). Jugular catheters were flushed twice daily with heparinized saline (10 IU heparin/1 mL saline) and after each blood collection (2 IU heparin/1 mL saline). The Cr-EDTA and Co-EDTA infusions for all cows occurred immediately after milking on d 3 of CHAL within each period (0630 h). Infusions were timed to occur 2 h before feed was offered (Horst et al., 2020). First, Co-EDTA was administered into the abomasum by locating the omasal orifice via the ruminal cannula without removing any digesta. A reinforced vinyl tube (o.d. 20 mm; Global Industries, Scarborough, ON, Canada) was maneuvered such that approximately 20 cm passed through the omasum to enter the abomasum. The tube contained 3-mm holes, spaced

20 mm apart, at the last 5 cm of the tube, to increase the ease of marker flow during infusions. The tube was attached to the end of a 400-mL container, and the Co-EDTA was carefully poured into the container. Following Co-EDTA infusion, 150 mL of water was used to flush residual Co-EDTA through the tube. The Cr-EDTA was delivered into the ruminal-fluid ruminal-mat interface using another, similar infusion device, followed by 150 mL of water. The exact time of Co-EDTA and Cr-EDTA infusion was recorded, and the midpoint between marker infusions was used as the sample collection time point for modeling the blood and urine marker concentrations. The time delay between Co-EDTA and Cr-EDTA infusions within a cow ranged from 30 to 60 s. The order of infusion was always consistent, starting with Co-EDTA and followed by Cr-EDTA. Time-zero samples for urine, feces, and blood were collected 15 min before marker infusions.

Sample Collection Following Infusions. Immediately after infusions, the urine drainage port of the bladder catheters was affixed to one end of Tygon tubing (i.d. 15 mm, o.d. 20 mm) using a tubing adaptor, and the other end of the tubing was connected to 25-L carboys to allow for total urine collection. The tubing was raised off the floor, to prevent cattle from stepping on the tubing, by passing the tube through an elastic rope that was suspended from the ceiling. In addition, all bedding was removed from the tiestalls, and individual fecal trays were placed behind each cow to allow for total fecal collection. Total urine and feces excreted were weighed and recorded every 8 h for 96 h. A 96-h collection duration was used because Zhang et al. (2013a) determined that, following a ruminal infusion of Cr-EDTA, more than 96% of the Cr-EDTA excreted in urine appeared within 48 h. Representative subsamples of feces (200 g, as-is basis) and urine (30 mL) were collected at each time point and stored at −20°C until processing. Fecal samples were thawed and dried in a forced-air oven at 55°C for 120 h for determination of DM. Dried samples were then ground to pass through a 1.0-mm screen using a Retsch ZM100 ultra centrifugal mill (Haan, Nordrhein-Wastfalen, Germany).

Blood samples (10 mL) were collected from the jugular catheter at 0, 1, 2, 3, 4, 6, 8, 12, 16, 20, 24, 32, 40, 48, and 64 h relative to the Cr-EDTA and Co-EDTA infusions, into tubes containing Na-heparin (BD, Franklin Lakes, NJ). Thereafter, blood samples were centrifuged at 2,500 × *g* for 15 min at 4°C, and plasma was harvested. Plasma samples were stored at −20°C. Milk samples representative of an entire milking were collected the morning before infusions and for 5 consecutive milkings (60 h) following Cr-EDTA and Co-EDTA infusions. Samples were stored at −20°C.

Plasma and urine samples were prepared for Cr and Co analysis as mentioned previously for the Cr-EDTA and Co-EDTA stock solution subsamples. However, dried fecal and milk samples required microwave digestion before ICP-MS. For milk, 1 mL of milk was combined with 2.25 mL of HNO_3 (70%), 0.5 mL HCl (0.5%), and 2.25 mL of ddH_2O. For feces, 0.3 g of dried feces was combined with 2.25 mL of HNO_3 (70%), 1 mL H_2O_2, 0.5 mL HCl (0.5%), and 2.25 mL of ddH_2O. Both fecal and milk samples were then digested in a Mars6 Microwave digestor (CEM Corporation, Matthews, NC) with increasing temperature to 200°C, which was held for 15 min, and then cooled over 40 min until the samples reached a temperature below 30°C. Digested samples were then diluted with 45 mL of ddH_2O and analyzed using an iCAP RQ plus ICP-MS (Thermo-Fisher Scientific, Bremen, Germany) as described above for the Cr-EDTA and Co-EDTA stock solutions. The final concentrations were corrected for the dilutions preformed.

Statistical Analysis and Calculations

Statistical power calculation was based on the ability to detected differences in urinary Cr and Co recovery of 100 mg/48 h when the standard deviation was 10% of the mean. This required 6 cows to exceed 80% power (actual nominal power was 85.7%) and to detect a difference ($\alpha = 0.05$).

All data were analyzed using SAS v9.4 (SAS Institute Inc., Cary, NC), with all analyses of variance conducted using the GLIMMIX procedure. The Kenward-Roger correction was used to computed adjusted denominator degrees of freedom for tests of fixed effects, and a Newton-Raphson optimization algorithm with ridging was requested using the NLOPTIONS statement, with an additional 10 iterations allowed to facilitate model convergence for some outcomes. The Tukey-Kramer adjustment was used for all multiple means comparisons. The treatment × period interaction was tested for all variables and models, but was not significant ($P > 0.17$). Studentized residuals were evaluated for every outcome to confirm that an appropriate distribution was used for each outcome. When a potentially influential interaction was detected, the simple effects of the interaction term were decomposed using the SLICE and SLICEDIFF options of the LSMEANS statement. Significance was declared at $P \leq 0.050$; 3 decimal places were shown when $P < 0.100$ and 2 decimal places when $P \geq$ to 0.10. The urine and fecal data from 1 cow during period 2 were removed from statistical analysis because of urinary straining, resulting in urine loss and urine contamination of feces.

Within CHAL. Outcomes measured at a single time point during CHAL were evaluated considering the fixed

effect of treatment and the random effects of cow and period. A compound symmetry covariance structure was used to accommodate negative covariance between periods. Those measured at multiple time points within CHAL additionally included the effects of time and the treatment × time interaction, with correlated errors from measurements on cows within periods modeled using a first-order autoregressive covariance structure.

Across Phases. Daily milk production outcomes were averaged by cow within phase before analysis. For all outcomes evaluated across phases (e.g., milk production, feed intake, rumen pH), the effects of treatment, phase (CHAL, REC1, or REC2), and their interaction were considered fixed, whereas the effects of cow and period were considered random. For each outcome, the mean value measured during BASE was included as a covariate. Correlated errors due to repeated measures on cows within periods were modeled using a first-order autoregressive covariance structure. For the majority of outcomes, it was determined that all variance due to both cow and period were captured by the repeated measures term, and as such these terms were removed from the model; however, a separate term for cow within period was retained in the model specifically for rumen pH outcomes. Data pertaining to SCC were determined to follow a lognormal distribution, and as such least squares means, standard errors, and confidence intervals were back-transformed before reporting. All other outcomes followed a normal distribution.

Within Recovery. Daily outcomes over time across the REC1 and REC2 phases were evaluated considering the effects of treatment, time, and their interaction as fixed, and the effects of cow and period as random. Correlated errors due to repeated measures on cows within periods were modeled using a first-order autoregressive covariance structure. It was determined that all variance due to both cow and period were captured by the repeated measures term, and as such these terms were removed from the model, with the exception of outcomes pertaining to rumen pH, for which a random effect of cow within period was included.

Plasma Marker Validation. An initial ANOVA was conducted using the GLIMMIX procedure to evaluate changes in plasma marker concentrations. The effects of treatment, time, and their interaction were considered fixed, with the effects of cow and period considered random. Correlated errors due to repeated sampling on cows within periods were modeled using a spatial power covariance structure. During analysis, it was observed that all variance due to cow and period were partitioned into the repeated measures component of the model, and as such these terms were removed from the model. Plasma marker AUC was calculated for each cow-treatment-marker combination using the trapezoidal rule as described by Shiang (2004). The relationship between plasma marker AUC and total urinary marker excretion was evaluated using the CORR procedure.

Before subsequent analyses, it was decided that a reduced sampling protocol should (1) use no more than 5 sampling time points, and (2) be capable of estimating AUC within 10% of the calculated baseline value. The preliminary ANOVA indicated that plasma concentrations of both markers in samples collected at 0 and 64 h did not differ from zero ($P \geq 0.93$); both of these time points were excluded from the selection process, resulting in a pool of 13 candidate samples. Using the PLAN procedure, every combination of 5 time points was generated, resulting in 1,287 sets of unique combinations of sampling times. Plasma marker AUC were recalculated as previously described within each set of time points, and the root mean square prediction error (**RMSPE**) was calculated as

$$\text{RMSPE} = \sqrt{\frac{\sum_{i=1}^{n}\left(AUC_{0i} - AUC_i\right)^2}{n}},$$

where AUC_{0i} is the reference AUC for the ith cow, AUC_i is the AUC calculated based on the given set of 5 time points for the ith cow, and n is the number of observations. For each marker, the geometric mean RMSPE was calculated across treatment groups within each set of time points to determine the optimal sampling times for that marker. It was observed that different sets of time points provided the best fit for each marker. However, in practice, it would be ideal to characterize the 2 markers from the same samples to meet the predetermined criteria of a maximum of 5 samples taken. As such, the geometric mean RMSPE was calculated across treatments and markers for each set of time points to estimate the average predictive ability, with the optimal set of time points having the lowest mean RMSPE.

The optimal set of time points for AUC estimation was defined as the set of time points that minimized the RMSPE. It was observed that different sets of time points yielded the optimal fit for different markers. To reconcile the fact that ideally both markers would be quantified in the same set of samples for both treatments, the geometric mean RMSPE was calculated across markers and treatments to identify the set of time points that yielded the best overall fit. After identification of the optimal set of refined sampling times, the refined and baseline AUC values were compared by ANOVA, considering the effects of treatment, set (baseline or refined), and the interaction between the 2 as fixed effects. The correlation between AUC values for each cow within period was

modeled using a compound symmetry covariance structure. The prediction error associated with each reduced AUC model was calculated as

$$\frac{\text{reduced AUC} - \text{full AUC}}{\text{full AUC}} \times 100,$$

and the SEM was calculated as

$$\frac{\text{SED between reduced and full AUC}}{\text{full AUC}} \times 100.$$

RESULTS

Dry Matter Intake and Ruminal Fermentation

After determination of TMR and refusal DM values, cows exposed to FR in periods 1 and 2 were offered 39.8% and 40.0% of ad libitum feed intake, respectively (data not shown). A treatment × phase interaction occurred, such that after returning to voluntary feed intake, cows exposed to FR continued to have lower DMI in REC1, but by REC2 no difference was detectable between FR and AL (Table 2) treatments. Dry matter intake for FR and AL cows was not different on d 1 of recovery but was lower for FR from d 2 to d 4 (Supplemental Figure S1, panel A; see Notes).

A treatment × phase interaction was detected for all ruminal pH characteristics (Table 2). During CHAL, daily minimum, mean, and maximum ruminal pH were greater and the duration that pH remained below 5.8 was the least for FR (Table 2). After returning to voluntary feed intake, FR had lesser minimum and maximum ruminal pH during REC1 and REC2 relative to FR in CHAL but were not different from AL. Additionally, during REC1, the duration that ruminal pH was <5.8 was the greatest and daily mean pH was the least for FR. However, by REC2, daily mean ruminal pH and the duration that pH was <5.8 no longer differed between FR and AL (Table 2). On d 1 and d 3 after returning to voluntary feed intake, mean ruminal pH was lower and the duration of pH <5.8 was greater for FR compared with AL (Supplemental Figure S2; see Notes). Additionally, the minimum ruminal pH for FR was lower on d 1 of recovery relative to AL and maximum ruminal pH was greater on d 1 and then lower from d 2 to d 5 when compared with AL (Supplemental Figure S2).

Milk Yield and Milk Components

Milk fat percentage was greatest for FR during CHAL, which was different from FR in REC1 and different from AL during CHAL and REC1 (Table 3). The main effect of treatment was detected for milk protein and lactose percentages, where FR reduced milk protein and lactose by 0.19 and 0.18 percentage units, respectively. A treatment × phase interaction was observed for milk yield, such that milk yield was less for FR than AL during CHAL and REC1, but values did not differ between treatments during REC2. The FR cows secreted 373 g less milk fat, 364 g less true protein, and 438 g less lactose. Milk urea nitrogen was the least for FR in REC1 compared with all other treatments. Milk SCC was unaffected by treatment, phase, or the interaction.

Total and Post-Ruminal Gastrointestinal Tract Permeability

As expected, exposure to FR reduced BW by 60 kg relative to AL cows (Table 4). Average daily urine output calculated during CHAL was not affected by treatment. On d 3 of CHAL, urinary recovery of Co was increased by 36% for FR relative to AL, and Cr recovery was numerically greater (16%) for FR ($P = 0.28$; Table 4). When Co was expressed as a percentage of Cr, no treatment differences were detected ($P = 0.38$). Plasma Cr AUC increased (34.4%; Figure 2A) and Co AUC tended to increase (35.3%; $P = 0.060$; Figure 2B) for FR. Supple-

Table 2. Effects of treatment (TRT, ad libitum feed intake [AL] or feed restriction [FR]) on DMI and ruminal pH characteristics of mid-lactation Holstein cows (n = 6 per treatment) during 5 d of treatment exposure (CHAL) and 2 consecutive weeks of recovery after returning to voluntary feed intake (REC1 and REC2)

	CHAL		REC1		REC2			*P*-value		
Variable	AL	FR	AL	FR	AL	FR	SEM	TRT	Phase	TRT × phase
DMI, kg/d	24.9^{a}	10.3^{c}	26.0^{a}	21.1^{b}	26.3^{a}	24.8^{a}	0.60	<0.001	<0.001	<0.001
Minimum pH	5.43^{b}	5.73^{a}	5.48^{ab}	5.30^{b}	5.44^{ab}	5.41^{b}	0.066	0.66	0.019	0.005
Mean pH	6.06^{b}	6.57^{a}	6.13^{b}	5.88^{c}	6.09^{b}	6.08^{b}	0.041	0.093	<0.001	<0.001
Maximum pH	6.68^{b}	7.19^{a}	6.69^{b}	6.61^{b}	6.71^{b}	6.72^{b}	0.036	0.003	<0.001	<0.001
Duration pH <5.8, min/d	353.2^{ab}	77.6^{c}	250.9^{bc}	590.2^{a}	322.7^{bc}	347.3^{ab}	59.76	0.63	0.008	<0.001
Area, pH × min/d	110.5^{ab}	25.2^{b}	55.3^{b}	164.5^{a}	95.9^{ab}	90.1^{ab}	24.32	0.77	0.24	0.009

$^{a–c}$Within a row, treatment means with different superscripts differ ($P \leq 0.05$).

Table 3. Effects of treatment (TRT, ad libitum feed intake [AL] or feed restriction [FR]) on milk composition, milk yield, and milk component yield for mid-lactation Holstein cows (n = 6 per treatment) during 5 d of treatment exposure (CHAL) and during the recovery after returning to voluntary feed intake (REC1 and REC2)

	CHAL		REC1		REC2			*P*-value		
Variable	AL	FR	AL	FR	AL	FR	SEM	TRT	Phase	TRT × phase
Composition, %										
Fat	4.62[b]	5.44[a]	4.52[b]	4.50[b]	—	—	0.210	0.092	0.013	0.035
True protein	3.39	3.21	3.44	3.26	—	—	0.055	0.013	0.25	0.97
Lactose	4.51	4.33	4.48	4.30	—	—	0.073	0.049	0.63	0.99
Yield, kg/d										
Milk	31.4[ab]	20.7[c]	32.0[a]	24.3[bc]	33.5[a]	31.9[a]	1.83	0.004	0.001	0.017
Fat	1.44	1.11	1.43	1.01	—	—	0.089	0.007	0.27	0.32
True protein	1.07	0.67	1.11	0.79	—	—	0.085	0.007	0.081	0.29
Lactose	1.42	0.90	1.43	1.07	—	—	0.108	0.007	0.16	0.24
MUN, mg/dL	16.3[a]	16.5[a]	15.3[a]	13.0[b]	—	—	0.59	0.16	<0.001	0.004
SCC, × 1,000 cells/mL	43	66	54	105	—	—	39.0	0.21	0.24	0.68

[a–c]Within a row, treatment means with different superscripts differ ($P \leq 0.05$).

mental Table S1 (see Notes) provides equations to predict urinary Cr and Co excretion using plasma AUC for lactating cows exposed to AL and FR treatments.

Validation of the Dual Marker Technique

More than 97% and 98% of the Cr-EDTA and Co-EDTA, respectively, that was excreted in urine at 96 h was recovered by 48 h, irrespective of treatment. Therefore, cumulative urinary marker excretion over 48 h and the corresponding plasma obtained 48 h after the marker infusions were used as the cutoff points. In addition, plasma Cr and Co concentrations were not different from zero by 48 h ($P > 0.90$; data not shown), indicating that marker concentrations returned to pre-infusion levels.

The Cr and Co plasma AUC calculated with the full model (15 time points) showed a strong positive correlation to 48-h urinary Cr and Co excretion (Table 5). Reducing the number of blood samples to a maximum of 5 time points using brute force estimations indicated that sampling at h 2, 8, 20, 40, and 48 were optimal when using the dual marker technique. This permutation of time points yielded the lowest RMSPE of 583 µg/L for both markers. Table 6 depicts the optimal permutation of blood sampling time points when the number of samples ranged from 1 to 13 and the associated prediction error for each marker-treatment combination, and Supplemental Table S2 (see Notes) shows the associated RMSPE for each permutation. For Cr, the reduced AUC model was not different from the full AUC model beyond 1 time point, and for Co, the reduced AUC model was not different from the full AUC model beyond 2 time points (Table 6). The final reduced model of 5 time points selected was not different from the full AUC for either marker. When the Co AUC was compared between the full and reduced models, the reduced model was able to predict AUC within 6.2% for FR and 5.0% for AL, and the Cr AUC was predicted within 1.9% for FR and 0.1% for AL (Table 6). Similar to the full AUC model, the reduced AUC predictions showed a strong positive correlation with 48-h urine excretion for both Cr and Co (Table 5). As previously reported, the plasma Cr AUC was greater for FR compared with AL on d 3 of CHAL for the full model and the reduced model (Figure 2A). Plasma Co

Table 4. Effects of treatment (ad libitum feed intake [AL] or feed restriction [FR]) on BW, daily urine output, and 48-h urinary excretion of chromium (Cr) and cobalt (Co) as indicators of total gastrointestinal tract and post-ruminal permeability, respectively, in mid-lactation Holstein cows (n = 6 per treatment) on d 3 of the challenge phase

	Treatment			
Item	AL	FR	SEM	*P*-value
BW,[1] kg	658	598	19.7	<0.001
Urine output, kg/d	26.6	21.3	4.49	0.37
48-h urinary excretion, % infused				
Chromium	2.23	2.59	0.186	0.28
Cobalt	1.30	1.77	0.113	0.044
Cobalt as % of chromium	60.6	69.1	6.11	0.38

[1]BW measurement taken on the first day of the first week of recovery (REC1) before morning feed delivery.

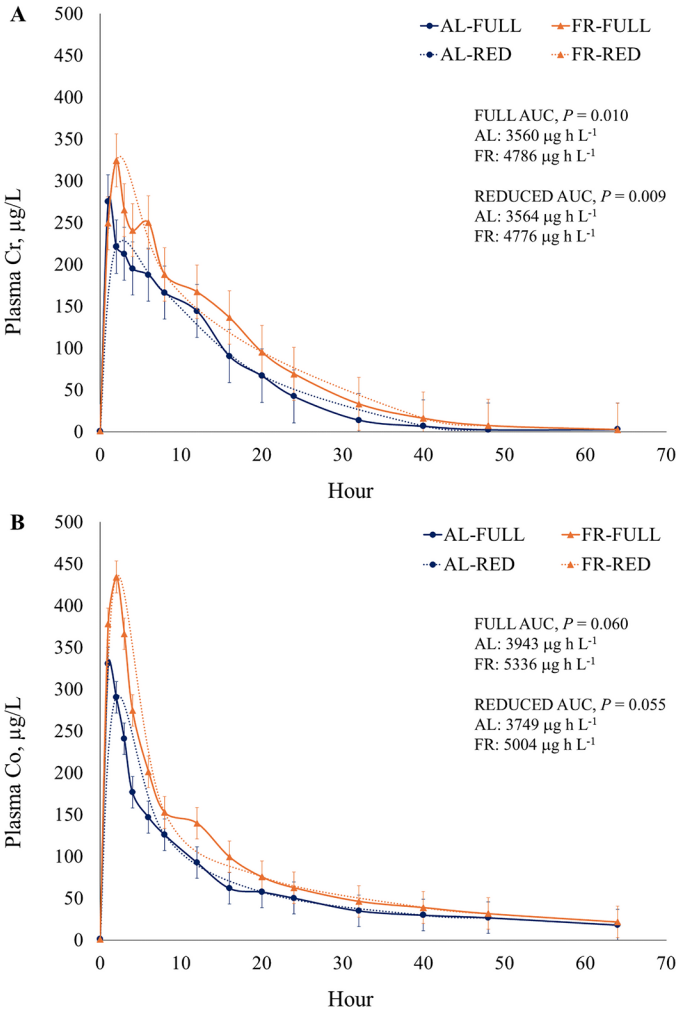

Figure 2. Effects of treatment (ad libitum [AL] or feed restriction [FR]) on plasma chromium concentrations (panel A) and plasma cobalt concentrations (panel B) and the comparison between the full AUC model (FULL; 15 blood sampling time points) and the reduced AUC model (RED; 5 blood sampling time points) following a ruminal dose of Cr-EDTA and an abomasal dose of Co-EDTA on d 3 of feed restriction (CHAL phase) in mid-lactation Holstein dairy cows (n = 6 per treatment). Hour 0 was the time point collected 15 min before the abomasal bolus dose of Co-EDTA and the ruminal bolus dose of Cr-EDTA. The results are presented as LSM ± SEM.

Table 5. Pearson correlation coefficients (r) to illustrate the relationship between 48-h urinary chromium (Cr) and cobalt (Co) excretion (expressed as % of Cr-EDTA and Co-EDTA infused into the rumen and abomasum, respectively) and the area under the curve (AUC) calculated from plotting plasma Cr and Co concentrations for the full AUC model (15 blood sampling time points) and reduced AUC model (5 blood sampling time points)

	48-h urine Cr		48-h urine Co	
Plasma AUC model	r	*P*-value	r	*P*-value
Full	0.76	0.005	0.87	<0.001
Reduced	0.75	0.005	0.92	<0.001

AUC tended to be greater for FR on d 3 of CHAL for the full model (35.3%, $P = 0.060$) and the reduced model (33.5%; $P = 0.055$; Figure 2B).

Supplemental Tables S3 and S4 (see Notes) present the optimal permutations of blood sampling time points independently for Cr and Co, respectively, when the number of samples included ranged from 1 to 13, along with the associated prediction errors and RMSPE for each reduced model.

Recovery of Cr and Co in Urine, Feces, and Milk

A treatment × time interaction was detectable for urinary Cr concentration, such that FR had greater concentrations than AL from h 24 to h 96 (Figure 3A). However, no treatment or treatment × time interaction occurred for cumulative urinary Cr recovery (Figure 3B). Fecal Cr concentrations were greater for AL at h 8, whereas from h 32 to h 96 fecal Cr concentrations were greater for FR (Figure 3C). Total fecal Cr recovery was greater for AL than FR starting from h 16 to h 96 (Figure 3D). Total recovery of Cr excreted in urine and feces after 96 h expressed as a percentage of Cr-EDTA infused was reduced 6.3 percentage units for FR (Table 7). Milk Cr concentrations were not affected by treatment or the treatment × time interaction (Figure 3E); however, milk Cr increased with time. No interaction effect (Figure 3F) or main effect of treatment on milk Cr recovery occurred; however, milk Cr recovery increased with time.

Urinary Co concentrations were greater for FR at h 24, 32, 40, 48, 64, 72, 88, and 96 (Figure 4A). Urinary Co recovery was not affected by the interaction of treatment and time (Figure 4B); however, main effects of treatment and time occurred, such that urinary Co recovery was greater for FR than AL, and Co recovery increased with time. Fecal Co concentrations were greater for AL than FR at h 8, but at h 24, 32, 40, 56, 64, 80, and 96 fecal Co concentrations were greater for FR than AL (Figure 4C). Cumulative fecal Co recovery was not affected by the interaction or the main effect of treatment (Figure 4D) but increased with time. Total recovery of Co excreted in urine and feces after 96 h of total collections was not affected by treatment (Table 7). Milk Co concentrations and recovery were not affected by the treatment × time interaction or the main effect of treatment (Figure 4E and Figure 4F); however, both increased with time.

DISCUSSION

Effects of Feed Restriction on Production, Milk and Milk Component Yields, and GIT Permeability

The apparent implications of short-term feed restriction on performance of lactating dairy cows include a

Table 6. Optimal permutations of sampling time points from 1 to 13 included time points for each marker–treatment combination and the associated prediction errors, following a ruminal infusion of Cr-EDTA and an abomasal infusion of Co-EDTA in mid-lactation Holstein dairy cows (n = 6 per treatment) offered 100% ad libitum feed intake (AL) or 40% of ad libitum feed intake (FR)

		Prediction error[1] (%)											
		Cr						Co					
No. of time points included (set)	Selected time points[2] (h)	AL	SEM[3]	*P*-value[4]	FR	SEM[3]	*P*-value[4]	AL	SEM[3]	*P*-value[4]	FR	SEM[3]	*P*-value[4]
1	12	−75.5	4.20	<0.001	−78.9	3.12	<0.001	−85.7	4.51	<0.001	−84.1	3.33	<0.001
2	8, 48	13.7	4.20	0.077	−2.4	3.12	1.00	−9.5	4.51	0.69	−19.2	3.33	<0.001
3	3, 20, 48	3.3	4.20	1.00	2.5	3.12	1.00	3.6	4.51	1.00	9.1	3.33	0.27
4	3, 12, 24, 48	0.8	4.20	1.00	−2.0	3.12	1.00	−7.4	4.51	0.93	−2.8	3.33	1.00
5	2, 8, 20, 40, 48	0.1	4.20	1.00	−0.2	3.12	1.00	−4.9	4.51	1.00	−6.2	3.33	0.84
6	2, 8, 12, 24, 40, 48	0.0	4.20	1.00	−0.3	3.12	1.00	−5.9	4.51	0.99	−4.4	3.33	0.99
7	2, 3, 12, 20, 24, 40, 48	−0.5	4.20	1.00	−1.2	3.12	1.00	−4.9	4.51	1.00	0.4	3.33	1.00
8	1, 2, 8, 12, 16, 20, 32, 48	0.5	4.20	1.00	1.0	3.12	1.00	−4.5	4.51	1.00	−3.7	3.33	1.00
9	1, 2, 6, 8, 12, 16, 20, 32, 48	0.8	4.20	1.00	2.1	3.12	1.00	−7.0	4.51	0.95	−6.3	3.33	0.83
10	1, 2, 3, 6, 8, 12, 16, 20, 32, 48	0.8	4.20	1.00	0.3	3.12	1.00	−7.7	4.51	0.91	−6.7	3.33	0.77
11	1, 2, 3, 6, 8, 12, 16, 20, 24, 32, 48	−0.5	4.20	1.00	−0.3	3.12	1.00	−7.7	4.51	0.91	−7.0	3.33	0.69
12	1, 2, 3, 4, 6, 12, 16, 20, 24, 32, 40, 48	−0.6	4.20	1.00	0.5	3.12	1.00	−8.9	4.51	0.78	−6.5	3.33	0.80
13	1, 2, 3, 4, 6, 8, 12, 16, 20, 24, 32, 40, 48	−1.2	4.20	1.00	−1.7	3.12	1.00	−9.1	4.51	0.75	−8.1	3.33	0.47

[1]The prediction errors reported (%) were calculated by $\frac{\text{reduced AUC} - \text{full AUC}}{\text{full AUC}} \times 100$, where negative values indicate underprediction and positive values indicate overprediction of the reduced AUC model in comparison to the full AUC model.

[2]Plasma concentrations of Cr and Co at h 0 and 64 were not statistically different from zero, and therefore during brute force calculations for determination of each reduced model, the plasma Cr and Co concentrations at h 0 were assumed to be zero to facilitate AUC calculations, and h 64 was removed. However, the full AUC model included h 64 in the AUC calculations.

[3]SEM were calculated as $\frac{\text{SED between reduced and full AUC}}{\text{full AUC}} \times 100$, where SED = standard error of the difference.

[4]*P*-values reported are for the treatment × set interaction, where each reduced AUC model was compared with the full AUC model (complete data set included).

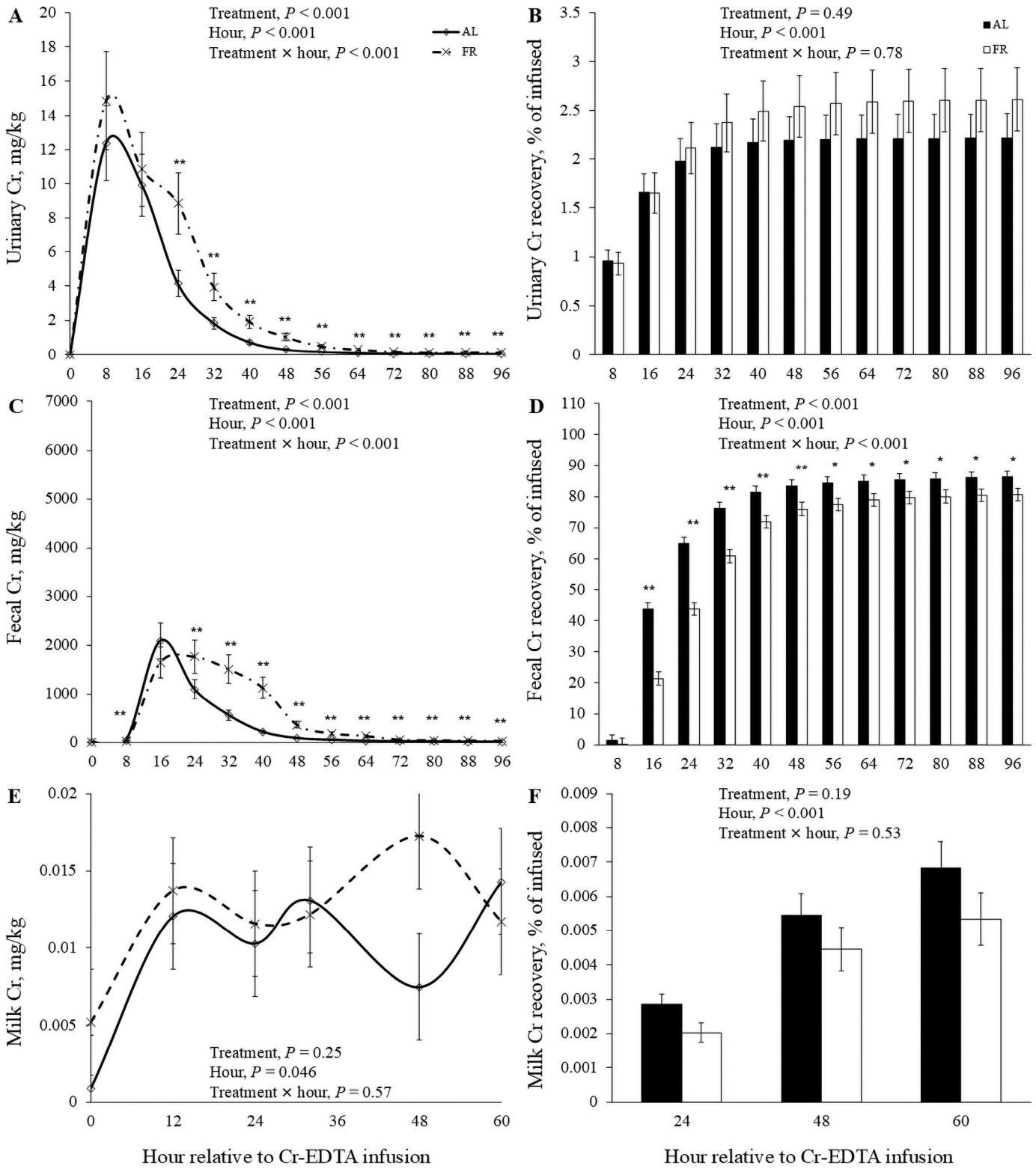

Figure 3. Effects of treatment (ad libitum [AL] or feed restriction [FR]) on urinary Cr concentrations (panel A), cumulative urinary Cr recovery (panel B), fecal Cr concentrations (panel C), cumulative fecal Cr recovery (panel D), milk Cr concentrations (panel E), and cumulative milk Cr recovery (panel F) following an abomasal bolus dose of Co-EDTA and a ruminal bolus dose of Cr-EDTA on d 3 of feed restriction (CHAL phase) in mid-lactation Holstein dairy cows (n = 6 for AL, n = 5 for FR due to removal of 1 animal for a single period). Values are LSM ± SEM. When a treatment by day interaction was detected, an asterisk (*) was used to indicate differences where $0.01 < P \leq 0.05$, and double asterisks (**) indicate differences where $P \leq 0.01$ between treatments at a given hour.

Table 7. Effects of treatment (ad libitum [AL] or feed restriction [FR]) on the sum of the cumulative recovery of Cr and Co excreted in urine and feces measured 24, 48, and 96 h after a ruminal dose of Cr-EDTA and an abomasal dose of Co-EDTA in mid-lactation Holstein dairy cows (n = 6 per treatment)

	Treatment			
Item	AL	FR	SEM	*P*-value
Total Cr recovery, % infused				
24 h	67.1	46.3	2.52	<0.001
48 h	85.7	77.7	2.46	0.029
96 h	88.5	82.2	2.20	0.031
Total Co recovery, % infused				
24 h	77.9	68.7	5.56	0.16
48 h	83.2	77.2	5.56	0.29
96 h	84.4	78.8	5.56	0.32

direct reduction in the consumption of fermentable substrates and the parallel decrease in available nutrients for absorption across the reticulorumen (Zhang et al., 2013a) and lower GIT (Kvidera et al., 2017). This was supported in the present study based on the increased mean, maximum, and minimum ruminal pH during 5 d of FR, likely a result of reduced short-chain fatty acid production (Zhang et al., 2013b) and altered microbial activity (Cole and Hutcheson, 1985). Consequently, it is likely that less nutrients were available for productive purposes, as evidenced by a reduction in milk yield of 9.3 kg/d, reduced fat yield of 373 g/d, reduced protein yield of 364 g/d, and reduced lactose yield of 438 g/d. In addition to direct implications of reduced nutrient intake, growing evidence suggests that GIT barrier dysfunction may be partly responsible for the adverse effects observed for the performance of lactating cows in response to transient exposure to low feed intake (Horst et al., 2020). A loss of GIT barrier function may allow bacteria and endotoxins to translocate across the GIT epithelium via paracellular pathways entering systemic or lymphatic circulation to elicit local (Kent-Dennis et al., 2020) and systemic inflammatory responses (Aschenbach et al., 2019).

Although the exact cause of compromised GIT barrier function during feed restriction in ruminants is unclear, feed restriction models have been used to investigate GIT permeability (Zhang et al., 2013a,b; Pederzolli et al., 2018) and potential mitigation strategies (Kvidera et al., 2017; Horst et al., 2020; Coleman et al., 2023). The in vivo technique using Cr-EDTA as a single permeability marker provides an indication of total-tract permeability; however, it is not possible to elucidate which region of the GIT the Cr-EDTA has translocated across. Understanding regional responses is important when considering that the epithelial structure differs along the GIT with pregastric compartments comprising a stratified squamous epithelium consisting of 4 distinct cell strata between the luminal contents and the lamina propria (Steele et al., 2016). In contrast, the lower GIT is made up of a single epithelial layer with an unstirred mucus layer, and secreted antimicrobial peptides and immunoglobulins protecting the apical side (Steele et al., 2016). Hence, the architectural and functional differences between the reticulorumen, omasum, and distal portions of the GIT indicate that differential permeability responses may occur in response to low feed intake (Penner et al., 2014; Pederzolli et al., 2018). Horst et al. (2020) demonstrated that lactating dairy cows exposed to feed restriction (40% of their ad libitum feed intake) had increased plasma Cr AUC after 24 h of low feed intake, and the magnitude of this response was diminished by d 5 of FR. These findings emphasize that the GIT is highly responsive to perturbations in nutrient supply (Aschenbach et al., 2019), and, following short-term exposure to low feed intake, the GIT has been suggested to respond by increasing tight junction protein expression (Pederzolli et al., 2018; Aschenbach et al., 2019) to prevent a chronic leaky gut. As noted, the use of orally administered Cr-EDTA as a GIT permeability marker does not provide the ability to decipher which regions are more permeable during feed restriction, and therefore previous studies have also performed postmortem GIT dissections (Kvidera et al., 2017; Horst et al., 2020). Morphological changes of the lower GIT, particularly in the jejunum and ileum, including reductions in villus width and depth (Kvidera et al., 2017), have provoked speculation that intestinal regions may be the location where majority of the Cr-EDTA permeates. However, approaches that evaluate structural changes of the GIT are invasive, requiring biopsies or euthanasia. Based on plasma AUC, the dual marker technique used in the present study generally demonstrated increased total GIT permeability and, more specifically, increased post-ruminal permeability during FR. The dual marker technique offers a less invasive approach to evaluate regional permeability responses in ruminally cannulated ruminants. These results support previous findings of altered ruminal (Pederzolli et al., 2018) and intestinal morphology, suggesting rapid changes that reduce absorptive surface area and increase permeability in response to 5 d of feed restriction (Kvidera et al., 2017; Pederzolli et al., 2018; Horst et al., 2020). The data from the present study are interpreted as supporting the suggestion that the lower GIT may be more susceptible to a loss of barrier function (Penner et al., 2014; Steele et al., 2016) and the consequential greater translocation of permeability markers.

As equal molar quantities of Cr-EDTA and Co-EDTA were administered and the markers are similar in size, Co excretion in urine can be expressed as a function of Cr excretion to understand the proportional GIT permeability occurring post-ruminally relative to the total tract. In

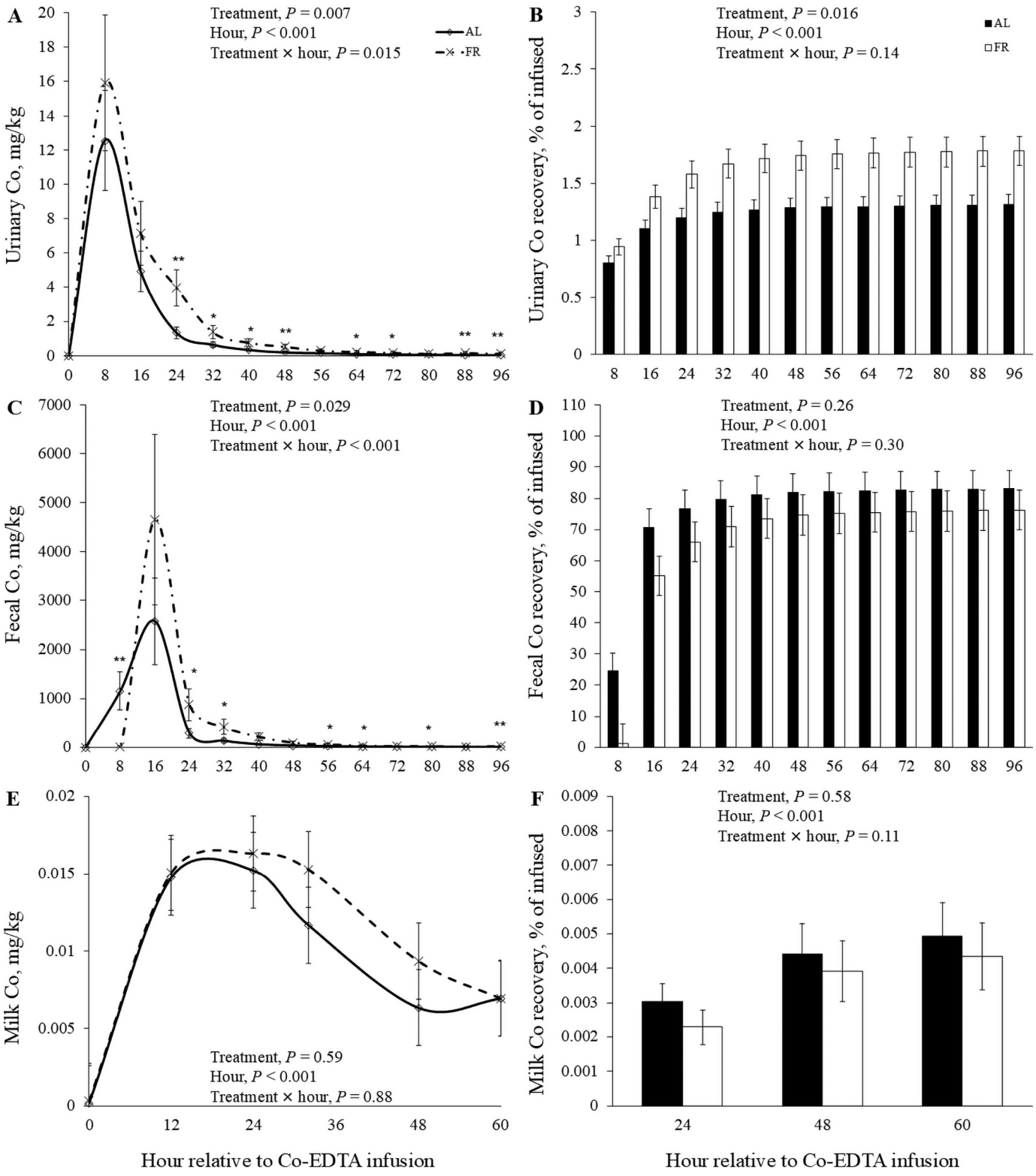

Figure 4. Effects of treatment (ad libitum [AL] or feed restriction [FR]) on urinary Co concentrations (panel A), cumulative urinary Co recovery (panel B), fecal Co concentrations (panel C), cumulative fecal Co recovery (panel D), milk Co concentrations (panel E), and cumulative milk Co recovery (panel F) following a abomasal bolus dose of Co-EDTA and a ruminal bolus dose of Cr-EDTA on d 3 of feed restriction (CHAL phase) in mid-lactation Holstein dairy cows (n = 6 for AL, n = 5 for FR due to removal of 1 animal for a single period). Values are LSM ± SEM. When a treatment by day interaction was detected, an asterisk (*) was used to indicate differences where $0.01 < P \leq 0.05$, and double asterisks (**) indicate differences where $P \leq 0.01$ between treatments at a given hour.

this study, 61% and 69% of the Cr excreted in urine were represented by Co for AL and FR, respectively. Although not statistically different between treatments, these data suggest that more than 60% of the markers permeate the post-ruminal regions of the GIT, supporting the greater innate leakiness of the intestines (Penner et al., 2014). Using epithelial tissues incubated in Ussing chambers, greater translocation of mannitol in the duodenum and jejunum relative to the rumen and omasum has been shown in healthy weaned calves (Penner et al., 2014) and calves after 5 d of low feed intake (Pederzolli et al., 2018). Our inability to detect statistical differences when Co was expressed as a percentage of Cr, even though differences were seen in urinary Co recovery alone, may be a consequence of the smaller magnitude of difference detected for Cr than Co. Additionally, the urinary data from 1 cow during FR was removed due to unquantifiable urine losses from straining likely associated with discomfort induced by the bladder catheter, highlighting a challenge with urinary catheters to measure urine volume and composition. Our group has shown similar responses of greater intestinal permeability as a function of total-tract permeability using a heat stress model in mid-lactation dairy cows (71%; Bertens et al., 2022) and a feed restriction model in beef heifers (AL = 60%, FR = 75%; Lambert and Penner, 2023). Although expressing Co as a function of Cr may provide insight into proportional permeability responses, it should be recognized that differential volumes of each marker were infused into their respective regions of the GIT (~0.7 L of Co-EDTA into the abomasum and 1.3 L Cr-EDTA into the rumen) and that regional differences in digesta volume and passage rate may affect surface area exposure, kinetics of marker translocation, and renal clearance.

Reasons for intestinal barrier dysfunction during FR are not fully elucidated but may be linked to reduced luminal nutrient sensing (Kvidera et al., 2017), altered microflora (Cole and Hutcheson, 1985), hunger-induced psychological stress (Vanuytsel et al., 2014), altered blood flow, or a nonspecific response to stress (Mayorga et al., 2020). Recently, our group has shown that sheep exposed to long-term (10-d) or short-term (5-d) feed restriction (fed 30% of ad libitum DMI) had reduced empty tissue weights along the entire GIT coupled with reduced liver weight (Lambert and Penner, 2023, unpublished). These findings suggest that low feed intake may reduce the metabolic activity of the liver and GIT, likely associated with decreased cellular proliferation and increased cellular apoptosis in response to restricted nutrient supply (Cant et al., 1996), and that these changes in cellular turnover may transiently increase permeability of the GIT. Under this context, differences in the duration and magnitude for FR models imposed may also alter the relative permeability response for the ruminal and post-ruminal regions.

Return to Voluntary Feed Intake and Effects on Ruminal pH and Milk and Milk Component Yields

Upon reintroduction to voluntary feed intake, the FR cows consumed a comparable amount of feed to AL cows on d 1 of recovery. The rapid increase in DMI on the first day of returning to full feed access relative to intake during CHAL induced conditions indicative of ruminal acidosis, as demonstrated by the daily minimum ruminal pH reaching 4.91, along with 15.4 h/d that pH was below 5.8. This sudden increase in DMI on d 1 of REC1 was followed by an 11-kg reduction on d 2. Retrogressive adaptation of GIT epithelium induced by short-term feed restriction occurs at an accelerated rate relative to proliferative adaptation (Wilson et al., 2012; Zhang et al., 2013b). Therefore, as cattle regain access to voluntary feed intake after exposure to transient low feed intake, the ability of the ruminal epithelium to absorb the sudden production of short-chain fatty acid is compromised (Albornoz et al., 2013; Schwaiger et al., 2013; Zhang et al., 2013b), resulting in a concurrent drop in ruminal pH and eliciting risk for secondary inflammation associated with ruminal acidosis (Khafipour et al., 2009). Ruminal acidosis has been associated with increased concentrations of circulating proinflammatory cytokines, which can have a direct effect on appetite centers in the brain, causing cattle to go off feed again (Brown et al., 2000). Similar to DMI, milk yield remained numerically lower for FR cows until d 14 after exposure to 5 d of low feed intake. Unfortunately, neither GIT barrier function nor systemic inflammatory markers were evaluated during the recovery phase; however, considering the prolonged return to baseline values for DMI and milk yield, it is plausible that nutrients were being redirected to support an immune response (Goetz et al., 2024). In the present study, it is notable that on d 2 of the recovery phase, 1 cow was diagnosed with sepsis by a veterinarian, likely from ruminal acidosis (minimum daily pH reached 4.56), and this cow received supplemental intervention, including antibiotics, electrolytes, and ruminal transfaunation.

Validation of the Dual Marker Technique

A single bolus dose of Cr-EDTA (Zhang et al., 2013a; Ceja et al., 2022) has been widely used to evaluate GIT permeability in milk-fed calves (Wood et al., 2015; Mellors et al., 2023), beef cattle (Briggs et al., 2021; Silva et al., 2023), and dairy cattle (Horst et al., 2020; Fontoura et al., 2022). The ability to differentiate regional permeability responses may be useful when devising potential

nutritional treatment or prevention strategies. In this study, a dual marker technique, using a single ruminal bolus dose of Cr-EDTA and an equimolar abomasal bolus dose of Co-EDTA, was used (Bertens et al., 2022) to characterize total GIT and post-ruminal permeability, respectively. Both Cr-EDTA and Co-EDTA have a similar molecular mass of approximately 340 g/mol and have a similar molecular size of 10Å (Zubkowski et al., 1995; García-Lafuente et al., 2001). Therefore, through size exclusion and a lack of known absorption mechanisms, limited movement of the markers takes place across the semipermeable GIT epithelium. Because both markers are considered indigestible and nonmetabolizable (Bjarnason et al., 1995), paracellular movement across the GIT of ruminants and monogastric species is currently the only known transport mechanism (García-Lafuente et al., 2001; Schweigel et al., 2005). Hence, the appearance of these markers in blood and the subsequent renal filtration and excretion in urine are suggestive of paracellular movement across the GIT, indicating increased epithelial permeability.

Alternatively, synthetic oligosaccharides (lactulose, sucralose, and polyethylene glycols [PEG]) and monosaccharides (mannitol and L-rhamnose) have been used to evaluate GIT permeability in preruminant calves (Mellors et al., 2023) and in adult ruminants (sucralose; Ellett et al., 2024). However, synthetic sugars possess limitations based on their ability to inhibit fermentation (sucralose) or be fermented (lactulose, mannitol, L-rhamnose; Bjarnason et al., 1995; Bischoff et al., 2014) and potential sensory stimulation with sweet taste receptors within the GIT epithelia (Schiffman and Rother, 2013; Moran et al., 2014). Hence, synthetic sugars are likely not biologically or chemically inert, bringing into question their appropriateness as permeability markers, at least in adult ruminants.

Comparison of Urinary Cr and Co Excretion and Plasma Concentrations. The strong positive correlation between total urinary excretion and the plasma AUC for both Cr and Co suggests that plasma AUC can be used as an acceptable predictor of total urinary marker excretion to evaluate permeability responses. Total urine collection has been viewed as the "gold standard" when using indigestible markers to assess permeability, as it enables quantification for the amount of marker excreted (Zhang et al., 2013b; Wood et al., 2015); however, concerns related to incomplete renal clearance, urine loss during total collection (as occurred in the present study), and risk for urinary tract infection are clear limitations. Blood sampling reduces the risk of potential renal interference and incomplete marker recovery and may reduce the risk of inflammation associated with bladder catheterization, at least when jugular catheters can be avoided and aseptic practices are implemented. However, determination of marker concentrations in blood does not provide information on total marker recovery without knowing total blood volume. The unknown effects of passage rate, marker exposure to the GIT, and renal filtration are challenges regardless of the sampling method used to determine marker translocation across the GIT (Bischoff et al., 2014; Horst et al., 2020; Goetz et al., 2024). Considering the previous statement, we observed greater plasma concentrations and AUC for Co than for Cr, despite the equal molar quantities of Cr-EDTA and Co-EDTA infused. In addition, Co appears to be retained in blood for longer than Cr. These differential findings for Co could be an outcome of Co dissociating from EDTA, allowing Co to bind to plasma proteins, primarily albumin, with high affinity (Paustenbach et al., 2013). In this scenario of Co-EDTA dissociating, it is unknown where the dissociation may be occurring. Dissociation could occur within the digesta of the post-gastric regions, challenging Co-EDTA as a suitable GIT permeability marker, or dissociation may occur in blood following GIT translocation, supporting Co-EDTA as a suitable permeability marker. Utilizing Co-EDTA as a liquid digesta marker in ruminants has been questioned in comparison to Cr-EDTA because of the potential for Co to dissociate from EDTA within the reducing conditions of the reticulorumen (Van Soest and Hall, 2020). However, the oxidation-reduction potential (**ORP**) of the ruminant GIT begins highly negative in the reticulorumen (−273 to −142 mV), changes to highly positive in the abomasum (+100 to +150 mV) and proximal small intestine (0 mV), becoming increasingly negative in the distal small intestine and then returning to highly negative in the large intestine (−220 mV; Marounek et al., 1987; Huang et al., 2018). The ORP whereby Co(III)-EDTA is prone to reducing to the less stable form Co(II)-EDTA begins at −60 mV (Ogino and Ogino, 1983), suggesting that at the primary site of exposure (abomasum and proximal small intestine) Co-EDTA is likely to remain intact. However, dissociation is possible in more distal segments of the lower GIT or following translocation into whole blood (ORP = −52 to −80mV; Daniels et al., 2018). Interestingly, following marker infusions, the urine collected during total collections appeared purple to the unaided eye and contained a pinkish crystalized sediment. These visual observations conceivably provide anecdotal evidence that Cr-EDTA and Co-EDTA were being excreted in their chelated forms (Dobson et al., 1976). Confirming the true quantities of Co-EDTA and Cr-EDTA in digesta, blood, or urine would require establishing alternative analyses able to differentiate between the 2 markers and evaluation of the chemical form.

Regardless of the higher plasma Co concentrations at 48 h when numerically compared with Cr, the concentrations were not statistically different from zero. As such,

our data are interpreted to suggest that use of marker concentrations in blood to calculate AUC can be used to assess GIT permeability. The data are interpreted to further suggest that evaluating marker concentrations at or among individual time points is not informative and could lead to erroneous conclusions. Further validation of the dual marker technique in different cattle breeds (beef vs. dairy), ages (calves vs. mature cattle), and physiological stages (lactating vs. non-lactating) during various potential GIT modulating challenges is warranted.

Recovery of Markers and Their Concentrations in Urine, Feces, and Milk. Absolute recovery of infused Cr- and Co-EDTA from urine and feces was not achieved after 96 h. The total Cr recovery was 6.3 percentage units lower for the FR cows than for AL-fed cows, whereas the total Co recovery was numerically reduced by 5.6 percentage units for FR. Udén et al. (1980) investigated the use of Cr-EDTA mordanted timothy fiber as a solid digesta passage marker and Co-EDTA as a liquid digesta marker in beef cattle fed ad libitum, and achieved 99.5% Cr recovery after 240 h and 90% Co recovery after 82 h. It is possible that the 96-h duration for total collections in the present study was inadequate to allow for complete fecal excretion and recovery of the markers; however, the present study was not designed to evaluate the time required for complete recovery of Cr and Co in urine, milk, and feces. It could be speculated that the incomplete marker recovery results may be an artifact of reduced digesta passage rate during feed restriction, as an outcome of reduced nutrient intake, less stimulation of gastric emptying, and reduced GIT motility (Fioramonti and Bueno, 1988). The presumption of reduced passage during feed restriction has not been evaluated in feed-restricted cattle; however, it has been reported that lactating dairy cows with lower feed intake have longer total-tract retention time compared with counterparts that have greater feed intake (Colucci et al., 1982).

The lower total recovery for Co compared with Cr regardless of treatment was unexpected, considering that equal doses were delivered and that Co-EDTA was administered distally in the GIT relative to Cr-EDTA. This may be related to the dissociation of Co from EDTA as mentioned previously. Unbound Co within the digesta of the lower GIT may be subject to microbial utilization for vitamin B_{12} synthesis in more distal regions of the small intestine and large intestine (Osman et al., 2021). In addition, free Co in circulation can bind to albumin (Paustenbach et al., 2013) and, over time, may deposit in organs or peripheral tissues (ATSDR, 2023). The lower total recovery of Co supports the greater plasma concentrations observed, as described previously. However, more than 98% of the Cr and 97% of the Co excreted in urine appeared within 48 h after administration, supporting the 48-h measurement duration recommended by Zhang et al. (2013a). These data are interpreted to suggest that, although Cr-EDTA and Co-EDTA may still be present in the GIT after 48 h, the residual quantity and epithelial surface area exposure are negligible contributors to the assessment of regional GIT permeability. Incomplete clearance of Cr-EDTA and Co-EDTA may limit the frequency of GIT permeability assessment for individual cows, at least when using urinary excretion and when reporting recovery of permeability markers as a percentage of that infused.

To the authors' knowledge, concentrations of Cr-EDTA and Co-EDTA have not been previously assessed in milk. Horst et al. (2020) identified milk as a potential excretion route for Cr-EDTA but did not report Cr concentrations. Concentrations of both Cr and Co were not statistically different from zero at h 0 but increased in milk following Cr- and Co-EDTA infusions, although they were not affected by feed restriction. The marker quantities recovered in milk represented 0.006% of the total Co recovered and 0.006% of the total Cr recovered. The changes in Co concentrations occurring 24, 48, and 60 h after infusion were 0.016, 0.008, and 0.007 mg/kg, respectively. Likewise, the changes in Cr concentrations occurring 24, 48, and 60 h after infusion were 0.008, 0.009, and 0.010 mg/kg, respectively.

Optimizing Timing of Blood Sampling to Estimate AUC. Refinement of research techniques to reduce invasiveness is promoted as part of the ethical responsibilities for animal use. We restricted sampling to 5 time points, to allow for future blood collection from the coccygeal vein and to avoid the need for jugular catheterization. The approach presented herein yielded AUC predictions that were ≤6.22% different from that measured using the complete data set, which met our predefined level of acceptability. The challenge imposed in this study, offering 100% ad libitum feed intake versus 40% of ad libitum feed intake, are arguably 2 extremely different treatments when comparing GIT permeability. Hence, considering that the reduced model using 5 time points adequately fits both extremes in lactating dairy cattle, it may also be suitable when assessing other metabolic, physiological, dietary, or environmental insults. However, confirmation of the applicability of the reduced model across challenges requires further validation.

CONCLUSIONS

Short-term low feed intake in lactating dairy cattle increases total GIT permeability and, more specifically, may increase permeability of the post-ruminal regions. In addition, upon return to voluntary feed intake, previously feed-restricted cows experienced ruminal acidosis and sustained reductions in DMI and milk yield. The novel dual marker technique reported in this study provides the

ability to differentiate regional permeability responses in ruminants. Blood sampling and subsequent plasma AUC calculations can be used as an acceptable indicator for total urinary recovery of Cr and Co. Moreover, blood sampling time points for both Cr and Co can be reduced to h 2, 8, 20, 40, and 48 following the dual permeability marker technique, at least when comparing GIT permeability for lactating dairy cows consuming 100% ad libitum feed intake or 40% of ad libitum feed intake.

NOTES

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Nonstandard abbreviations used: AL = ad libitum feed intake treatment; AUC = area under the curve; BASE = baseline phase; CHAL = challenge phase; ddH_2O = double-distilled water; FR = feed restriction treatment; GIT = gastrointestinal tract; ICP-MS = inductively coupled plasma-mass spectroscopy; ORP = oxidation-reduction potential; REC1, REC2 = 2 consecutive weeks of recovery; RMSPE = root mean square prediction error.

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