Multi-dose misoprostol pharmacokinetics and its effect on the fecal microbiome in healthy, adult horses

by

Rachel Lynn Pfeifle

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 5, 2023

Keywords: misoprostol, pharmacokinetics, fecal microbiome, horses

Approved by

Kara M. Lascola, Chair, Associate Professor, Department of Clinical Sciences
Erin S. Groover, Clinical Professor, Department of Clinical Sciences
Anne A. Wooldridge, Professor and Department Head, Department of Clinical Sciences
Aaron Ericsson, Assistant Professor, Department of Veterinary Pathobiology, University of Missouri

Abstract

Misoprostol, a synthetic prostaglandin E_1 (PGE_1) analogue, is administered to treat glandular gastric ulcers in horses and may possess anti-inflammatory properties. However, misoprostol's multi-dose pharmacokinetics and effects on the fecal microbiome in horses require investigation. Our objectives were to compare the pharmacokinetics between repeated doses and to characterize changes in the fecal microbiome after oral and rectal multi-dose misoprostol administration in 6 healthy university-owned geldings. In a randomized, cross-over study, misoprostol (5 μg/kg) was administered orally or rectally every 8 hours for 10 doses, or not administered (control), with a 21-day washout between treatments. Concentration-versus-time data for dose 1 and dose 10 were subject to non-compartmental analysis. For microbiota analysis using 16sRNA amplicon sequencing, manure was collected at -7 days, immediately prior to dose 1, then 6 hours, 7 days, and 14 days after dose 10, with time-matched points in controls. Repeated dosing related differences in pharmacokinetic parameters were not detected for either administration route. Area under the concentration-versus-time curve was greater ($p < 0.04$) after oral versus rectal administration. Relative bioavailability of rectal administration was 4-86% that of oral administration. Microbial composition, richness, and β-diversity differed among subjects ($p < 0.001$ all) while only composition differed between treatments ($p \leq 0.01$). Richness was decreased 6 hours after dose 10 and at the control-matched timepoint ($p = 0.0109$) in all subjects. No other differences for timepoints, treatments, or their interactions were observed. Overall, differences in systemic exposure were associated with route of administration, but were not detected after repeated administration of misoprostol. Differences in microbiota parameters were primarily associated with inter-individual variation and management rather than misoprostol administration.

Acknowledgments

This project was funded by the **2019 Animal Health and Disease Research Grant** and the **2020 American College of Veterinary Internal Medicine Resident Research Grant**.

This project would not have been possible without help from so many different individuals and teams. A heartfelt thank you goes out to **Dr. Kara Lascola**, for her direction, guidance, and mentorship while I was planning, organizing, and completing this clinical research project. To **Dr. Dawn Boothe**, for her expertise and help in analyzing our pharmacokinetic data. To **Dr. Aaron Ericsson**, for his knowledge and instruction in handling and interpreting our microbiome data. To **Drs. Tamara Sierra-Rodriquez, Annette McCoy, and Jennifer Reinhart** for their guidance and appraisal on this project and our published manuscript. To **the Auburn University equine technicians, staff, and student workers**, for their help looking after my study subjects throughout sample collection. To **Jessica Brown, Taylor Moss, and Haejin Kim** for their technical help during sample collection and initial processing. To **all of the staff at the Auburn University Clinical Pharmacology Laboratory**, for their technical help with our pharmacokinetic sample processing. To **all of the staff at the University of Missouri DNA Core Facility and Informatics Research Core Facility** for their technical help with our microbiome sample processing. To my mentors in equine medicine, **Dr. Rocky Mason**, and in equine internal medicine, **Drs. Erin Groover, Anne Wooldridge, and Mariano Mora** for their tremendous impact on me as a clinician. To all of my resident-mates, **Shun Kimura, Serena Ceriotti, Patricia Egli, and Lana Dedecker**, for their friendship and encouragement throughout my project and residency program. To my parents, **Mike and Sharon Roberson**, who demonstrated to me the meaning of hard work and have supported me in every single one of my endeavors. To my brother, **Alex Roberson**, for setting an example in persevering to achieve your

dreams. To my twin sister, **Dana Pryor**, for being right by my side through every stage of life and always inspiring me to more caring, kind, and compassionate like her. To my husband, **Adam Pfeifle**, for his loving support and continuous encouragement as I followed my dreams. You have seen me through every challenge and frustration, and also through every success and celebration. Finally, above all else, I am thankful to **God** for his immeasurable blessing upon my life and work. May each step I take reflect Your instruction in Colossians 3:23 “Whatever you do, work at it with all your heart, as though you are working for the Lord and not for people.”

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ANOVA Analysis of variance

AUC Area under the curve

$AUC_{0\rightarrow last}$ Area under the concentration-versus-time curve to the last sample timepoint

$AUC_{0\rightarrow\infty}$ Area under the concentration-versus-time curve to infinity

AUC_{tao} Area under the concentration-versus-time curve to the dosing interval

AUC_{extrap} The percentage extrapolated from the terminal component of the $AUC_{0\rightarrow\infty}$ curve

$AUC_{taoextrap}$ The percentage extrapolated from the terminal component of the AUC_{tao} curve

c-AMP Cyclic adenosine monophosphate

C_{max} Peak plasma drug concentration

COX Cyclooxygenase

CV Coefficient of variation

EGGD Equine glandular gastric disease

F Relative bioavailability

LC-MS/MS Liquid-chromatography-tandem mass spectrometry

LLOD Lower limit of detection

LLOQ Lower limit of quantification

LPS Lipopolysaccaride, endotoxin

MFA Misoprostol free acid

MRT Mean residence time

MU University of Missouri

NSAID Non-steroidal anti-inflammatory drug

PCoA Principle coordinate analysis

PERMANOVA	Permutation multivariate analysis of variance
PGE_1	Prostaglandin E_1
PO	Per os, oral administration
PR	Per rectum, rectal administration
SD	Standard deviation
SIRS	Systemic inflammatory response syndrome
t_{max}	Time to peak plasma drug concentration
$t_{1/2}$	Half-life (disappearance value)
$1/\lambda$	Elimination rate constant (disappearance value)

Chapter 1: Literature Review

Section 1: Introduction and Justification

Endotoxemia, defined as the presence of endotoxin (lipopolysaccharide, LPS) in the blood after proliferation or death of Gram-negative bacteria,[1-6] and/or bacteremia, defined as bacteria in the blood, can lead to the release of inflammatory mediators. In horses, these inflammatory mediators can trigger a life-threatening state called systemic inflammatory response syndrome (SIRS).[1,2,4,5] This condition is associated with multiple diseases in horses, particularly gastrointestinal diseases.[1,3,4] Significant increases in morbidity and mortality are associated with SIRS, because this condition often results in life-threatening secondary conditions.[1,3,5,6]

Preventing or reducing inflammation is essential for managing SIRS. Unfortunately, treatment options for horses are limited, with flunixin meglumine and polymyxin B representing the mainstays of therapy.[1,4,5] Flunixin meglumine is a non-selective cyclooxygenase (COX) inhibitor that inhibits prostanoid synthesis to provide anti-inflammatory and analgesic effects.[7] While flunixin meglumine improves some consequences of SIRS, it does not inhibit production of key inflammatory mediators,[1,5,8,9] and is associated with adverse effects including gastrointestinal ulceration and nephrotoxicity.[7,9,10] Polymyxin B, a cationic polypeptide antibiotic, binds LPS and prevents the inflammatory cascade.[4,5,8,11] However, since polymyxin B only targets free LPS, its anti-inflammatory effects are limited once LPS initiates the inflammatory cascade. While these medications are useful in some regards, there remains a clear need to identify alternative or complementary medications with effective *in vivo* anti-inflammatory activity.

Misoprostol, a synthetic prostaglandin E_1 (PGE_1) analogue and E2, E3, and E4 prostanoid receptor subtype agonist, is approved in humans for the prevention of gastric and duodenal injury related to nonsteroidal anti-inflammatory drug (NSAID) use,[12] and is recommended in horses for treatment of equine gastric glandular disease (EGGD) and NSAID-induced colitis.[13,14] In addition to its recommended uses, misoprostol may also hold promise as a potential anti-inflammatory medication for the management of SIRS in horses. While numerous *in vitro* and *ex vivo* studies have described the anti-inflammatory effects of misoprostol through cytokine and cyclic adenosine monophosphate (c-AMP)-mediated pathways,[15-19] its anti-inflammatory potential in horses is less clear. A recent study investigating the administration of single dose misoprostol orally or rectally in healthy adult horses challenged with LPS identified appreciable changes in select inflammatory cytokines.[20] A critical next step in the evaluation of misoprostol involves obtaining clinically useful multi-dose pharmacokinetic data in order to make more informed decisions regarding misoprostol dosing regimens. Additionally, because the drug has the potential to alter gastrointestinal physiology, misoprostol's potential to affect the gastrointestinal microbiota should be considered and further evaluated.

Section 2: Misoprostol Pharmacokinetics

Subsection 2a: Misoprostol Pharmacokinetics in Humans

The pharmacokinetics of misoprostol have been described in humans for a variety of routes of administration, including oral, sublingual, buccal, transvaginal, and transrectal, with pharmacokinetic profiles varying widely based on the route.[21-24] Following administration, misoprostol is quickly de-esterified to its active metabolite, misoprostol free acid (MFA).[21,22] MFA has varying bioavailability depending on the route of administration and degree of protein-binding. MFA is typically 81-89% protein-bound, with the majority of protein-binding occurring with albumin.

Following oral administration in humans, misoprostol appears to be rapidly absorbed with a maximum plasma concentration (C_{max}) occurring within 12-30 minutes and a reported half-life of 20-40 minutes. However, it appears that a fed versus fasted state affects the pharmacokinetic properties of orally administered misoprostol in people. In particular, the rate of absorption and bioavailability can decrease in fed people as compared to fasted people.[25]

The route of administration also has an impact on misoprostol pharmacokinetics in humans. One study revealed that sublingual administration had higher bioavailability as compared to oral and transvaginal administration.[23] Additional studies demonstrated that oral administration had a faster time to C_{max} (T_{max}) and shorter disappearance half-life ($T_{1/2}$) as compared to trans-rectal administration.[24] Although transrectal and transvaginal administration yielded longer T_{max} and $T_{1/2}$ compared to other routes, they did not lead to a significant change in bioavailability compared to oral administration.[21,22]

Subsections 2b: Misoprostol Pharmacokinetics in Horses

Single dose pharmacokinetics of misoprostol at a dose of 5 µg/kg have been described after oral administration (per os, PO) in corn oil in fasted horses, and after PO and rectal administration (per rectum, PR) in water in fed and fasted healthy horses.[15,26] The dose of 5 µg/kg is extrapolated from human misoprostol pharmacokinetic studies, in which adult subjects are most often administered 400 µg (total dose, not per kilogram).[23,27] The dose of 5 µg/kg has also been commonly accepted to be effective for treating certain gastrointestinal conditions in horses when given two to three times per day.[14,28] Comparisons between the single dose pharmacokinetic studies in healthy horses are presented in **Table 1**. The reported C_{max} in fasted horses receiving misoprostol PO in corn oil was much lower than in fasted horses receiving misoprostol PO in water.[15,26] Additionally, the C_{max} in both fasted and non-fasted horses receiving misoprostol PO in water was much lower than in horses receiving misoprostol PR in water.[26] T_{max} values reported in horses administered misoprostol PO in water and corn oil were similar, while horses administered misoprostol PR in water had a significantly shorter T_{max}.[15,26] Finally, when evaluating area under the concentration-versus-time curve (AUC), values were highest in fasted horses administered misoprostol PO in water, followed by fed horses administered misoprostol PO in water, and then lowest in fasted horses administered misoprostol PO in corn oil and horses administered misoprostol PR in water.[15,26] While pharmacokinetic profiles vary between routes of administration and carrier vehicle, reported plasma concentrations for both routes and vehicles were comparable or superior to those reported in humans.

Overall, the presence of food in the gastrointestinal tract appears to have a strong influence on the absorption and pharmacokinetic profile of orally administered misoprostol.

Fasted horses receiving the drug in water demonstrated a greater C_{max}, $T_{1/2}$, and AUC as compared to fed horses receiving the drug in water or even fasted horses receiving the drug in corn oil.[15,26] This suggests that the presence of food (such as hay or corn oil) reduces the bioavailability of orally administered misoprostol. This could be due to alterations in the gastric emptying/gastrointestinal motility, drug binding to food, and/or changes in physiologic factors such as gastrointestinal pH between the fasted and fed states, which also happens with other medications such as gastroprotectants, anthelminthics, antimicrobials, and NSAIDs.[29-33]

A more recent study evaluated the pharmacokinetics of a single 5 μg/kg dose of misoprostol administered to fasted horses PO in water and horses PR in water that were experimentally challenged with endotoxin (LPS) at a 20 ng/kg dose. Select pharmacokinetic parameters as compared to the earlier single-dose study are presented in **Table 2.** Similar differences in parameters occurred between PO and PR administration in both studies. However, C_{max} concentrations and AUC were interestingly increased in the horses experimentally challenged with LPS that were receiving misoprostol PO as compared to healthy horses receiving misoprostol PO, suggesting that inflammation may also influence oral drug absorption.[20,26]

In equine clinical practice, misoprostol is typically administered for several days to weeks at a suggested dose of 5 μg/kg orally or rectally every 8 to 12 hours.[14] To date, the pharmacokinetics of multi-dose misoprostol have yet to be described in the horse. Since the pharmacokinetic behavior of a drug can change with repeated administration, describing multi-dose pharmacokinetics of misoprostol administered orally and rectally is an essential step for optimizing misoprostol administration protocols in horses.

	Martin et al. EVJ 2019	Lopp et al. AJVR 2019		
Route/Vehicle	PO-Fasted Water + Corn Syrup	PO-Fasted Water	PO-Fed Water	PR Water
T_{max} (hr)	0.39 ± 0.04	0.25 (0.17-0.75)	0.30 (0.08-1.5)	0.08
C_{max} (pg/mL)	290 ± 70	655 ± 259	352 ± 109	967 ± 492
$AUC_{0\rightarrow\infty}$ (pg*hr/mL)	400 ± 120	2,217 ± 955	1358 ± 891	385 ± 153
$t_{1/2}$ (hr)	0.67 ± 0.20	4.13 ± 3.4	2.53 ± 1.73	0.53 ± 0.27

Table 1: Comparison of select pharmacokinetic parameters from two investigations of single-dose misoprostol administration to healthy horses by a variety of delivery strategies.[15,26]

	Kimura et al. AJVR 2022		Lopp et al. AJVR 2019	
	PO-Fasted	PR	PO-Fasted	PR
T_{max} (min)	25 (10-45)	3 (3-5)	21 (10-45)	5
C_{max} (pg/mL)	5209 ± 3487	854 ± 855	655 ± 259	967 ± 492
$AUC_{0\rightarrow\infty}$ (hr*pg/mL)	4999 ± 3665	179 ± 155	2217 ± 955	385 ± 153
$t_{1/2}$ (min)	40 ± 21	9 ± 7	170 ± 129	21 ± 24

Table 2: Comparison of select pharmacokinetic parameters from two investigations of single-dose misoprostol administration to healthy horses and LPS-challenged horses by a variety of delivery strategies.[20,26]

Section 3: The Equine Gastrointestinal Microbiome

In order to review and comprehend previous publications relating to the equine gastrointestinal microbiome, a general understanding of some common microbial ecology concepts is vital. **Appendix I** reviews the definitions of a few of the most important terms utilized in the literature and throughout the remainder of this work.

The gastrointestinal microbiota and microbiome have been previously recognized for their importance in the breakdown and utilization of complex nutrients as well as protection from overgrowth of pathogenic organisms. As hindgut fermenters, horses have a unique digestive tract, which relies on anaerobic fermentation within the large intestine. Although polymicrobial populations reside throughout the entire gastrointestinal tract, the complex microbial community responsible for the aforementioned fermentation primarily exists within the cecum and large colon. The bacterial population of the large intestines is thought to be most important in maintaining the homeostasis of this environment.[34] Particularly important in the horse, fibrolytic bacteria breakdown complex carbohydrates to produce short-chain fatty acids that supply most of the animal's energy requirements.[35,36]

It is important to glean an understanding of the normal, healthy microbiota in horses, in order to allow for assessment of the abnormal. However, it is important to realize that this "normal" population can have marked inter-horse variation. Additionally, a wide variety of factors such as geographic region, nutrition, breed, age, and management, can influence the composition of the microbiota in healthy horses. As a result, a generalized assessment of the microbial population is required.

Similar to other mammals, Firmicutes is the primary bacterial phylum noted in the distal gastrointestinal tract of horses. In the literature, some studies show Bacteroidetes as the second

most abundant phylum,[37-40] while others show Verrucomicrobia,[41-43] as depicted in **Figure 1**. Shifts in Firmicutes and Bacteroidetes populations have been observed in the literature in response to certain factors such as nutritional changes, housing and management changes, various gastrointestinal pathologies, and stress-related factors like transportation, although these shifts are often inconsistent.[44]

As evaluation of phyla only provides a high-level overview of the microbiota, concurrent evaluation of lower taxonomic levels should also take place. Decreases in *Lachnospiraceae*, *Ruminococcaceae*, and other butyrate-producing bacteria (important contributors to intestinal homeostasis) or increases in *Lactobacillus* and *Streptococcus* (lactic acid-producing bacteria), have been considered undesirable changes in the microbiota because they are often associated with gastrointestinal dysbiosis. Additionally, overall decreases in microbial richness and/or diversity are also considered an undesirable change.[44]

As with many species, the intestinal microbial population of horses is influenced by many factors. The membership and structure, especially at lower taxonomic levels (e.g., species and genus) can be affected by age, diet, environment (geographic location, climate, and season), management (continuous vs. meal feeding, fasting, exercise, and transportation), medication administration, and multiple disease states.[34,44] For the purposes of this review, the effects of diet, gastrointestinal disease, and drug administration will be discussed.

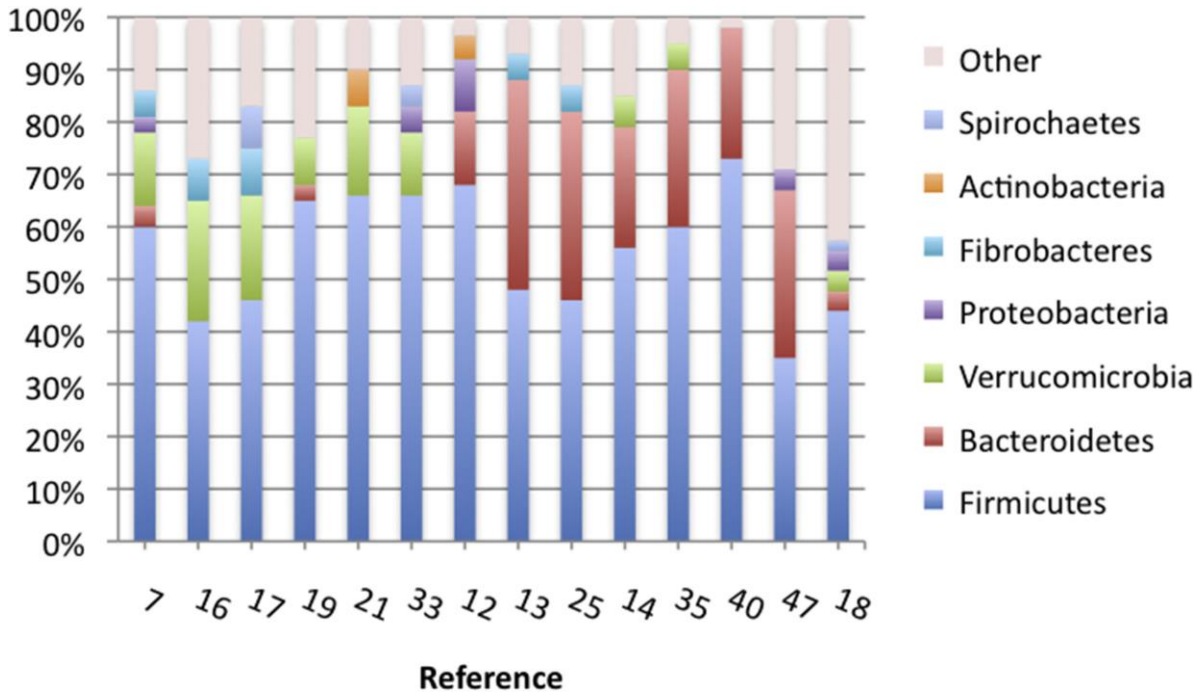

Figure 1: Relative abundance of the main phyla reported in feces of healthy horses in numerous studies. Reprinted from Costa & Weese 2018,[34] with permission from Elsevier.

Subsection 3a: Diet and the Microbiome

Evolutionarily, horses are adapted to graze continuously, allowing for a continuous intake of complex carbohydrates as roughage. However, active performance horses often require supplementation with more readily digestible carbohydrates. As expected, marked differences are reported in the microbiota of horses on forage-based diets versus carbohydrate-supplemented diets.[45-49] Forage based, less nutrient dense diets are reported to increase microbial diversity and stability.[49,50] In contrast, the high-nutrient availability of carbohydrate supplemented diets is reported to reduce microbial diversity.[48-50] Ecological theories suggest this decreased diversity leads to a microbial community that is less stable and more prone to gastrointestinal dysbiosis.[51] It is proposed that this is due to a decrease in the proportion of fibrolytic bacteria (genus *Fibrobacter* and family *Ruminococcaceae*), where lactic-acid producing bacteria (group *Bacillus-Lactobacillus-Streptococcus*) were increased in horses on a concentrate supplemented diet.[45,48,52-54] *Streptococci* are lactic-acid producing bacteria associated with hind-gut acidosis.[53,54] It seems that in horses fed high carbohydrate diets, a large amount of those starches pass by enzymatic digestion in the foregut.[55] When those starches reach the hindgut, they are fermented and increased volumes of lactic acid are produced.[56] The resulting drop in pH is thought to create conditions unfavorable for fibrolytic bacteria, representing a risk factor for disease.[56]

In addition to the inclusion of starch in the diet, it also appears that the source of starch has an effect on changes in the microbial population.[57] For instance, in one study, supplementation of oats in the diet resulted in increased *Lactobacilli* and decreased Gram-positive cocci, while supplementation with corn reversed these effects.[58] This may be explained by increased digestibility of oats in the foregut as compared to corn.[59] It could also be attributed

to other properties of these starches, including differences in amylose/amylopectin ratio, starch granule morphology, presence of other nonstarch components in oats (beta-glucans in oats have prebiotic effect), or others.[60] Finally, abrupt changes in the diet are also strongly associated with gastrointestinal microbial disturbance in the horse.[47,61-64]

Subsection 3b: Gastrointestinal Disease and the Microbiome

Numerous diseases have been associated with alterations in the equine gastrointestinal microbiota. This includes colic,[65,66] acidosis, diarrhea, colitis,[37,67] equine grass sickness,[68,69] equine metabolic syndrome,[70] laminitis,[53,54,71-73] asthma,[74] and parasitism.[75,76] However, with many of these findings, it is important to note that the differentiation of cause versus effect can be difficult, if not impossible, given how difficult it is to control sporadic disease for study conditions. This means that we cannot tell which shapes the other, disease or altered microbiota. It is possible and likely that each shapes the other in a cycle.

As gastrointestinal disease is a major contributor to morbidity and mortality in horses, the effect of both colic and colitis on the fecal microbiome will be the focus of this review.

Despite the importance of colic, and the expectation that the microbiota would experience marked changes with colic, few studies have been completed to evaluate this hypothesis. One such study reported increased Bacteroidetes and *Clostridium phytofermentans* in samples from horses experiencing non-surgical large intestinal colic versus 30-90 days post colic resolution.[65] Another study found consistently decreased Firmicutes and increased Proteobacteria in horses with colic, and suggested that the ratio of Firmicutes to Proteobacteria could potentially be used to predict colic (the higher the ratio, the less likely colic is to develop).[66] Finally, in a third study, horses presenting for colic had reduced microbial richness and diversity compared with horses presenting for elective procedures. This included decreased prevalence of commensal bacteria including *Prevotella* and *Lachnospiraceae*, and increased pathobionts *Streptococcus* and S*phaerochaeta*.[77] It has been proposed that decreased ability of the microbiota to adapt to environmental or diet changes (such as season, weather, or forage types) may be related to

development of certain types of colic.[78] This could explain why these changes predispose some but not all horses to colic.

In cases of colitis, the recognized infectious bacterial causes include *Clostridium difficile, Clostridium perfringens, Salmonella* spp, and *Neorickettsia risticii*.[34] Still, in many cases of colitis, the underling etiology remains unknown, so the role of other pathogenic bacteria as a cause continues to be an area that requires investigation. Despite the importance of colitis as a disease process in the horse, few studies have investigated this further. In one study, a decrease in the normally abundant Firmicutes phylum and increase in the Bacteroidetes phylum was noted in horses with colitis as compared to healthy horses.[37] Additionally, horses with colitis had more *Fusobacteria*, but less *Actinobacteria, Sprirochaetes*, and *Clostridia* members. Another study reported that species richness and evenness (distribution of different bacterial species present) were lower in horses with diarrhea.[67] Additionally, both studies discovered increased levels of *Fusobacteria*, but none in healthy horses. Further evaluation of the role of *Fusobacteria* in the microbiota of horses with and without diarrhea is warranted, as it may serve as an equine enteric pathogen. However, it is also possible that these *Fusobacteria* are just able to proliferate more easily in response to intestinal changes that occur with diarrhea. For instance, it has been proposed that increased intestinal permeability may contribute to some of the changes in the microbiota.[79] However, the overall findings suggest colitis is likely more associated with major intestinal disturbances rather than to any one single, specific pathogen.[37]

Subsection 3c: Drugs and the Microbiome

Among the numerous external factors leading to changes in the microbiome, antimicrobial drug administration may have some of the most profound consequences.[80] While treatment with antibiotics is one of the most important therapies in equine medicine, it can induce dysbiosis. This can lead to overgrowth of more pathogenic organisms (such as *Clostridium difficile*),[81] and result in antimicrobial induced diarrhea/colitis.[82-86] It is also proposed that changes in microbial communities can promote deterioration of the mucosal protective barrier.[87] One study of three antibiotics administered for five days in horses revealed specific changes in fecal microbiota community membership (including reduced richness and diversity).[41] The most significant effect was noted with trimethoprim sulfonamide administered orally, as compared to procaine penicillin and ceftiofur sodium administered intramuscularly. However, all drugs induced some effect. Another study demonstrated that trimethoprim sulfadiazine oral administration was associated with decreased cellulolytic bacteria concentrations and increased amylolytic bacteria.[84] Similarly, a third study found horses administered trimethoprim sulfadiazine or ceftiofur exhibited decreased cellulolytic bacteria in fecal samples.[86] Furthermore, it demonstrated that disruption of the microbiota was associated with proliferation of *Salmonella* and *Clostridium difficile* commonly associated with diarrhea, even after treatment with antibiotics was discontinued. A fourth study revealed that metronidazole administered intracecally in cannulated horses decreased alpha diversity metrics from both cecal and fecal samples.[88] Finally, a recent study demonstrated that horses administered oral doxycycline experienced significant decrease of both alpha and beta diversity.[89] Overall, antimicrobial drug administration is associated with decreased celluloytic bacteria, suggesting these treatments could decrease the total energy derived from forage in the

diet.[84,86] Moreover, the decline in diversity may allow for propagation of more pathogenic bacteria.

In addition to antimicrobials, NSAIDs are commonly utilized in the treatment of various conditions in horses. These medications have previously been associated with the development of gastropathy, enteropathy, or NSAID-induced colitis,[9,90,91] as well as decreased recovery of the mucosal barrier function.[92] One study evaluating the effects of therapeutic doses of phenylbutazone and firocoxib on the fecal microbiota revealed decreased members of the Firmicutes phylum, and the *Lachnospiraceae, Clostridiaceae*, and *Ruminococcaceae* families with both medications.[91] While this demonstrates dysbiosis, the implications of these findings are still unknown.

Finally, another commonly overlooked but critical consideration is the potential of the gastrointestinal microbiota to modulate the bioavailability of drugs, particularly orally administered drugs that are absorbed in the lower gastrointestinal tract. The microbiota may influence drug bioavailability directly through microbial enzyme activity, or indirectly through changes in the gastrointestinal environment or drug transport.[93] Although these effects have not been specifically investigated in equine medicine, their possibility should still be taken into consideration when evaluating the pharmacokinetics and bioavailability of medications in horses.

Section 4: Misoprostol and the Gastrointestinal Microbiome

Administration of misoprostol has the potential to alter gastrointestinal physiology through interactions with prostanoid receptors throughout the gastrointestinal tract. These effects can be beneficial with respect to the role of misoprostol in mucosal protection and repair, [15-19,92,94] but can also result in adverse events including alterations in smooth muscle contractility, colonic transit time, or intestinal fluid movement leading to reported side effects of abdominal cramping and diarrhea.[95] To date, there is limited information available regarding whether misoprostol-mediated effects on gastrointestinal physiology have the potential to cause alterations in the gastrointestinal microbiome. In mice, misoprostol improved colonic barrier function and promoted the recovery of microbiome homeostasis after disruption with antibiotics, although the exact mechanism of these benefits remains unknown.[96] This could be of therapeutic significance in horses where gastrointestinal disease[37,65,66,77,97,98] and administration of antibiotics or NSAIDs[41,86,91,99] have the potential to create dysbiosis, if misoprostol could be prescribed to help restore the microbiome population. In contrast, it is possible that administration of misoprostol and the subsequent changes in gastrointestinal physiology previously described could result in enough changes to the local intestinal environment to cause dysbiosis itself. Given the essential role of the gastrointestinal microbiome in health and disease of horses, as well as the increased frequency with which misoprostol is used to treat horses with gastrointestinal disease, investigation into its potential to alter the gastrointestinal microbiome in horses is warranted.

Section 5: Objectives and Hypotheses

The objectives of this study were to determine the pharmacokinetics of misoprostol and to describe changes in the fecal microbiome after oral and rectal repeated dose misoprostol administration to healthy, adult horses. It was hypothesized that pharmacokinetic parameters would differ by route of administration, but not after repeated dose administration. Furthermore, it was hypothesized that changes in the composition, richness, or diversity of the fecal microbiome would not be observed after oral or rectal repeated dose misoprostol administration.

Chapter 2: Materials and Methods

Section 1: Animals

Six university-owned healthy adult (13-18 years old) mixed-breed geldings, ranging in body weight from 468 to 609 kg, were used for the study. All horses had no history of illness or antimicrobial administration within the previous six months and were deemed healthy on the basis of physical examination findings and biochemistry panel screenings. Horses were housed individually in stalls for a minimum of 12 hours prior to and for the duration of each experimental condition. Horses were maintained on group-specific pasture prior to study onset, during all washout periods, and after study completion. Fresh clean water was provided *ad libitum*. Horses were offered grass hay (three flakes twice daily) and a senior concentrate mash (three pounds once daily) leading up to and throughout the entire study, including washout periods. Physical examinations were performed prior to and then every 12 hours for the duration of each experimental condition. Horses were monitored hourly during each experimental condition and daily during each washout period. All procedures were reviewed and approved by the Auburn University Institutional Care and Use Committee (Protocol #2020-3736).

Section 2: Experimental Design

A prospective, three treatment randomized crossover study design was used. Treatment order was assigned by simple randomization using a random number generator (random.org). Horses were administered a 5 µg/kg dose of misoprostol orally (ORAL) or rectally (RECTAL) every 8 hours for a total of 10 doses, or no medication (CONTROL) for the same duration, with a minimum 21-day washout period between each experimental condition. For the ORAL and RECTAL conditions, horses were instrumented with a 14-gauge over-the-needle catheter in the left or right jugular vein to facilitate repeated blood sample collection for the measurement of plasma MFA concentrations. For all conditions, manure samples were collected by manual evacuation of the rectum for fecal microbiome analysis. Horses were maintained on a standard feeding schedule throughout the study with feed provided a minimum of 5 hours prior to and 1 hour following drug administration for the ORAL and RECTAL conditions and at time-matched points for the CONTROL condition.

Section 3: Misoprostol Administration

For oral administration (ORAL condition), misoprostol hydrochloride tablets (Greenstone®, 100 µg) were dissolved in 30 mL water, administered via oral syringe, and followed immediately by administration of 30 mL water through the same syringe to ensure delivery of the total dose. For rectal administration (RECTAL condition), manure was manually evacuated prior to drug administration. Misoprostol tablets were dissolved in 30 mL water, administered via syringe attached to a 40 cm, 18 French red rubber catheter advanced approximately 30 cm into the rectum, and followed immediately by infusion of 30 mL water through the same syringe and catheter to ensure delivery of the total dose.

Section 4: Blood Sample Collection

For measurement of plasma MFA concentrations for the ORAL condition, blood samples were collected immediately prior to (time 0), and at 10, 15, 20, 30, 60, 90, and 120 minutes following drug administration for doses 1 (first) and 10 (last) as well as at time 0, and at 15 and 30 minutes for doses 2 and 8. For the RECTAL condition, blood samples were collected at time 0, and at 3, 5, 10, 15, 30, 60, and 90 minutes after drug administration for doses 1 and 10, as well as at time 0, and at 3 and 5 minutes after drug administration for doses 2 and 8. Timing of sample collection was based on previously reported pharmacokinetics for single-dose misoprostol administered by the oral and rectal routes.[9,26] Samples were immediately placed in sodium heparin tubes, placed on ice, and then centrifuged (400 × g for 10 minutes at 4°C) within 15 minutes of collection. Plasma was separated into 2 mL aliquots and stored at -80°C until analysis.

Section 5: Fecal Sample Collection

For all horses, a manure sample was collected 7 days prior to study onset (Baseline). Samples were then collected immediately prior to dose 1 (Start Tx), and then 6 hours (End Tx), 7 days (D7), and 14 days (D14) after dose 10 for the ORAL and RECTAL conditions, and at time-matched points for the CONTROL condition. Samples were refrigerated within 10 minutes of collection. Aliquots of feces from the center of each fecal ball were then transferred to 2 mL Eppendorf tubes within 30 minutes of collection and stored at -80°C until analysis.

Section 6: Measurement of Plasma MFA Concentration

Plasma MFA concentrations were analyzed by liquid-chromatography-tandem mass spectrometry (LC-MS/MS) with a triple quadrupole system (Thermo Altis, Thermo Fisher Scientific) and software (TraceFinder 4.1) designed for data acquisition and analysis. The LC-MS/MS protocol and data acquisition parameters have been previously described for equine plasma.[20,26] Briefly, 500 µL plasma sample was mixed with 1 mL acetonitrile spiked with 5 µL D5-misoprostol acid (100 ng/mL), centrifuged and the supernatant dried and then reconstituted into 100 µL solvent prior to LC-MS/MS instrument injection. LC separation was performed on a C18+ column (2.1 x 100mm, 1.5µm; Thermo Accucore Vanquish, Thermo Scientific) with a mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). For method validation, the calibrated concentration range was 2 pg/ml to 5000 pg/ml with a lower limit of quantification (LLOQ) of 2 pg/mL and standard curve correlation coefficient values (R2) > 0.993. Percent MFA recovery and within and between-run accuracy and precision data are presented in **Table 3**.

MFA concentration (ng/mL)	Recovery (%)	Within run precision (n = 15)	Between run precision (n = 15)	Within run accuracy (n = 5)			Between run accuracy (n = 15)
				Day 1	Day 2	Day 3	
0.3	67 ± 9	7.8	5.4	96 ± 8	100 ± 6	101 ± 10	99 ± 7
1	71 ± 6	5.3	4.3	116 ± 3	115 ± 8	113 ± 6	115 ± 6
3	73 ± 3	4.3	3.7	102 ± 3	101 ± 5	101 ± 5	101 ± 4

Table 3: Recovery, accuracy, and precision data for validation of method used to detect MFA concentrations via LC/MS/MS. Recovery is calculated by the signal ratio of spiked standard in serum to spiked standard in 50% acetonitrile. Values for recovery and accuracy are presented as mean ± standard deviation.

Section 7: Pharmacokinetic Analysis

Plasma MFA concentration-versus-time data were subjected to noncompartmental pharmacokinetic modeling using Phoenix WinNonlin, v8.1 (Certara, St Louis MO) software. Peak plasma concentration (C_{max}) and its respective time (T_{max}) were reported. The area under the MFA concentration-versus-time curve to the last sample timepoint ($AUC_{0\rightarrow last}$), to infinity ($AUC_{0\rightarrow\infty}$), and to the dosing interval (AUC_{tao}) after dose 1 (first) and dose 10 (last) were calculated using the log-linear trapezoidal method. For $AUC_{0\rightarrow\infty}$ and AUC_{tao} the percentage extrapolated from the terminal component of the curve was calculated and reported as AUC_{extrap} and $AUC_{taoextrap}$ respectively. Nonlinear regression was used to determine the slope of the terminal component of the drug-elimination time curve. Because intravenous drug administration was not performed, both the elimination rate constant ($1/\lambda$) and half-life ($t_{1/2}$) are reported as disappearance values. Additional reported parameters include mean residence time (MRT), relative bioavailability (F) of rectal compared to oral administration ($F=AUC_{RECTAL}/AUC_{ORAL}$), and the accumulation index. The coefficient of variation (CV) for selected values was calculated as the standard deviation (SD) divided by the mean.

Section 8: Fecal DNA Extraction

DNA was extracted from fecal samples using QIAamp PowerFecal kits (Qiagen) according to the manufacturer's instructions, with minor adaptations in sample and lysis buffer homogenization as previously described.[100] DNA, eluted in 100 µL of buffer, was quantified via fluorometry (Qubit 2.0, Invitrogen, Carlsbad, CA) using quant-iT BR dsDNA reagent kits (Invitrogen) and normalized to a uniform concentration and volume.

Section 9: 16S rRNA Amplicon Library Preparation and Sequencing

Construction and sequencing of bacterial 16S rRNA amplicon libraries was performed at the University of Missouri (MU) DNA Core Facility according to previously described methodology.[100] Briefly, 16S rRNA amplicons were generated via amplification of the V4 hypervariable region of the 16S rRNA gene using universal primers (U515F/806R) flanked by Illumina standard adapter sequences[101,102] and the following amplification parameters: $98°C^{(3\ min)}$ + [$98°C^{(15\ sec)}$ + $50°C^{(30\ sec)}$ + $72°C^{(30\ sec)}$] × 25 cycles + $72°C^{(7\ min)}$. Amplicons were pooled, purified, and then washed. The final amplicon pool was quantified (quant-iT HS dsDNA reagent kit) and sequenced using a standard 2×250 bp paired-end reads protocol for sequencing on the Illumina MiSeq instrument.

Section 10: Bioinformatics Analysis

Bioinformatics on DNA sequences was performed at the MU Informatics Research Core Facility. Primers designed to match the 5' ends of forward and reverse reads were removed from the 5' end of the forward read using Cutadapt[103] (version 2.6; https://github.com/marcelm/cutadapt). When identified, reverse complements of the primer and all bases downstream were then removed from the forward read. For the reverse reads the approach was similar but opposite. Two passes were made over each read with an allowable error-rate of 0.1. Read pairs were rejected if both did not match a 5' primer. The QIIME2[104] DADA2[105] plugin (version 1.10.0) with R version 3.5.1 and Biom version 2.1.7 was used to denoise, de-replicate, and count amplicon sequence variants (ASVs). Finally, taxonomies were assigned using the Silva.v132[106] database.

Section 11: Statistical Analysis

Analysis of pharmacokinetic data was performed using Graphpad Prism 9. Distribution of data was evaluated for normality using Shapiro-Wilk and Kolmogorov-Smirnov methods. Data are reported as mean ± standard deviation apart from T_{max} and $T_{1/2}$ which are reported as median (range) and harmonic mean ± pseudo-standard deviation, respectively. Comparisons between the first and last (tenth) doses for each route of administration were evaluated using Wilcoxon signed rank test for T_{max} and paired T-tests for all other parameters. Comparisons between routes of administration (ORAL versus RECTAL) were evaluated using mixed effects analysis with Sidák's post hoc multiple comparisons for T_{max} and repeated measures two-way analysis of variance (ANOVA) with Tukey's post-hoc multiple comparisons test for all other parameters.

Analysis of fecal microbiota data was performed using SigmaPlot version 14.0. Differences in overall microbiota composition (beta-diversity) between horses and treatments were tested via two-way permutation multivariate analysis of variance (PERMANOVA) using Bray-Curtis and Jaccard similarities. Principal coordinate analysis (PCoA) was performed on ¼ root-transformed data. Effects of horse and treatment on change in intra-subject beta-diversity over time were assessed for normality and equal variance using the Shapiro-Wilk and Brown-Forsythe methods, respectively, and then tested using a two-way ANOVA with Holm-Sidak post hoc multiple comparisons. Univariate data including detected and predicted richness of ASVs (Taxa_S and Chao-1 respectively), and alpha-diversity (Shannon index and Simpson index) were assessed for normality and equal variance using the Shapiro-Wilk and Brown-Forsythe methods, respectively, and then tested via three-way ANOVA with Holm-Sidak post hoc multiple comparisons. Significance for all statistical comparisons was defined as $p < 0.05$.

Chapter 3: Results

Section 1: Study Completion

Physical examination parameters remained normal and no clinical evidence of abdominal discomfort or changes in appetite, fecal consistency, or fecal output were noted for any horse during the study. One horse developed mild cellulitis of one distal hindlimb just prior to the third experimental condition necessitating administration of a single dose each of flunixin meglumine (1.1 mg/kg IV) and ceftiofur crystalline free acid (6.6 mg/kg IM). The horse was maintained on group-specific pasture until after resolution of the cellulitis. Since dysbiosis following NSAID and antibiotic administration has been demonstrated in horses,[21,23,24] an additional four-week wash-out period (from the time of medication administration) was instituted prior to the horse completing the third experimental condition. The fecal microbiome data for the horse's third experimental condition was ultimately included in the final analysis for this study because significant differences were not identified between the horse's fecal samples at baseline and the Start Tx time-point of the third experimental condition.

Section 2: Pharmacokinetic Results

Plasma MFA concentrations remained above the LLOQ for all horses at 120 minutes after doses 1 and 10 in the ORAL condition, and at 90 minutes in the RECTAL condition for 5 of 6 horses after dose 1 and 4 of 6 horses after dose 10. Plasma MFA concentrations fell below the lower limit of detection (LLOD) in all horses at time 0 for doses 1, 2, 8, and 10 in both ORAL and RECTAL conditions. Differences in time-matched plasma MFA concentrations were not observed across doses within both the ORAL and RECTAL conditions.

Plasma concentration-versus-time curves generated for dose 1 and dose 10 of the ORAL and RECTAL conditions are displayed in **Figure 2** and pharmacokinetic parameters are summarized in **Table 4**. The mean ± standard deviation percentage of the $AUC_{0\rightarrow\infty}$ that was extrapolated after dose 1 and 10 for the ORAL condition was 17 ± 10% and 18 ± 12%, and for the RECTAL condition was 14 ± 15% and 15 ± 11% respectively. For this reason, both $AUC_{0\rightarrow last}$ and $AUC_{0\rightarrow\infty}$ are reported and included in statistical comparisons. The mean percentage of AUC_{tao} extrapolated was < 5% for all measurements. Within the ORAL and RECTAL conditions, differences between $AUC_{0\rightarrow last}$, $AUC_{0\rightarrow\infty}$, and AUC_{tao} were not detected for either dose 1 or dose 10 ($p > 0.5$ all). Differences in pharmacokinetic parameters were not detected between dose 1 and dose 10 for either the ORAL or RECTAL conditions.

Several differences in pharmacokinetic parameters were identified between the ORAL and RECTAL conditions. T_{max} ($p < 0.03$) was significantly longer, and both $AUC_{0\rightarrow last}$ ($p = 0.01$) and AUC_{tau} ($p = 0.01$) were significantly greater for the ORAL condition after dose 1, but not after dose 10 ($p > 0.06$ all). $AUC_{0\rightarrow\infty}$ ($p = 0.01$ both) was significantly greater and $T_{1/2}$ ($p \leq 0.02$ both) was significantly longer for the ORAL condition after both doses 1 and 10. No other differences in pharmacokinetic parameters were detected between routes of administration.

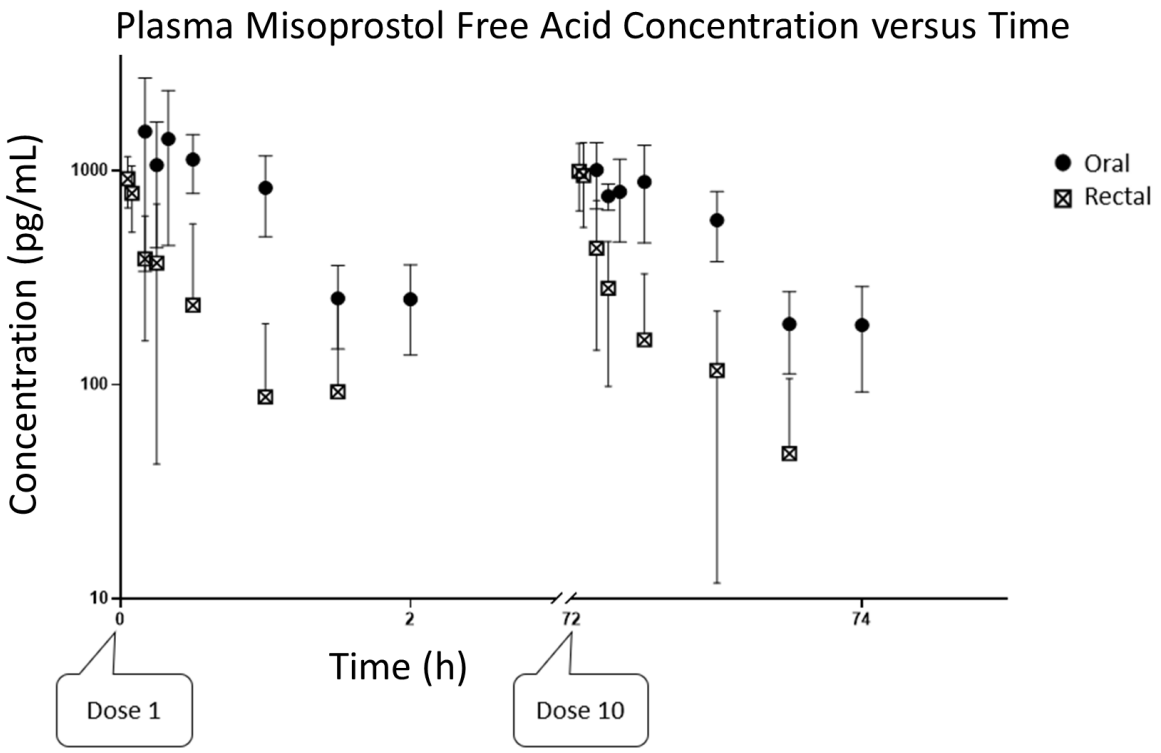

Figure 2: Mean plasma misoprostol free acid (MFA) concentration-versus-time curve for six healthy, adult horses that received misoprostol (5 µg/kg q8h for 10 doses) after dose 1 and dose 10 ORAL administration (solid circles) as well as dose 1 and dose 10 RECTAL administration (cross-hatched boxes). Error bars represent standard deviation.

Parameter	Units	ORAL		RECTAL	
		Dose 1	Dose 10	Dose 1	Dose 10
C_{max} (CV)	pg/mL (%)	1648 ± 1084 (66)	1138 ± 324 (29)	957 ± 225 (24)	1117 ± 402 (36)
T_{max}	min	20 (10 – 60)[a]	12.5 (10 – 30)	3 (3 – 5)[a]	3 (3 – 5)
$AUC_{0\rightarrow last}$ (CV)	pg*min/mL (%)	75,720 ± 29,060[a] (38)	54,040 ± 15,060 (29)	17,630 ± 16,160[a] (92)	16,080 ± 11,600 (72)
$AUC_{0\rightarrow\infty}$ (CV)	pg*min/mL (%)	90,860 ± 32,620[a] (36)	66,400 ± 17,010[b] (26)	23,960 ± 28,310[a] (118)	19,290 ± 13,990[b] (73)
AUC_{tau}	pg*min/ml	77,820 ± 29,490[a]	55,610 ± 15,180	17,930 ± 16,560[a]	16,460 ± 11,520
MRT_{inf}	min	61.92 ± 19.76	65.36 ± 24.20	38.81 ± 30.64	36.36 ± 21.08
$T_{1/2}$	min	38 ± 12[a]	41 ± 16[b]	28 ± 16[a]	30 ± 20[b]
λ_z	min^{-1}	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.04 ± 0.03
Accumulation Index		1.14 ± 0.10	1.17 ± 0.13	1.15 ± 0.17	1.19 ± 0.22
F	%	NA	NA	28 ± 30	32 ± 27

Table 4: Plasma pharmacokinetic parameters for misoprostol free acid (MFA) following dose 1 and dose 10 for ORAL and RECTAL administration in six healthy, adult horses. Values are reported as mean ± SD for all parameters except T_{max} which is reported as median (range) and $T_{½dis}$ which is reported as harmonic mean ± pseudoSD.

C_{max}: maximum observed plasma concentration; T_{max}: time to C_{max}; $AUC_{0\rightarrow last}$: area under the concentration-versus-time curve from time 0 to the last measured concentration; $AUC_{0\rightarrow\infty}$: area under the concentration-versus-time curve from time 0 extrapolated to infinity; AUC_{tau}: area under to concentration-versus-time curve from time 0 to the dosing interval (eight hours); CV: coefficient of variation presented for select variables; F: relative bioavailability of rectal compared to oral administration; MRT_{inf}: mean residence time extrapolated to infinity; NA: not applicable; $T_{1/2:}$ disappearance half-life; λ_z: disappearance rate constant

[a] within a row denotes a significant difference ($p < 0.05$) between ORAL and RECTAL for dose 1.
[b] within a row denotes a significant difference ($p < 0.05$) between ORAL and RECTAL for dose 10.

Section 3: Microbiome Results

Subsection 3a: Assessment of Microbial Community

All samples yielded acceptable coverage with a mean read count of 87,662 per sample (range 46,652 – 106,767). A total of 31 phyla, 249 families, 411 genera, and 15,647 species were detected. Major phyla and families identified and their associated mean relative abundances (%) included Firmicutes (55%), Bacteroidota (28%), and Verrucomicrobiota (5%) for phyla and *Clostridia* (11%), *Lachnospiraceae* (9%), and *p-251-o5* (8%) for families. Significant differences in relative abundance of major identified taxa were not detected among experimental conditions or timepoints at the phylum, family, genus, or species level as depicted in **Figure 3**.

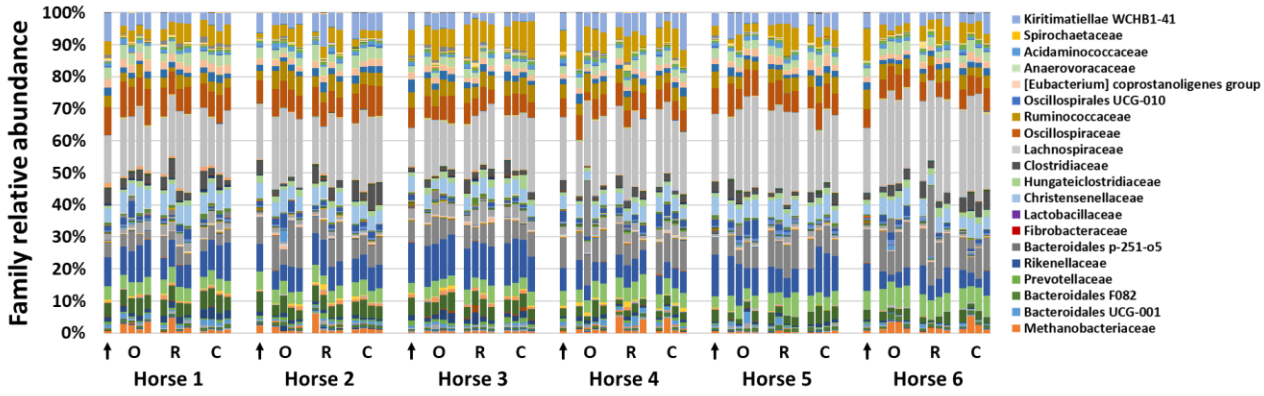

Figure 3: Stacked bar chart depicting the major families detected in fecal samples from horses at baseline (arrow), undergoing ORAL (O) or RECTAL (R) treatments, or the CONTROL (C) experimental condition.

Subsection 3b: Beta-diversity

Principal coordinate analysis comparing composition of fecal microbiota between all 6 study horses and conditions (ORAL, RECTAL, CONTROL) is depicted for the Bray-Curtis and Jaccard similarity indices in **Figure 4** and **Figure 5**, respectively. A strong significant effect of horse on beta-diversity was detected by two-way PERMANOVA (Bray-Curtis $p < 0.001$, $F = 10.8$; Jaccard $p < 0.001$, $F = 5.7$) with samples clearly clustering according to horse. A weak significant effect of experimental condition on beta-diversity was also detected (Bray-Curtis $p = 0.002$, $F = 1.8$; Jaccard $p = 0.01$, $F = 1.4$), with minimal separation of samples identified according to condition.

To further evaluate the effect of experimental condition over time, samples within each condition and for each timepoint were compared to the baseline samples collected 7 days prior to study onset. Two-way ANOVA to assess the effect of experimental condition and timepoint on intra-horse similarity to baseline found that, when controlling for the individual horse, there were significant effects of condition ($p < 0.001$, $F = 8.5$) and timepoint ($p < 0.001$, $F = 7.8$) on fecal beta-diversity, and no significant interaction between factors ($p = 0.778$, $F = 0.5$; **Figure 6**). Specifically, intra-subject similarity to baseline was greater during the CONTROL condition than during either the RECTAL ($p = 0.009$) or ORAL ($p < 0.001$) conditions and was lower at the End Tx timepoint compared to other timepoints ($p < 0.019$ all). When experimental condition within each timepoint was considered, significance was only identified between the CONTROL and ORAL conditions at the End Tx timepoint ($p = 0.016$).

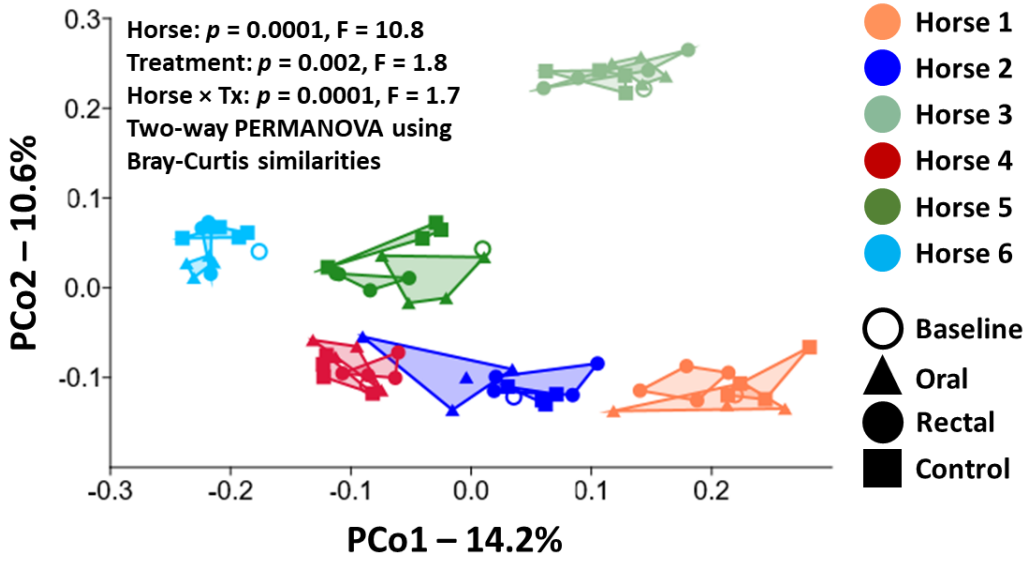

Figure 4: Principal coordinate analysis based on Bray-Curtis similarities with results of two-way permutational multivariate analysis of variance shown in upper left. Samples cluster according to horse, with minimal to no separation of experimental conditions within horse (legend at right), showing clear inter-horse differences in beta-diversity.

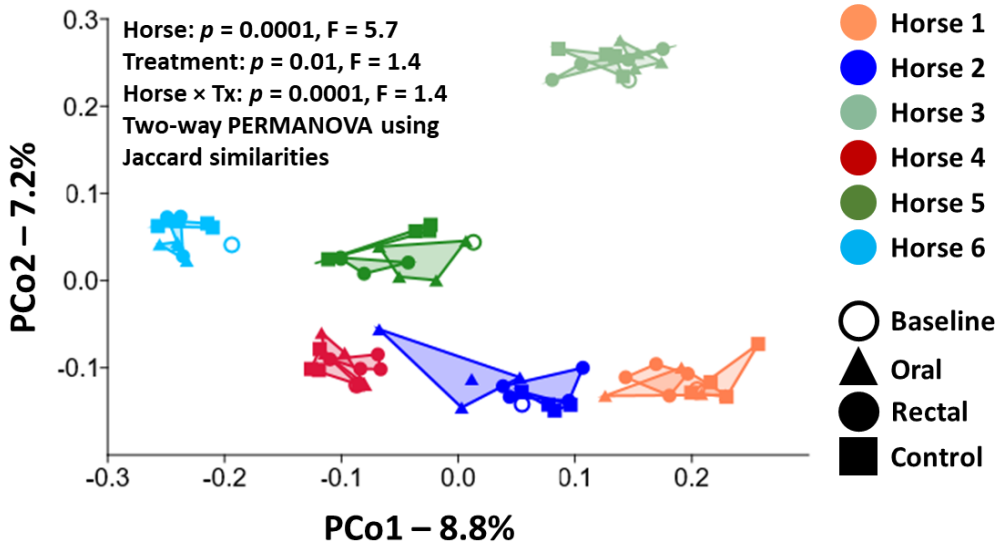

Figure 5: Principal coordinate analysis based on Jaccard similarities with results of two-way permutational multivariate analysis of variance shown in upper left. Samples cluster according to horse, with minimal to no separation of experimental conditions within horse (legend at right), showing clear inter-horse differences in beta-diversity.

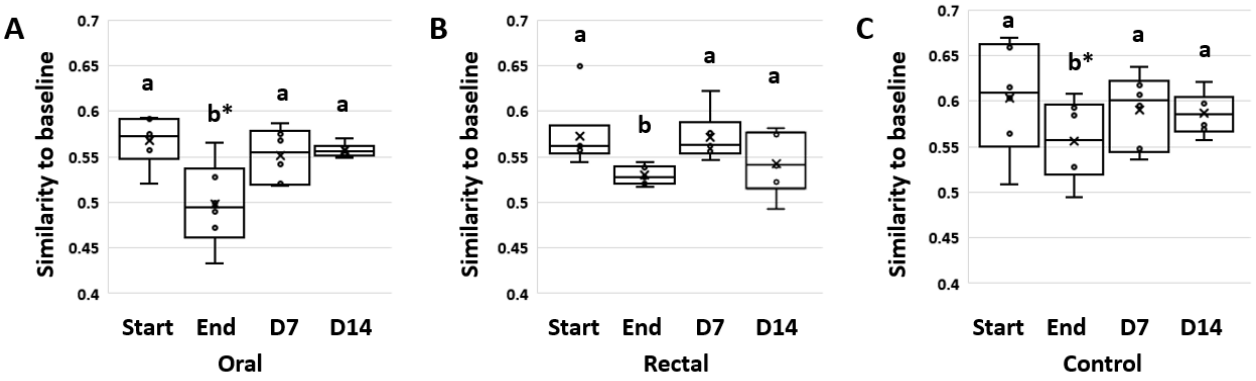

Figure 6: Intra-horse similarity to baseline at each subsequent time-point, according to treatment group. Tukey box plots showing intra-horse similarity to their baseline composition at each time-point during ORAL (**A**) or RECTAL (**B**) administration of misoprostol, or during the CONTROL period (**C**). Different letters indicate significant differences in pairwise comparisons within each panel. An asterisk (*) indicates a significant difference between the CONTROL and ORAL conditions at the End Tx timepoint.

Subsection 3c: Richness and Alpha Diversity

Univariate data including inter-horse and time-dependent effects on observed ASV richness and alpha-diversity are presented in **Figure 7**. Results of the three-way ANOVA identified differences in observed richness (Taxa_S) by horse ($p < 0.001$, $F = 12.2$) and timepoint ($p = 0.002$, $F = 5.6$) particularly when the End Tx timepoint was compared to the Start Tx ($p = 0.001$) and D14 ($p = 0.037$) timepoints. Differences were not detected among experimental condition ($p = 0.181$, $F = 1.7$; **Figure 7 A & B**). Similarly, differences were detected in predicted richness (Chao-1) by horse ($p < 0.001$, $F = 3.9$) and timepoint ($p = 0.014$, $F = 4.9$), but not by experimental condition ($p = 0.689$, $F = 0.4$). Evaluation of diversity using the Shannon Index detected differences by horse ($p < 0.001$, $F = 6.8$) but not according to timepoint ($p = 0.130$, $F = 2.0$) or experimental condition ($p = 0.077$, $F = 2.7$; **Figure 7 C & D**). Evaluation using the Simpson index resulted in similar findings for diversity by horse ($p < 0.001$, $F = 4.4$), timepoint ($p = 0.339$, $F = 1.2$) and experimental condition ($p = 0.5$, $F = 0.7$).

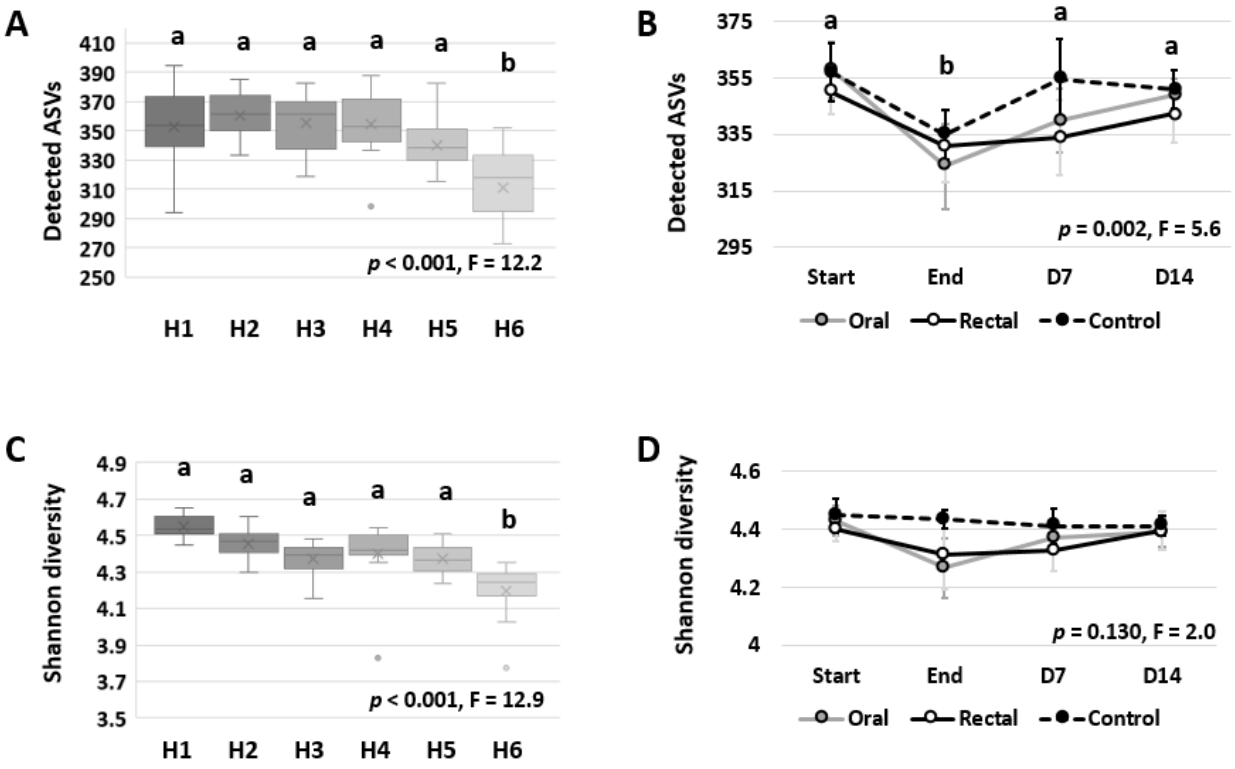

Figure 7: Inter-horse and time-dependent effects on richness and alpha-diversity. Box plot and line graph showing the number of detected amplicon sequence variants (ASVs) as a measure of richness, grouped by horse (**A**) or treatment and time-point (**B**). Main effects associated with horse and time-point (three-way ANOVA) are included in **A** and **B**, respectively. Box plot and line graph representing Shannon alpha-diversity in the same fashion (**C & D**). Different letters indicate significant differences in pairwise comparisons within each panel.

Chapter 4: Discussion

To date, the present study is the first to describe the pharmacokinetics and detail the fecal microbiome in horses following repeated dose oral and rectal administration of misoprostol. As predicted, differences in pharmacokinetic parameters were noted between routes of administration but not in response to repeated drug administration. With respect to fecal microbiome assessment, changes in the microbiome composition were observed over the course of the study. While associations with treatment and timepoint were identified, these were far outweighed by observed inter-horse differences in fecal microbiome composition.

At the dose and dose interval described in this study, misoprostol did not appear to accumulate with repeated-dose administration or reach steady state plasma concentrations. The mean disappearance $t_{1/2}$ was well below one-hour for both oral and rectal administration, and MFA plasma concentrations were below the LLOD when measured immediately prior to drug administration. Additionally, repeated dose administration did not appear to alter misoprostol absorption or metabolism as suggested by the lack of detectable differences in pharmacokinetic parameters between the first and last dose for either route of administration. Considerable variability in misoprostol absorption (C_{max}) and systemic exposure (AUC) has been recognized in horses[15,20,26] and was also noted among horses in the present study. This is evident by the large CV observed for these parameters for both routes of administration (**Table 4**). Notable variability between the first and last dose was also observed when individual horses were considered, with differences for C_{max} and AUC between the first and last dose exceeding 50% for some horses. Thus, it is possible that both between and within-horse variability could have precluded the detection of small differences in pharmacokinetic parameters associated with repeated dose administration.

The pharmacokinetics of misoprostol in horses are described after single dose administration by the oral or rectal route in fasted and fed horses and in horses challenged with LPS.[15,20,26] Rapid absorption of misoprostol is observed for this and all previous studies with reported values for T_{max} of 3-5 minutes after rectal and 10-45 minutes for oral administration.[15,20,26] Additional similarities are particularly apparent when considering rectal administration of misoprostol. The mean C_{max} and AUC values reported in the present repeated-dose study demonstrate notable overlap with what has been reported for these parameters in single-dose studies both with and without LPS challenge.[20,26] The relative differences between oral and rectal pharmacokinetics observed in this study are also similar to those previously reported where the larger AUC and longer disappearance $t_{1/2}$ observed after oral administration corresponds to greater systemic drug exposure with oral administration and decreased relative bioavailability for rectal administration.[20,26] Thus while parenteral administration can provide an opportunity to improve relative bioavailability by bypassing hepatic first-pass metabolism, it is less predictable in horses[107] and does not appear to have improved the bioavailability of misoprostol in this or previous studies.[9,10]

The variability in drug pharmacokinetics noted across studies with oral drug administration most likely reflects the impact that differences in experimental protocols such as fasting times or endotoxin challenge and inherent physiologic differences among horses may have on drug absorption and systemic exposure after oral administration. The C_{max} and AUC values reported in this study are most similar to those reported by Lopp et al for fasted versus fed horses after single-dose oral misoprostol administration.[26] While horses in this study were not specifically fasted, their similarity to the fasted horses in the Lopp et al study most likely reflects the feeding schedule relative to timing of drug administration used in this repeated-dose

study. Similar to what has been reported in comparisons to previous single-dose studies, the mean C_{max} and AUC values reported in the present study were approximately 4-5 fold lower than those reported by Kimura et al in LPS-challenged horses.[20] The study population in this repeated-dose study is identical to that used by Kimura et al with a 12-month gap between studies.[20] However, comparisons between separate studies must be made with caution and therefore the influence of LPS or inflammation on misoprostol pharmacokinetics in horses remains to be determined.

With respect to the fecal microbiome, the overall composition and major taxa present at the phylum, family, and genus levels in the horses in this study were similar to what has been reported previously in healthy adult horses.[34] When comparing microbiome composition using beta diversity indices, although significant differences were detected among individual horses and experimental conditions, marked clustering was only noted between individual horses and not between experimental conditions (**Figures 4 and 5**). Furthermore, intra-horse similarity to baseline decreased at the End Tx timepoint, particularly in the ORAL condition (**Figure 6**). Taken altogether, these results suggest that while inter-horse differences outweigh other factors, there are nonetheless subtle effects of treatment and timepoint on fecal microbiome composition. Although the exact mechanism(s) underlying these effects remains unknown, it is proposed that prostanoid receptor interactions leading to alterations in intestinal smooth muscle contractility, ingesta transit time, and intestinal fluid movement likely alter the local intestinal environment enough to impact the survival and composition of the microbiome. Since changes in the relative abundance of specific taxa were not identified, it is impossible to predict whether these differences in microbiome composition represent clinically or biologically significant changes.

When considering the univariate data, the decrease in observed (Taxa_S) and predicted (Chao-1) richness at the End Tx time-point (**Figure 7A & B**) might be considered an undesirable change.[44] However, since the decline in richness occurred equally across all experimental conditions, including the CONTROL, this decline is likely in response to management changes during each experimental condition. All horses were fed the same hay and concentrate throughout the entire study and were housed on group-specific grass pasture prior to, in between, and after all experimental periods. However, they were brought into individual stalls with pine shavings during each experimental condition. Similar changes in diet and environment have been associated with a decline in richness of the microbiome in previous studies.[45,49,50] Given that the decline in richness affected all experimental conditions similarly, it is likely that study-specific management changes, rather than misoprostol administration, resulted in the decreased microbial richness observed at the End TX time-point.

Finally, comparison of the Shannon and Simpson indices (**Figure 7C & D**) suggests no significant impact of treatment or time-point on fecal microbiome diversity. It is important to note that a considerable amount of variability was observed in these indices at the End Tx timepoint. This variability between horses, combined with the small number of horses included in the study, could have limited our ability to detect differences in these α-diversity indices. Regardless, the overall impact of misoprostol on the microbiome composition was minor, and specific effects of misoprostol on richness or α-diversity were not appreciated in the current study.

There are limitations to this study. The small sample size, particularly in consideration of the observed variability in several measured parameters, may have precluded the ability to detect subtle differences in pharmacokinetic or microbiome parameters associated with repeated-dose

misoprostol administration. Little information exists regarding therapeutic plasma concentrations for misoprostol. Current dosing recommendations in horses are largely extrapolated from human literature, and are described for the treatment of EGGD,[14] and single-dose pharmacokinetic studies in healthy horses.[15,26] To date, evaluation of a lower drug dose or shorter dosing interval for misoprostol has not been investigated in horses but is described in humans for the prevention of NSAID-induced gastric ulcers. While the 10-dose course of drug administration used in this study may be similar to that used for treatment of acute colitis, it is shorter than what is recommended for treatment of EGGD[3] and may have been of insufficient duration to detect changes in drug pharmacokinetic behavior or alterations in microbiome composition associated with a longer course of treatment. Furthermore, only healthy horses were included in this study and thus the potential impact that systemic illness or inflammation could have on misoprostol pharmacokinetics or the fecal microbiome, or the interaction of the two, remains unknown at this time.

Finally, unavoidable changes in management between the experimental and washout periods was a limitation of the study. Multiple inherent factors such as age,[46] breed,[98,108] and sex or pregnancy status;[66,109] as well as external factors such as diet,[46,48,49,98] exercise,[110,111] transport,[112,113] geographic location,[98] and season[98,114] can all affect the fecal microbiome. While a study designed to avoid any management or environmental changes would have been preferred, this was not possible given the limitations of the facility. The CONTROL group was included to limit over-interpretation of these confounding factors' effects on the fecal microbiome.

In conclusion, this study did not identify differences in systemic exposure after repeated dose administration of misoprostol, although increased systemic exposure was observed after oral as compared to rectal administration. Additionally, the observed differences in the fecal

microbiome's composition, richness, and alpha-diversity appeared to be primarily related to large inter-individual variation and changes in management, with only minor effects on composition associated with misoprostol administration. Further evaluation of misoprostol after long-term administration to horses with gastrointestinal disease or systemic inflammation and with or without concurrent administration of anti-inflammatory or antimicrobial medications is warranted, as this may provide clinically relevant information regarding changes in drug pharmacokinetics and pharmacodynamics or the potential for misoprostol to impact the gastrointestinal microbiome.

References

1. Moore JN, Vandenplas ML. Is it the systemic inflammatory response syndrome or endotoxemia in horses with colic? *Veterinary Clinics: Equine Practice* 2014;30:337-351.
2. Werners A, Bull S, Fink-Gremmels J. Endotoxaemia: a review with implications for the horse. *Equine veterinary journal* 2005;37:371-383.
3. Taylor S. A review of equine sepsis. *Equine Veterinary Education* 2015;27:99-109.
4. Moore JN, Barton MH. Treatment of endotoxemia. *Veterinary Clinics: Equine Practice* 2003;19:681-695.
5. Werners A. Treatment of endotoxaemia and septicaemia in the equine patient. *Journal of Veterinary Pharmacology and Therapeutics* 2017;40:1-15.
6. Hart K, MacKay R. Endotoxemia and Sepsis In: Smith B, ed. *Large Animal Internal Medicine*. 5th ed, 2015;682-695.
7. Cook VL, Blikslager AT. The use of nonsteroidal anti-inflammatory drugs in critically ill horses. *Journal of Veterinary Emergency and Critical Care* 2015;25:76-88.
8. Kelmer G. Update on treatments for endotoxemia. *Veterinary Clinics: Equine Practice* 2009;25:259-270.
9. Marshall J, Blikslager A. The effect of nonsteroidal anti-inflammatory drugs on the equine intestine. *Equine Veterinary Journal* 2011;43:140-144.
10. Rao P, Knaus EE. Evolution of nonsteroidal anti-inflammatory drugs (NSAIDs): cyclooxygenase (COX) inhibition and beyond. *Journal of pharmacy & pharmaceutical sciences* 2008;11:81s-110s.
11. Barton M, Parviainen A, Norton N. Polymyxin B protects horses against induced endotoxaemia in vivo. *Equine veterinary journal* 2004;36:397-401.
12. Dollery C. Misoprostol. *Therapeutic Drugs*. Ediburgh, UK: Churchill Livingstone, 1999;193-197.
13. Sangiah S, MacAllister C, Amouzadeh H. Effects of misoprostol and omeprazole on basal gastric pH and free acid content in horses. *Research in veterinary science* 1989;47:350-354.
14. Varley G, Bowen I, Habershon-Butcher J, et al. Misoprostol is superior to combined omeprazole-sucralfate for the treatment of equine gastric glandular disease. *Equine veterinary journal* 2019;51:575-580.
15. Martin E, Schirmer J, Jones SL, et al. Pharmacokinetics and ex vivo anti-inflammatory effects of oral misoprostol in horses. *Equine veterinary journal* 2019;51:415-421.
16. Martin EM, Messenger KM, Sheats MK, et al. Misoprostol inhibits lipopolysaccharide-induced pro-inflammatory cytokine production by equine leukocytes. *Frontiers in veterinary science* 2017;4:160.
17. Martin EM, Till RL, Sheats MK, et al. Misoprostol inhibits equine neutrophil adhesion, migration, and respiratory burst in an in vitro model of inflammation. *Frontiers in veterinary science* 2017;4:159.
18. Gobejishvili L, Ghare S, Khan R, et al. Misoprostol modulates cytokine expression through a cAMP pathway: Potential therapeutic implication for liver disease. *Clinical Immunology* 2015;161:291-299.

19. Chilcoat CD, Rowlingson KA, Jones SL. The effects of cAMP modulation upon the adhesion and respiratory burst activity of immune complex-stimulated equine neutrophils. *Veterinary immunology and immunopathology* 2002;88:65-77.
20. Kimura S, McCoy AM, Boothe DM, et al. Effects of a single dose of orally and rectally administered misoprostol in an in vivo endotoxemia model in healthy adult horses. *American Journal of Veterinary Research* 2022;83.
21. Tang O, Gemzell-Danielsson K, Ho P. Misoprostol: pharmacokinetic profiles, effects on the uterus and side-effects. *International Journal of Gynecology & Obstetrics* 2007;99:S160-S167.
22. Meckstroth KR, Whitaker AK, Bertisch S, et al. Misoprostol administered by epithelial routes: drug absorption and uterine response. *Obstetrics & Gynecology* 2006;108:582-590.
23. Tang OS, Schweer H, Seyberth H, et al. Pharmacokinetics of different routes of administration of misoprostol. *Human reproduction* 2002;17:332-336.
24. Khan R-U, El-Refaey H, Sharma S, et al. Oral, rectal, and vaginal pharmacokinetics of misoprostol. *Obstetrics & Gynecology* 2004;103:866-870.
25. Bharathi DV, Jagadeesh B, Hotha KK, et al. Development and validation of highly sensitive method for determination of misoprostol free acid in human plasma by liquid chromatography–electrospray ionization tandem mass spectrometry: application to a clinical pharmacokinetic study. *Journal of Chromatography B* 2011;879:2827-2833.
26. Lopp CT, McCoy AM, Boothe D, et al. Single-dose pharmacokinetics of orally and rectally administered misoprostol in adult horses. *American journal of veterinary research* 2019;80:1026-1033.
27. Khan R-U, El-Refaey H, Sharma S, et al. Oral, rectal, and vaginal pharmacokinetics of misoprostol. *Obstetrics & Gynecology* 2004;103:866-870.
28. Varley G, Bowen I, Nicholls V, et al. Misoprostol is superior to combined omeprazole and sucralfate for healing glandular gastric lesions. *Equine Vet J* 2016;48:11-12.
29. Britzi M, Gross M, Lavy E, et al. Bioavailability and pharmacokinetics of metronidazole in fed and fasted horses. *Journal of veterinary pharmacology and therapeutics* 2010;33:511-514.
30. Echeverria K, Lascola K, Giguère S, et al. Effect of feeding on the pharmacokinetics of oral minocycline in healthy adult horses. *Journal of veterinary pharmacology and therapeutics* 2018;41:e53-e56.
31. Alvinerie M, Sutra J, Cabezas I, et al. Enhanced plasma availability of moxidectin in fasted horses. *Journal of Equine Veterinary Science* 2000;20:575-578.
32. Sykes B, Hewetson M, Hepburn R, et al. European College of Equine Internal Medicine Consensus Statement—equine gastric ulcer syndrome in adult horses. *Journal of Veterinary Internal Medicine* 2015;29:1288.
33. Welsh J, Lees P, Stodulski G, et al. Influence of feeding schedule on the absorption of orally administered flunixin in the horse. *Equine Veterinary Journal* 1992;24:62-65.
34. Costa MC, Weese JS. Understanding the intestinal microbiome in health and disease. *Veterinary Clinics: Equine Practice* 2018;34:1-12.
35. Al Jassim RA, Andrews FM. The bacterial community of the horse gastrointestinal tract and its relation to fermentative acidosis, laminitis, colic, and stomach ulcers. *Veterinary Clinics: Equine Practice* 2009;25:199-215.

36. Argenzio R, Southworth M, Stevens C. Sites of organic acid production and absorption in the equine gastrointestinal tract. *American Journal of Physiology-Legacy Content* 1974;226:1043-1050.
37. Costa MC, Arroyo LG, Allen-Vercoe E, et al. Comparison of the fecal microbiota of healthy horses and horses with colitis by high throughput sequencing of the V3-V5 region of the 16S rRNA gene. 2012.
38. Daly K, Stewart CS, Flint HJ, et al. Bacterial diversity within the equine large intestine as revealed by molecular analysis of cloned 16S rRNA genes. *FEMS microbiology ecology* 2001;38:141-151.
39. Dougal K, de la Fuente G, Harris PA, et al. Identification of a core bacterial community within the large intestine of the horse. *PloS one* 2013;8:e77660.
40. O'Donnell M, Harris H, Jeffery I, et al. The core faecal bacterial microbiome of Irish Thoroughbred racehorses. *Letters in applied microbiology* 2013;57:492-501.
41. Costa MC, Stämpfli HR, Arroyo LG, et al. Changes in the equine fecal microbiota associated with the use of systemic antimicrobial drugs. *BMC veterinary research* 2015;11:1-12.
42. Shepherd ML, Swecker Jr WS, Jensen RV, et al. Characterization of the fecal bacteria communities of forage-fed horses by pyrosequencing of 16S rRNA V4 gene amplicons. *FEMS microbiology letters* 2012;326:62-68.
43. Steelman SM, Chowdhary BP, Dowd S, et al. Pyrosequencing of 16S rRNA genes in fecal samples reveals high diversity of hindgut microflora in horses and potential links to chronic laminitis. *BMC Veterinary Research* 2012;8:231.
44. Garber A, Hastie P, Murray J-A. Factors influencing equine gut microbiota: Current knowledge. *Journal of Equine Veterinary Science* 2020;88:102943.
45. Daly K, Proudman CJ, Duncan SH, et al. Alterations in microbiota and fermentation products in equine large intestine in response to dietary variation and intestinal disease. *British Journal of Nutrition* 2012;107:989-995.
46. Dougal K, de la Fuente G, Harris PA, et al. Characterisation of the faecal bacterial community in adult and elderly horses fed a high fibre, high oil or high starch diet using 454 pyrosequencing. *PloS one* 2014;9:e87424.
47. Fernandes KA, Kittelmann S, Rogers CW, et al. Faecal microbiota of forage-fed horses in New Zealand and the population dynamics of microbial communities following dietary change. *PloS one* 2014;9:e112846.
48. Warzecha C, Coverdale J, Janecka J, et al. Influence of short-term dietary starch inclusion on the equine cecal microbiome. *Journal of animal science* 2017;95:5077-5090.
49. Willing B, Vörös A, Roos S, et al. Changes in faecal bacteria associated with concentrate and forage-only diets fed to horses in training. *Equine veterinary journal* 2009;41:908-914.
50. Hansen NC, Avershina E, Mydland LT, et al. High nutrient availability reduces the diversity and stability of the equine caecal microbiota. *Microbial ecology in health and disease* 2015;26:27216.
51. Murcia P. Clinical insights: The equine microbiome. *Equine veterinary journal* 2019;51:714-715.
52. Berg J, Chambers B, Siegel H, et al. Equine microbiome project: understanding differences in the horse gut microbiome related to diet. *Journal of Equine Veterinary Science* 2017;100:94.

53. Milinovich G, Trott D, Burrell P, et al. Changes in equine hindgut bacterial populations during oligofructose-induced laminitis. *Environmental microbiology* 2006;8:885-898.
54. Milinovich GJ, Klieve AV, Pollitt CC, et al. Microbial events in the hindgut during carbohydrate-induced equine laminitis. *Veterinary Clinics: Equine Practice* 2010;26:79-94.
55. Harris P, Dunnett C. Nutritional tips for veterinarians. *Equine Veterinary Education* 2018;30:486-496.
56. Coenen M, Vervuert I. A minimum of roughage and a maximum of starch-necessary benchmarks for equine diets. *Pferdeheilkunde* 2010;26:147-151.
57. Harlow B, Donley T, Lawrence L, et al. Effect of starch source (corn, oats or wheat) and concentration on fermentation by equine faecal microbiota in vitro. *Journal of applied microbiology* 2015;119:1234-1244.
58. Harlow BE, Lawrence LM, Hayes SH, et al. Effect of dietary starch source and concentration on equine fecal microbiota. *PloS one* 2016;11:e0154037.
59. Kienzle E, Pohlenz J, Radicke S. Morphology of starch digestion in the horse. *Journal of Veterinary Medicine Series A* 1997;44:207-221.
60. Mishra S, Hardacre A, Monro J. Food structure and carbohydrate digestibility. *Carbohydrates-Comprehensive studies on glycobiology and glycotechnology* 2012:289-316.
61. De Fombelle A, Julliand V, Drogoul C, et al. Feeding and microbial disorders in horses: 1-Effects of an abrupt incorporation of two levels of barley in a hay diet on microbial profile and activities. *Journal of Equine Veterinary Science* 2001;21:439-445.
62. Grimm P, Julliand V, Philippeau C, et al. Effect of yeast supplementation on hindgut microbiota and digestibility of horses subjected to an abrupt change of hays. *Livestock Science* 2016;186:34-40.
63. Muhonen S, Connysson M, Lindberg J, et al. Effects of crude protein intake from grass silage-only diets on the equine colon ecosystem after an abrupt feed change. *Journal of animal science* 2008;86:3465-3472.
64. Muhonen S, Julliand V, Lindberg J, et al. Effects on the equine colon ecosystem of grass silage and haylage diets after an abrupt change from hay. *Journal of animal science* 2009;87:2291-2298.
65. Venable E, Kerley M, Raub R. Assessment of equine fecal microbial profiles during and after a colic episode using pyrosequencing. *Journal of Equine Veterinary Science* 2013;5:347-348.
66. Weese JS, Holcombe S, Embertson R, et al. Changes in the faecal microbiota of mares precede the development of post partum colic. *Equine veterinary journal* 2015;47:641-649.
67. Rodriguez C, Taminiau B, Brévers B, et al. Faecal microbiota characterisation of horses using 16 rdna barcoded pyrosequencing, and carriage rate of clostridium difficile at hospital admission. *BMC microbiology* 2015;15:1-14.
68. Garrett LA, Brown R, Poxton IR. A comparative study of the intestinal microbiota of healthy horses and those suffering from equine grass sickness. *Veterinary microbiology* 2002;87:81-88.
69. Leng J, Proudman C, Blow F, et al. Understanding intestinal microbiota in equine grass sickness: next generation sequencing of faecal bacterial DNA. *Equine Veterinary Journal* 2015;47:9-9.

70. Elzinga SE, Weese JS, Adams AA. Comparison of the fecal microbiota in horses with equine metabolic syndrome and metabolically normal controls fed a similar all-forage diet. *Journal of Equine Veterinary Science* 2016;44:9-16.
71. Steelman SM, Chowdhary BP, Dowd S, et al. Pyrosequencing of 16S rRNA genes in fecal samples reveals high diversity of hindgut microflora in horses and potential links to chronic laminitis. *BMC veterinary research* 2012;8:1-11.
72. Garner H, Moore J, Johnson J, et al. Changes in the caecal flora associated with the onset of laminitis. *Equine Veterinary Journal* 1978;10:249-252.
73. Moreau MM, Eades SC, Reinemeyer CR, et al. Illumina sequencing of the V4 hypervariable region 16S rRNA gene reveals extensive changes in bacterial communities in the cecum following carbohydrate oral infusion and development of early-stage acute laminitis in the horse. *Veterinary microbiology* 2014;168:436-441.
74. Leclere M, Costa MC. Fecal microbiota in horses with asthma. *Journal of veterinary internal medicine* 2020;34:996-1006.
75. Peachey LE, Castro C, Molena RA, et al. Dysbiosis associated with acute helminth infections in herbivorous youngstock–observations and implications. *Scientific reports* 2019;9:1-16.
76. Kunz IG, Reed KJ, Metcalf JL, et al. Equine fecal microbiota changes associated with anthelmintic administration. *Journal of equine veterinary science* 2019;77:98-106.
77. Stewart H, Southwood L, Indugu N, et al. Differences in the equine faecal microbiota between horses presenting to a tertiary referral hospital for colic compared with an elective surgical procedure. *Equine veterinary journal* 2019;51:336-342.
78. Salem S. Epidemiological and Microbiome Studies of Equine Colic: University of Liverpool, 2017.
79. Stewart AS, Pratt-Phillips S, Gonzalez LM. Alterations in intestinal permeability: the role of the “leaky gut” in health and disease. *Journal of equine veterinary science* 2017;52:10-22.
80. Jalanka-Tuovinen J, Salonen A, Nikkilä J, et al. Intestinal microbiota in healthy adults: temporal analysis reveals individual and common core and relation to intestinal symptoms. *PloS one* 2011;6:e23035.
81. Barr B, Waldridge B, Morresey P, et al. Antimicrobial-associated diarrhoea in three equine referral practices. *Equine Veterinary Journal* 2013;45:154-158.
82. Barr BS, Waldridge BM, Morresey PR, et al. Antimicrobial-associated diarrhoea in three equine referral practices. *Equine veterinary journal* 2013;45:154-158.
83. Cohen N, Woods A. Characteristics and risk factors for failure of horses with acute diarrhea to survive: 122 cases (1990-1996). *Journal of the American Veterinary Medical Association* 1999;214:382-390.
84. Collinet A, Grimm P, Julliand S, et al. Oral administration of antibiotics alters fecal ecosystem of adult horses in the long-term. *Journal of Equine Veterinary Science* 2019;76:94.
85. Gustafsson A, Båverud V, Gunnarsson A, et al. Study of faecal shedding of Clostridium difficile in horses treated with penicillin. *Equine veterinary journal* 2004;36:180-182.
86. Harlow BE, Lawrence LM, Flythe MD. Diarrhea-associated pathogens, lactobacilli and cellulolytic bacteria in equine feces: Responses to antibiotic challenge. *Veterinary microbiology* 2013;166:225-232.

87. Jakobsson HE, Rodríguez-Piñeiro AM, Schütte A, et al. The composition of the gut microbiota shapes the colon mucus barrier. *EMBO reports* 2015;16:164-177.
88. Arnold C, Isaiah A, Coverdale J, et al. The effects of Metronidazole on the equine caecal and faecal microbiome and metabolome. *Equine Veterinary Education* 2017;29:6.
89. Chapuis RJ, Becker AA, Dowling PM, et al. Characterisation of faecal microbiota in horses medicated with oral doxycycline hyclate. *Equine Veterinary Journal* 2023;55:129-141.
90. Chapman AM. Acute diarrhea in hospitalized horses. *Veterinary Clinics: Equine Practice* 2009;25:363-380.
91. Whitfield-Cargile CM, Chamoun-Emanuelli AM, Cohen ND, et al. Differential effects of selective and non-selective cyclooxygenase inhibitors on fecal microbiota in adult horses. *PLoS One* 2018;13:e0202527.
92. Tomlinson J, Blikslager A. Effects of cyclooxygenase inhibitors flunixin and deracoxib on permeability of ischaemic-injured equine jejunum. *Equine veterinary journal* 2005;37:75-80.
93. Zhang X, Han Y, Huang W, et al. The influence of the gut microbiota on the bioavailability of oral drugs. *Acta Pharmaceutica Sinica B* 2021;11:1789-1812.
94. Davis J. Nonsteroidal anti-inflammatory drug associated right dorsal colitis in the horse. *Equine Veterinary Education* 2017;29:104-113.
95. Ratnaike RN, Jones TE. Mechanisms of drug-induced diarrhoea in the elderly. *Drugs & aging* 1998;13:245-253.
96. Zackular JP, Kirk L, Trindade BC, et al. Misoprostol protects mice against severe Clostridium difficile infection and promotes recovery of the gut microbiota after antibiotic perturbation. *Anaerobe* 2019;58:89-94.
97. Stewart HL, Pitta D, Indugu N, et al. Changes in the faecal bacterial microbiota during hospitalisation of horses with colic and the effect of different causes of colic. *Equine Veterinary Journal* 2021;53:1119-1131.
98. Arnold CE, Pilla R, Chaffin MK, et al. The effects of signalment, diet, geographic location, season, and colitis associated with antimicrobial use or Salmonella infection on the fecal microbiome of horses. *Journal of Veterinary Internal Medicine* 2021.
99. Arnold CE, Isaiah A, Pilla R, et al. The cecal and fecal microbiomes and metabolomes of horses before and after metronidazole administration. *Plos one* 2020;15:e0232905.
100. Ericsson AC, Johnson PJ, Gieche LM, et al. The Influence of Diet Change and Oral Metformin on Blood Glucose Regulation and the Fecal Microbiota of Healthy Horses. *Animals* 2021;11:976.
101. Walters WA, Caporaso JG, Lauber CL, et al. PrimerProspector: de novo design and taxonomic analysis of barcoded polymerase chain reaction primers. *Bioinformatics* 2011;27:1159-1161.
102. Caporaso JG, Lauber CL, Walters WA, et al. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci U S A* 2011;108 Suppl 1:4516-4522.
103. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnetjournal* 2011;17:10-12.
104. Bolyen E, Rideout JR, Dillon MR, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* 2019;37:852-857.

105. Callahan BJ, McMurdie PJ, Rosen MJ, et al. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* 2016;13:581-583.
106. Pruesse E, Quast C, Knittel K, et al. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* 2007;35:7188-7196.
107. Baggot JD. The Physiological Basis of Veterinary Clinical Pathology: Blackwell Science Ltd, 2001;55-91.
108. Massacci FR, Clark A, Ruet A, et al. Inter-breed diversity and temporal dynamics of the faecal microbiota in healthy horses. *Journal of Animal Breeding and Genetics* 2020;137:103-120.
109. Mshelia ES, Adamu L, Wakil Y, et al. The association between gut microbiome, sex, age and body condition scores of horses in Maiduguri and its environs. *Microbial pathogenesis* 2018;118:81-86.
110. Janabi AHD, Biddle AS, Klein DJ, et al. The effects of acute strenuous exercise on the faecal microbiota in Standardbred racehorses. *Comparative Exercise Physiology* 2017;13:13-24.
111. Plancade S, Clark A, Philippe C, et al. Unraveling the effects of the gut microbiota composition and function on horse endurance physiology. *Scientific Reports* 2019;9:9620.
112. Faubladier C, Chaucheyras-Durand F, Da Veiga L, et al. Effect of transportation on fecal bacterial communities and fermentative activities in horses: Impact of Saccharomyces cerevisiae CNCM I-1077 supplementation. *Journal of animal science* 2013;91:1736-1744.
113. Schoster A, Mosing M, Jalali M, et al. Effects of transport, fasting and anaesthesia on the faecal microbiota of healthy adult horses. *Equine veterinary journal* 2016;48:595-602.
114. Salem SE, Maddox TW, Berg A, et al. Variation in faecal microbiota in a group of horses managed at pasture over a 12-month period. *Scientific reports* 2018;8:1-10.

Appendix I

Definitions adapted from Costa & Weese, 2018.[34]

Microbiota: All microorganisms of a particular environment

Microbiome: All microorganisms along with their genetic material and their interaction with an environment

Alpha Diversity: Describes characteristics of individual samples; indices include richness, evenness, and diversity

- Richness: Total number of taxa (eg, species, genera, families, phyla) present in an environment, either through direct measure (observed richness) or through calculations to estimate the true richness that would hae been detected if the entire population had been studied (estimated richness)
- Evenness: Distribution of species (eg, prevalence or relative abundance of each population within a microbial community)
- Diversity: Mathematical equation that takes into account richness and evenness (ie, it quantifies how equal a microbial community is)

Beta Diversity: Comparisons of the overall composition of the microbiota between samples or groups

- Membership: Members (eg, species, etc) that are or are not present
- Structure: Broader comparison that takes into account the members that are or are not present, as well as their relative abundance