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Effects of phenylbutazone alone or in combination with a nutritional therapeutic on gastric ulcers, intestinal permeability, and fecal microbiota in horses

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Abstract

Background: Gastrointestinal (GI) injury and dysbiosis are adverse events associated with nonsteroidal anti-inflammatory drug (NSAID) use in horses. Phenylbutazone has been shown to alter GI barrier function both in vitro and ex vivo, but its effects on barrier function have not been assessed in vivo. In addition, the ability of nutritional therapeutics to prevent these changes is not known.

Objective: Our objectives were to determine whether (a) phenylbutazone affected barrier function in vivo and (b) if phenylbutazone-induced GI injury could be ameliorated by the use of a nutritional therapeutic.

Animals: Thirty healthy horses were randomly assigned to 3 groups (n = 10 per group): control, phenylbutazone, or phenylbutazone plus nutritional therapeutic.

Methods: This study was conducted as a blinded, randomized block design. All horses were managed identically throughout the study period. Samples were collected throughout the study period to monitor fecal microbiota changes and gastric ulcers before and after treatment. Quantification of the bacterial 16S rRNA gene in blood was used as a marker of intestinal permeability.

Results: Phenylbutazone increased amounts of bacterial 16S rDNA in circulation 3.02-fold (95% confidence interval [CI], 0.1.89-4.17), increased gastric ulceration score by a mean of 1.1 grade (P = .02), and induced specific changes in the microbiota, including loss of *Pseudobutyrivibrio* of family Lachnospiraceae. These changes were attenuated by nutritional treatment.

Abbreviations: AMP, antimicrobial peptide; ASV, amplicon sequence variant; CF, cell free; COX, cyclooxygenase; EGGD, equine glandular gastric disease; EGUS, equine gastric ulcer syndrome; GI, gastrointestinal; IACUC, Institutional Animal Care and Use Committee; IEC, intestinal epithelial cells; NSAID, nonsteroidal anti-inflammatory drugs; PCoA, principal coordinate analysis; PERMANOVA, permutational multivariate analysis of variance; PG, prostaglandin; RDC, right dorsal colon; ZO1, zonulin-1.

Conclusions and Clinical Importance: Collectively, these findings suggest that phenylbutazone induces GI injury, including impaired barrier function, and that nutritional treatment could attenuate these changes.

KEYWORDS
barrier function, gastric ulcers, horses, inflammation, NSAIDs

1 | INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most frequently used pharmaceuticals in the world.[1] In equine veterinary medicine, NSAIDs are commonly prescribed, with approximately 29% of horses across North America and the United Kingdom being prescribed an NSAID at some time.[2] Unfortunately, NSAID use in both people and animals is associated with numerous adverse events, with their negative effects on the gastrointestinal (GI) tract being among the most concerning. Specifically, within the GI tract of horses, NSAID use is associated with delayed healing after injury, gastric ulcers, and NSAID enteropathy, primarily affecting the right dorsal colon.[3-5] Routine dosages of both cyclooxygenase (COX) selective and nonselective NSAIDs can induce gastric ulceration, increase markers of intestinal injury, and alter the microbiota, even in the absence of clinical evidence of GI injury.[4,6]

The intestinal mucosal barrier is a critical feature of the GI tract that allows for selective passage of and barrier to luminal contents. In horses, mucosal disruption, and subsequent increased GI permeability, has been recognized in clinical syndromes such as intestinal ischemia and severe colitis. Increased intestinal permeability can occur in many conditions not necessarily associated with devitalized intestine or loss of GI structural integrity, and often is associated with GI inflammation. Increased intestinal permeability in people has been associated with several important health conditions including inflammatory bowel disease, liver disease, type 1 and type 2 diabetes, kidney diseases, chronic heart failure, and depression.[7] Whether increased intestinal permeability also occurs in the horse is unknown. Intestinal injury induced by NSAIDs, however, is a proposed mechanism by which increased permeability may occur even in the absence of clinical signs consistent with right dorsal colitis. In horses, this mild disruption has been demonstrated ex vivo using tissue harvested from horses and in vitro with cell culture techniques but not in vivo.[8,9] Therefore, our initial objective was to determine if routine use of phenylbutazone resulted in increased intestinal permeability in horses.

Administration of therapeutic doses of NSAIDs has been shown to result in dysbiosis, altering the GI microbiota of people and laboratory animals.[10-13] Furthermore, we have determined that both phenylbutazone and firocoxib induce GI inflammation, gastric ulceration, and dysbiosis in horses.[4,6] In addition, specific bacteria and their metabolites have been shown to enhance intestinal barrier function in both homeostasis and disease, and conversely, loss of such bacteria can result in increased intestinal permeability. Specific to NSAID administration, evidence indicates that both probiotics and microbiota-derived metabolites can prevent NSAID-induced intestinal inflammation in people and animal models.[13-15] It is unknown, however, if a similar approach could be successfully employed in horses. Therefore, our second objective was to document the effects of a commercially available nutritional therapeutic that targets the microbiota, among other features, on NSAID-induced intestinal injury and dysbiosis in horses.

2 | MATERIALS AND METHODS

2.1 | Study design

The protocol for this study was approved by the University's Institutional Animal Care and Use Committee (IACUC 2018-003). Thirty healthy adult horses from the university herd were utilized for this study. Triplets of horses were matched based on breed, age (±2 years), weight (±45 kg), and sex. One horse from each triplet was randomly assigned to 1 of 3 groups (n = 10 per group): control, phenylbutazone (ButaPaste, Henry Schein, Dublin, Ohio), or phenylbutazone plus nutritional therapeutic (Platinum Performance GI, Buellton, California; Supplemental Figure 1). Horses were separated, based on group assignment, and housed in separate but neighboring pastures. Feeding and pasture management practices were identical for all horses. Beginning 14 days before the start of the study, all horses were fed the same hay (coastal bermuda) from the same cutting, and continued to do so for the duration of the study. After this initial period of acclimation, the study spanned 68 days divided into 3 distinct phases: a 50-day pretreatment period (days 0-50), a 9-day treatment period (days 50-59), and a 10-day posttreatment period (days 59-68). For the entire duration of the study, horses in the nutritional therapeutic group were brought into individual stalls once daily, the nutritional therapeutic alone (147 g PO q24h) was provided in a bucket, and the amount consumed was recorded. After the 50-day pretreatment period, the treatment period began. Beginning on day 50, phenylbutazone paste (ButaPaste, Henry Schein, Dublin, Ohio) was administered (4.4 mg/kg PO q24h) for 9 days to both the phenylbutazone group and the phenylbutazone plus nutritional therapeutic group. The horses assigned to the control group were given an equivalent volume of placebo (base of phenylbutazone paste). The dosage of phenylbutazone was chosen based on label directions because this dosage frequently is used to manage common inflammatory conditions in horses (eg, osteoarthritis).[16-18] The posttreatment period continued for 10 additional days (ie, days 59-68). Physical examination variables including rectal temperature, heart rate, respiratory rate, and body weight using a calibrated electric scale were recorded on days 0, 50, 59, and 68 for all horses. Serum

albumin concentrations were determined at the beginning and end of the treatment period. Similarly, right dorsal colon (RDC) wall thickness was measured at the beginning and end of the treatment period by transabdominal ultrasonography. Specifically, a portable ultrasound system (Hitachi Aloka Noblus, Hitachi Healthcare Americas, 1995 Summit Commerce Park, Twinsburg, Ohio) and a convex array (1-5 MHz) probe with frequency set to 4 MHz was used. The RDC was imaged in the mid-abdomen (13–14th intercostal space) medial to the right lobe of the liver. Images were coded and the sonographer measured the thickness of the RDC offline using electronic calipers blinded to group, time point, and individual horse identity. Three images of the RDC were obtained for each time point and individual and the average of the 3 measurements used for analysis.

2.2 | Gastroscopy, fecal collection, and blood collection

Fecal samples were collected by rectal palpation using 1 rectal sleeve per animal on days 0, 50, 59, and 68. These days represented before the start of supplementation (day 0), after 50 days of supplement and before phenylbutazone (day 50), after 10 days of phenylbutazone (day 59), and 10 days after discontinuation of phenylbutazone (day 68). Feces were collected in a sterile container, immediately placed on dry ice, and transferred to a −80°C freezer for long-term storage.

Gastroscopy was performed for all horses on day 50 and day 59 as previously described.[19] Briefly, each horse was held off feed for 18 hours and water for 3 hours before gastroscopy. Horses were sedated using xylazine hydrochloride (0.4 mg/kg IV) and a 3-m endoscope was passed into the stomach. The entire stomach was examined, including the pylorus, and assigned a score by a single observer board certified in large animal internal medicine and blinded to treatment group. Visualization was improved by insufflating the stomach using room air and rinsing adherent food material from the mucosa. Squamous scoring was based on a previously published scoring system: 0 = intact normal mucosa; 1 = intact mucosa with reddening, hyperkeratosis or both; 2 = small single or small multifocal ulcers; 3 = large single or large multifocal ulcers; 4 = extensive (often coalescing) ulcers with areas of deep ulceration.[20] Glandular ulcers were scored using the same criteria as described for squamous ulcers (without consideration of lesion depth).

Whole blood (20 mL) was collected on days 50, 54, and 59 (ie, during phenylbutazone administration) from an aseptically prepared jugular vein. Blood was collected in EDTA and serum separator tubes and stored at 4°C until processing.

2.3 | Sample processing and data analysis

2.3.1 | Evidence of compromised GI barrier

Quantification of the bacterial 16S rDNA gene in blood has been used as a marker for loss of GI barrier function and bacterial translocation in people with inflammatory bowel disease and in animal models of GI disease.[21-23] We adapted this approach to examine bacterial translocation in horses during phenylbutazone administration. Briefly, cell-free (cf) DNA was isolated from 200 μL whole blood using a commercially available bead-based cfDNA isolation kit (Omega Mag-Bind cfDNA kit, BIO-TEK) according to manufacturer protocol. The 16S rDNA was quantified (relative abundance) using SYBR green qPCR according to established protocols with universal 16S rDNA primers (16S-27F and 16S-338R). The effects of treatment and day on the ratio of 16S rDNA relative to day 50 values were compared using linear mixed-effects modeling in S-PLUS using the lme package with an exchangeable correlation structure. All analyses were performed using S-PLUS (Version 8.2, TIBCO, Inc.) with significance set at $P < .05$.

2.3.2 | Gastric ulcer score analysis

Gastric ulcers were scored by a single observer blinded to treatment group. Squamous and glandular ulcer scores were analyzed using descriptive and inferential methods. For descriptive purposes, ulcer scores were plotted by horse, grouped by treatment in separate panels for each day. For inferential analysis, score data were rank transformed and analyzed using linear mixed-effects models with rank of squamous or glandular ulcer score as the dependent variable, and independent variables including fixed effects of day, treatment, and their bivariate interaction, and individual horse as random effects. Model fit was assessed by inspection of residual plots and by the Akaike information criterion (AIC) value. Post hoc comparisons of effects of day and treatment were determined using a previously described method.[24]

2.3.3 | Microbiota

The DNA extraction, 16S rRNA gene PCR, and sequencing were performed as previously described.[25] Briefly, fecal samples were removed from storage at −80°C and 200 mg of feces wereharvested from the frozen sample. Genomic DNA was extracted from fecal samples using a commercially available fecal DNA extraction kit (QIAamp Fast DNA Stool Mini Kit, Qiagen) according to manufacturer's protocol with slight modifications. Briefly, 200 mg of frozen feces were placed in a 2 mL tube containing 1 mL Inhibitex buffer and 50 mg each of sterile DNAase-free 0.1- and 0.5-mm silica zirconium beads. This material then was homogenized for 90 seconds at 6.5 m/sec using a FastPrep FP120 cell disrupter (Qbiogene, Carlsbad, California). The sample then was heated at 70°C for 10 minutes before following the manufacturer's protocol for DNA extraction. The DNA was suspended in tris-EDTA buffer (Integrated DNA Technologies, Coralville, Iowa) and stored at −80°C.

Amplification and sequencing of the V3-V4 variable region of the 16S rRNA gene were performed by Zymo Research. Briefly, a

library was prepared using a commercially available 16S rDNA prep kit (Zymo Research quick-16S NGS Prep Kit) according to the manufacturer's protocol. Samples were barcoded and PCR primers for the V3-V4 hypervariable region of the 16S rRNA gene were used. Sequencing was performed on a MiSeq (Illumina) following the manufacturer's guidelines. The software Quantitative Insights Into Microbial Ecology (QIIME2—ver 2019.1) (https://qiime2.org), dada2 (ver 1.6), and phyloseq (ver 1.28.0) were used for data processing and analysis.[26-28] Sequences were quality filtered and assigned to amplicon sequence variant (ASV) using dada2. Qiime2 was used to assign taxonomy to these ASVs against the Greengenes database (ver. gg_13_8) filtered at 97% identity for 16S rDNA sequences. Count tables with assigned taxonomy and phylogenetic trees constructed in QIIME2 were exported to R (ver. 3.6.1) where phyloseq, a software package that utilizes raw (ie, unrarefied) data, which preserves data from all samples thus retaining data even from samples with fewer reads, was used for further analysis.[27,29] Diversity metrics were calculated in phyloseq. Beta diversity was analyzed by visual assessment of principal coordinate analysis (PCoA) plots and by permutational multivariate analysis of variance (PERMANOVA) using phylogenetic (eg, UniFrac) distance metrics using the r package vegan (ver 2.5.5).[30,31] The ASV tables were collapsed to the genera level and differentially abundant genera were determined using count regression for correlated observations with the beta-binomial (corncob ver 0.1.0).[32] These analyses were performed for each

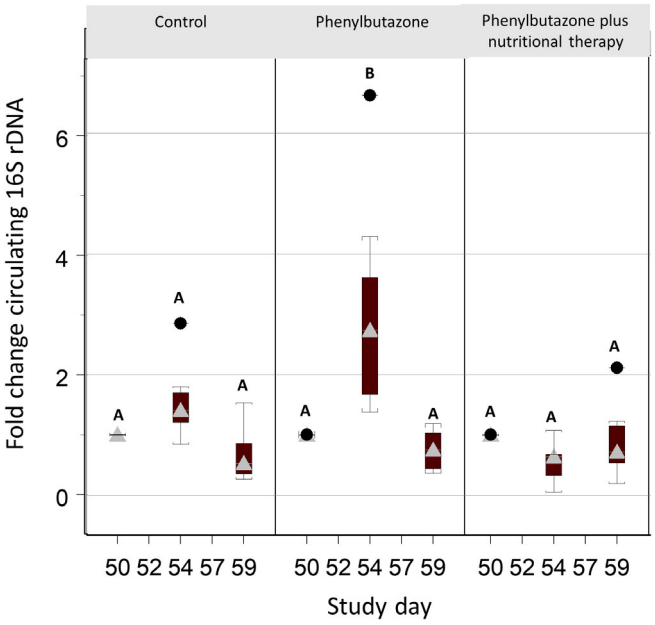

FIGURE 2 Box and whisker plots depicting fold change, relative to day 50) of 16S rDNA over the 10 days of phenylbutazone administration (study days 50-59). Triangles represent median values and horizontal black bars represent mean values. Values within columns with different letters differ significantly. There was a significant increase in the fold change of circulating 16S rDNA in the phenylbutazone group at day 54. No other time points were different among the groups

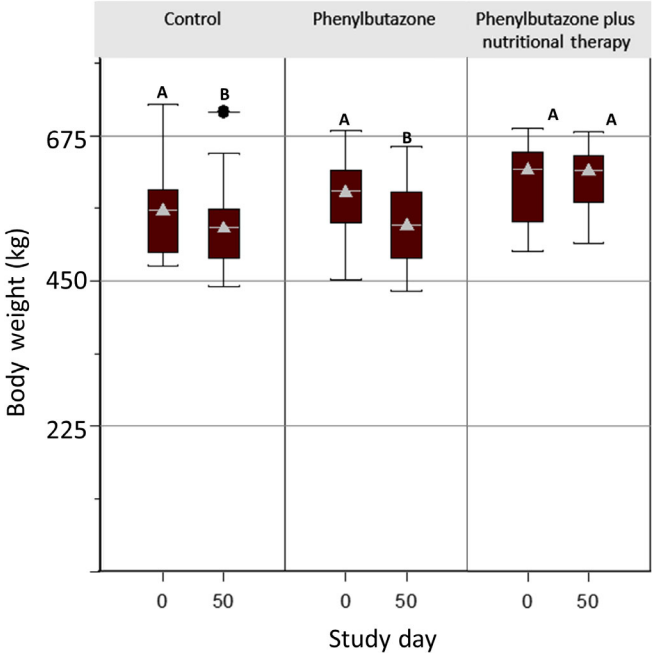

FIGURE 1 Box and whisker plots depicting body weights of horses in each group at days 0 and 50. Triangles represent median values and horizontal black bars represent mean values. Values within columns with different letters differ significantly. There was a significant decrease in the body weight of all groups except the phenylbutazone plus nutritional therapeutic group

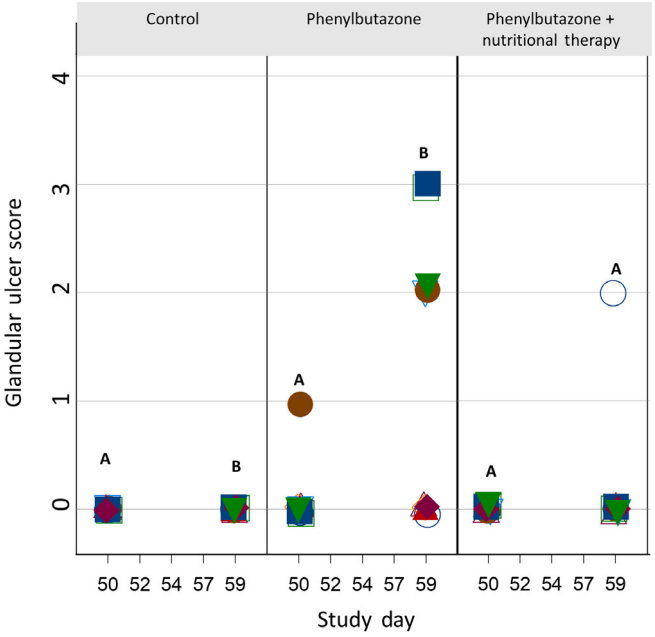

FIGURE 3 Scatter plot showing distribution of glandular ulcer scores with each point colored by the individual animal. Values within columns with different letters differ significantly. There was a significant increase in the gastric glandular ulcer scores in the phenylbutazone treated animals. No other changes were significant

group separately so as to follow changes over time and in response to treatments.

3 | RESULTS

3.1 | Horses

All horses included in the study were geldings. There was no difference in age among the groups ($P > .05$). The mean age in years ± SD for the control group, phenylbutazone group, and the nutritional therapeutic group were 14.7 ± 3.5, 14.8 ± 3.2, and 13.5 ± 3.4, respectively. The majority of horses were Quarter Horses, with the exception of 1 Warmblood in each group.

All horses completed the study with no evidence of negative effects related to phenylbutazone administration, with all vital parameters within normal limits at all recorded time points. All horses consumed 100% of the nutritional therapeutic each day. No differences were found in serum albumin concentrations associated with NSAID administration ($P > .05$). In addition, no significant differences were found in the thickness of the RDC wall among treatment groups associated with NSAID administration ($P > .05$; Supplemental Figure 2). The only significant change noted with animal health at any point in the study was body weight in the pretreatment period. Interestingly, in both the control group and phenylbutazone-treated group, a significant ($P = .001$ and $P = .002$, respectively) decrease in body weight was identified. Mean weight loss in the control group was 23.2 kg (95% CI, 17.6-28.8 kg) and 36.2 kg (95% CI, 16.1-56.2 kg) in the phenylbutazone-treated animals (Figure 1). Interestingly, no significant difference in body weight was found for the horses receiving the nutritional therapeutic during this time ($P > .05$; Figure 1).

3.2 | Circulating 16S rDNA

Relative amounts of 16S rDNA in circulation were determined by RT qPCR of bacterial 16S rDNA in whole blood during the treatment period (ie, days 50, 54, and 59, representing days 0, 5, and 9 of phenylbutazone administration). A transient increase in the fold change of circulating 16S rDNA was found in the phenylbutazone group on day 54 (ie, after 5 days of phenylbutazone; Figure 2). Relative to day 50, there was a 3.03-fold increase (95% CI, 1.89-4.17) in the amount of 16S rDNA in circulation, and this difference was significant ($P = .001$). No other significant changes over time were observed in any other groups.

3.3 | Gastric ulceration

Gastroscopy determined that phenylbutazone-induced gastric ulceration in the glandular mucosa, as expected and previously described. Specifically, 6 of the 10 horses developed glandular ulceration with a mean increase of 1.1 grades (range, 0-3; Figure 3).[4,33] Only 1 horse in the nutritional therapeutic group developed phenylbutazone-induced glandular ulceration. The difference in glandular ulcer scores was significant in the phenylbutazone-treated horses ($P = .02$) but not in the control and phenylbutazone plus nutritional therapeutic groups (Figure 3).

3.4 | Fecal microbiota

Very low frequency ASVs (ie, ASVs represented an average of fewer than 10 times across all samples) were removed. Data were not

TABLE 1 Beta diversity

Group	*F*-statistic	*P* value
Control	0.09	.02
Phenylbutazone	0.08	.098
Phenylbutazone plus nutritional therapeutic	0.1	.007

Note: Results of permutational multivariate analysis of variance on the unweighted UniFrac distance metric for each treatment group over time. Note the small magnitude of the *F*-statistic suggesting minimal change in the beta diversity.

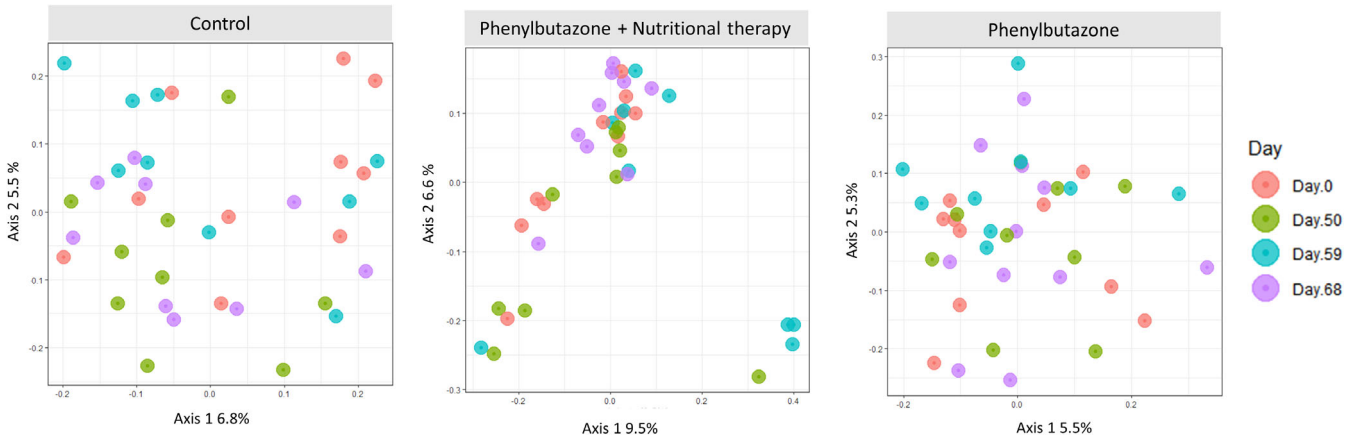

FIGURE 4 Principal coordinate analysis plots based on the unweighted UniFrac distance measure for each sample within each treatment group as indicated. Each time point is colored based on study day. Permutational multivariate analysis of variance based on this distance metric revealed significant ($P < .05$) differences over time although the magnitude of these differences was very small

TABLE 2 Differentially abundant taxa

Bacterial family and genus	Control	Bute	Bute + N.T.
Pre-treatment			
Lachnospiraceae_g__*Pseudobutyrivibrio*			*2.93E-02*
			−0.77
Lachnospiraceae_g__*Roseburia*			*8.54E-04*
			−1.48
Anaeroplasmataceae_g__*Anaeroplasma*	**2.00E-03**		
	1.23		
Bacteroidaceae_g__*BF311*		*1.94E-02*	
		−1.34	
Treatment			
Anaeroplasmataceae_g__*Anaeroplasma*	*9.99E-03*	*5.58E-03*	
	−0.28	*−0.39*	
Verrucomicrobiaceae_g__*Akkermansia*	*2.83E-06*	**1.36E-03**	
	−0.97	**2.46**	
Spirochaetaceae_g__*Treponema*	*1.12E-04*	*4.69E-02*	
	−0.84	*−1.50*	
[Paraprevotellaceae]_g__*CF231*	*3.06E-02*		
	−0.43		
Lachnospiraceae_g__*Roseburia*	*1.20E-04*		
	−1.60		
Lachnospiraceae_g__*Epulopiscium*		**4.69E-02**	
		0.92	
Lachnospiraceae_g__*Pseudobutyrivibrio*		*6.99E-04*	
		−2.37	
Post-treatment			
Anaeroplasmataceae_g__*Anaeroplasma*	*1.23E-02*	*3.57E-02*	
	−0.50	*−1.19*	
Verrucomicrobiaceae_g__*Akkermansia*	*5.83E-05*	*2.41E-02*	
	−2.08	*−1.79*	
[Paraprevotellaceae]_g__*CF231*	**1.67E-05**	**2.86E-02**	**3.49E-02**
	2.14	**1.58**	**1.39**
Lachnospiraceae_g__*Roseburia*	**5.83E-05**	**2.41E-02**	**3.06E-02**
	1.95	**1.84**	**0.58**
Lachnospiraceae_g__*Epulopiscium*		**4.69E-02**	
		0.92	
Lachnospiraceae_g__*Pseudobutyrivibrio*		*6.99E-04*	**4.30E-05**
		−2.37	**2.11**
Clostridiaceae_g__*Sarcina*		*3.57E-02*	
		−4.58	
Veillonellaceae_g__*Phascolarctobacterium*	**9.37E-03**		
	0.80		
Prevotellaceae_g__*Prevotella*	*2.68E-02*		
	−0.43		

Note: Significantly differentially abundant genera (and associated family) within each treatment group for each phase of the study. The top number within each cell represents the FDR *P* value and the lower number indicates the log(2)-fold change. The bold and italic values represent whether there was an increase (bold) of a specific genera or loss (italics) from the beginning to end of each study phase.

normalized (ie, rarefied) before analysis based on current recommendations.[29] Each treatment group was analyzed independently to assess how the fecal microbiota changed over time in response to treatment (ie, control, phenylbutazone, or phenylbutazone plus nutritional therapeutic).

Principal coordinate analysis (PCoA) plots based on the unweighted UniFrac distance metric indicated no apparent visual clustering of the samples by time within any group (Figure 4). Permutational multivariate analysis of variance on the unweighted UniFrac distance metric was performed for each treatment group to quantitatively assess beta diversity. This analysis identified a significant difference in the beta diversity over time in both the control and phenylbutazone plus nutritional therapeutic groups. The magnitude of these findings, however, was very small (Table 1) and related to day 0 vs day 50 rather than the NSAID treatment period (day 50 vs day 59). The same analyses were performed on the weighted UniFrac distance metric and no differences were found among groups. Similarly, no differences were found in the Shannon alpha diversity index over time within any group ($P > .05$).

We next examined the microbiota data to determine if any genera of bacteria were differentially abundant between the beginning and end of each phase of our study (ie, pretreatment—days 0 to 50, treatment—days 50 to 59, and posttreatment—days 59 to 68). Across all groups, only 4 genera were differentially abundant in the pretreatment period. Seven genera were differentially abundant in the treatment period, with continued changes in the posttreatment period. Interestingly, no differentially abundant genera were found in the phenylbutazone plus nutritional therapeutic group in the treatment phase, and minimal changes were identified in both the pretreatment and posttreatment phases (Table 2).

4 | DISCUSSION

We determined that a clinically recommended dosage and duration of phenylbutazone resulted in evidence of transient impaired intestinal barrier function. The clinical implications of this finding remain unclear but, given the known importance of impaired intestinal barrier function in people, this transient impairment could have important health implications for the horse. Importantly, we used healthy adult horses; the same impairment in compromised or very young horses may have serious clinical implications. Coadministration of the nutritional therapeutic prevented the phenylbutazone-associated increase in intestinal permeability.

The intestinal barrier is a complex system with several features that provide protection against microbial invasion and other unwanted components of the GI lumen. These features include antimicrobial peptides (AMPs), a mucus layer, commensal bacteria, and a single layer of intestinal epithelial cells (IECs) that are sealed by tight junction proteins including zonulin-1 (ZO-1), which prevent paracellular passage. Although not well studied in horses, strong evidence exists that NSAIDs can disrupt nearly all components of the mucosal barrier of both the upper and lower GI tract. For example, NSAIDs are well known to induce IEC cell death because of their topical effects. Loss of IECs then leads to increased intestinal permeability.[34] In addition, the mucus layer of the GI tract is a major component of the intestinal barrier. Mucus production is regulated in part by prostaglandins (PG), specifically PGE2. Cyclooxygenase (COX)-1 contributes to PGE2 production, and therefore nonselective NSAIDs, such as phenylbutazone, may induce increased mucosal permeability by loss of PGE2-mediated mucus production.[35] In addition, NSAIDs may decrease intestinal AMP secretion, an important component of the intestinal barrier, by altering the function of Paneth cells.[36] Finally, NSAIDs also have been shown to interfere with barrier function by altering the proteins involved with tight cell junctions. One such example is related to the production of reactive oxygen species (ROS). The ROS produced by mitochondria have been shown to alter the expression of ZO-1, resulting in increased mucosal permeability.[37]

Quantifying the relative amounts of 16S rDNA in circulation has been used as a marker of intestinal barrier function in both clinical and research models of GI diseases including inflammatory bowel disease (IBD).[21-23] To our knowledge, this approach has not been used previously in horses. The transient (day 54 only) increase in mucosal permeability is interesting. We expected increased mucosal permeability to be most severe after the full 10 days of phenylbutazone administration. The reasons for the transient nature of this finding are unclear. One possibility is that increased mucosal permeability resulted in adaptive responses by the host to repair the mucosal barrier (ie, increased production of mucus, AMPs, or increased expression of tight cell junction proteins). Others have identified adaptive responses of both the host and the microbiome to NSAIDs that prevent worsening disease severity.[38] This adaptive response and repair of mucosal barrier may have occurred in the healthy horses in our study. The horses used in our study had no clinical signs of GI injury or evidence of GI injury based on ultrasonographic findings and serum albumin concentration. It is possible that horses with more severe NSAID-induced injury could have had prolonged evidence of increased mucosal permeability.

Equine gastric ulcer syndrome (EGUS) is a frequently encountered disease in horses that results in high morbidity, loss of use, and high cost of diagnosis and treatment to the owner.[20] Although the causes of EGUS are unknown, NSAIDs have long been associated with inducing gastric ulcers in people and other animal species, specifically equine glandular gastric disease (EGGD) in horses.[4,33] The best approach to prevent NSAID-induced ulcers in both people and animals is unclear. One proposed strategy is the administration of proton-pump inhibitors (PPIs; eg, omeprazole) concurrent with NSAIDs. Although not yet investigated in horses, in people evidence exists that coadministration of PPIs with NSAIDs exacerbates the severity of NSAID-induced lower GI injury.[39] Use of COX-selective inhibitors is another commonly used approach and, although COX-2 selective NSAIDs are associated with less severe gastric ulcers in horses, they do in fact still induce gastric injury.[4] Here, we determined that the nutritional therapeutic used significantly decreased phenylbutazone-induced glandular ulcers, but the mechanism by which this occurs is unclear.

The nutritional therapeutic we administered contains many components, including omega-3 fatty acids, antioxidants, vitamins, trace

minerals, di-tri-octahedral smectite (ie, Bio-Sponge), prebiotics (eg, mannan oligosaccharides, probiotics such as *Saccharomyces boulardii* and *Saccharomyces cerevisiae*), and glutamine among other components. Given the complexity of the mucosal barrier, the many features of the barrier thought to be disrupted by NSAIDs, and the many bioactive molecules in the therapeutic we provided, it is challenging to identify the mechanism by which loss of barrier function was prevented in these horses, but several plausible hypotheses exist. For example, biologics targeting tumor necrosis factor-α (TNF-α) have been shown to decrease the severity of NSAID-induced intestinal injury.[40] Omega-3 fatty acids have been shown to decrease proinflammatory cytokines, specifically TNF-α, in animal models of IBD and some studies have shown improvement in clinical measures of IBD in people.[41,42] Similarly, substantial data support the anti-inflammatory properties of glutamine in both people and animal models of GI inflammation, including NSAID-induced intestinal inflammation and intestinal barrier function of horses independent of NSAIDs.[43-45] Finally, synergistic effects related to the combination of the components of the nutritional therapeutic may have played a protective role.

We also identified phenylbutazone-induced alterations in the fecal microbiota, as previously reported.[6] Interestingly, coadministration of the nutritional therapeutic appeared to stabilize the fecal microbiota during the period of phenylbutazone administration. In our study, microbiota changes were not limited only to the phenylbutazone group but also were noted in the control group, albeit with differences in specific genera and direction of change (ie, gain or loss). The reasons for the changes in the control group are unclear but may be related to the fact that these horses were held off feed for gastroscopy. Diet, and specifically withholding food, has been shown to alter the fecal microbiota.[46,47] These same changes were not observed in the group receiving the nutritional therapeutic. The clinical implications of these changes remain unclear but hold merit for further study. Previous studies have shown that the effects of NSAIDs on the microbiota are characterized by loss of members of the Firmicutes phylum and notably the family Lachnospiraceae.[6] Of the changes observed in our study, *Pseudobutyivbrio* of family Lachnospiraceae is of particular interest. Loss of this genus has been associated with inflammatory diseases in people and it is a known producer of butyrate, a short chain fatty acid that is an important energy source for IECs and has anti-inflammatory activity in the GI tract.[48-50] Therefore, loss of this genus may have contributed to NSAID-induced intestinal inflammation.

The mechanisms by which phenylbutazone and other NSAIDs alter the microbiota are unknown. One possibility is that ROS released from IECs after NSAID-induced mitochondrial damage contribute to this dysbiosis, because ROS molecules released during intestinal inflammation have been shown to induce dysbiosis.[51] With this mechanism, any treatment that inhibits or decreases NSAID-induced inflammation and cell death will prevent these changes. Thus, if the nutritional therapeutic we provided prevented inflammation, then effects on the microbiota also would be attenuated.

Our study had several limitations. First, our study involved a relatively small sample size of healthy horses with similar signalment. Whether or not the same findings would be observed in a larger study and in horses with illness is unknown. Second, we used relative amounts of 16S rDNA in circulation as a marker of impaired intestinal barrier function. This assay has been used in people and other animal models to indicate increased intestinal permeability, but our study represents the first use of this assay in horses. In addition, the source of the increased 16S rDNA that we observed is unknown, and may reflect either upper or lower GI injury. We performed gastroscopy to document the effects on the upper GI tract but, to our knowledge, there is currently no effective or validated means of documenting subclinical lower GI mucosal injury in a noninvasive manner. Therefore, the source of 16S rDNA is unknown and it could be solely related to upper GI injury, because EGGD was prevented in horses receiving the nutritional therapeutic and that group also had decreased 16S rDNA as compared with phenylbutazone-treated horses. Also, we scored EGGD using the same scoring system used for EGUS because the relationship between any of the reported EGGD scoring systems to histopathologic diagnosis of EGGD remains unknown, and our previous studies have had similar findings, regardless of the scoring system used.[4,52] Additionally, we used fecal samples to examine the microbiota. Some data suggest that fecal samples fail to reflect the microbiota of the upper GI tract and the mucosal associated microbiota, both of which are important in the context of NSAID-induced GI injury. Finally, we used 16S rDNA sequencing that has several well-recognized limitations primarily related to deeper taxonomic resolution. Other approaches to query the microbiota (ie, whole genome sequencing) would have allowed a more complete understanding of the community and function of the microbiota in our study.

Taken together, our findings suggest that phenylbutazone resulted in increased permeability of the GI mucosa leading to transient bacterial translocation, induced gastric ulcers, and altered the fecal microbiota. Each of these effects was prevented or attenuated by feeding a commercially available nutritional therapeutic before and during phenylbutazone administration. The clinical relevance of the phenylbutazone-induced changes we observed is unclear. The exact cellular and molecular mechanisms by which the nutritional therapeutic prevented these changes also is unclear. Deciphering the complex interaction of phenylbutazone, GI inflammation, and components of the nutritional therapeutic is the focus of ongoing and future work.

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CONFLICT OF INTEREST DECLARATION

The potential conflicts related to this study include:

1) Platinum Performance has provided financial support to our laboratory in the form of donations for gastroenterology research.
2) Dr. Whitfield-Cargile has spoken at educational meetings that Platinum Performance has organized, and received honoraria for participation in 1 in-person meeting and 1 webinar.
3) Platinum Performance provided gifts to members of our laboratory and routinely donates these items to our VMTH.

These potential conflicts we managed in the following ways:

1) Platinum Performance has provided financial support (as a research gift) to our laboratory. They have had no control of this study or any other research conducted in our laboratory. Study design and execution were the sole responsibility of the authors of this study. In addition, Platinum Performance has had no input on what we chose to publish regardless of our laboratory's findings.
2) All horses and samples were blinded for all data in this manuscript. For example, the author performing gastroscopy and scoring gastric ulcers was blinded to treatment group and has no personal conflicts of interest with Platinum Performance.
3) All samples (blood and feces) were coded in a way that prevented anyone from knowing the group assignment until data were analyzed.
4) A placebo paste was used to further blind all study personnel in the study as to group assignment.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by Texas A&M University IACUC (number 2018-003).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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