The objective of this pilot study was to generate data to support the development of an experimental model of hindgut acidosis to further understand its systemic consequences independently of rumen acidosis. Four ruminally fistulated multiparous Holstein cows (213 ± 11 d in milk) were subjected to 2 consecutive experimental periods (P1 and P2), separated by a 3-d washout. Experimental periods were 96 h long from the baseline to the final measurements but expanded over 5 calendar days (d 0–4). Abomasal infusions of saline and corn starch (2.8 kg/d) were performed for the first 72 h (d 0–3) of P1 and P2, respectively. Final measurements were performed 24 h after the end of the infusions (d 4). Each cow was used as its own control by comparing P2 to P1. Postruminal-intestinal permeability was assessed by Cr appearance in blood after a pulse dose administration of Cr-EDTA into the abomasum on d 2 (48 h after infusion initiation) of each period. Starch infusion during P2 was associated with a milk protein yield increase (3.3%) and a decrease in milk urea nitrogen (11%). Fecal dry matter increased (8.8%), and starch content tended to increase (~2 fold) during P2. There was a period-by-day interaction for fecal pH as it decreased during starch infusion (1.3 pH points) but remained constant during P1. Although fecal lactate was not detectable during P1, it consistently increased during starch infusion. Fecal alkaline phosphatase activity also increased (~17 fold) in association with starch infusion. Two hours after Cr-EDTA administration, blood Cr concentration was higher during starch infusion, resulting in a tendency for a treatment-by-hour interaction. Furthermore, blood d-lactate increased (~2.5 fold), serum Cu decreased (18%), and blood urea nitrogen, cholesterol, and Ca tended to decrease (9.4%, 1.2%, and 2.4%, respectively), relative to P1. The current results suggest that hindgut acidosis was successfully induced by postruminal starch infusion, leading to gut damage and increased intestinal permeability. However, indications of systemic inflammation were not observed. The herein described preliminary results will require confirmation in a properly powered study. Key words: leaky gut, hindgut acidosis, transition period

INTRODUCTION

The effects of highly and rapidly fermentable diets (e.g., lactation diets) on rumen dynamics and health have been the focus of extensive research (Plaizier et al., 2008). However, it is increasingly evident that the effect of such diets on other sections of the gastrointestinal tract might substantially contribute to the pathophysiology of SARA (Sanz-Fernandez et al., 2020). Increasing the proportion of grains in the diet subsequently increases the proportion of starch and other fermentable components that escape rumen digestion and reach the small intestine and the hindgut (Offner and Sauvant, 2004). This increased flow can be further exacerbated by rumen acidosis, under which starch digestibility might be partially impaired (Gressley et al., 2011). As in the rumen, the combination of VFA and LPS accumulation, low pH, and increased osmotic pressure due to excessive fermentation can impair the barrier function and increase permeability in the hindgut (Emmanuel et al., 2007; Gressley et al., 2011). Further, some authors hypothesize that differences in histological structure, buffering capacity, and immunity make the hindgut more vulnerable than the rumen to acidotic insults (Steele et al., 2016).

Regardless of the anatomical site, a defective intestinal barrier function can result in leaky gut or the translocation of luminal immunogenic substances across the epithelium with the potential to activate a local or even a systemic inflammatory response (Kvidera et al., 2017). Certainly, several studies have detected an increase in blood inflammatory markers in response to diets aiming at inducing SARA (Gozho et al., 2005; Emmanuel...
et al., 2008). Although frequently overlooked, hindgut acidosis concomitantly develops in these studies with the potential to contribute to systemic inflammation (Li et al., 2012; Minuti et al., 2014). Inflammation is an animal welfare concern but also an economic burden, as immune activation induces whole-body adaptations intended to funnel energy and nutrients toward the immune system at the expense of milk synthesis (Horst et al., 2021). Further, enhanced inflammation has been associated with a higher incidence of metabolic diseases during the transition period (Trevisi et al., 2011; Eckel and Ametaj, 2016).

In contrast to monogastric species, hindgut health has received far less attention in ruminants. The aim of the current pilot study was to support the development of a model to study the contribution of the hindgut to acidosis as a systemic syndrome in high-grain diets in ruminants, by isolating postruminal digestion of starch. We hypothesized that hindgut acidosis on its own negatively affects intestinal barrier function with systemic consequences.

**MATERIALS AND METHODS**

**Animals and Experimental Design**

All experimental procedures were approved by the Central Authority for Scientific Procedures on Animals (CCD) and conducted under the Dutch Act on Animal Experiments, which complies with the European Directive 2010/63/EU. Four ruminally fistulated Holstein cows (213 ± 11 DIM; 744 ± 54 kg of BW; parity 3 ± 1) were housed individually in tiestalls in the metabolic unit of the Trouw Nutrition Ruminant Research Facility (Kempenhof, Boxmeer, the Netherlands). Because this was a pilot study aimed at compiling data to support the design of a fully powered study, we used 3 cows, being the minimum number of experimental replicates that allowed running statistics, +1, to compensate in case a cow reached a humane endpoint requiring exclusion from the study. The number of animals was chosen assuming the experiment would be underpowered. A summary of the experimental design is illustrated in Figure 1. Cows were allowed to acclimate for 7 d before being subjected to 2 consecutive experimental periods (P1 and P2) separated by a 3-d washout. Experimental periods were 96 h long from the baseline to the final measurements but expanded over 5 calendar days (d 0–4). Period 1 served as a control for P2 to study the association between the intervention and the observed parameters. Within each period, baseline measurements were obtained before the initiation of the infusions (d 0). Then, abomasal infusions were performed for 72 h (d 0–3) via an infusion line manually placed through the rumen fistula as previously described (Doelman et al., 2019). Final measurements were taken 24 h after the end of the infusions (d 4). Infusates were prepared fresh daily. During P1, saline (20 L/d) was infused continuously starting at 0900 h. During P2, a suspension of 2.8 kg/d of corn starch (Roquette, Lestrem, France) in 25 L of water was infused, aiming at inducing hindgut acidosis. Differences in the total daily infusion volumes between P1 and P2 were due to technical challenges with the starch suspension’s viscosity. Continuous infusion of the starch was not possible and, instead, it was infused for approximately 4 h, twice a day, 12 h apart (i.e., 0900 ± 2 h and 2100 ± 2 h). The potential consequences of the differences in the infusion volumes and schedules between experimental periods are taken into consideration in the discussion section. The corn starch dose was established based on a literature review where it was determined that fecal starch content sharply increases at around 13 g/kg BW of total dietary starch input (Supplemental Figure S1; https://data.mendeley.com/datasets/c679smj535/1; Sanz-Fernandez, 2023). The actual starch content of the experimental diets was lower than estimated, and total starch input (i.e., dietary + infused) during P2 averaged 8.9 ± 0.4 g/kg BW.

Cows had free access to water, and fresh feed was offered daily at ~0900 h after orts were weighed to determine daily intake. The diet consisted of a for-
age mixture offered ad libitum and a fixed amount of compound feed that was top dressed, allowing for sorting (Table 1). Compound feed was assumed to be consumed in full for daily DMI calculations. The main ingredients in the compound feed as fed were ground corn (16.5%), wheat (16.0%), beet pulp (7.8%), rumen-protected rapeseed meal (7.7%), palm kernel flakes (7.5%), rumen-protected soybean meal (7.0%), and rapeseed meal (6.7%). The amount of compound feed allocated to each cow was based on their milk production and ranged between 28% and 40% of their DMI. As a result, the starch content of the diets (i.e., excluding starch infusion) ranged between 16.7% and 18.1% DM. The rationale for not fixing the compound feed allocation among cows was to create a certain degree of variability in the total starch input to calibrate the starch content in future studies. Cows were milked twice a.m. and p.m. daily during the experimental periods, stored frozen (−20°C) until analyses. Fecal samples were collected for 10 min, transferred to cryotubes, and stored frozen (−20°C) until analyses. Blood samples were collected via coccygeal venipuncture into preservative-free tubes on d 0, 2, and 3 a.m. and p.m. daily during the experimental periods, stored frozen (−20°C) until analyses. Blood Cr appearance was analyzed in serum at 0, 2, 4, and 6 h postadministration.

### Sample Analyses

Composition of the different ingredients in the diet was analyzed individually by Eurofins Agro (Wageningen, the Netherlands) by near-infrared spectroscopy, and the results were combined to calculate the final diet composition. Part of the blood analyses were performed at Synlab.vet GmbH (Leverkusen, Germany). Colorimetric assays were used to measure blood urea, cholesterol, BHB, glucose, D- and L-lactate, and blood Ca, Cu, and Zn were analyzed by flame atomic absorption spectrometry. Additional blood analyses were performed at the University of Nottingham (NUvetNA laboratory, United Kingdom). Blood nonesterified fatty acids (NEFA) were determined using a Randox kit and an RX imola analyzer (Randox Laboratories Ltd., Antrim, UK). Blood insulin was analyzed with an ELISA kit (Mercodia, Uppsala, Sweden) and a Varioskan Flash plate reader (Thermo Fisher Scientific, Loughborough, UK). Blood Cr was measured using inductively coupled plasma mass spectrometry (Thermo Fisher XSeriesII, Thermo Fisher Scientific, Waltham, MA). Blood haptoglobin was determined at Elanco’s R&D laboratory (Monheim, Germany) with a colorimetric assay according to the manufacturer’s specifications (Tridelta Development Limited, Maynooth, Ireland). Fecal composition was analyzed by Masterlab (Boxmeer, the Netherlands). Dry matter content was determined by drying to a constant weight in a 103°C oven for 4 h (EC 152/2009; EC, 2009). Starch was determined enzymatically using amylloglucosidase (ISO 15914; ISO, 2004). Milk samples were analyzed by Qlip NV (Zutphen, the Netherlands). Fat, CP, lactose, and urea concentrations were measured using a Foss Milkscan FT6000 (ISO 9622; ISO, 2013). For fatty acid profile analysis, we prepared FAME from fat fractions (ISO 15884; ISO, 2002) and determined by gas chromatography (ISO 16958; ISO, 2015). Fecal VFA and lactate were determined by HPLC, but excessive dilution of the samples coupled with limitations with the detection thresholds in the laboratory prevented an accurate determination of their absolute values. However, the proportions between fecal acetate and propionate coincided with values previously reported (Li et al., 2012; Seymour et
al., 2021), and therefore only relative changes from each period’s baseline are presented. Fecal alkaline phosphatase (ALKP) activity was measured by Elanco’s R&D laboratory. Fecal extracts were generated by diluting 50 mg of feces in 500 µL of ice-cold PBS containing 2% octylglucoside (Sigma-Aldrich, St. Louis, MO). This mixture was incubated for 1 h in a shaking device at 8°C and 1,000 rpm, and subsequently centrifuged at 16,000 × g at 4°C for 15 min. The supernatant (fecal extract) was transferred into a new vial and stored at -20°C until used. Alkaline phosphate activity was determined in quadruplicate in a 384-well plate. Per well, 10 µL of fecal extract were mixed with a 90-µL solution of 5 mM 4-nitrophenyl phosphate disodium salt hexahydrate (A1442,0050, Applichem, Darmstadt, Germany) in carbonate buffer (50 mM NaHCO3/Na2CO3, 2 mM MgCl2, pH 9.6). The optical density (OD) at 405 nm was measured kinetically in a plate reader, and the curve’s slope in a linear range (ΔOD/min) was used. When needed, fecal extracts were diluted 1:10 in PBS.

Calculations and Statistics

Due to large daily variability, blood metabolites measured on d 2 and 3 (48 and 72 h after the initiation of the infusions) of each period were averaged, except for D- and L-lactate which were only measured on d 2. Fat- and protein-corrected milk (corrected to 4.0% fat and 3.3% protein; kg/d) was calculated as: milk yield (kg/d) × [0.377 + 0.116 × fat (%)] + 0.06 × protein (%)].

All data were statistically analyzed using SAS Studio 3.81 (SAS Institute Inc., Cary, NC). Daily DMI, and milk and fecal parameters, were analyzed by repeated measures using PROC MIXED with an autoregressive covariance structure, day relative to the infusion’s initiation as the repeated effect and cow as the subject. The model included period, day, and their interaction as fixed effects. The interaction was nonsignificant for any of the milk parameters or for voluntary DMI (P > 0.5) and subsequently dropped from the model. Cow was included as a random effect in the model. For each cow, the baseline of each period measurement (i.e., d 0 measurement, before the initiation of the infusions) was used as a covariate and not included in the repeated measures analysis, except for blood Cr and fecal VFA concentrations where baselines for both periods virtually equaled 0. Chromium appearance in blood during the intestinal permeability test was analyzed similarly, but hour relative to dosing was used as the repeated statement. Additionally, blood Cr was summarized as an area under curve (AUC) calculated by the linear trapezoidal summation between successive pairs of Cr concentrations and time coordinates. Blood Cr appearance expressed as AUC, the rest of the blood parameters and milk fatty acid content were analyzed using PROC MIXED, including period as fixed and cow as random effects. When available, the baseline measurement of each period was used as a covariate. When deemed informative, a post hoc analysis was performed to determine differences between d 0 (covariate) and d 1, within period.

For each variable in each model, normal distribution of residuals was tested using PROC UNIVARIATE. Logarithmic transformation was performed when necessary. Confidence intervals were created in the log form and back transformed into the original scale for reporting. Data are reported as least squares means and considered significant if P ≤ 0.05 and a tendency if 0.05 < P ≤ 0.15.

RESULTS

Voluntary DMI and milk composition results are summarized in Table 2. The period-by-day interaction was not significant (P > 0.50) for any of the measured parameters. Voluntary DMI (without taking the starch infusion into account) did not differ between periods but changed overtime (P < 0.01), decreasing the first 48 h of the infusions and 24 h after their cessation (Supplemental Figure S2A; https://data.mendeley.com/datasets/c679smj535/1; Sanz-Fernandez, 2023).

Period had a significant effect on protein yield (Table 2) and MUN (Figure 2), as they were increased (3.3%; P = 0.03) and decreased (11%; P = 0.01), respectively with starch infusion.

Most parameters were significantly affected by day, independently of the period. Milk (Supplemental Figure S2A), milk protein, and lactose yields (data not shown) increased in the first 24 h after the initiation of the infusions (post hoc analysis: P ≤ 0.03) and decreased progressively from d 1 to 4 (P < 0.01). Fat and

<table>
<thead>
<tr>
<th>Item</th>
<th>P1</th>
<th>P2</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voluntary DMI, kg/d</td>
<td>25.5</td>
<td>25.5</td>
<td>0.3</td>
<td>0.96</td>
</tr>
<tr>
<td>Milk yield, kg/d</td>
<td>31.7</td>
<td>32.6</td>
<td>0.5</td>
<td>0.27</td>
</tr>
<tr>
<td>Fat, %</td>
<td>4.65</td>
<td>4.39</td>
<td>0.12</td>
<td>0.22</td>
</tr>
<tr>
<td>Protein, %</td>
<td>3.63</td>
<td>3.64</td>
<td>0.02</td>
<td>0.76</td>
</tr>
<tr>
<td>Lactose, %</td>
<td>4.58</td>
<td>4.57</td>
<td>0.04</td>
<td>0.60</td>
</tr>
<tr>
<td>Fat yield, kg/d</td>
<td>1.48</td>
<td>1.43</td>
<td>0.02</td>
<td>0.18</td>
</tr>
<tr>
<td>Protein yield, kg/d</td>
<td>1.15</td>
<td>1.18</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>Lactose yield, kg/d</td>
<td>1.45</td>
<td>1.49</td>
<td>0.03</td>
<td>0.37</td>
</tr>
<tr>
<td>FPCM, kg/d</td>
<td>36.0</td>
<td>36.1</td>
<td>0.5</td>
<td>0.93</td>
</tr>
<tr>
<td>MUN, mg/dL</td>
<td>9.85</td>
<td>8.79</td>
<td>0.49</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Voluntary DMI excluding the infusates.

3Fat- and protein-corrected milk = milk yield (kg/d) × [0.377 + 0.116 × fat (2%) + 0.06 × protein (%)].
protein-corrected milk was increased 24 h after infusion initiation, compared with the rest of the days ($P = 0.01$; Supplemental Figure S1A). Milk urea nitrogen (Supplemental Figure S2B) decreased after 24 h of infusion (post hoc analysis: $P < 0.01$) and increased thereafter ($P < 0.01$). Both protein and fat content increased ($P < 0.01$) and tended to increase ($P = 0.07$) at the end of the periods (Supplemental Figure S2C).

Milk fatty acid content results are summarized in Table 3. Despite milk butyrate tending to decrease (6.9%; $P = 0.09$), overall, de novo fatty acid content increased (8.6%; $P < 0.01$) in P2. Mixed fatty acid content tended to increase (5.2%; $P = 0.06$) and preformed fatty acids decreased (12%; $P = 0.01$), during starch infusion. During P2, total UFA content, as well as n-3 and MUFA, tended to decrease (4.4%, 6.2%, 6.1%, respectively; $P < 0.14$). Oppositely, n-6 increased (8.9%; $P = 0.01$), and PUFA content tended to increase (3.6%; $P = 0.11$). No differences were detected in CLA content between periods.

Fecal DM content increased (8.8%; $P = 0.02$; Figure 3), and starch content tended to increase (~2 fold; $P = 0.08$; Figure 4A), in P2 compared with P1. We observed a significant period-by-day interaction in fecal pH ($P = 0.002$; Figure 4B) as it tended to decrease on d 1 and decreased on d 2 with the starch infusion. Fecal lactate was not detectable during P1, but it increased during starch infusion and decreased back to baseline 24 h after infusion cessation ($P = 0.02$; Figure 4C). We observed a significant period-by-day interaction for fecal acetate expressed relative to baseline ($P < 0.001$), as it progressively increased in P1 but decreased in P2 (Supplemental Figure S3; https://data.mendeley.com/datasets/c679smj535/1; Sanz-Fernandez, 2023). Fecal propionate behaved similarly (data not shown). Fecal ALKP activity was increased in P2 relative to P1 (~17 fold; $P = 0.003$; Figure 5A).

Blood metabolites are presented in Table 4. During P2, d-lactate increased (~2.5 fold; $P = 0.01$; Figure 5B), Cu decreased (16%; $P = 0.02$), and BUN, cholesterol, and Ca tended to decrease (9.4%, 1.2%, and 2.4%, respectively; $P \leq 0.11$; Figure 1), relative to P1. No differences were detected in other blood parameters.

Regarding the intestinal permeability measurements, we observed a tendency for a period-by-hour interaction in blood Cr-EDTA appearance, as it was increased ($P = 0.02$) 2 h after Cr-EDTA administration during P2 (Figure 5C). Blood Cr-EDTA expressed as AUC did not differ between periods (Figure 5D).

**DISCUSSION**

The current pilot study was intended to set the basis to develop an experimental model of hindgut acidosis and investigate its local and systemic consequences,
in isolation, without the concomitant effects of rumen acidosis. Infusing 2.8 kg of corn starch directly into the abomasum led to a large increase in fecal lactate and a decrease in fecal pH suggestive of increased hindgut fermentation. In contrast to rumen pH, there is little characterization of fecal pH and no threshold defined for hindgut acidosis. However, the 1.26 pH point decrease in the current study is in line with recent reports where abomasal infusion of 3 to 4 kg of cornstarch reduced fecal pH 1.20 to 1.34 points (van Gastelen et al., 2021; Abeyta et al., 2023c). Because cows received different amounts of compound feed depending on production level, daily starch supply differed among cows resulting in variable responses. Anecdotally, cows 2 and 3 in this pilot study received the highest compound feed allowance (+1.3 and 2.9 kg of DM relative to cows 1 and 4, respectively) and had the largest increase in fecal lactate and starch with the lowest fecal pH. In contrast, cow 1 had the highest total starch intake due to a larger forage intake (+0.31, 0.46, 0.91 kg of DM/d relative to cow 2, 3, and 4, respectively) but experienced moderate responses. This may represent differences in the starch fermentation site and highlights how small dietary variations may drastically change starch digestion dynamics. Interestingly, despite these apparent individual differences in hindgut fermentation, most of the other parameters measured in this study behaved similarly among individuals.

Due to the small sample size, changes in milk performance were not expected in response to the starch infusion, in agreement with similarly sized studies (Gressley and Armentano, 2007; van Gastelen et al., 2021; Abeyta et al., 2023c). Although, increases in milk yield have been previously reported corresponding to the increase in metabolizable energy supply (Reynolds et al., 2001), lactation responses to postrumen starch digestion greatly vary in the literature (Nocek and Tamminga, 1991). In the current study, a small but statistically significant increase in milk protein yield was observed concurrent with numerical increases in milk yield and protein content. The mechanism behind this response remains unknown, but it might be the result of AA sparing from the portal-drained viscera and the liver due to the increased availability of glucose and VFA from starch digestion (Reynolds et al., 2001). A decrease in milk fat content or yield is frequently reported as a result of postrumen carbohydrate digestion, along with a decrease in milk preformed fatty acid (>C16) content (Reynolds et al., 2001; Gressley and Armentano, 2007; van Gastelen et al., 2021), suggestive of decreased lipolysis. In agreement, Abeyta et al. (2023c) observed a decrease in circulating NEFA, likely explained by an increase in insulin (i.e., an antilipolytic hormone) after an abomasal infusion of corn starch.
Figure 5. Effects of infusing saline (period 1 = P1) or corn starch (period 2 = P2) into the abomasum on fecal alkaline phosphatase activity (ALKP); within each period, infusions were performed for 72 h (d 0–3), and final measurements were taken 24 h after the end of the infusions (d 4). Baseline values before the initiation of the infusions (d 0) were statistically used as covariate (A), blood d-lactate concentrations on d 2 of each period (48 h after the initiation of the infusions (B), blood Cr appearance over time after Cr-EDTA abomasal administration on d 2 of each period (48 h after the initiation of the infusions (C), and blood Cr appearance summarized as area under the curve (AUC; D). *Statistical differences within a given hour ( \( P < 0.05 \)). \( P \times H = \) period \( \times \) hour interaction. Error bars represent SEM.

Table 4. Blood metabolite concentrations of mid-lactation cows receiving abomasal infusions of saline (P1) or corn starch (P2)

<table>
<thead>
<tr>
<th>Item</th>
<th>d 0(^1)</th>
<th>d 2–3(^2)</th>
<th>d 0</th>
<th>d 2–3</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUN, mmol/L</td>
<td>2.55</td>
<td>2.16</td>
<td>2.33</td>
<td>1.96</td>
<td>0.05</td>
<td>0.07</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>6.64</td>
<td>6.55</td>
<td>6.55</td>
<td>6.47</td>
<td>0.09</td>
<td>0.11</td>
</tr>
<tr>
<td>NEFA, (^3) mmol/L</td>
<td>0.035</td>
<td>0.062</td>
<td>0.049</td>
<td>0.054</td>
<td>0.004</td>
<td>0.21</td>
</tr>
<tr>
<td>BHB, (^3) mmol/L</td>
<td>657</td>
<td>847</td>
<td>866</td>
<td>766</td>
<td>94</td>
<td>0.59</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>3.75</td>
<td>3.65</td>
<td>3.61</td>
<td>3.61</td>
<td>0.04</td>
<td>0.34</td>
</tr>
<tr>
<td>L-lactate, (^4) mmol/L</td>
<td>0.018</td>
<td>0.066</td>
<td>0.066</td>
<td>0.009</td>
<td>0.009</td>
<td>0.01</td>
</tr>
<tr>
<td>Haptoglobin, (^4) µg/mL</td>
<td>304</td>
<td>401</td>
<td>189</td>
<td>316</td>
<td>157</td>
<td>0.73</td>
</tr>
</tbody>
</table>

\(^1\)Baseline values before the initiation of the infusions (d 0) were used as a covariate in the statistical model and are shown only as a reference.

\(^2\)Average of d 2 and 3 values (48 and 72 h after the initiation of the infusions) within each period.

\(^3\)Nonesterified fatty acids.

\(^4\)d- and L-lactate were only measured on d 2 of each period.
In the current study, milk preformed fatty acid (i.e., ≥C18) content decreased, whereas the de novo fraction (C4–C14) increased. Concomitantly, although nonsignificant, milk fat content and yield decreased, blood NEFA decreased, and insulin increased. Ultimately, insulin secretion might have been stimulated by the increased glucose and VFA absorption from starch hydrolysis and fermentation. Finally, most milk parameters were affected by day independently of the experimental period suggesting that the infusions per se had an effect. As discussed later in this section, the fact that saline was used as control in P1, whereas starch was diluted in water in P2 in addition to differences in the infusion regimens, could have had influenced these results. Alternatively, increasing the volume of fluids could have also affected milk performance, similar to the responses to increased water intake. For instance, an increase in water intake consistently decreases MUN due to increase urination and urinary urea excretion (Spek et al., 2013). Regardless, it is necessary to consider the potential effects of the infusions independently of their composition when interpreting the results of the current study.

Infusing starch postruminally led to intestinal damage and leaky gut. Fecal ALKP activity sharply increased during starch infusion, which might reflect sloughing of enterocytes or colonocytes carrying the enzyme due to mucosal damage. In the current study, total fecal ALKP activity was determined, which includes all host isozymes as well as microbial ALKP. Although their relative contribution is not known in cattle (Celi et al., 2019), in humans, the host-derived enzyme represents the largest proportion in feces (Malo, 2015). Increased fecal ALKP activity could also be partially due to mucosal inflammation and neutrophil recruitment, as neutrophils are another source of ALKP (Lallès, 2014). In contrast to most of the measured parameters, fecal ALKP activity did not return to baseline 24 h after the cessation of the starch infusion, which probably was too short of a period for mucosal recovery. Along with the increase in fecal ALKP activity, blood Cr recovery was increased early after Cr-EDTA dosing in the abomasum, resulting in a numerical increase (34%) in Cr AUC. In addition, blood d-lactate, which is almost exclusively produced by bacteria and likely originating from the intestinal microbiota, was also increased. Taken together, these data may represent increased translocation of intestinal content from the lumen to the bloodstream suggesting that intestinal permeability was increased in response to postruminal starch infusion.

Despite the apparent induction of leaky gut, no evident signs of systemic inflammation were observed based on blood concentrations of haptoglobin and minerals, which are frequently altered during immune activation (i.e., decreased Ca and Zn, and increased Cu), other than a mild decrease in blood Ca. This is in agreement with previous reports where hindgut acidosis did not affect blood acute phase proteins (Mainardi et al., 2011, van Gastelen et al., 2021; Abeyta et al., 2023c) and contrasts with models of subacute and acute rumen acidosis where the increase in inflammatory parameters is frequently reported (as reviewed by Plaizier et al., 2018). Intriguingly, in a recent report by Abeyta et al. (2023a), inducing subacute rumen acidosis led to a delayed inflammatory response, only evident 24 h postchallenge and coinciding in time with a decrease in fecal pH and the development of metabolic acidosis. Based on the temporal pattern of events, the authors speculated that hindgut hyperpermeability was responsible for the increase in circulating inflammatory pattern. The lack of detectable systemic inflammation in the current study might indicate that, if existing, immune activation arising from hindgut acidosis is resolved either locally or at hepatic level, at least when the challenge occurs in isolation. Possibly, the actual consequences of hindgut acidosis might be dictated by the context as repeated and concomitant inflammatory stimuli, as typically observed during the transition period (e.g., uterine and mammary gland inflammation, oxidative stress), are known to have additive effects (Bradford et al., 2015). However, in a recent report, hindgut acidosis did not further increase inflammatory parameters in previously immune-activated dairy cows (Abeyta et al., 2023b). Nevertheless, nutritional strategies intended to support hindgut health have shown beneficial effects on performance in lactating cows (Seymour et al., 2021), suggesting that the energetic cost of such an insult is still considerable.

Increased postruminal carbohydrate fermentation consistently decreased BUN and MUN in this and other studies (Gressley and Armentano, 2007; Rojen et al., 2012). This is typically attributed to increased retention of N in the hindgut due to enhanced microbial proliferation and subsequent protein synthesis, resulting in increased fecal N excretion and decreased CP apparent digestibility (Orskov et al., 1970; Oncuer et al., 1990; Reynolds et al., 2001). The increase in the fecal excretion of microbial mass explains the increase in fecal DM observed in this and other studies in response to hindgut carbohydrate fermentation (Gressley and Armentano, 2007; Rojen et al., 2012). However, estimations based on fecal purines excretion suggest that only 11% to 77% of the increase in fecal N is due to increased fecal microbial N (Gressley et al., 2011, Westreicher-Kristen et al., 2018). The remaining excess has been attributed to either increased endogenous losses or the physical interference (e.g., increased vis-
creased Na⁺ should not represent a limiting factor for the increase in endogenous losses due to mucosal inflammation and damage contributing to the increase in fecal N.

In the current study, saline infusion was used as control during P1, whereas corn starch in P2 was diluted in water, which in retrospect we identify as a flaw in the experimental design, and something to consider when designing these experimental models. Although this could potentially affect the comparison between periods, the changes induced by the starch fermentation are of such magnitude that the influence of the solvent is likely negligible for most, if not all, of the parameters. Nevertheless, unexpected findings resulted from this set up, such as the substantial increase in fecal acetate and propionate concentrations during saline infusion. Without an apparent increase in fermentation, the increase in VFA during P1 could be understood as the result of decreased VFA absorption. In general terms, Na⁺ is a major counter-cation for VFA⁻, so increased Na⁺ should not represent a limiting factor for VFA absorption (Sellin, 1999; Stumpf, 2018). However, different mechanisms potentially explain how increased Cl⁻ could compete with VFA⁻ uptake. Increasing Cl⁻ concentrations have been shown to decrease acetate and propionate disappearance in washed reticulorumen experiments (Aschenbach et al., 2009). Further, Cl⁻ and VFA⁻ could be absorbed through the same anion transporters, so increased Cl⁻ concentrations could compete with VFA⁻ (Stumpf, 2018). Regardless of the underlying mechanism, the accumulation of VFA in the hindgut might have implications on intestinal health due to increased osmolarity (Aschenbach et al., 2019). In agreement, fecal DM content was decreased during saline infusion when compared with P2, suggesting that osmotic pressure was enough to pull water into the lumen. Incidentally, luminal hyperosmolarity increases paracellular permeability in the epithelium (Aschenbach et al., 2019). Whether saline infusion resulted in increased intestinal permeability in the current experiment and whether this led to systemic consequences is unknown. Without a proper negative control, it is not possible to determine if blood Cr recovery was increased during P1, as a basal uptake in response to the Cr-EDTA dosing in the abomasum was expected (Horst et al., 2020). However, if confirmed, the herein reported effects of hindgut acidosis could have been underestimated. Moreover, saline is commonly used in the literature as control and solvent in infusion studies, which calls to review the interpretation of the existing reports.

This small-scale study was designed to evaluate the suitability of the model to induce hindgut acidosis and obtain preliminary data to adapt future studies aiming to evaluate the consequences of high postruminal starch on hindgut health. In this pilot study, period and treatments were confounded, and thus only associations can be inferred between treatment and the observed responses. Further, technical challenges due to the starch suspension’s viscosity resulted in differences in daily infusion volumes and infusion regimens between experimental periods that likely affected the current results. Nevertheless, the magnitude of some of the changes (i.e., fecal parameters) and agreement with existing literature, provide confidence in the interpretation of the data. A properly controlled and powered study will be required to confirm if the observed increase in gut permeability can be solely attributed to increased postruminal starch digestion. In addition, the relative contribution of the different sections of the gut (e.g., small vs. large intestine) to this increase in permeability cannot be established based on the current experimental design. Some authors hypothesize that the hindgut is more vulnerable than the rumen to acidic conditions (Steele et al., 2016), which in combination with the drastic changes observed in this study on fecal parameters would support a substantial contribution from the hindgut. However, although traditionally overlooked and scarcely studied, there is a considerable microbial population in the small intestine, especially in the distal ileum, equally susceptible to acidosis (Plaizier et al., 2022). Similar to the hindgut, establishing the involvement of the small intestine to the systemic syndrome is critical to fully characterized the pathophysiology of SARA.

CONCLUSIONS

We herein describe the outcome of a pilot study evaluating the association between postruminal starch infusion and hindgut acidosis, characterized by local intestinal damage and increased permeability. We did not observe any evidence of systemic inflammation; however, the local consequences of hindgut acidosis are likely sufficient to affect whole-body energetics and nutrient partitioning. As previously described, BUN and MUN decreased during starch infusion likely as a results of increased N retention in the hindgut due to increased microbial proliferation. However, as previously pointed by other, increased endogenous N losses possibly contribute to the increase in fecal N, as suggested by the increase in fecal ALKP activity, a proxy for mucosal damage. Finally, the characterization of the temporal changes relative
to infusion showed the interference of saline with VFA absorption, which questions its value as control. Based on these findings, the use of saline as a control in future similar research should be carefully evaluated.

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