Evaluation of feed restriction and abomasal infusion of resistant starch as models to induce intestinal barrier dysfunction in healthy lactating cows

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ABSTRACT

Intestinal hyperpermeability and subsequent immune activation alters nutrient partitioning and thus, decreases productivity. Developing experimental models of intestinal barrier dysfunction in healthy cows is a prerequisite in identifying nutritional strategies to mitigate it. Six cannulated Holstein cows (mean ± standard deviation, 37 ± 10 kg/d milk yield; 219 ± 97 d in milk; 691 ± 70 kg body weight) were used in a replicated 3 × 3 Latin square design experiment with 21-d periods (16-d wash-out and 5-d challenge) to evaluate either feed restriction or hindgut acidosis as potential models for inducing intestinal hyperpermeability. Cows were randomly assigned to treatment sequence within square and treatment sequences were balanced for carryover effects. Treatments during the challenge were (1) control (CTR; ad libitum feeding); (2) feed restriction (FR; total mixed ration fed at 50% of ad libitum feed intake); and (3) resistant starch (RS; 500 g of resistant starch infused in abomasum once a day as a pulse-dose 30 min before morning feeding). The RS (ActiStar RT 75330, Cargill Inc.) was tapioca starch that was expected to be resistant to enzymatic digestion in the small intestine and highly fermentable in the hindgut. Blood samples were collected 4 h after feeding on d 13 and 14 of the wash-out periods (baseline data used as covariate), and on d 1, 3, and 5 of the challenge periods. Fecal samples were collected 4 and 8 h after the morning feeding on d 14 of the wash-out periods and d 5 of the challenge periods. Blood samples were collected 4 h after feeding on d 13 and 14 of the wash-out periods (baseline data used as covariate), and on d 1, 3, and 5 of the challenge periods. Fecal samples were collected 4 and 8 h after the morning feeding on d 14 of the wash-out periods and d 5 of the challenge periods. By design, FR decreased dry matter intake (48%) relative to CTR and RS, and this resulted in marked reductions in milk and 3.5% FCM yields over time, with the most pronounced decrease occurring on d 5 of the challenge (34 and 27%, respectively). Further, FR increased somatic cell count by 115% on d 5 of the challenge relative to CTR and RS. Overall, FR increased nonesterified fatty acids (159 vs. 79 mEq/L) and decreased BHB (8.5 vs. 11.2 mg/dL), but did not change circulating glucose relative to CTR. However, RS had no effect on production or metabolism metrics. Resistant starch decreased fecal pH 8 h after the morning feeding (6.26 vs. 6.81) relative to CTR and FR. Further, RS increased circulating lipo-poly saccharide binding protein (4.26 vs. 2.74 µg/mL) compared with FR only on d 1 of the challenge. Resistant starch also increased Hp (1.52 vs. 0.48 µg/mL) compared with CTR, but only on d 5 of the challenge. However, neither RS or FR affected concentrations of serum amyloid A, IL1b, or circulating endotoxin compared with CTR. The lack of consistent responses in inflammatory biomarkers suggests that FR and RS did not meaningfully affect intestinal barrier function. Thus, future research evaluating the effects of hindgut acidosis and FR using more intense insults and direct metrics of intestinal barrier function is warranted.

Key words: challenge model, gut health, gut permeability

INTRODUCTION

Intestinal barrier dysfunction (i.e., leaky gut) refers to enhanced permeability of the gastrointestinal tract (GIT) barrier, a scenario that triggers an inflammatory response accompanied by considerable alterations to metabolism and production (Eckel and Ametaj, 2016; Kvidera et al., 2017a). Immunoactivation is largely mediated by the infiltration of luminal derived antigens (i.e., LPS, the immunogenic component of gram-negative bacteria), into splanchnic tissues and local and systemic circulation, where they are recognized by cells with toll-like receptor 4 (TLR4; Ibeagha-Awemu et al., 2008) such as leukocytes (Kumar et al., 2011). Consequently, the activated immune system consumes copious AA and glucose at the expense of productive phenotypes (e.g., milk yield, growth), and this nutrient deficit is further compounded by inflammation-induced hypophagia (Kvidera et al., 2017a; Kuhla, 2020). Farm animals are exposed to stressors related to management (i.e., grouping, weaning, transport), physiological state...
(i.e., periparturient metabolic stress), and environment (i.e., heat stress) capable of inducing intestinal hyperpermeability (Mayorga et al., 2020). Thus, there is merit in the considerable attention barrier dysfunction and general “gut health” have received recently, as they likely play a central role in stress-induced poor performance in animal agriculture (Mayorga et al., 2020; Horst et al., 2021).

A prerequisite to developing nutritional mitigation strategies is to establish effective and repeatable models of GIT barrier dysfunction. Previously, feed restriction (FR) has been confirmed as an effective leaky gut model in monogastrics (Carey et al., 1994; Pearce et al., 2013) and ruminants (Zhang et al., 2013; Kvidera et al., 2017b; Horst et al., 2020). Similarly, SARA increases circulating LPS and inflammatory biomarkers (Gozho et al., 2005; Khafipour et al., 2009; Bilal et al., 2016). However, the site(s) at which barrier dysfunction occurs (rumen vs. hindgut) remains uncertain. During grain-induced SARA, some starch bypasses ruminal and small intestinal degradation and is fermented in the large intestine, which could result in a noxious luminal environment (i.e., decreased pH, increased free LPS; Li et al., 2012). Due to seemingly inferior anatomical fortifications (single layer of columnar epithelial cells and 2 mucous layers; Steele et al., 2016) and a relative lack of buffering capacities (Gressley et al., 2011), the hindgut epithelium is ostensibly more susceptible to injury (physical, chemical, pathogen, toxin) by increased microbial fermentation than the rumen wall (Sanz-Fernandez et al., 2020), which is reinforced with 4 multilayered strata of epithelium (Steele et al., 2016). The effects of hindgut acidosis (HGA) on barrier function have yet to be extensively characterized in lactating dairy cows but may be a valuable intestinal hyperpermeability empirical model if it can be experimentally induced. Thus, the objective of this experiment was to evaluate the effectiveness of HGA and FR as GIT barrier dysfunction models. We hypothesized that both FR and HGA would similarly induce immune activation and cause inflammation.

**MATERIALS AND METHODS**

**Animals and Treatments**

The experiment was performed at the Cargill Animal Nutrition and Health Innovation Campus in Elk River, Minnesota, and all procedures were approved by Cargill Animal Care and Use Committee. Six cannulated Holstein cows (37 ± 10 kg/d milk; 219 ± 97 DIM; 691 ± 70 kg BW) were used in a replicated balanced 3 × 3 Latin square design experiment with 21-d periods (16 d wash-out and 5 d challenge). Cows were blocked by parity [primiparous: n = 3; multiparous (cows from third, fourth, and fifth lactation): n = 3] and randomly assigned to treatment sequence within square. Treatment sequences were balanced for carryover effects. Treatments during the challenge period were (1) control (CTR; TMR fed ad libitum), (2) FR (TMR fed at 50% of ad libitum feed intake), or (3) TMR fed ad libitum plus 500 g of DM resistant starch (RS; Actistar RT 75330 resistant tapioca starch, Cargill Inc.) abomasally infused once a day as a pulse-dose before the morning feeding. The RS infused was tapioca starch and was expected to be resistant to digestion in the small intestine but highly fermentable by microbes in the hindgut (Supplemental Figure S1; https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/QUHSN; Piantoni, 2022). Previous work utilizing an in vitro digestion model sequentially mimicking 3 phases of the digestive tract (saliva, gastric, and small intestine) showed that less than 10% of the glucose from Actistar RT 75330 was released after 6 h of incubation, indicating that approximately 90% of the starch was undigestible. The in vitro digestion model used was based on that reported by Garcia-Campayo et al. (2018). Briefly, Actistar RT 75330 was mixed with a saliva cocktail including salivary α amylase, the pH was adjusted to 7, and the solution was incubated for 5 min at room temperature during the saliva phase. During the gastric phase, an acidic gastric cocktail containing pepsin and lipase was added to the saliva phase solution, the pH was adjusted to 4 if necessary, and the mix was incubated under head of heels rotation for 2 h at 37°C. Finally, during the small intestinal phase, a cocktail containing lipase, pancreatin, rat intestinal powder extract, and bile salts was added to the gastric phase solution, the pH was adjusted to 6.5 if necessary, and the mix was incubated under head over heels rotation for 4 h at 37°C. Glucose concentration in solution was determined as described in Garcia-Campayo et al. (2018). Also in previous work, the fermentability of ActiStar RT 75330 was compared with corn starch in vitro using the ANKOM RF gas production system and rumen fluid as the inoculum. Results indicated that corn starch increased gas production more quickly compared with Actistar RT 75330 during the first 60 min of incubation, and that starch sources were not different during the remainder of the incubation (Supplemental Figure S1). All cows were fed a diet formulated to meet or exceed requirements for energy, protein, minerals, and vitamins for lactating dairy cows (Table 1; NRC, 2001). Cows were allowed ad libitum access to water throughout the experiment, and except during FR, cows were allowed ad libitum access to feed.

Cows were fed twice a day (0630 and 1500 h) and milked 3 times a day (0500, 1200, and 1900 h). During
the challenge period (last 5 d of each period), daily feed offered to cows on the FR treatment was split evenly into 3 feedings delivered after each milking. Resistant starch was administered via an infusion line as previously described by Gressley et al. (2006). In brief, lines were placed in the abomasum on the afternoon of d 14 of the wash-out period to ensure cows were adapted to them before the challenge. Infusions were conducted daily during the challenge period at 0600 h, before the morning feeding. Infusion lines were checked for correct position in the abomasum and flushed with ~100 mL of warm water to confirm patency. After flushing, 500 g DM of starch dissolved in 1 kg of warm water (1:2 dilution) were infused as a pulse-dose into the abomasum. After the infusion, lines were flushed with ~250 mL of warm water to guarantee all starch reached the abomasum and avoid clogging of the line.

### Data and Sample Collection and Analysis

Individual feed intake and milk yield were recorded daily throughout the experiment. Nutrient composition of individual ingredients was determined by near-infrared spectroscopy (Cargill Animal Nutrition and Health Laboratory, Elk River, MN) before the experiment, and results were used for diet formulation. Samples of corn and alfalfa silages were collected weekly throughout the experiment. Samples of dry ingredients were collected at least 3 times over the course of the experiment. In- gredient samples were oven-dried at 100°C for 48 h to estimate DMI. Individual milk samples were collected with preservative at each milking on d 13 and 14 of the wash-out and 1, 3, and 5 of the challenge period for component analysis using mid-infrared spectroscopy (Zumbrota Lab, Zumbrota, MN). Body weights were recorded on d 14 of the wash-out and d 5 of the challenge period. Body condition score was recorded at the beginning of the experiment and at the end of each challenge period by 2 trained technicians on a 5-point scale, as described by Wildman et al. (1982), and averaged. Blood samples were collected by coccygeal venipuncture 4 h after the morning feeding (1030 h) on d 13 and 14 of the wash-out and 1, 3, and 5 of the challenge period. Blood samples were collected into 3 evacuated tubes. One of the tubes contained EDTA as anticoagulant, and another potassium oxalate with fluoride as a glyco- lytic inhibitor. Tubes were kept in ice until centrifuged at 1,500 × g for 15 min at 4°C. After centrifugation, plasma was aliquoted into microfuge tubes and stored at −80°C until further analysis. The third evacuated tube had no anticoagulant. This sample was kept at room temperature for ~1 h to allow for coagulation, centrifuged at 1,500 × g for 15 min, aliquoted, and stored at −80°C until further analysis. Before analyses, plasma and serum samples from d 13 and 14 were composited across the 2 d into a single baseline sample per cow per period. Plasma samples were analyzed for nonesterified fatty acids (NEFA; Wako Chemicals USA Inc.), BHB (Pointe Scientific Inc., Canton, MI), glucose (Wako Chemicals USA Inc.), LPS binding protein (LBP; Hycult Biotech), haptoglobin (Hp; Immunology Consultants Laboratory Inc.), and serum amyloid A (SAA; Tridelta Development Ltd.). Serum samples were analyzed for IL1β (ELISA, MyBioSource # MBS2887468), and endotoxin (ELISA, MyBioSource # MBS735050). Endotoxin and IL1β were determined only on baseline and d 5 samples, and intra-assay coefficients of variation were 3.9 and 2.3%, respectively. The inter- and intra-assay coefficients of variation for NEFA, BHB, glucose, LBP, Hp, and SAA were 13.5 and 2.7, 2.0 and 3.9, 7.2 and 3.9, 20.0 and 4.9, 2.9 and 3.4, 13.0 and 13.1%, respectively.

Fecal samples were collected at 1030 and 1500 h (~4 and 8 h after morning feeding, respectively) on d 14 of the wash-out and d 5 of the challenge period. A subsample of 5 g of fresh feces was manually mixed into 5 g of distilled water at room temperature to determine pH (Oakton pHTestr 50S), and the remaining sample was stored at −20°C. Fecal samples collected on d 5 of the challenge period were analyzed for starch content by wet chemistry (method 2014.10; AOAC International, 2019), by Cargill Animal Nutrition and Health Laboratory (Elk River, MN).

### Table 1. Ingredient and nutrient composition of the formulated diet (% of DM unless otherwise indicated)

<table>
<thead>
<tr>
<th>Item</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient</td>
<td></td>
</tr>
<tr>
<td>Corn silage</td>
<td>25.7</td>
</tr>
<tr>
<td>Alfalfa silage</td>
<td>13.9</td>
</tr>
<tr>
<td>Chopped alfalfa hay</td>
<td>7.8</td>
</tr>
<tr>
<td>Cottonseed with lint</td>
<td>3.5</td>
</tr>
<tr>
<td>Chopped wheat straw</td>
<td>1.8</td>
</tr>
<tr>
<td>Concentrate pellet</td>
<td>47.4</td>
</tr>
<tr>
<td>Nutrient composition</td>
<td></td>
</tr>
<tr>
<td>DM, %</td>
<td>55.6</td>
</tr>
<tr>
<td>NDF</td>
<td>29.9</td>
</tr>
<tr>
<td>Forage NDF</td>
<td>18.6</td>
</tr>
<tr>
<td>Starch</td>
<td>25.0</td>
</tr>
<tr>
<td>CP</td>
<td>16.6</td>
</tr>
<tr>
<td>Fat</td>
<td>3.9</td>
</tr>
</tbody>
</table>

1Concentrate pellet contained (as is basis): dry ground corn (41.3%), distillers grains with solubles (33.8%), malt sprouts (15.0%), wheat middlings (4.47%), calcium carbonate (3.34%), salt (1.01%), sodium sesquicarbonate (0.62%), magnesium oxide (0.19%), trace minerals premix (0.16%), selenium (0.06%) (0.08%), and vitamin premix (0.03%).

2Nutrient composition of individual ingredients was determined by near-infrared spectroscopy (Cargill Animal Nutrition and Health Laboratory, Elk River, MN) before the start of the experiment and results were used for diet formulation.
**Statistical Analysis**

Data from d 13 and 14 of each wash-out period were averaged and used as a covariate. Data collected on d 1, 3, and 5 of each challenge period were used to evaluate treatment effects. All data were analyzed using the GLIMMIX procedure of SAS v.9.4 (SAS Institute Inc.) according to the following model with repeated measures:

\[
Y_{ijklm} = \mu + \alpha_i + S_j + P_k + C_l + \delta + \varepsilon_{ijkl} + \tau_m + \alpha\tau_{im} + \varepsilon_{ijklm}
\]

where \(Y_{ijklm}\) = a specific trait per experimental unit (subject), \(\mu\) = overall mean of the population, \(\alpha_i\) = fixed effect of treatment \((i = 1 \text{ to } 3)\), \(S_j\) = fixed effect of square \((j = 1 \text{ to } 2)\), \(P_k\) = fixed effect of period \((k = 1 \text{ to } 3)\), \(C_l\) = random effect of cow \((l = 1 \text{ to } 6)\), \(\delta\) = covariate (baseline measurements for each period), \(\varepsilon_{ijkl}\) = random error representing the variance among subjects within treatment \(i\), \(\tau_m\) = fixed effect of day \((m = 1 \text{ to } 3)\), \(\alpha\tau_{im}\) = treatment by day interaction, and \(\varepsilon_{ijklm}\) = random error representing the variance among measurements within subject. The covariance structure used was autoregressive. Nonrepeated measures data such as BW, BW change, BCS, BCS change, endotoxin, and IL1β were analyzed with the model described above, without \(\tau_m\) and its interaction with treatment. Fecal starch was the only variable with no covariate.

Normality of residuals and homogeneity of variance were checked with normal probability and box plots and plots of residuals versus predicted values, respectively. Data were transformed as needed and transformed LSM were back-transformed for interpretation purposes and included on the results tables. Significance was declared at \(P \leq 0.05\) for main effect of treatment and at \(P \leq 0.10\) for treatment by day interactions. Tendencies were declared at \(P \leq 0.10\) for main effect of treatment and at \(P \leq 0.15\) for treatment by day interactions. When the main effect of treatment was significant, all pairwise comparisons were conducted and adjusted according to Tukey’s HSD test. All pairwise comparisons within day were also evaluated when interactions between treatment and day were significant or tended to be significant. For all pairwise comparisons, significance was declared at \(P \leq 0.05\). Cows were monitored weekly during the entire experiment for infectious diseases (i.e., mastitis, lameness). One cow on the RS treatment developed diarrhea the week before the first challenge period, and therefore, data from her first period were not included in the analysis.

A carryover effect assessment was conducted by performing a chi-square test on the goodness-of-fit parameters \((-2\text{ residual log-likelihood})\) of 2 models: with and without carryover effects. The model without the addition of carryover effects (model A) was described above, and the model with carryover effects (model B) was model A with the addition of continuous variables X1 and X2, which refer to the preceding treatment in the sequence of treatments. Chi-square test was performed on the \(-2\text{ residual log-likelihood}^2\) to compare models A and B. A chi-squared test \(P\)-value <0.05 indicated the presence of carryover effects for a particular response variable (Supplemental Tables S1 and S2; [https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/QUHISN](https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/QUHISN), Piantoni, 2022).

**RESULTS AND DISCUSSION**

Intestinal barrier dysfunction likely contributes to stress-induced production losses and metabolic disorders in dairy cows (Sanz-Fernandez et al., 2020; Horst et al., 2021). Specifically, inflammation originating from the translocation of luminally derived antigens (e.g., LPS) markedly shifts nutrient partitioning toward the activated immune system and away from productive phenotypes (Johnson, 2012; Kvidera et al., 2017a). There are several common stressors production animals experience that can increase intestinal permeability including psychological or emotional stress (Li et al., 2017; Proudfoot et al., 2018), transportation stress (Meddings and Swain, 2000), heat stress (Baumgard and Rhoads, 2013), and weaning (Moese et al., 2007). Further, SARA increases systemic concentrations of endotoxin and inflammatory biomarkers (Gozho et al., 2005; Khafipour et al., 2009; Bilal et al., 2016). However, the location(s) at which luminal antigens are entering circulation (rumen vs. hindgut) remain uncertain (Gressley et al., 2011; Plaizier et al., 2018). Intentionally inducing HGA may be a valuable model to employ when attempting to identify nutritional mitigation strategies targeting intestinal hyperpermeability as it is seemingly structurally more vulnerable against luminal insults (e.g., decreased pH, increased osmolarity, LPS; Li et al., 2012) relative to the rumen epithelium (Gressley et al., 2011; Plaizier et al., 2018). However, the effects of HGA on barrier permeability have yet to be fully characterized in lactating dairy cows, as some HGA experiments indicate adverse effects (Zust et al., 2000; Bissell and Hall, 2010) and others have not (Mainardi et al., 2011; van Gastelen et al., 2021a,b; Abeyta et al., 2023). Thus, the overall objectives of this experiment were to compare the effectiveness of HGA against a known model of intestinal barrier dysfunction (FR; Zhang et al., 2013; Kvidera et al., 2017b; Pisoni et al., 2022) and establish a potential model for future nutritional or
pharmaceutical mitigation research in lactating dairy cows.

By design, FR decreased DMI relative to CTR and RS animals (48%; \( P < 0.01 \); Table 2) and this resulted in reductions in milk, 3.5% FCM, protein, and lactose yields over time with the most pronounced decreases occurring on d 5 of the challenge period (34, 27, 35, 35, and 3%, respectively, relative to CTR and RS; all interactions \( P < 0.12 \); Table 2 and Figure 1A–D). Further, FR increased 3.5% FCM feed efficiency (72%; \( P < 0.01 \)) and milk fat concentration (3.98 vs. 3.57; \( P = 0.04 \); Table 2) throughout the challenge period relative to CTR and RS cows. The effect of FR on feed efficiency was larger earlier in the challenge period, when 3.5% FCM yield was still high (interaction \( P = 0.04 \); Figure 1E). These production and milk composition responses have been shown in previous FR experiments (Lapierre et al., 1995; Carlson et al., 2006; Ferraretto et al., 2014; Kvidera et al., 2017b). Interestingly, FR tended to increase SCC over time (115% increase on d 5 of the challenge; interaction \( P = 0.11 \); Figure 1F) relative to CTR and RS treatments, which corroborates other FR experiments (Gabbi et al., 2016; Kvidera et al., 2017b; Herve et al., 2019). Identifying why FR appears to increase milk SCC has pragmatic implications because epithelia apparently communicate with each other (gut–lung axis; Enaud et al., 2020) and it is likely that the gut and mammary epithelia also liaise (Rodríguez et al., 2021).

In addition to the observed production responses, FR altered metabolism such that it increased NEFA (159 vs. 79 mEq/L; \( P < 0.01 \); Table 3), decreased BHB (8.5 vs. 11.2 mg/dL; \( P = 0.02 \); Table 3), but did not change circulating glucose relative to CTR (Table 3). The increase in plasma NEFA concentrations herein was small compared with other FR reports (Kvidera et al., 2017b; Horst et al., 2020). This is likely explained by the timing of the blood sample relative to feeding (4 h after morning feeding), as postprandial insulin is a potent antilipolytic signal (Baumgard et al., 2017). Similarly, although most feed restricted dairy cows have elevated or unchanged BHB concentrations (Gross et al., 2011; Kvidera et al., 2017b; Herve et al., 2019). Identifying why FR appears to increase milk SCC has pragmatic implications because epithelia apparently communicate with each other (gut–lung axis; Enaud et al., 2020) and it is likely that the gut and mammary epithelia also liaise (Rodríguez et al., 2021).

### Table 2. Effects of feed restriction or abomasal resistant starch infusions on production metrics in lactating dairy cows

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment(^1)</th>
<th>SEM</th>
<th>( P)-value</th>
<th>Trt</th>
<th>Trt × Day(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMI, kg/d</td>
<td>CTR (n = 6)</td>
<td>24.0(^a)</td>
<td>12.2(^b)</td>
<td>22.6(^a)</td>
<td>0.8</td>
</tr>
<tr>
<td>Yield, kg/d</td>
<td>FR (n = 6)</td>
<td>31.9</td>
<td>26.1</td>
<td>30.8</td>
<td>1.7</td>
</tr>
<tr>
<td>Fat, %</td>
<td>RS (n = 5)</td>
<td>1.10</td>
<td>1.03</td>
<td>1.13</td>
<td>0.07</td>
</tr>
<tr>
<td>Protein, %</td>
<td></td>
<td>0.96(^a)</td>
<td>0.79(^b)</td>
<td>0.95(^a)</td>
<td>0.05</td>
</tr>
<tr>
<td>Lactose, %</td>
<td></td>
<td>1.53</td>
<td>1.24</td>
<td>1.49</td>
<td>0.08</td>
</tr>
<tr>
<td>3.5% FCM(^3)</td>
<td></td>
<td>31.7</td>
<td>28.1</td>
<td>31.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Milk variable</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat, %</td>
<td></td>
<td>3.48(^b)</td>
<td>3.98(^a)</td>
<td>3.66(^b)</td>
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</tr>
<tr>
<td>Protein, %</td>
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<td>3.08</td>
<td>3.06</td>
<td>3.13</td>
<td>0.03</td>
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<tr>
<td>Lactose, %</td>
<td></td>
<td>4.82</td>
<td>4.74</td>
<td>4.81</td>
<td>0.04</td>
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<tr>
<td>SCC, (\times) 1,000/mL</td>
<td></td>
<td>77</td>
<td>105</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>Log-transformed</td>
<td></td>
<td>1.89</td>
<td>2.02</td>
<td>1.89</td>
<td>0.12</td>
</tr>
<tr>
<td>MUN, mg/dL</td>
<td></td>
<td>14.1</td>
<td>14.1</td>
<td>13.8</td>
<td>0.4</td>
</tr>
<tr>
<td>3.5% FCM/DMI</td>
<td></td>
<td>1.29(^a)</td>
<td>2.31(^b)</td>
<td>1.40(^b)</td>
<td>0.08</td>
</tr>
<tr>
<td>BW, kg</td>
<td></td>
<td>703</td>
<td>702</td>
<td>713</td>
<td>20</td>
</tr>
<tr>
<td>BW change, kg</td>
<td></td>
<td>5.0(^a)</td>
<td>–58.1(^b)</td>
<td>–1.1(^b)</td>
<td>5.0</td>
</tr>
<tr>
<td>BCS(^7)</td>
<td></td>
<td>2.88</td>
<td>2.69</td>
<td>2.87</td>
<td>0.30</td>
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<tr>
<td>BCS change(^8)</td>
<td></td>
<td>0.153</td>
<td>–0.125</td>
<td>–0.002</td>
<td>0.130</td>
</tr>
</tbody>
</table>

\(^{a,b}\)Different superscripts within a row indicate \( P \leq 0.05 \).

\(^{1}\)Treatments (Trt) were control (CTR; fed ad libitum), 50% feed restriction (FR), and TMR fed ad libitum plus 500 g of DM resistant starch (RS; abomasally infused as a pulse-dose once daily 30 min before morning feeding).

\(^{2}\)NA = not applicable.

\(^{3}\)3.5% FCM yield = (0.4255 × milk yield) + [16.425 × (milk fat % ÷ 100) × milk yield].

\(^{4}\)Back-transformed means (from the transformed means shown in the following row) on the original data scale used for interpretation and discussion.

\(^{5}\)Measured on d 14 of the wash-out period.

\(^{6}\)Change measured between d 5 of the challenge and d 14 of the wash-out period.

\(^{7}\)Measured on d 5 of the challenge period.

\(^{8}\)Change in a 21-d period.
Leduc et al., 2021), some have decreased BHB (Carlson et al., 2006; Abeyta et al., 2021), which may relate to stage of lactation or severity of FR (Bjerre-Harpøth et al., 2012). Most circulating ketones in a well-fed rumenant originate from ketogenesis in the rumen epithelium (Pennington, 1952); thus, the reduction in BHB herein is likely a reflection of reduced ketone synthesis by the GIT upon nutrient restriction. Regardless, the minor alterations in circulating NEFA and BHB concentrations, along with unaltered BCS (Table 3), suggest that the BW loss in FR cows (58.1 kg; \( P < 0.01 \); Table 3) was primarily due to reduced rumen content from nutrient restriction and not from substantial body tissue loss.

Hindgut acidosis is generally defined as an increase in large intestine (i.e., cecum and colon) fermentation that results in a noxious luminal environment that is strikingly similar to the constituents of acidotic rumen fluid (i.e., increased VFA and decreased pH, increased free LPS and osmolarity; Gressley et al., 2011; Li et al., 2012). In the present study, we hypothesized that a pulse-dose of 500 g of resistant starch (i.e., starch immune to enzymatic degradation in the small intestine; Supplemental Figure S1) would ferment near the ileoce-
cal junction and thus decrease luminal pH and cause intestinal hyperpermeability and its associated sequelae. During the challenge period, fecal pH was not different among treatments during the morning sampling (4 h after morning feeding; Table 4) but was moderately decreased in RS cows at 8 h after morning feeding compared with CTR and FR (6.26 vs. 6.81; \( P < 0.01 \)). Additionally, RS tended to decrease fecal pH by ~0.4 units between 8 and 4 h after morning feeding compared with CTR (−0.39 vs. 0.15; \( P = 0.07 \)), suggesting starch was at least partially fermented in the hindgut. Resistant starch infusions tended to increase fecal starch content between 8 and 4 h after morning feeding compared with CTR (1.63 vs. 1.34 g; \( P = 0.15 \)). Utilizing an equation developed by Nennich et al. (2005), based upon DMI, it appears that approximately 80% of the resistant starch infuse in abomasum was not fermented, but instead excreted into the manure. Thus, utilizing RS to establish extensive HGA was mostly unsuccessful. Regardless, RS decreased fecal pH below 6.6, which has previously been described as a threshold for HGA in ruminants (Plaizier et al., 2018), although this remains to be well characterized.

Several indirect metrics of intestinal barrier function were evaluated to assess the effectiveness of FR and RS as hyperpermeability models including acute phase proteins (APP; LBP, SAA, and Hp), inflammatory cytokines (IL1β) and circulating endotoxin. Resistant starch increased circulating LBP on d 1 of the challenge (4.26 vs. 2.74 µg/mL; \( P < 0.05 \); Figure 2A) relative to FR, and Hp on d 5 of the challenge (1.52 vs. 0.48 µg/mL; \( P < 0.05 \); Figure 2B) relative to CTR. However, there were no differences in SAA, IL1β, or circulating endotoxin due to starch infusions (\( P > 0.40 \)). It is possible that the initial HGA insult was sufficient to induce intestinal hyperpermeability and elicit the increase in LBP and Hp. However, no other inflammatory or production metrics (i.e., milk yield) were altered, and the LBP and Hp responses were seemingly transient and inconsistent by day. Further, the magnitude of increase for these APP (especially Hp) were small compared with other models of immune activation and intestinal barrier dysfunction (Kvidera et al., 2017b,c; Horst et al., 2020; Opgenorth et al., 2021), putting their biological relevance into question. Regardless, the minor elevations in these APP, although likely not indicative of meaningful intestinal barrier dysfunction, suggest future work is warranted using a more aggressive HGA model.

Carryover effects were significant for several parameters (Supplemental Table S1), indicating that the wash-out period length (16 d) was insufficient to allow full recovery between insults. This also indicates that cows may respond differently with repeated insults to the intestinal wall, which may explain the discrepancies observed in immune metrics herein. Therefore,
depending on objectives, a Latin square design is likely not the most appropriate experimental approach for studies with repeated challenges to the GIT barrier. However, we ran an additional analysis to compare results between the model presented above and a model adjusting for carryover effects and found no significant alterations in our data, interpretation, or conclusions from the experiment (Supplemental Table S2). Thus, we decided to report data from the model not adjusting for carryover effects.

Results from indirect metrics of intestinal barrier function indicate that FR and RS infusions did not meaningfully affect intestinal barrier function in healthy postpeak cows. Late lactation cows (219 ± 97 DIM) are not as metabolically challenged as early lactation cows, and this might explain the lack of an inflammatory response at 50% FR compared with responses observed by Kvidera et al. (2017b) in earlier lactation cows (157 ± 9 DIM) at 40% of ad libitum feed intake. Bissell and Hall (2010) observed an increase in Hp following HGA from the abomasal infusion of 4 kg/d of corn starch in dry cows. Although starch used herein was resistant to enzymatic degradation in the small intestine, and thus presumably maximized dosage availability for hindgut fermentation, the selected dosage herein (500 g) was markedly less than that of the Bissell and Hall (2010) and apparently mostly impervious to large intestine fermentation, scenarios that likely explain discrepancies between experiments. It should be noted that although inflammatory biomarkers such as APP and cytokines have been utilized in previous intestinal barrier dysfunction experiments (Kvidera et al., 2017b,c; Abuajamieh et al., 2018; Horst et al., 2020), these metrics are nonspecific responses to imunoactivation regardless of origin, and the scientific field of intestinal barrier health is currently lacking a reliable biomarker specific

![Figure 2](image-url) Effects of treatments [control (CTR; fed ad libitum; black line), 50% feed restriction (FR; black dashed line), and TMR fed ad libitum plus 500 g DM resistant starch (RS; abomasally infused as a pulse-dose once daily 30 min before morning feeding; gray solid line)] on plasma concentrations of LPS binding protein (LBP) and haptoglobin during challenge periods. Back-transformed LSM were used to build the plots, and therefore, SEM were not included. LBP = LPS binding protein. Different letters within day indicate \( P \leq 0.05 \).

### Table 4. Effects of feed restriction or abomasal resistant starch infusions on fecal parameters in lactating dairy cows determined on d 5 of the challenge period

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTR (n = 6)</td>
</tr>
<tr>
<td>Fecal pH</td>
<td></td>
</tr>
<tr>
<td>4 h after morning feeding</td>
<td>6.76</td>
</tr>
<tr>
<td>8 h after morning feeding</td>
<td>6.91(^a)</td>
</tr>
<tr>
<td>8 − 4 h after morning feeding</td>
<td>0.15</td>
</tr>
<tr>
<td>Fecal starch, % of DM</td>
<td></td>
</tr>
<tr>
<td>4 h after morning feeding</td>
<td>2.47</td>
</tr>
<tr>
<td>8 h after morning feeding</td>
<td>2.72</td>
</tr>
<tr>
<td>8 − 4 h after morning feeding</td>
<td>0.25</td>
</tr>
</tbody>
</table>

\(^a\)^Different letters within a row indicate \( P \leq 0.05 \).

\(^b\)Treatments (Trt) were control (CTR; fed ad libitum), 50% feed restriction (FR), TMR fed ad libitum plus 500 g DM resistant starch (RS; abomasally infused as a pulse-dose once daily 30 min before morning feeding).
to gastrointestinal barrier dysfunction in ruminants. Thus, future experiments may benefit from the use of in vivo paracellular permeability markers, such as Cr-EDTA or mannitol, which would more accurately indicate intestinal hyperpermeability.

CONCLUSIONS

Feed restriction and RS infusions failed to meaningfully induce intestinal hyperpermeability as indicated by the lack of pertinent inflammatory and production responses. Future research may benefit from intensifying the severity of the challenges by either increasing the level of FR or RS dosage. A limitation of the experiment was that we did not directly measure leaky gut, and therefore, further research may benefit from incorporating additional makers of intestinal barrier dysfunction. Further, considering the presence of carryover effects, it is likely that the Latin square design is not an ideal experimental design for intestinal barrier function research. Hindgut acidosis may have direct implications on intestinal barrier function and the inflammatory profile of early lactation cows; thus, future research increasing the severity of HGA with the use of higher dosages of starch is warranted.

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REFERENCES


Herve, L., H. Quesnel, M. Veron, J. Portagnen, J. J. Gross, R. M. Bruckmaier, and M. Boutinaud. 2019. Milk yield loss in response to feed restriction is associated with mammary epithelial cell exfo-


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