THE EQUINE FECAL MICROBIOME - EFFECTS OF DIET, ANTIMICROBIALS, AND COLITIS

A Thesis

by

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ABSTRACT

The microbiome of the equine gastrointestinal tract (GIT) form a symbiotic relationship with the horse, and play a critical role in nutrition, metabolism, and immunity. This work utilized 16S rRNA sequencing and metabolomics to characterize the fecal microbiome of healthy horses and to measure the dysbiosis caused by antimicrobial use and colitis.

The fecal microbiome of healthy horses was comprised of the following phyla: Firmicutes (45%), Bacteroidetes (31.5%), Verrucomicrobia (9%), Spirochaetes (6%), Tenericutes (1.8%) with the remainder (6.7%) unclassified. Amongst healthy horses, diet had more influence on the fecal microbiome than other variables, such as age, breed, sex, geographic location, or season. Feeding concentrate at > 1-2% BW in kg/day altered the community composition but not diversity.

The presence of colitis had a marked effect on the fecal microbiome. Horses with acute colitis caused by antimicrobial use (AAD) or infection with *Salmonella* showed a decreased richness and evenness compared to healthy horses. Horses with colitis showed an increased abundance of Bacteroidetes (q=0.002) and Proteobacteria (q=0.001), while horses with *Salmonella* infection showed a decrease in Firmicutes (q=0.001) and AAD horses showed a decreased abundance of Verrucomicrobia (q=0.001).

Metronidazole administration decreased species richness and evenness and altered the bacterial community composition (ANOSIM, p=0.008). The most abundant phyla were Bacteroidetes and Firmicutes, but significant changes in Actinobacteria,

Spirochaetes, Lentisphaerae, and Verrucomicrobia occurred during metronidazole administration and coincided with clinical signs of GIT disease. Metronidazole altered the metabolism of amino acids, carbohydrates, lipids, nucleic acids and cofactors, and vitamins.

Antimicrobial agents induced a severe dysbiosis, regardless of whether horses developed diarrhea. Antibiotics markedly reduced diversity measures compared to control horses. All horses on antibiotics, including diarrhea and non-diarrhea controls, had changes in phyla compared to non-antibiotic-treated control horses. The phylum Verrucomicrobia distinguished horses with antibiotic induced colitis from antibiotic-treated and non-antibiotic-treated control horses.

In summary, diet can affect the fecal microbiome of healthy horses by feeding high amounts of concentrate. The effects of antibiotic administration in itself, antibiotic-induced colitis, or *Salmonella* infection, all dramatically reduced diversity and altered bacterial community composition. The effects of antibiotics and disease are larger than those of diet.

DEDICATION

This work would not have been possible without the support of my family and friends. To Keith, who thought of the PhD in the first place and had to live with me every day until I finished. To my Mom whose encouragement never wavered. And to my sister Catherine, who always thought I could, even when I doubted it myself.

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Contributors

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NOMENCLATURE

AAD	Antimicrobial associated diarrhea
ABX	Antibiotic
ANOSIM	Analysis of similarities
ASV	Amplicon sequence variant
BW	Body weight
CON	Control horse
DI	Dysbiosis index
GIT	Gastrointestinal tract
NAHMS	National Animal Health Monitoring System
KEGG	Kyoto Encyclopedia of Genes and Genomes
PCA	Principal Component Analysis
PCoA	Principal Coordinate Analysis
LEfSe	Linear discriminant analysis effects size
OTU	Operational taxonomic unit
SCFA	Short chain fatty acids
USDA	United States Department of Agriculture

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1. INTRODUCTION

1.1 Equine Gastrointestinal Disease

Despite the fact that there are multiple types of GIT diseases in horses, the non-specific term colic is used to describe the behavioral manifestations of horses with abdominal pain from all forms of GIT disease. It is difficult to estimate the true prevalence of GIT disease in the general equine population, but the National Animal Health Monitoring System (NAHMS) of the United States Department of Agriculture (USDA) performed three surveys of horse owners in 1998, 2005, and 2015 (1-3). The study from 1998 estimated that horses in the US experienced 4.2 episodes of GIT disease per 100 horses per year, although similar studies by academic institutions have estimated the incidence of colic in horses as high as 10 episodes of colic per 100 horses per year (4, 5). For horses that have previously experienced one episode of colic, this rate increases to 50 episodes per 100 horses per year, indicating that a subset of horses with recurrent or chronic GIT disease exists within the general population (6).

Equine GIT disease is associated with poor health outcome and increased veterinary medical expense. The NAMHS studies have identified a fatality of 15.2% in 1998, rising to 31% in the most recent 2015 survey (2, 3). While these statistics do not indicate the reasons for fatality, such as unfavorable response to treatment or financial limitations imposed by owners, they do reflect a persistence and perhaps increasing prevalence of GIT disease in horses. The cost of GIT disease to owners is also substantial,

both emotionally as well as economically with a reported loss of $115 million per year (7). For these reasons, further research into the cause of GIT disease in horses is warranted.

1.1.1 Anatomical and Physiological Risk Factors for Gastrointestinal Disease in Horses

Researchers have speculated that horses are predisposed to colic because of their fermentative method of degradation and the anatomic makeup of their GIT. The horse is a herbivorous hindgut fermenter, and does not possess the endogenous enzymes required to break down cellulosic plant material (8). As such, the horse is completely reliant upon the activity of the microbial populations of the GIT to convert long stem forages into energy. While cattle perform this same process in their rumen or fore stomach, in the horse, the hindgut is the site of fermentation. By grazing continuously, the horse supplies its large colon with a steady substrate supply that meets approximately 75% of total energy needs for the horse (9).

The horse's GIT has evolved for hindgut fermentation, and may have developed some unique structural adaptations that predispose the horse to colic. Specifically, the horse's GIT has segments of intestine that have a decreasing lumenal diameter, abrupt turns or folds upon itself, and is freely mobile within the abdomen due to the lack of mesenteric attachments to the body wall (10). While the horse has similar GIT compartments compared to other monogastric species (i.e., oral cavity, esophagus, stomach, small intestine, colon, and rectum), their small and large intestines are often implicated in colic. The small intestine (i.e., duodenum, jejunum, and ileum) is roughly 60 feet long and is suspended from the dorsal body wall by a connecting mesentery. The

singularity of the mesenteric attachment and mobility of the small intestine within the abdomen make it susceptible to torsion upon its mesenteric access or incarceration within other abdominal viscera or spaces. The small intestine is the primary site of amino acid and fatty acid absorption from protein and lipid digestion. Small intestinal enzymes are responsible for breaking down hydrolysable carbohydrates, but cannot break down fermentable carbohydrates such as the majority of those found in plant material.

The large intestine includes the cecum, colon (ascending or large colon and descending or small colon), and rectum (10). It is a voluminous organ that can hold over 100 L of ingesta, approximately two-thirds of the total capacity of the equine gastrointestinal tract (11). Also referred to the hindgut, the large colon is critical to the health of the horse. The large colon consists of the right and left dorsal and ventral colon, which are positioned as a two-layered, U-shaped structure within the abdomen. As the large colon is tethered at its sites of origin and termination, the majority of the organ is freely mobile within the abdomen. The large colon is a common site of obstructive or ischemic lesions due to its anatomic topography (e.g., decreasing lumenal diameters, 180 degree turns upon itself, and lack of mesenteric attachments) and is also impacted by inflammatory conditions such as colitis. In the large intestine, the fermentation process is driven by the commensal bacteria or microbiota. The resident microbes degrade the insoluble fiber components of plant material (i.e., cellulose and hemicellulose) into monosaccharides, which are then fermented to produce short chain fatty acids (i.e., acetate, propionate, and butyrate) (8). The fermentation process also results in the production of gasses such as carbon dioxide (CO_2), hydrogen (H_2), and methane (CH_4).

The proportion of SCFA and gasses produced is dependent on the horse's diet (plant intake), its commensal bacteria, and the specific metabolic pathways utilized. Due to the metabolic importance of this organ in horses, diseases of the large colon can have a significant impact on the health of the horse (10).

Current husbandry practices may also exacerbate the horse's tendency for the development of a colic and contribute to the prevalence of GIT disease. Unlike wild horses that continuously exercise while grazing large expanses of native forages, the modern horse is stabled with considerably less acreage to roam and is fed regular meals of hay (dried grass) and grain. This decrease in exercise and change in meal size and frequency, combined with a poor anatomical design may account for an increase in the prevalence of colic. Epidemiological studies have confirmed that increased stabling or decreased pasture turn out (12-14) and feeding high quantities of grain or poor-quality forages (4, 12, 14) are risk factors for the development of GIT disease in horses.

1.1.2 Treatment of Equine GIT Disease

Historically, GIT disease in horses was associated with a poor prognosis, and therapy was not commonly attempted. Following the Vietnam war, techniques used to improve survival in critically-injured soldiers fed back into civilian medicine. Veterinarians adapted this knowledge to treat horses with life-threatening GIT diseases, such as colic and colitis. As a result, early intervention with rapid referral, hemodynamic stabilization, recognition and treatment of endotoxemia, and improved surgical technique were applied to horses with GIT disease. These techniques were effective, and the survival rates of individual horses with GIT disease improved greatly (15, 16).

In the 1990's, outbreaks of colitis due to *Salmonella* and *Clostridia* in veterinary referral centers brought attention to infectious colitis, nosocomial spread of infection, and a greater awareness of antimicrobial use in horses (17-20). In addition to the mortality of affected horses, these referral centers sustained a significant loss of revenue due to institutional closures during decontamination in addition to claims of liability (21-23). As a result, biosecurity practices, such as routine screening for pathogens, isolation criteria, use of personal protective equipment (e.g., gloves, gowns, and booties), and disinfection protocols were implemented that changed the daily management of horses on the farm and in the veterinary medical centers.

While these two eras of investigation reduced the morbidity and mortality of individual horses with GIT disease and prevented spread of infectious forms of colitis, the incidence of GIT disease in the equine population remains high. Furthermore, the underlying cause of equine GIT disease has not yet been elucidated and remains an important clinical concern.

1.1. The Equine Fecal Microbiome and the Impact of Dysbiosis

The relationship between the gut bacteria and health has been well-established in both human and companion animal medicine (24, 25). In humans, the identification of the microbiome and its functional role in ulcerative colitis, Crohn's disease, obesity, and metabolic disease has enabled advances in the understanding, diagnosis, and treatment of various GIT diseases (26). Similar efforts have been made in companion animals with acute and chronic enteropathies (27-29). The identification of the GIT bacteria was made possible by use of next generation sequencing technologies, platforms that can rapidly

identify the DNA sequence of many bacterial species in a sample simultaneously. Prior to the advent of next generation sequencing, the bacteria of the GIT were investigated using traditional culture-based techniques, which greatly under-represented the number of species and diversity of the bacteria in the GIT. With the advent of next generation sequencing, the GIT bacteria are more more completely elucidated and bacteria can now be identified at the level of genus and species.

The bacteria that reside in the gut play a functional role in nutrient breakdown and absorption, the production of short chain fatty acids, the conversion of primary to secondary bile acids, the biosynthesis of vitamins and amino acids, the regulation of the inflammatory environment of the gut, and immune modulation in response to pathogens (24). The presence of a stable microbiota in the GIT is crucial for maintenance of health, and alterations in the identity or abundance of those bacteria are noted in states of disease. Changes in the bacterial communities of the gut that occur in association with disease states, termed dysbiosis, affect the physiologically important metabolic processes performed by the bacteria.

In hopes of reducing the significant morbidity and mortality attributed to GIT disease in the horse, researchers have begun to utilize similar molecular techniques to better characterize the microbiome and its functions in the equine GIT. There is strong preliminary evidence that many factors influence the equine fecal microbiome in states of health, while other factors are associated with dysbiosis and states of disease. While the microbiome is technically composed of bacteria, archaea, fungi, protozoa, and viruses, the term microbiome will refer only to the bacteria as assessed by 16S rRNA sequencing in

this thesis. The objective of this work was to first characterize the fecal microbiome in healthy horses and then describe the impact of diet, antimicrobial use, and colitis on said fecal microbiome.

1.1.1.1. **Hypothesis and Objectives**

The hypotheses of this study are:

1. The fecal microbiota of healthy horses will be affected by diet.
2. Horses with colitis will show evidence of dysbiosis.
3. Antibiotic administration will affect the fecal microbiome and metabolome of healthy horses.
4. Horses that develop diarrhea while on antimicrobials will have a greater degree of dysbiosis and metabolomic changes compared to horses on antimicrobials that maintained normal fecal character or healthy horses not on antimicrobial therapy.

The objectives and specific aims of this study are to:

1. Characterize the fecal microbiota in a large, diverse population of healthy horses and examine the impact of the following variables:
    a. Age
    b. Breed
    c. Sex
    d. State of origin or geographic location
    e. Season
    f. Dietary variables

2. Characterize the fecal microbiota in horses with colitis caused by
    a. Infection with *Salmonella*
    b. Antimicrobial use

3. Compare the cecal and fecal microbiomes and metabolomes in horses receiving metronidazole

4. Compare the fecal microbiome in horses with AAD to that in horses that remained healthy on antimicrobial therapy and to that of a population of healthy controls horses

2. CHARACTERIZATION OF THE FECAL MICROBIOTA IN HEALTHY HORSES

2.1 Overview

This study characterized the fecal microbiota in a large diverse population of healthy horses (n=80) with the aim of evaluating the influence of age, breed, gender, geographic location, season, and diet. Eighty horses, representing 14 breeds from 14 different states within the United States were used as subjects for this study. The fecal microbiota of healthy horses was comprised of the following phyla in order of decreasing abundance: Firmicutes (45%), Bacteroidetes (31.5%), Verrucomicrobia (9%), Spirochaetes (6%), and Tenericutes (1.8%). The following phyla were also present, representing less than 1% of the total bacteria: Euryarchaeota, Fibrobacteres, Proteobacteria, Actinobacteria, Planctomycetes, SR1, and Synergistes. Individual factors had, at most, minor influences on either microbial community composition (i.e., breed, state of residence, season, percentage maximum fiber in the concentrate, amount of concentrate fed, and time spent in pasture) or diversity (gender, time spent in pasture), but not both. When analyzed by a dietary scale that included both forages and concentrates, horses fed higher amounts of grain (1-2% of body weight in kg/day) had different microbial community composition compared to those eating forages alone or forages in combination with smaller amounts of grain (<0.5% of body weight in kg/day). These differences resulted in horses on Diet E (1-2% concentrate) clustering from those on Diets A-D (<0.5% concentrate) due to an increase in the phylum Actinobacteria.

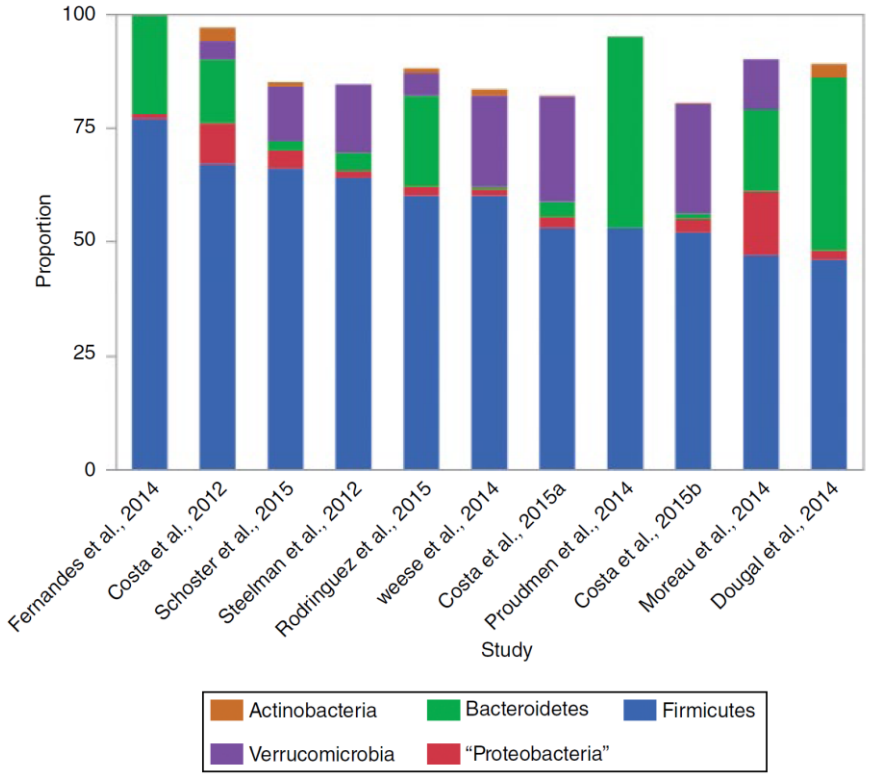
2.2 Introduction

As with humans and companion animals, the fecal microbiota appears to be highly relevant to the health of horses. Gastrointestinal bacteria play a functional role in feedstuff breakdown and absorption, the production of short chain fatty acids, the conversion of primary to secondary bile acids, the biosynthesis of vitamins and amino acids, the regulation of the inflammatory environment of the gut, and immune modulation in response to pathogens (24). In order to understand how the microbiome is altered by disease, it is critical to first define the microbiome in healthy horses.

Initial efforts to characterize the equine microbiome began by identifying the bacterial communities in each portion of the GIT in healthy horses. Early studies found that the microbiome of each GIT compartment is unique, with significant differences noted between the stomach, small, and large intestine (30, 31). Studies confirmed that feces are an adequate proxy for the hindgut of the horse (30, 31), allowing for minimally-invasive sampling from a portion of the GIT frequently affected by disease.

Multiple studies have been performed in healthy horses and 17-20 phyla with varying abundances have been identified in equine feces (32-34). The major phyla that appear consistently across studies include Firmicutes (gram positive anaerobes or facultative anaerobes), Bacteroidetes (gram negative largely anaerobes), Verucomicrobia (gram negative anaerobes), Actinobacteria (gram positive anaerobes), and Proteobacteria (gram negative anaerobes). An example of the wide variation of the microbiota in healthy horses from 11 studies performed from 2012-2015 is found in Figure 2.1 (35).

Figure 2.1 Phylum distribution of the equine intestinal microbiota reported in 11 different studies (35). Reprinted with permission. Copyright © 2017 by Wiley Blackwell.



Speculated cause for this variability includes factors related to the subjects enrolled and also the methodology employed. Horse-related variables include signalment, such breed (32, 34, 36), age (37, 38), and pregnancy status (39) as well as external factors, such as geographic location (32), transport (40), exercise intensity (41-43), fasting (44, 45), and season (46) appear to have some influence on the fecal microbiome. Dietary variables such

as exposure to pasture (32, 47), abrupt feed change (47, 48), and feeding concentrate versus forage (49) can alter the microbiome to some degree, while the feeding of high starch concentrates can alter the colonic environment and induce laminitis (37, 50).

Initial efforts to characterize the microbiome may not adequately represent the diversity of the equine population due to their small sample size, the use of university teaching herds, or the use of horses housed at a singular location (51, 52). Also, early studies often employed multiple sequencing methodologies (i.e., terminal-restriction fragment length polymorphism (49), Illumina, 454-pyrosequencing (34, 37, 50), as well as a variety of methods for sample collection, storage, and DNA extraction, which makes comparison across studies difficult. Furthermore, because these factors have been described independently across studies, it remains unclear how large the effect of these individual factors is.

As the importance of the intestinal microbiota in states of health and disease continues to emerge, having clearly established ranges for the abundances of taxa with measured responses to normal variation, such as diet, location, and season or internal factors, such as age, breed, and gender is essential. This foundational knowledge will be critical for understanding the effects of disease or measuring the response to therapeutic efforts. The objective of this study, therefore, was to define the fecal microbiota in a large diverse population of healthy horses.

2.3 Materials and Methods

2.3.1 Subjects and sampling

Healthy horses were sampled from non-hospital environments across multiple geographical locations in the United States. Veterinarians (n=17) were asked to collect feces from horses during routine wellness exams. Veterinarians were instructed to sample no more than 2 horses per farm and collect feces from a total of 10 horses. The inclusion criteria for healthy horses consisted of the following: one year of age or older, no antibiotic or non-steroidal anti-inflammatory administration within 6 months, no history of colic or diarrhea within 6 months, and a normal physical examination on the day of sample collection. Feces was collected after natural defecation and stored at -18°C until all samples were ready for shipping. Samples were shipped frozen overnight to the GI Laboratory at Texas A&M University and kept frozen at -80°C until DNA extraction. Approximately 200 fecal samples from healthy horses were collected, and 80 samples were selected for sequencing in order to have representation from each geographic location, age, breed, sex, and diet.

The following information was collected for all horses: age, breed, gender, weight (estimated by the veterinarian), season during fecal collection, geographical location (state of origin), and diet. The diet was categorized by a variety of factors, such as hay type (i.e., warm season grass, cool season grass, warm season grass plus legume, cool season grass plus legume, legume, or no hay), pasture type (warm, transition, or cool grasses), time spent in pasture (none, some, or continuous), percentage maximum crude fiber in the concentrate (low; 5-8%, medium; 10-15%, high; 18-33%), and amount of concentrate (none, 0.5%, and 1-2% of body weight in kilograms per day). Finally, a dietary scale was created that was inclusive for all aspects of the diet detailed in Table 2.1.

Table 2.1 Dietary scale based upon consumption of forages and concentrate.

Diet	Forage	% Maximum Crude Fiber	Amount (%BW in kg/day)
A	Hay and/or pasture	None	None
B	Hay and/or pasture	5-8	≤0.5
C	Hay and/or pasture	10-15	≤0.5
D	Hay and/or pasture	18-33	≤0.5
E	Hay and/or pasture	10-15	1-2

2.3.2 DNA extraction

One hundred mg of feces from the center of each fecal ball or liquid fecal sample was aliquoted into a sterile 1.7 ml tube (Microtube, Sarstedt AG & Co, Numbrecht, Germany) containing 150 µl of 0.1 mm zirconia-silica beads and 100 µl of 0.5 mm zirconia-silica beads (BioSpec Products Inc., Bartlesville, OK, USA). Samples were then homogenized (FastPrep-24, MP Biomedicals, USA) for a duration of 1 minute at a speed of 4 m/s. DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) following the manufacturer's instructions.

2.3.3 Sequencing of 16S rRNA genes

Sequencing of the V4 region of the 16S rRNA gene was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq platform (Illumina Inc., San Diego, CA). Following the manufacturer's instructions, 2x300 paired-end reads were produced using 515F (5'-GTG YCA GCM GCC GCG GTA A-3') and 806R (5-

GGA CTA CNV GGG TWT CTA AT-3') primers (53, 54). The PCR reaction was performed in a single-step 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles (5 cycles used on PCR products) of 94°C for 30 seconds, 53°C for 40 seconds, and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. Using Illumina TruSeq DNA's protocol, a DNA library was set up and Illumina MiSeq was used for sequencing according the manufacturer's guidelines.

2.3.4 Analysis of sequences

A total of 80 samples were analyzed, which generated 4,386,598 quality sequences. Sequences were analyzed using a QIIME 2 (Quantitative Insights into Microbial Ecology)(55) v.2019.7 pipeline as described elsewhere.(56, 57). Briefly, barcodes and primers were removed and short (<150bp), ambiguous, homopolymeric sequences were depleted from the dataset. DADA2 was used to identify and remove chimeric sequences.(58) The amplicon sequence variant (ASV) table was created using DADA2 (59), and rarefied to 41,383 sequences per sample based on the lowest read depth in all samples for an even depth of analysis. Sequences determined to be belonging to mitochondria, chloroplasts, unassigned, or those belonging to the phylum cyanobacteria were excluded from further analysis. Data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession number SRP228480 and the BioProject number PRJNA580257.

Alpha diversity metrics Chao1 (richness), observed ASVs (species richness), and Shannon diversity (evenness) were generated in QIIME2. Beta diversity was evaluated

with the phylogeny based weighted and unweighted UniFrac distance metric, and Principal Coordinate Analysis (PCoA) plots for visualization were generated in QIIME2.

2.3.5 Statistical analysis

Prior to analysis, data were tested for normality using the Shapiro-Wilk test (JMP Pro 14, SAS, Marlow, Buckinghamshire). As the data were not normally distributed, non-parametric measures were used throughout the study.

Statistical analysis of alpha diversity indices (i.e., Chao 1, observed ASVs, and Shannon) was performed using the software package PRISM (PRISM 7, GraphPad Software Inc., San Diego, CA). A Kruskal Wallis test with a Dunn's multiple comparison test was used to test alpha diversity measures for the variables age range (1-5, 6-10, 11-15, 16-20, 21-25 years old), gender, season, and dietary variables (i.e., percentage maximum crude fiber in the concentrate, amount of concentrate, time in pasture, hay type, grass type in pasture), and diet classification scale. A one-way ANOVA and Tukey's post-test were used to analyze breed and state of origin.

Beta diversity (bacterial community composition) was measured with weighted and unweighted UniFrac metrics and visualized for clustering with Principle Coordinate Analysis (PCoA) plots. An Analysis of Similarity test (ANOSIM) within the PRIMER 6 (PRIMER-E Ltd. Luton, UK) software package was performed on the beta diversity distance matrices to assess the significance of the differences in the bacterial community composition based upon the variables noted above.

Univariate analysis of the bacterial taxa in the fecal samples was evaluated using a Kruskal-Wallis test (PRISM 7, GraphPad Software Inc., San Diego, CA) followed by a

Dunn's multiple comparison post-test. Only bacterial taxa present in at least 50% of the samples were included in the analysis.

Linear discriminant analysis effect size (LEfSe) using the web-based program Calypso v8.62 (http://cgenome.net/wiki/index.php/Calypso) was performed to analyze the abundance of bacterial taxa and their associations with any of the 5 diet categories. A cut-off threshold of 3.5 was set for significance.

2.4 Results

Information regarding the signalment, weight, diet, state of residence, and season of sampling in healthy horses is shown in Appendix A.

2.4.1 The effect of diet A-E on the fecal microbiome of healthy horses

2.4.1.1 Beta diversity measures

A principal coordinate analysis plot of unweighted Unifrac distances in normal horses by diet category is shown in Figure 2.2. Figure 2.2 A shows some clustering among horses on each diet (ANOSIM, unweighted, R=.156, P=.052), whereas Figure 2.2 B indicates clustering between horses on Diet E and those combined on Diets A-D (ANOSIM, R=.15, P=.025).

Figure 2.2 PCoA plots of unweighted Unifrac distances in normal horses. A) Diets A-E. B) Diets A-D versus E.

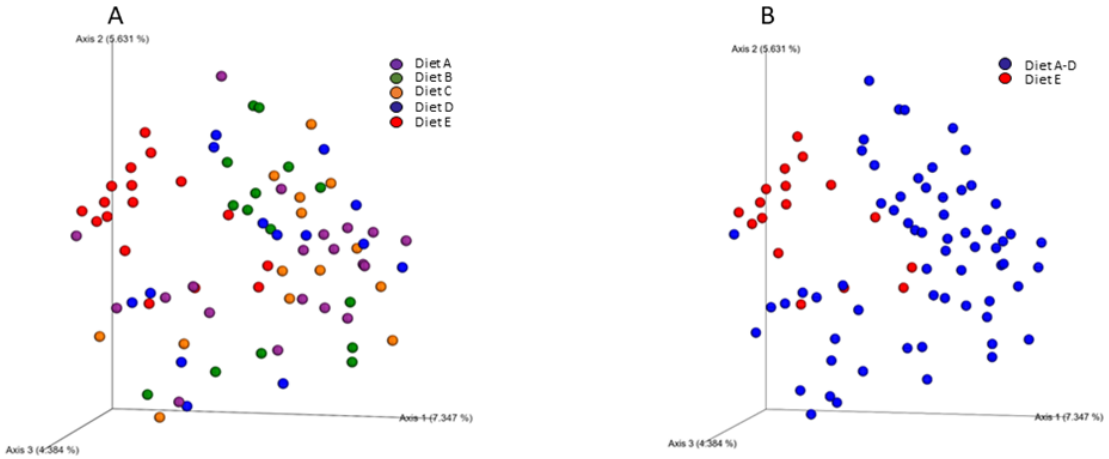


Table 2.3 contains the pairwise comparison of the unweighted and weighted Unifrac distances by diet category.

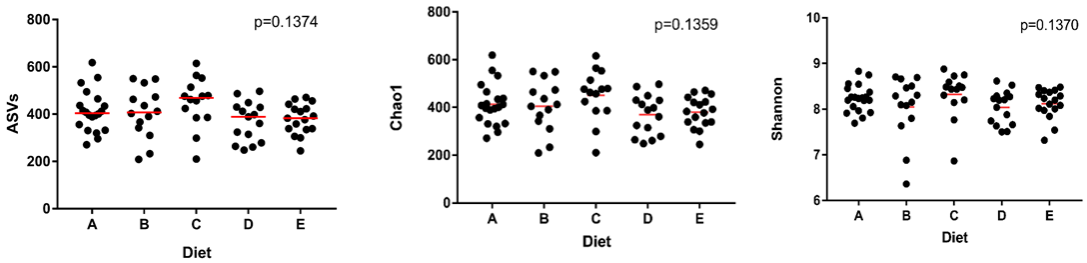
Table 2.2 Pairwise and overall comparisons of weighted and unweighted Unifrac distances by diet category.

	Unweighted		**Weighted**	
	R Statistic	**P value**	**R Statistic**	**P value**
Overall	0.156	0.052	0.076	0.0003
A, B	0.088	0.0001	0.084	0.076
A, C	-0.01	0.492	-0.012	0.52
A, D	0.106	0.106	0.055	0.114
A, E	0.356	0.001	0.167	0.002
B, C	-0.019	0.623	0.007	0.348
B, D	0.061	0. 119	0.036	0.171
B, E	0.201	0.002	0.128	0.008
C, D	0.017	0.35	-0.022	0.686
C, E	0.365	0.001	0.146	0.003

2.4.1.2 Alpha diversity measures

The alpha diversity metrics of healthy horses as stratified by diet category is displayed in Figure 2.3. Neither ASVs, Chao 1, nor Shannon metrics showed statistically significant differences between diet categories.

Figure 2.3 Alpha diversity metrics of healthy horses by diet. There were no significant differences between diets A-E for the ASV, Chao 1, or Shannon metrics.



2.4.1.3 Taxonomy

Information regarding the taxa of bacteria at the phylum level in feces of healthy horses, stratified by diet, can be found in Table 2.4. At the phylum level, four taxa were significantly altered by diet, but only Actinobacteria remained statistically significant after adjustments for multiple comparisons were made. The abundance of Actinobacteria was decreased in horses on Diet E when compared to those on Diets B, C, or D (p=0.003,

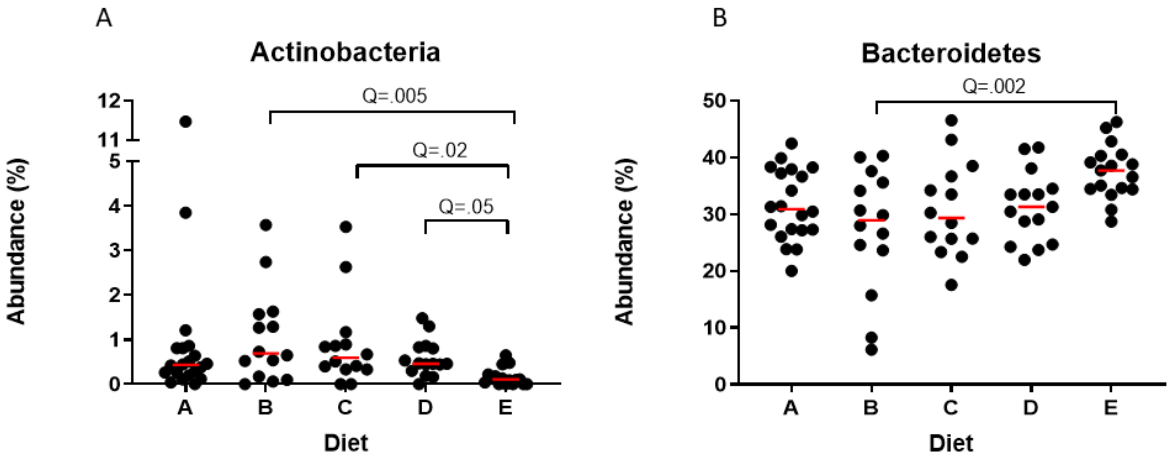
q=0.04). At the family level, only *Micrococcaceae* from the phyla Actinobacteria, class Actinobacteria was significantly decreased in the feces from horses on Diet E (p=0.01, q=0.03) whereas the class Coriobacteriia, family *Coriobacteriaceae* was not significantly different after adjustments for multiple comparisons (p=0.04, q=0.10).

Table 2.3 Taxa of bacteria at the phylum level in the feces of healthy horses, stratified by diet A-E. Superscripts denote significant differences between groups.

Bacterial group	Diet A		Diet B		Diet C		Diet D		Diet E			
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value
Euryarchaeota	0.9	0-4.7	1.4	0.4-6.8	0.6	0-2.1	0.5	0.1-5.6	1.1	0.4-4	0.1521	0.3205
Actinobacteria	$0.4^{a,b}$	0-11.5	0.7^{b}	0-3.6	0.6^{b}	0-3.5	0.5^{b}	0-1.5	0.1^{a}	0-0.6	0.0031	0.0403
Bacteroidetes	$30.9^{a,b}$	20-42.5	28.9^{a}	6.1-40.3	$29.4^{a,b}$	17.6-46.6	$31.3^{a,b}$	22-41.8	37.7^{b}	28.7-46.3	0.0094	0.0611
Fibrobacteres	0.9	0-4.6	0.2	0-3.4	1.1	0.2-2.6	0.6	0-8.9	0.7	0.1-3	0.1726	0.3205
Firmicutes	44	28.8-55.4	45.8	22-74.1	48.3	24.4-60.3	45.6	26.4-65.3	40.6	25.2-56.2	0.3324	0.5402
Planctomycetes	$0^{a,b}$	0-0.1	$0^{a,b}$	0-0.1	0^{a}	0-0.4	$0^{a,b}$	0-0.4	0^{b}	0-0	0.0427	0.1388
Proteobacteria	$0.6^{a,b}$	0.1-7.5	$0.9^{a,b}$	0.1-59.8	$0.6^{a,b}$	0.2-29.5	0.4^{a}	0.1-3	1.6^{b}	0.2-9	0.0278	0.1205
SR1	0	0-0.3	0	0-0.1	0	0-0.1	0	0-1.2	0	0-0.1	0.1007	0.2618
Spirochaetes	5.6	2-16.2	5.8	0.4-12.8	5.6	2.2-11.1	6.1	1.1-12.5	6.9	2.8-9.6	0.9122	0.9122
Synergistetes	0	0-0.6	0	0-0.1	0	0-0.1	0	0-0.2	0	0-0.1	0.8424	0.9122
Tenericutes	2	0.5-3.9	2.1	0.7-7.1	1.5	1-6	1.7	0-5.2	2.2	0.7-5.6	0.4766	0.6884
Verrucomicrobia	10.3	1.4-16.2	9.9	2.5-24.1	9.1	1.8-18	9	1.6-18.2	7	2.1-17.5	0.7559	0.9122

Linear discriminant effects size analysis (LEfSe) at the phylum level indicated that increased abundance of Actinobacteria was associated with Diet A whereas increased abundances of Bacteroidetes and WPS2 were associated with Diet E. Figure 2.4 indicates the median abundances of Actinobacteria and Bacteroidetes in each horse in the respective dietary groups. WPS2 is not included because of its low prevalence in the population despite significance on LEfSe analysis.

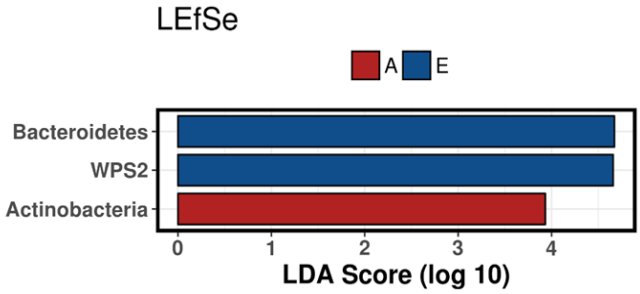
Figure 2.4 The median abundances (%) of significantly altered phyla according to linear discriminant effects size analysis of healthy horses on Diets A-E. A). Horses on Diet E have a significantly lower abundance of Actinobacteria than those on Diets B, C, and D. B). Horses on Diet E have a significantly higher abundance of Bacteroidetes than those on Diet B.



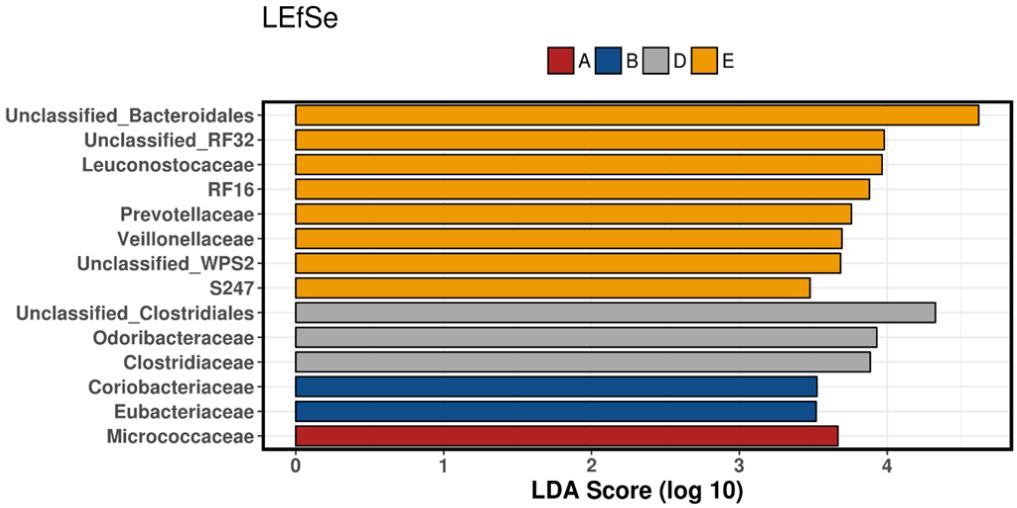
The results of linear discriminant effects analysis of the fecal microbiota in healthy horses as stratified by diet at the phylum and family level are shown in Figure 2.5.

Figure 2.5 Linear discriminant analysis of the fecal microbiota on the phylum (A) and family (B) level in healthy horses as stratified by diet. Capital letters represent different diets fed.

A.



B.

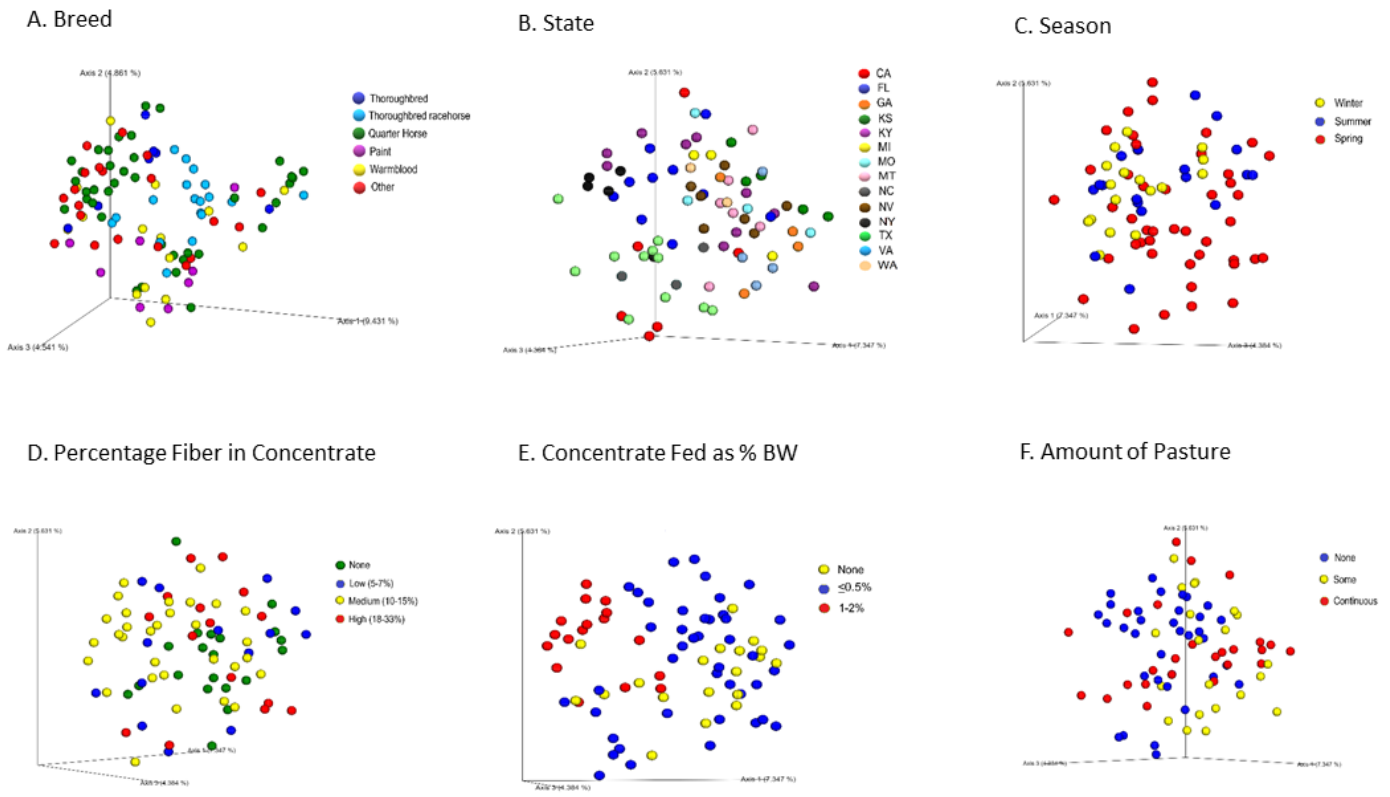


2.4.2 The effect of signalment and external factors on the fecal microbiome of healthy horses.

2.4.2.1 Beta-diversity measures

The following variables achieved significance: breed (unweighted, R=0.19, p=0.0001), state (unweighted, R=0.29, p=0.001; weighted, R=0.17, p=0.002), season (unweighted, R=0.02, p=0.002; weighted, R=0.10, p=0.04), percentage maximum crude fiber in the concentrate (unweighted, R=0.05, p=0.05), amount of concentrate (unweighted, R=0.287, p=0.002; weighted, R=0.132, p=0.05), and time in pasture (unweighted R=0.06, p=0.02). A principal coordinate analysis plot of unweighted Unifrac distances in normal horses by these external factors is shown in Figure 2.6.

Figure 2.6 Principal coordinate analysis plot of individual factors that demonstrated significant clustering by A. breed (Thoroughbred, dark blue; Thoroughbred racehorse, light blue; Quarter Horse, green; Paint, purple; Warmblood, yellow; other, red); B. state of residence (CA, red; FL, royal blue; GA, orange; KS, dark green; KY, purple; MI, yellow; MO, teal; MT pink; NC, gray; NV, brown; NY, black; TX, lime green; VA, light blue; WA, tan); C. season (Winter, yellow; Summer, blue; Spring, red); D. percentage of maximum crude fiber in the concentrate; E. amount of concentrate (None, green; Low, green; Medium, yellow, High, red), and F. time in pasture (None, blue; Some, yellow; Continuous, red).



Results of ANOSIM analysis of unweighted and weighted Unifrac distances for all individual variables are listed in Table 2.5.

Table 2.4 The results of ANOSIM testing for individual variables in healthy horses using unweighted and weighted Unifrac distances.

	Unweighted		**Weighted**	
	R Statistic	**P value**	**R Statistic**	**P value**
Age range	0.006	0.43	0.029	0.25
Breed	**0.191**	**<.001**	0.162	0.08
Sex	0.007	0.40	0.004	0.39
State	**0.29**	**0.001**	**0.172**	**0.002**
Season	**0.017**	**0.002**	**0.1**	**0.035**
% Fiber	**0.058**	**0.05**	0.047	0.094
Amount Concentrate	**0.287**	**0.002**	0.132	0.049
Time in Pasture	**0.061**	**0.015**	0.023	0.19
Pasture Zone	0.042	0.102	0.038	.013
Hay Type	0.008	0.43	-0.08	0.54

2.4.2.2 *Alpha diversity measures*

Of all the variables analyzed, only gender and time in pasture had any significant effect on alpha diversity measures (Figures 2.7 and 2.8). Mares had lower diversity than geldings but not stallions for the Chao 1 (p=0.05) and observed ASVs (p=0.05) metrics, but the significance was lost after correction for multiple comparisons (q=0.07) for both.

The lack of pasture exposure decreased all 3 diversity indices compared to some pasture (Chao 1, p=p.01, q=0.03; observed ASVs, p=0.01, q=0.03; Shannon, p=0.02, q=0.03). The effect of other individual diet-related variables was not significant.

Figure 2.7 Alpha diversity metrics by gender. Mares have significantly lower richness as measured by the ASVs and Chao 1 metrics as compared to geldings.

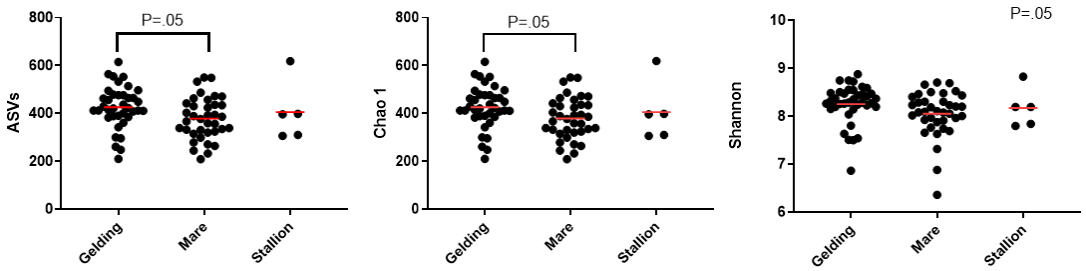
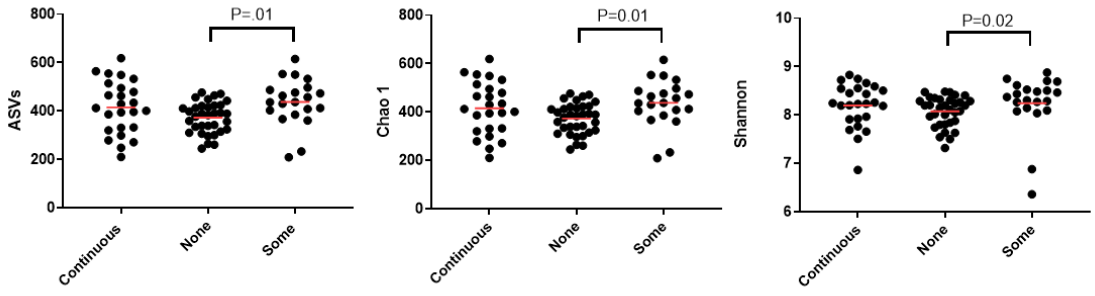


Figure 2.8 Alpha diversity metrics of healthy horses by time spent in pasture**.** Horses with some exposure to pasture have significantly more richness or evenness than those with no pasture exposure as measured by the ASVs, Chao1 and Shannon diversity metrics.



2.5 Discussion

This study characterized the fecal microbiome of adult healthy horses by sampling from a large, diverse equine population. A stringent set of inclusion criteria was applied to ensure that only healthy individuals were included. A veterinarian familiar with the horse was asked to perform a physical exam, estimate weight, confirm dietary information and exclude those with recent history of NSAID or antibiotic use, and gastrointestinal disease. The veterinarian collected samples and stored them prior to shipping to the laboratory. Inclusion of veterinarians in this process likely enhanced the accuracy of medical information and sample handling as opposed to using crowd-sourced samples. As such, this study avoided potential sources of bias such as using university teaching herds or horses from singular geographical locations.

Overall, the results of this study found that fecal microbiome of healthy horses was composed of Firmicutes, Bacteroidetes, Verrocomicrobia, Spirochaetes, Tenericutes, Proteobaceria, Fibrobacteria and Actinobacteria. These results are similar to other studies, with the exception of the abundances of each phylum. To date, this is one of the largest and most diverse sample sets used for investigation of the healthy horse fecal microbiome, which allows for investigation into factors that may influence the bacterial community diversity and composition.

Statistical analysis of this healthy horse population indicated that the inherent factors such as age, breed, and gender had a minor impact on the fecal microbiome compared to disease states. Unlike previous studies, advanced age (horses over 20 years

of age) (37, 38) did not significantly affect the fecal microbiome of the horses in this study. Breed showed a mild but statistically significant clustering on PCoA plot with no significant effect noted on alpha diversity metrics. Gender had an impact on richness indices (mares had decreased alpha diversity metrics compared to geldings), but no effect was seen on beta diversity. Similar results have been reported in the human literature with females having less richness than males.(60) As geldings have very low levels of testosterone due to castration, this effect may be due to the influence of estrogen and progesterone. Due to the limited impact of these variables on diversity measures, their effect on taxonomy was not investigated.

Environmental factors that were examined included state of residence, season and diet. Healthy horses displayed mild but significant clustering by state on PCoA plot, with horses from Texas appearing to cluster together. There was a significant lack of clustering by season of sample collection on a PCoA plot. Samples from healthy horses were collected only during the winter, spring and summer months. The authors speculate that this lack of effect in 3 seasons would also apply to the fall. Diet was examined by individual variables that included the type and amount of forages and concentrate prior to the development of the inclusive dietary scale. Three dietary factors showed significance on ANOSIM testing, the amount of concentrate, the maximum percentage of fiber in the concentrate and the time spent in pasture. Only the amount of concentrate demonstrated significant clustering on PCoA plots with horses fed high amounts of grain (1-2% of body weight in kg/day) showing separation from those horses fed either no grain or <0.5% of body weight in kg/day. The variable time spent in pasture did have significant effects on

both alpha (horses on some pasture had increased richness and evenness compared to those on no pasture) and beta diversity (no significant clustering), but as these effects were mixed they are difficult to interpret.

A dietary scale that accounted for the feeding of forages and concentrates was created to stratify horses based upon common feeding practices. Horses in Diet A ate only forages, whereas those on Diets B-D ate a combination of forages and amounts of concentrate suitable for hoses in light to moderate athletic activity. These groups ate concentrate containing a low, moderate and high percentage of maximum crude fiber. Horses fed Diet E were elite athletes in intense exercise training, specifically, Thoroughbred racehorses. These horses were fed a medium fiber containing concentrate similar to group C, but at 2-4 times the amount. Stratification of healthy horses by dietary group resulted in mild differences in beta diversity and taxonomy between Diet E and B-D. Horses in Diet E tended to cluster separately from the other groups on PCoA plot of unweighted Unifrac distances, and had decreased abundance of Actinobacteria, a finding which has been reported in horses fed high starch diets (37, 61). Horses fed Diet E had increased amounts of Bacteroidetes on LEfSe analysis, an effect which nearly reached significance on univariate analysis. These results suggest that horses fed concentrates at 1-2 mg/kg per body weight per day have differences in bacterial community composition.

The group of horses fed Diet E comprised a much more homogenous population compared to those on Diets A-D. Horses in Group E were young Thoroughbreds at training centers or racetracks in New York, Kentucky and Florida, consuming 2-4 times the amount of concentrate fed to other horses. It is possible that differences in the fecal

microbiome of horses on Diet E were due not to diet alone, but to similarity in breed, age or exercise intensity. In order to investigate if breed and age accounted for these differences, ANOSIM testing was performed separately for breed (Thoroughbred) and age group (1-5 years) between horses on Diets A-D versus Diet E. The lack of significant differences between these variables in the two groups indicates that fecal microbiome differences may be due to diet or exercise intensity. As previous studies have found that the bacterial community composition changes are associated with the initiation of exercise that return to baseline upon adaption (41-43) it is likely that diet alone is responsible for the changes in beta diversity and taxonomy of horses fed Diet E.

2.6 Conclusion

The healthy horse fecal microbiome is composed in order of decreasing abundance: Firmicutes, Bacteroidetes, Verrocomicrobia, Spirochaetes, Tenericutes, Proteobaceria, Fibrobacteria and Actinobacteria. While some individual (sex, breed) or external (state of residence) factors may have minor influence on fecal microbiome, diet and specifically the amount of concentrate fed, appear to influence the bacterial community composition. Feeding concentrate at 1-2% of body weight per kilogram per day is associated with an increase in the amount of Actinobacteria in fecal samples.

3. A COMPARISON OF THE FECAL MICROBIOME OF HORSES WITH COLITIS CAUSED BY ANTIMICROBIAL USE AND SALMONELLA TO HEALTHY HORSES

3.1 Overview

Horses with acute colitis caused by antimicrobial use (AAD) and infection with Salmonella have decreased richness and evenness compared to healthy horses. Each form of colitis has a unique microbial signature. Horses with both forms of colitis have increased abundance of Bacteroidetes (q=0.002) and Protebacteria (q=0.001). Horses with Salmonella have decreases in Firmcutes (q=0.001) while horses with AAD have decreases in Verrucomicrobia (q=0.001).

3.2 Introduction

Colitis is a subset of GIT disease, and is classified as an acute or chronic common inflammatory condition of the large colon (62). While there are no statistics that estimate the prevalence of this particular subset of GIT disease in the equine population, roughly one third to half of all horses admitted for GIT disease to the Texas A&M VMTH hospital population are diagnosed with colitis (personal communication). Early in the disease process, horses often present with non-specific symptoms of colic due to ileus, distention of the bowel and inflammation. Eventually, horses develop diarrhea accompanied by fever, lethargy, inappetence, and neutropenia. The physiological effects of colonic inflammation results in a loss of colonization resistance, overgrowth of pathogenic bacteria, toxin production, mucosal inflammation, alterations in the metabolism of carbohydrates, volatile fatty acids and bile acids, enhancement of secretory mechanisms

and reduced absorption of water and electrolytes. The morbidity and mortality of horses with colitis is related to the primary damage to the colon (including the colonic mucosa or infarction of a portion of the colon) or to secondary effects such as endotoxic shock, laminitis, coagulation deficits, hypovolemia, electrolyte derangement, peritonitis, bacteremia, and renal failure (63).

Colitis has may been further categorized by the inciting cause, such as infectious agents (e.g., *Salmonella*, *Clostridia*, *Lawsonia*, Potomac horse fever, or parasites), antimicrobial-induced, non-steroidal anti-inflammatory medication or infiltration with inflammatory cells (IBD) (64). It is not uncommon for horses to have overlap between categories, such as those with antimicrobial associated diarrhea (AAD) who also test positive for pathogenic strains of enteric bacteria such as Salmonella.

Over the past 20 years, the use of antibiotics and outbreaks of infectious forms of colitis have garnered particular attention. Antibiotic use has been linked to fecal shedding of enteric pathogens and AAD, a form of colitis with the 4.5 times the mortality rate of other types of colitis (65). AAD has a reported incidence of 22-94% with subsequent mortality rates of 15-50%. Even when colitis does not result from antimicrobial use, it has been associated with fecal shedding of Salmonella, Clostridial perfringens and difficile (20, 23, 66). Outbreaks of infectious colitis in veterinary referral centers have prompted concerns (21-23, 67) due to their prevalence, infectious potential to affect herds of horses, impact on equine mortality, and associated costs.

There is evidence that humans and companion animals with diarrhea have significant dysbiosis, and this relationship also seems true for horses. Finally, effects of

only one type of colitis (undifferentiated) on the microbiota has been described in the literature, which may not be true for other types of colitis, which constitute approximately 60% of all colitis cases (68). To date, the factors with the greatest impact on the fecal microbiome appear to be diet, antibiotic use,(69, 70) and the presence of gastrointestinal disease itself, such as colic or colitis (71, 72).In addition to the effects of disease on individual horses, there is potential for horses infected with zoonotic agents such as Salmonella and Clostridia to infect herds of horses.

3.3 Materials and Methods

Fecal samples were collected from clinical patients with colitis admitted to the Veterinary Medical Teaching Hospital at Texas A&M University in an effort to biobank fecal samples from horses with gastrointestinal disease. Horses with antimicrobial-associated diarrhea (AAD) were defined as those receiving antimicrobial prophylaxis before elective surgery or to treat a suspected or known infection prior to the development of diarrhea. Horses in the AAD group had no history of gastrointestinal disease prior to antibiotic administration, and the clinician of record classified the cause of colitis as antibiotic-associated. Horses with *Salmonella* colitis were defined as those with a presenting complaint of colitis with no history of antimicrobial administration or prior gastrointestinal disease such as colic, a positive PCR test for *Salmonella* (73) and were classified by the clinician of record as having Salmonella colitis.

3.3.1 DNA extraction

One hundred mg of feces from the center of each fecal ball or liquid fecal sample was aliquoted into a sterile 1.7 ml tube (Microtube, Sarstedt AG & Co, Numbrecht,

Germany) containing 150 ul of 0.1 mm zirconia-silica beads and 100 ul of 0.5 mm zirconia-silica beads (BioSpec Products Inc., Barlesville, OK, USA). Samples were then homogenized (FastPrep-24, MP Biomedicals, USA) for a duration of 1 minute at a speed of 4 m/s. DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) following the manufacturer's instructions.

3.3.2 Sequencing of 16S rRNA genes

Sequencing of the V4 region of the 16S rRNA gene was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq platform (Illumina Inc., San Diego, CA). Following the manufacturer's guidelines, 2x300 paired-end reads were produced using 515F (5'-GTG YCA GCM GCC GCG GTA A-3') and 806R (5-GGA CTA CNV GGG TWT CTA AT-3') primers. (Apprill, McNally et al. 2015, Parada, Needham et al. 2016) The PCR reaction was performed in a single-step 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles (5 cycles used on PCR products) of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. Using Illumina TruSeq DNA's protocol, a DNA library was set up and Illumina MiSeq was used for sequencing according the manufacturer's guidelines.

3.3.3 Analysis of sequences

A total of 106 samples were analyzed, which generated 4,386,598 quality sequences. Sequences were analyzed using a QIIME 2 (Quantitative Insights into Microbial Ecology) (Bolyen, Rideout et al. 2019) v.2019.7 pipeline as described

elsewhere (Marsilio, Pilla et al. 2019, Park, Pilla et al. 2019). Briefly, barcodes and primers were removed and short (<150bp), ambiguous, homopolymeric sequences were depleted from the dataset. DADA2 was used to identify and remove chimeric sequences (Callahan, McMurdie et al. 2016). The amplicon sequence variant (ASV) table was created using DADA2 (Callahan, McMurdie et al. 2016), and rarefied to 41,383 sequences per sample based on the lowest read depth in all samples for even depth of analysis. Sequences determined to be mitochondria, chloroplasts, unassigned, or those belonging to the phylum cyanobacteria were excluded from further analysis. Data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession number SRP228480 and the BioProject number PRJNA580257.

Alpha diversity metrics Chao1 (richness), observed ASVs (species richness), and Shannon diversity (evenness) were generated in QIIME2. Beta diversity was evaluated with the phylogeny based weighted and unweighted UniFrac distance metric, and Principal Coordinate Analysis (PCoA) plots for visualization were generated in QIIME2.

3.3.4 Statistical analysis

Prior to analysis, data was tested for normality using the Shapiro-Wilk test (JMP Pro 14, SAS, Marlow, Buckinghamshire). As data was not normally distributed, non-parametric measures were used throughout the study.

Statistical analysis of alpha diversity indices (Chao 1, observed ASVs, and Shannon) was performed using the software package PRISM (PRISM 7, GraphPad Software Inc., San Diego, CA). A Kruskal Wallis test with a Dunn's multiple comparison test was used to compare healthy horses to those with colitis.

Beta diversity (bacterial community composition) was measured with weighted and unweighted UniFrac metrics and visualized for clustering with Principle Coordinate Analysis (PCoA) plots. An Analysis of Similarity test (ANOSIM) within the PRIMER 6 (PRIMER-E Ltd. Luton, UK) software package was performed on the beta diversity distance matrices to assess the significance of the differences in the bacterial community composition based upon the variables noted above.

Univariate analysis of the bacterial taxa in the fecal samples was evaluated using a Kruskal-Wallis test (PRISM 7, GraphPad Software Inc., San Diego, CA) followed by a Dunn's multiple comparison post-test. Only bacterial taxa present in at least 50% of the samples were included in the analysis.

Linear discriminant analysis effect size (LEfSe) using the web-based program Calypso v8.62 (http://cgenome.net/wiki/index.php/Calypso) was performed to analyze the abundance of bacterial taxa and their associations with health status. A cut off threshold of 3.5 was set for significance.

3.4 Results

A population of clinical patients with colitis due to AAD (n=14) and *Salmonella* (n=12), was compared to the healthy horses (n=80) described in Chapter II. Information on the signalment, weight, diet, state of residence and season of horses with colitis is included in Appendix B.

Horses in the AAD group received the following antimicrobial agents: ceftiofur crystalline (n=4); metronidazole (n=1); doxycycline (n=1); penicillin and gentocin (n=3); penicillin, gentocin and doxycycline (n=2); penicillin, gentocin and metronidazole (n=2);

chloramphenicol (n=1). Antimicrobials were used as surgical prophylaxis in 3 horses and to treat known infections in 11 horses (respiratory, n=8; lacerations, n=2; cellulitis, n=1).

3.4.1 Beta diversity (between samples)

A principal coordinate analysis plot of unweighted Unifrac distances is displayed in Figure 3.1, and indicates significant clustering between healthy horses and those with colitis (overall ANOSIM, R=0.565, p=0.0001). Pairwise tests indicate that AAD horses were significantly separated from healthy horses on Diets A-D (ANOSIM, R=0.861, p=0.0001) and Diet E (ANOSIM, R=0.679, p=0.0001). Healthy horses on Diets A-D (ANOSIM, R=0.608, p=0.0001) and Diet E (ANOSIM, R=0.581, p=0.0001) had significantly different microbial community composition from horses with *Salmonella*. There was less separation between horses with AAD and *Salmonella* colitis (R=0.0226, p=0.009). Results of ANOSIM testing for weighted Unifrac values are listed in Table 3.2.

Figure 3.1 Beta diversity of unweighted UniFrac distances in healthy horses and those with colitis caused by AAD and SAL. There is distinct clustering of horses with antimicrobial associated (AAD) (red) and *Salmonella* (yellow) colitis from healthy horses on Diets A-D (royal blue) and Diet E (light blue) (overall ANOSIM, R=0.565, $p<0.001$). There is statistically significant but less distinct separation of the two types of colitis horses from each other (AAD vs Salmonella, ANOSIM, R=.226, $p<0.001$) and healthy horses on Diets A-D from those on Diet E (ANOSIM, R=.287, $p<0.001$).

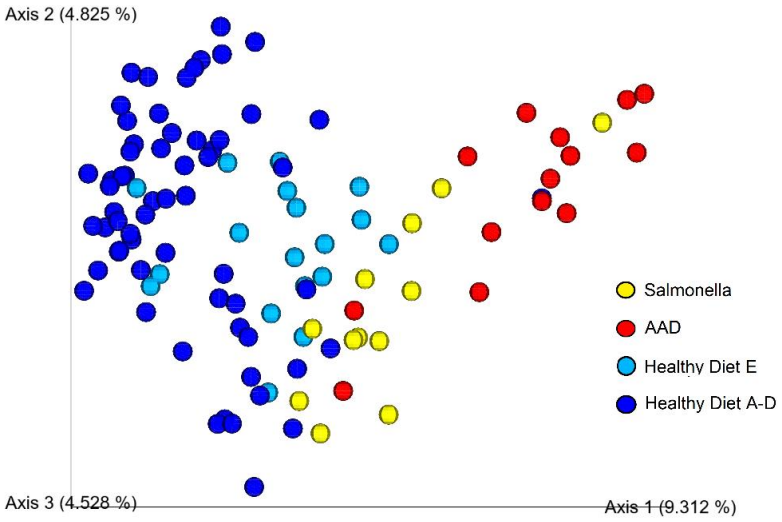


Table 3.1 Pairwise ANOSIM analysis of unweighted and weighted Unifrac distances in healthy horses (Diets A-D and Diet E) and those with antimicrobial-associated (AAD) and *Salmonella* colitis.

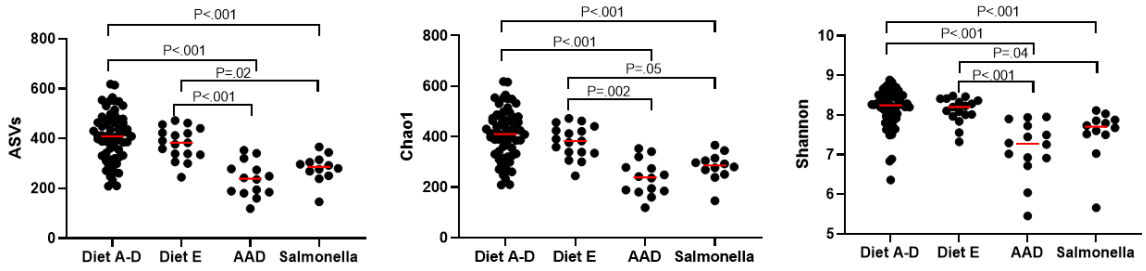
	Unweighted		**Weighted**	
Groups	**R Statistic**	**P value**	**R Statistic**	**P value**
Overall	0.565	0.0001	0.488	0.0001
Healthy A-D, AAD	0.861	0.0001	0.781	.0001
Healthy E, AAD	0.679	0.0001	0.503	0.0001
Healthy A-D, *Salmonella*	0.608	0.0001	.467	.0001
Healthy E. *Salmonella*	0.581	0.0003	0.363	0.001
AAD, *Salmonella*	0.226	0.0009	0.201	0.0007
Healthy A-D, Healthy E	0.287	0.0001	0.135	0,047

3.4.2 Alpha diversity (within samples)

Alpha diversity indices were significantly lower in horses with colitis compared to healthy horses (Figure 3.2), but horses with AAD and *Salmonella* were not different from each other. Observed ASVs were decreased in healthy horses compared to horses with AAD (Diet A-D, P<.0001; Diet E, p=0.001) and *Salmonella* (Diet A-D, p=0.001; Diet E, p=0.02), but there was no difference between horses with each type of colitis. Chao 1 was significantly different between healthy horses and those with AAD (Diet A-D, p<0.0001; Diet E, p=0.02) and *Salmonella* (Diet A-D, p=0.0004; Diet E, p=0.05), while no difference was detected between the horses with either form of colitis. Finally, the Shannon metric

was decreased between healthy horses and those AAD (Diet A-D, p<0.0001; Diet E, p=0.009) and *Salmonella* (Diet A-D, p<0.0009; Diet E, p=0.38) but not between the two colitis groups (p=0.10).

Figure 3.2 Alpha diversity measures of healthy horses and those with antimicrobial-associated (AAD) and *Salmonella* colitis. Amplicon sequence variants (ASVs) (A), Chao1 (B) and Shannon (C) indices are significantly reduced in both colitis groups (antimicrobial-associated diarrhea or AAD and *Salmonella*) compared to both Dietary groups of healthy horses, but not to each other.



3.4.3 Taxonomy

Information regarding the bacterial taxa at the phylum level can be found in Table 3.2 and the family level can be found in Table 8. At the phylum level, there were 7 phyla that had significant changes between healthy horses and those with colitis.

Horses with *Salmonella* had increased Euryarchaeota compared to healthy horses (p=0.002, q=0.02) but not compared to horses with AAD. The family *Methanomassilliococcaceae* was increased in the AAD and Salmonella group (p=0.0001, q=0.0007) compared to normal horses.

Actinobacteria was not significantly different across groups (p=0.03, q=0.05), however the family *Micrococcaceae* was increased in normal horses (p=0.0051, q=0.0130).

Horses with AAD had significantly more Bacteroidetes (p=0.0004, q=0.0015) compared to healthy horses but not those with *Salmonella*. Within the phylum Bacteroidetes, the majority of this change occurred within the order Bacteroidales and an unassigned family (p=0.0056, q=0.0136) that was decreased in the AAD horses compared to healthy horses and those with *Salmonella*. Other more minor changes at the family level involved an increase in *Bacteroidaceae* (p=0.0001, q=0.0007), *Rikenellaceae* (p=0.0001, q=0.0007) and *Porphyromonadaceae* (p=0.0001, q=0.0007) in the two colitis groups compared to normal. In contrast, the family BS11 was decreased in the AAD group compared to normal horses (p=0.0001, q=0.0007).

Horses with *Salmonella* had significantly less Firmicutes than horses in the normal groups (p=0.008, q=0.0176). Within the class Bacilli, the family *Enterococceceae* was increased in AAD horses (p=0.0001, q=0.0007), while *Lactobacillaceae* was increased in both the normal group compared to horses with *Salmonella* (p=0.05, q=0.07). Within the class Clostridia an unknown group within the order Clostridiales was decreased in the *Salmonella* group but not the normal or AAD groups (p=0.01, q=0.01). An unknown family within the order Clostridiales (p=0.002, q=0.01) and *Mogibacteriaceae* (p=0.0002, q=0.001) were significantly decreased in the AAD group but not the healthy or *Salmonella* groups. *Lachnospiraceae* was significantly decreased within the *Salmonella* group

compared to healthy controls and AAD horses (p=0.0006, q=0.003). *Eubacteriaceae* was increased in healthy horses compared to those with AAD (p=0.0004, q=0.002).

Lentisphaerae was increased in the *Salmonella* group (p=0.0013, q=0.0036) compared to healthy horses and those with AAD. Two families within the class [Lentisphaeria], *Victivallaceae* (p=0.037, q=0.06) and *R4-45B* (p=0.003, q=0.009) accounted for this increase.

Overall, Proteobacteria was increased in the AAD group compared to *Salmonella* and Healthy groups (p=0.0002, q=0.001). Within the class Betaproteobacteria, the family *Alcaligenaceae* was increased in the AAD and *Salmonella* groups compared to normal horses (p=0.0013, q=0.005), whereas the *Campylobacteriaceae* from the class Epsilonproeobacteria was increased only in the *Salmonella* groups (p=p.01, q=p.03). Within the class Gammaproteobacteria, *Enterobacteriaceae* was increased in AAD horses (p=p.0001, q=p.0007) compared to healthy horses and *Moraxellaceae* (p=p.01, q=p.03) was decreased in AAD horses compared to healthy horses.

The phylum Verrucomicrobia was significantly decreased in the AAD group compared to healthy horses and those with *Salmonella* (p=p.0001, q=p.001). This change is a result in a severe depletion of *RFP12* (p=p.0001, q=p.0007) from the class Verrucomicrobiae in horses with AAD compared to healthy horses and those with *Salmonella*, while there was and a small increase in the family *Verrucomicrobiaceae* within the class Verrucomicrobiae (p=p.005, q=p.01) in horses with both types of colitis compared to healthy horses.

Table 3.2 Distribution of taxa at the phylum level in healthy horses and those with colitis due to antimicrobial use (AAD) and *Salmonella*.

Phyla	**AAD**		**Normal**		**Salmonella**		**AAD vs Normal vs Salmonella**	
	Median	**Range**	**Median**	**Range**	**Median**	**Range**	**P value**	**Q value**
Euryarchaeota	1.12[a,b]	0.07-4.08	1[a]	0-6.79	2.43[b]	0.22-5.76	**0.0118**	**0.0216**
Actinobacteria	0.09[a]	0-2.09	0.47[a]	0-11.49	0.15[a]	0-2.82	**0.0292**	**0.0459**
Bacteroidetes	51.35[a]	23.77-85.58	33.21[b]	6.27-46.91	35.03[a,b]	30.06-60.76	**0.0004**	**0.0015**
Fibrobacteres	0.13	0-2.17	0.81	0-9.04	0.58	0-4.08	0.0525	0.0722
Firmicutes	37.84[a,b]	11.81-51.07	44.32[a]	22.18-73.84	36.11[b]	26.25-43.77	**0.008**	**0.0176**
Lentisphaerae	0[a]	0-0.3	0[a]	0-0.58	0.05[b]	0-0.95	**0.0013**	**0.0036**
Proteobacteria	3.24[a]	0.67-16.41	0.66[b]	0.07-59.68	1.65[b]	0.29-17.21	**0.0002**	**0.0011**
Spirochaetes	3.52	0-17.84	5.94	0.41-16.68	7.01	1.3-13.24	0.1616	0.1778
Synergistetes	0	0-0.16	0	0-0.6	0.02	0-0.41	0.1085	0.1326
Tenericutes	1.5	0-5.14	2	0-6.95	1.82	0-5.1	0.4414	0.4414
Verrucomicrobia	0.72[a]	0-4.23	9.15[b]	1.49-23.63	7.55[b]	0-24.67	**0.0001**	**0.0011**

Table 3.3 Distribution of taxa at the family level in healthy horses and those with colitis due to antimicrobial use (AAD) and *Salmonella*.

Family	AAD		Healthy		Salmonella			
	Median	**Range**	**Median**	**Range**	**Median**	**Range**	**P value**	**Q value**
Methanobacteriaceae	0.71	0-3.8	0.56	0-4.59	0.91	0.13-3.15	0.259	0.2837
[Methanomassiliicoccaceae]	0.23[a]	0-1.62	0[b]	0-2.57	0.58[a]	0-1.14	0.0001	0.0007
Micrococcaceae	0a	0-0.03	0.01b	0-10.08	0a,b	0-0.81	0.0051	0.0130
Coriobacteriaceae	0.05[a]	0-2.06	0.33[a]	0-2.65	0.14[a]	0-2	0.0322	0.0557
Bacteroidales	0.2	0-6.93	0.47	0-6.79	0.65	0-7.34	0.1161	0.1526
Bacteroidales;f__	12.1[a]	0.15-48.21	23.9[b]	5.61-37.43	20.39[a,b]	4.14-24.73	0.0056	0.0136
BS11	0.44[a]	0-24.33	1.47[b]	0-14.75	1.78[a,b]	0-20.19	0.0221	0.0407
Bacteroidaceae	4.03[a]	1.32-49.87	0.43[b]	0-6	2.27[a]	0.16-11.98	0.0001	0.0007
Porphyromonadaceae	1.02[a]	0.13-6.84	0.12[b]	0-2.39	0.78[a]	0-12.58	0.0001	0.0007
Prevotellaceae	1.03	0-6.83	0.53	0-2.75	0.28	0.12-1.91	0.0839	0.1170
RF16	1.09	0.16-8.28	0.52	0-6.87	1.65	0-5.19	0.0604	0.0926
Rikenellaceae	0.15[a]	0-57.07	0[b]	0-0.71	0.13[a]	0-2.67	0.0001	0.0007
S24-7	0.11	0-8.68	0.07	0-1.71	0	0-0.36	0.0987	0.1335
[Paraprevotellaceae]	3.66	0.18-26.92	2.67	0.28-5.64	2.8	1.13-51.75	0.2437	0.2734
Fibrobacteraceae	0.13	0-2.17	0.81	0-9.04	0.58	0-4.08	0.0525	0.0833
Planococcaceae	0	0-9.84	0	0-35.3	0.04	0-8.58	0.1746	0.2033
Enterococcaceae	0.26[a]	0-2.04	0[b]	0-1.18	0[b]	0-0	0.0001	0.0007
Lactobacillaceae	0.46[a,b]	0-7.82	0.35[a]	0-3.3	0.1[b]	0-1	0.0452	0.0743
Streptococcaceae	0.16	0-4.54	0.1	0-13.92	0.18	0-1.16	0.9088	0.9088
o__Clostridiales;__	0.74[a,b]	0-3.57	1.11[a]	0-3.83	0.56[b]	0-1.45	0.0051	0.0130
o__Clostridiales;f__	3.22[a]	0.16-10.03	7.99[b]	1.93-15.7	6.33[a,b]	3.1-9.55	0.002	0.0071
Christensenellaceae	0.08	0-1.52	0.21	0-1.57	0.35	0-1.8	0.1768	0.2033
Clostridiaceae	0.51[a]	0-3.04	1.39[a]	0.03-4.6	0.92[a]	0.37-1.87	0.0165	0.0316
Eubacteriaceae	0[a]	0-0.16	0.11[b]	0-1.09	0[a,b]	0-0.39	0.0004	0.0018
Lachnospiraceae	6.93[a]	3.2-17.75	8.11[a]	1.32-21.78	2.76[b]	1.43-8.99	0.0006	0.0025
Peptococcaceae	0.03	0-0.44	0	0-0.35	0	0-0.36	0.6758	0.6908
Ruminococcaceae	13.4	2.36-27.34	16.27	3.11-36.98	15.17	9.21-29.45	0.3871	0.4047
Veillonellaceae	0.69	0-2.67	0.42	0-2.4	0.63	0.25-2.9	0.1202	0.1536
[Mogibacteriaceae]	0.45[a]	0.08-2.3	1.46[b]	0.12-3.87	0.94[a,b]	0.18-3.62	0.0002	0.0010
Erysipelotrichaceae	1.35	0.21-5.32	1.71	0.37-5.41	3.45	0.72-6.58	0.0759	0.1126
Victivallaceae	0[a]	0-0.3	0[a,b]	0-0.54	0.01[b]	0-0.95	0.0327	0.0557

Table 3.3 Continued.

Family	AAD		Healthy		Salmonella			
	Median	**Range**	**Median**	**Range**	**Median**	**Range**	**P value**	**Q value**
Alcaligenaceae	0.04[a]	0-1.84	0[b]	0-1.47	0.08[a]	0-0.62	0.0013	0.0050
Desulfovibrionaceae	0.19	0-1.81	0.09	0-0.68	0.12	0-2.05	0.1691	0.2033
Campylobacteraceae	0.02[a,b]	0-1.21	0[a]	0-0.62	0.07[b]	0-0.96	0.013	0.0284
Succinivibrionaceae	0.1	0-7.01	0	0-7.5	0	0-1.63	0.0797	0.1146
Enterobacteriaceae	0.81[a]	0-3.62	0[b]	0-32.63	0.09[a,b]	0-4.17	0.0001	0.0007
Moraxellaceae	0[a]	0-0.66	0.06[b]	0-26.68	0[a,b]	0-12.67	0.0136	0.0284
Spirochaetaceae	3.52	0-17.49	5.85	0.41-16.08	6.17	1.3-12.39	0.1501	0.1866
Synergistaceae	0[a]	0-0.01	0[b]	0-0.6	0[a,b]	0-0.08	0.0067	0.0154
Anaeroplasmataceae	0.36[a]	0-1.7	0[b]	0-1.11	0.13[a,b]	0-3.05	0.0038	0.0117
Mycoplasmataceae	0[a]	0-0.86	0.06[b]	0-2.23	0.08[b]	0-3.11	0.0142	0.0284
o__RF39;f__	0.03[a]	0-2.77	1.41[b]	0-6.93	0.82[a,b]	0-2.16	0.0002	0.0010
o__ML615J-28;f__	0.03	0-0.83	0	0-0.78	0.03	0-1.21	0.3808	0.4047
RFP12	0.44[a]	0-3.99	9.1[b]	1.23-23.6	6.95[b]	0-24.67	0.0001	0.0007
Verrucomicrobiaceae	0.26[a]	0-1.13	0[b]	0-0.66	0.09[a]	0-1.25	0.0047	0.0130

The results of LEfSe analysis are found in Figure 3.3. Healthy horses on Diets A-D have more Actinobacteria than those on Diet E those with *Salmonella* colitis. Horses with AAD have more Bacteroidetes and Proteobacteria, whereas horses with *Salmonella* have more Fusobacteria, Euryarchaeota and Lentisphaerae. Normal horses have more Firmicutes, Verrucomicrobia, WPS2 and SR1.

Figure 3.3 The results of linear discriminant effects size analysis in healthy horses and those with antimicrobial-associated diarrhea (AAD) and Salmonella. A) Phylum level

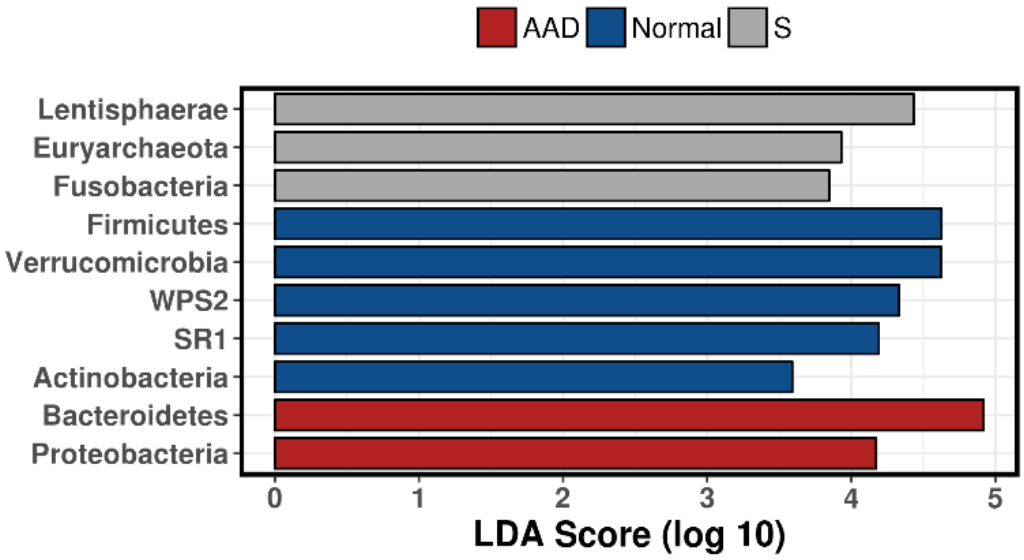
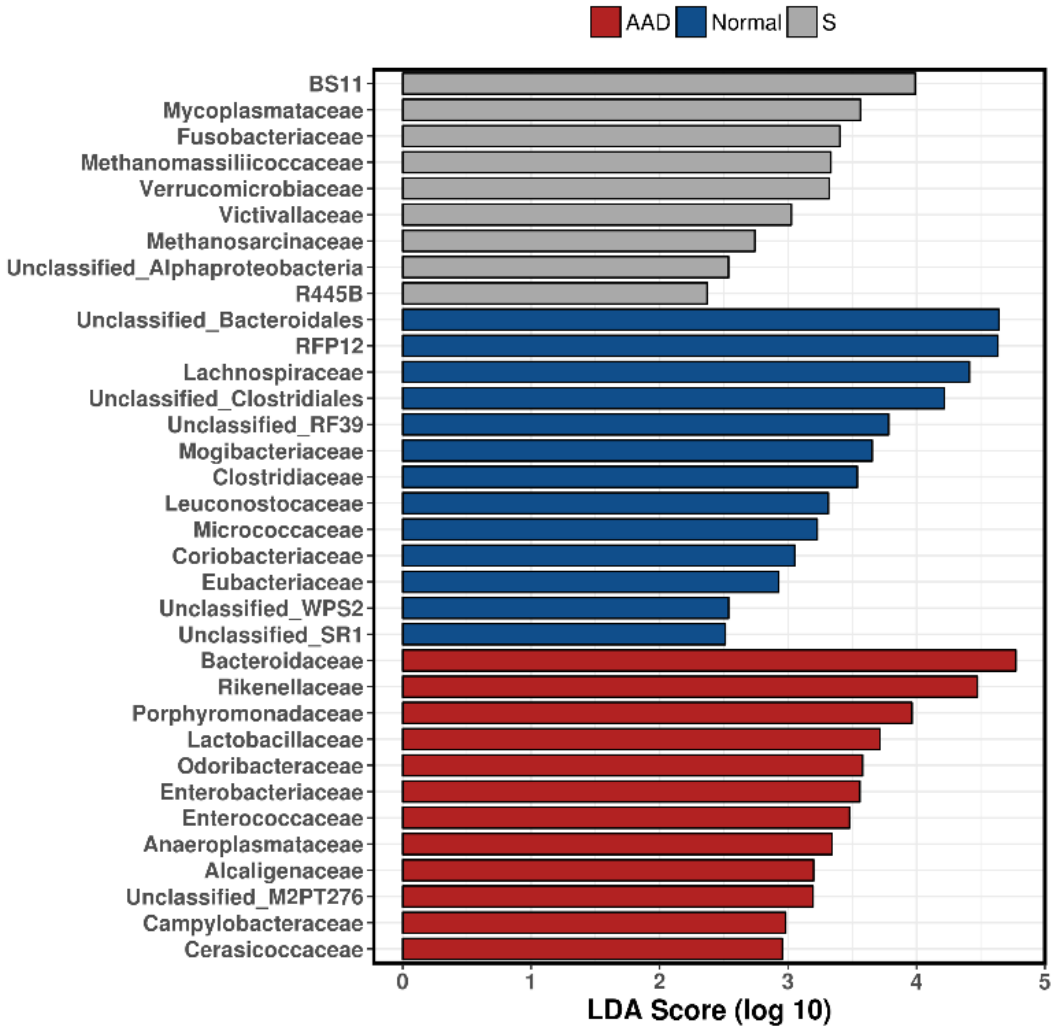
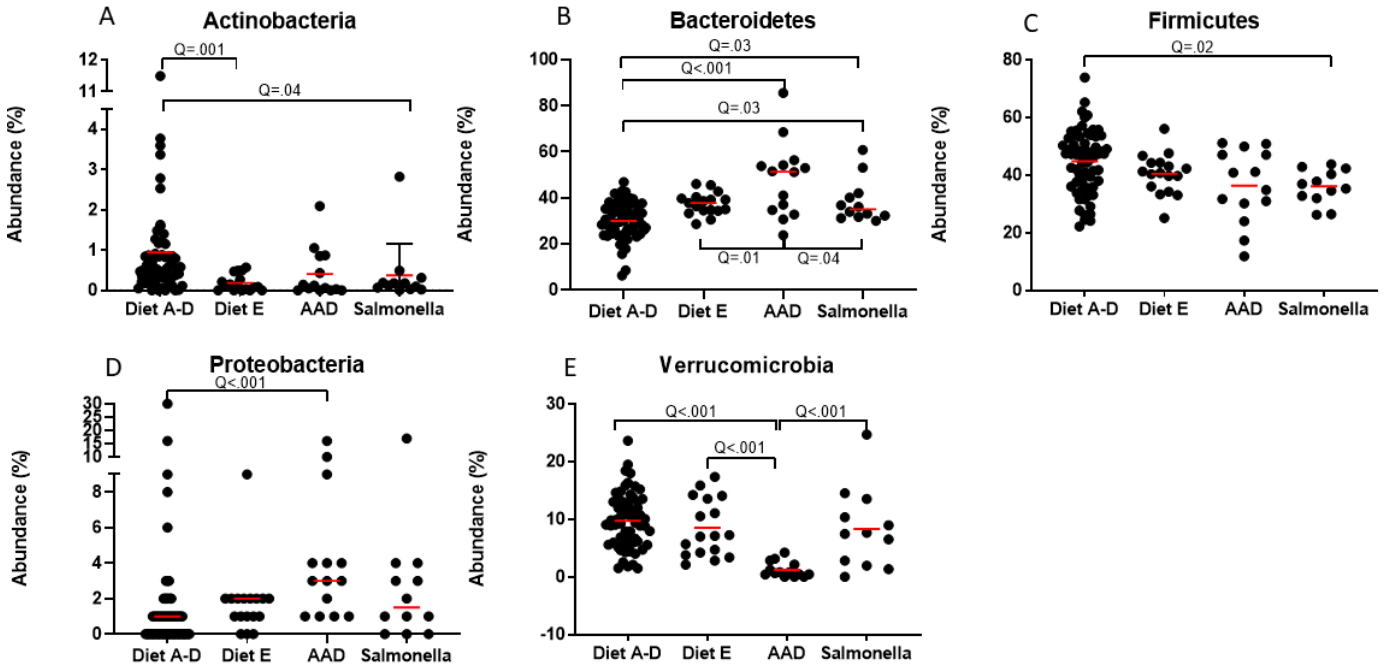


Figure 3.3 Continued. B) Family level



The median abundances of those phyla significantly different between healthy horses and those with colitis due to AAD and Salmonella are displayed in scatter plots in Figure 3.4 below.

Figure 3.4 The median abundance (%) of significantly altered phyla according to linear discriminant effects size analysis in healthy horses and those with antimicrobial-associated diarrhea (AAD) and Salmonella. Healthy horses are separated by Diets A-D and Diet E. A). The abundance of Actinobacteria is significantly greater in healthy horses on Diets A-D than those on Diet E and for those with *Salmonella* colitis. B). Horse with AAD have significantly more Bacteroidetes than healthy horses on Diets A-D or Diet E and those *Salmonella*. C). Horses with *Salmonella* colitis have significantly less Firmicutes than healthy horses on Diets A-D. D). Horses with AAD have significantly more Proteobacteria than healthy horses on Diets A-D. E). Horses with AAD have a marked reduction in the amount of Verrucomicrobia compared to healthy horses on Diets A-D and Diet E and Salmonella colitis.



3.5 Discussion

Having characterized the fecal microbiome of healthy horses, the authors used this group as a control for horses with colitis. The authors chose two phenotypes of colitis, AAD and *Salmonella*, as they are common variants of colitis in our hospital population and their effects on the fecal microbiome are yet undescribed in the literature. By categorizing colitis according to inciting cause, some of the AAD horses were positive for *Salmonella* on qPCR testing. The difference between the two groups is that the horses in the AAD group developed colitis secondary to antibiotic administration whereas horses in the *Salmonella* group had a spontaneous onset of colitis. Colitis horses were compared separately to healthy horses on Diets A-D and Diet E due to our previous findings regarding the effect of diet on the fecal microbiome and the fact that all of the colitis horses were fed Diets A, B, C or D.

The presence of gastrointestinal disease greatly affected the diversity and composition of the fecal microbiome. Horses with each type of colitis had marked decreases in each of the alpha diversity measures (observed ASVs, Chao 1 and Shannon) compared to the two groups of healthy horses. However, each subset of healthy horses and colitis horses were not significantly different from each other in regards to richness and evenness. Microbial community composition, however, indicated large differences between healthy horses and those with colitis, with smaller yet significant differences within the subsets of healthy (Diet A-D vs E) and colitis (AAD versus *Salmonella*) horses. This can be seen on PCoA plots with AAD and *Salmonella* horses clustering distinctly

from healthy horses, with the AAD group having greater distance (i.e. had a more different microbiome composition) from healthy horses than those with *Salmonella*.

Horses with colitis had significant changes in the bacterial community composition of the fecal microbiome with 7 major phyla affected (Euryarchaeota, Actinobacteria, Bacteroidetes, Firmicutes, Lentisphaerae, Proteobacteria, Verrucomicrobia). The results of univariate analysis found distinct microbial patterns associated with each type of colitis. Horses with AAD had increases in Bacteroidetes and Proteobacteria and decreases in Verrucomicrobia compared to healthy horses. Horses with *Salmonella* had decreases in Firmicutes and increases in Euryarcheota and Lentisphera compared to healthy horses. These results were supported by the LEfSe analysis. To date, the fecal microbiome of horses with colitis has only been described in a population of horses with undifferentiated colitis. While these horses also had significant decreases in richness and evenness, they experienced changes in the percentages of Bacteroidetes and Firmicutes simultaneously. These results suggest that colitis due to antimicrobial use or *Salmonella* may have differing effects on the fecal microbiome composition.

3.6 Conclusion

Horses with colitis caused by AAD and *Salmonella* experience decreases in diversity and changes in bacterial community composition. The occurence of colitis due to AAD and *Salmonella* colonization produced a marked dysbiosis on the fecal microbiome with differing effects upon major phyla. While both colitis groups clustered apart from healthy horses, horses with AAD had a larger shift in microbiome composition

than did horses with *Salmonella*, compared to healthy controls. The effect of gastrointestinal disease was greater than diet.

4. THE EFFECT OF METRONIDAZOLE ON THE EQUINE CECAL AND FECAL MICROBIOMES AND METABOLOMES

4.1 Overview

Metronidazole administration altered the bacterial composition of the horse's cecal and fecal content, and the metabolome of fecal samples. Richness and evenness indices were significantly decreased by metronidazole administration in both cecal and fecal samples, but the overall composition was only significantly changed in fecal samples on Day 3 (ANOSIM, p=0.008). The most dominant phyla were Bacteroidetes and Firmicutes in all groups examined. In fecal samples, significant changes in Actinobacteria, Spirochaetes, Lentisphaerae, and Verrucomicrobia occurred on Day 3, which correlated with clinical signs of gastrointestinal disease. The fecal metabolites affected represent diverse metabolic pathways, such as the metabolism of amino acids, carbohydrates, lipids, nucleic acids and cofactors and vitamins.

4.2 Introduction

Antimicrobial agents, which are commonly administered to horses to treat established infections and to provide prophylaxis for surgically created wounds, affect the microbiome of the hindgut of the horse. Currently, tetracyclines, macrolides, cephalosporins, fluoroquinolones, trimethoprim-sulphonamides, aminoglycosides, chloramphenicol, and β-lactams all have been reported to cause colitis in horses (70, 74).

Paradoxically, some antimicrobial agents, such as metronidazole, have been found useful in the treatment of colitis. Metronidazole is a bactericidal nitroimidazole with activity against anaerobic bacteria and protozoa. It is commonly used as a primary treatment for diarrhea in dogs (75) and has been used to treat foals with hemorrhagic enteritis caused by Clostridium *difficile* (76). Metronidazole is also prescribed to Clostridial diarrhea in adult horses. Although historically metronidazole has been associated with a low incidence of diarrhea when used in adult horses for purposes other than to treat diarrhea (77), its effect on the bacterial communities of the GIT has not yet been reported. While culture-based methods have traditionally been used to assess the effects of antibiotics, the use of Next Generation Sequencing and metabolomics can potentially provide greater insight regarding the changes in the microbial populations and their functional effect on the metabolism of the horse's hindgut (69, 70). The objective of this study was to characterize the changes in the cecal and fecal microbiome and metabolome of the horse before and after metronidazole administration.

4.3 Materials and Methods

This study was approved by Texas A&M University Institutional Animal Care and Use Committee (IACUC; Protocol number 2014-0123).

4.3.1 Study population

Five horses belonging to the Department of Animal Science at Texas A&M University with indwelling cecal cannulas were used as subjects. The horses had been cannulated approximately 10 years prior to this study, and had received no antibiotics or other medications (excluding routine vaccinations and anthelminthics) for the previous 12 months. The horses were housed on a dry lot, fed free choice coastal hay and a commercial pelleted concentrate (Nutrena Safe Choice, Cargill, Minnetonka, MN) at 0.5% of body weight for the duration of the study. Age, breed, sex, and weight of the study participants is summarized in Table 4.1

Table 4.1 Age, breed, sex and weight of study participants.

Horse	Age (years)	Breed	Sex	Weight (kg)
1	19	Quarter Horse	Gelding	500
2	12	Mixed	Gelding	512
3	24	Quarter Horse	Gelding	413
4	13	Quarter Horse	Gelding	602
5	15	Mixed	Gelding	654

4.3.2 Sample collection

Fecal samples were collected immediately after natural elimination. Cecal samples were collected by removing the plug of the cannula while horses were restrained in stocks and siphoning 5 mls of cecal ingesta, including both the particulate and liquid fractions. Samples were placed on ice for transport to the laboratory, where they were refrigerated at 4°C prior to DNA extraction. Samples were collected before (study days referred to as Dminus52, Dm28, Dm14, and 0) and after metronidazole administration (referred to as D7, D14, D28, and D52). Metronidazole (Unichem Pharmaceuticals, Hasbrouck Heights, NJ) was administered after sample collection on Day 0 at a dose of 15 mg/kg PO BID for 3 days instead of 7 days as planned in the study protocol due to development of complications. This resulted in an additional sampling point for feces on Day 3.

4.3.3 DNA extraction

One hundred mg of feces or cecal contents (particulate and liquid fractions) was aliquoted into a sterile 1.7 ml tube (Microtube, Sarstedt AG & Co, Numbrecht, Germany) containing 150 µl of 0.1 mm zirconia-silica beads and 100 µl of 0.5 mm zirconia-silica beads (BioSpec Products Inc., Bartlesville, OK, USA). Samples were then homogenized (FastPrep-24, MP Biomedicals, Irvine, CA, USA) for a duration of 1 minute at a speed of 4 m/s. DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) following the manufacturer's instructions.

4.3.4 Sequencing of 16S rRNA genes

Sequencing of the V4 region of the 16S rRNA gene was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq platform (Illumina Inc., San Diego, CA) following the manufacturer's guidelines using 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'- GGACTACVSGGGTATCTAAT-3'). Briefly, the PCR reaction was performed in a single-step 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles (5 cycles used on PCR products) of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. Using Illumina TruSeq DNA's protocol, a DNA library was set up and Illumina MiSeq was used for sequencing according the manufacturer's guidelines.

4.3.5 Analysis of sequences

QIIME v1.9 (Quantitative Insights into Microbial Ecology) was used for analysis of the sequences. After sequencing, barcodes and primers were removed and short (<150 bp), ambiguous, homopolymeric sequences were depleted from the dataset. USEARCH was used to identify and remove chimeric sequences. Operational taxonomic units (OTUs) were assigned based on at least 97% sequence similarity to the Greengenes database (v13.5) using an open reference approach. Sequences determined to be mitochondria, chloroplasts, unassigned, or those belonging to the phylum cyanobacteria were excluded from further analysis.

4.3.6 Enteric pathogen testing

Both cecal and fecal samples were tested for the presence of Salmonella and Clostridial toxins. For Salmonella PCR, a tetrathionate green broth enrichment method

was used prior to extraction, and qPCR performed as previously described (73)(Kurowski, Traub-Dargatz et al. 2002). Commercially available ELISAs for Clostridium perfringens enterotoxin and Clostridium difficile toxins A and B were performed according to the manufacturer's instructions (TechLab, Blacksburg, VA, USA).

4.3.7 Metabolomic analysis

Samples were stored at −80°C until shipped on dry ice to the West Coast Metabolomics Core (University of California, Davis, CA, USA) for untargeted analysis (78). Samples were lyophilized for 24 hours and weighed in a 1.5 ml Eppendorf tube. One ml of a 3:3:2 extraction mixture of degassed acetonitrile (Fisher, Ottawa, Canada, A9554), isopropanol (Fisher, Ottawa, Canada, A461212) and water (Fisher, Ottawa, Canada, 7732-18-5) was used to re-suspend 4 mg of lyophilized sample. Three millimeter grinding beads (Next Advance, Troy, NY, USA) were added to disrupt the sample at 1500 rpm for 30 seconds followed by shaking at 4°C for 5 minutes and centrifugation for 2 minutes at 14,000 rcf. The supernatant was separated into two aliquots of 475 ul for dry down in a Centrivap cold trap vacuum overnight at room temperature (Labconco, Kansas City, MO, USA). Dried supernatant was resuspended in 500 ul of 50% aqueous acetonitrile (Fisher, Ottawa, Canada, A9554) and centrifuged at 14,000 rpm for 2 minutes. The supernatant was dried down overnight in a Centrivap overnight at room temperature. A volume of 10 ul of 40 mg/ml solution of methoxyamine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA, 89803) in pyridine (99.99%) (Sigma-Aldrich, St. Louis, MO, USA 270970) was added to the samples and shaken for 90 minutes at 30°C. Next, 90 uL of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS)

(Sigma-Aldrich, St. Louis, MO, USA 69479) and Fatty Acid Methyl Esters (FAMEs) retention indexing markers (see next paragraph) were added with a ratio 100:0.001 was added to each sample and shaken for 37°C for 30 minutes for trimethylsilylation of acidic protons. The reaction mixture was placed in a 2 ml clear glass auto-sampler vial with micro-insert (Agilent, Santa Clara, CA, USA, 5185-5946) and closed with a 11mm T/S/T crimp cap (Thomas Scientific, Swedesboro, NJ, USA, 11-0038A).

Quality control was assessed by retention indexing with Fatty Acid Methyl Esters (FAMEs) and the use of blanks and quality control samples. A mixture of internal retention index markers using 13 FAME markers dissolved in chloroform (0.8mg/ml C8-C16 or 0.4 mg/ml C18-C30) (Acros, Morris Plains, New Jersey, USA, AC4235500100) was added to each sample as listed above during derivatization. During data acquisition, both blank samples and a quality control mix were injected every 10 samples. Blank samples were used to check for carry over and also in data processing for blank subtraction. The quality control mix included 28 compounds used in 6 concentrations that were dried down and derivatized as previously described. (78).

An Agilent 6890 GC equipped with a Gerstel automatic liner exchange system (ALEX) that includes a multipurpose sample (MPS2) dual rail, and a Gerstel CIS cold injection system (Gerstel, Muehlheim, Germany) with temperature program was used as follows: 50°C to 275°C final temperature at a rate of 12°C/s and hold for 3 minutes. Injection volume is 0.5 µl with 10 µl/s injection speed on a splitless injector with purge time of 25 seconds. Liner (Gerstel #011711-010-00) is changed after every 10 samples, (using the Maestro1 Gerstel software vs. 1.1.4.18). Before and after each injection, the 10

µl injection syringe is washed three times with 10 µl ethyl acetate. For gas chromatography, a 30 m long, 0.25 mm i.d. Rtx-5Sil MS column (0.25 µm 95% dimethyl 5% diphenyl polysiloxane film) with additional 10 m integrated guard column was used (Restek, Bellefonte, PA, USA). 99.9999% pure Helium with built-in purifier (Airgas, Radnor Pennsylvania, USA) was set at constant flow of 1 ml/min. The oven temperature was held constant at 50°C for 1 min and then ramped at 20°C/min to 330°C for 5 minutes. A Leco Pegasus IV time of flight mass spectrometer controlled by the Leco ChromaTOF software vs. 2.32 (St. Joseph, MI, USA) was used for mass spectrometry. The transfer line temperature between gas chromatograph and mass spectrometer was set to 280°C. Electron impact ionization at 70V was employed with an ion source temperature of 250°C. Acquisition rate was 17 spectra/second, with a scan mass range of 85-500 Da.

Raw data files were processed using ChromaTOF v.2.32. BinBase algorithm matched spectra to database compounds, and quantification was reported by peak height of an ion at the specific retention index characteristic of the compound across all samples. Peak heights were normalized by average total peak-sums for detected compounds across each sample group. Metabolites were cross referenced using their compound number to the KEGG database (https://www.genome.jp/kegg/pathway.html) in order to identify their metabolic pathways such as metabolism, cellular processes, and others. Metabolomic data has been submitted to metabolomicsworkbench.org under the submission ST001248.

4.3.8 Statistical analysis

4.3.8.1Microbiome analysis

Prior to analysis, data were tested for normality using the Shapiro-Wilk test (JMP Pro 14, SAS, Marlow, Buckinghamshire). As data followed a non-normal distribution, non-parametric measures were used throughout the study. Adjustments for multiple comparisons were made with either a Dunn's post-test or Fischer's least significant differences. P- and q-values <0.05 were considered statistically significant.

Alpha diversity was calculated using observed OTUs, Shannon, and Chao1 metrics to compare species richness and evenness. Statistical analysis of alpha diversity indices was performed using the software package PRISM (PRISM 7, GraphPad Software Inc., San Diego, CA). A Friedman's test followed by a Dunn's multiple comparison post-test were performed to assess differences in alpha diversity metrics between study days.

Beta diversity (bacterial community composition) was calculated using both weighted and unweighted UniFrac metrics to measure similarity between samples, and visualized for clustering with Principle Coordinate Analysis (PCoA) plots. An Analysis of Similarity test (ANOSIM) within the PRIMER 6 (PRIMER-E Ltd. Luton, UK) software package was performed on the beta diversity distance matrices to assess the significance of the differences in the bacterial community composition.

The abundance of bacterial taxa in the cecal and fecal samples was evaluated using a Friedman's test (PRISM 7, GraphPad Software Inc., San Diego, CA) followed by a Dunn's multiple comparison post-test. Only bacterial taxa present in at least 50% of the samples of at least one time point were included in the analysis. P values of <0.05 and q values of <0.1 were considered significant.

Linear discriminant analysis effect size (LEfSe) using the web-based program Calypso v8.62 (http://cgenome.net/wiki/index.php/Calypso) was performed to analyze the abundance of bacterial taxa and their associations with study day. A cut-off threshold of 3.5 was set for significance.

4.3.8.2 Metabolomic analysis

MetaboAnalyst 4.0 (Xia Lab, McGill University, Canada) was used to analyze metabolomics data. The peak intensity data table contained peak heights normalized against the average total peak sums. Data was not filtered, normalized or transformed, but was subjected to Pareto scaling. Principle Component Analysis (PCA) plots were used to display metabolic composition of individual horses and a heat map was used to display hierarchical clustering of the significant metabolites. An ANOVA test followed by a Fisher's least significant differences was used to determine, which time points in the cecal or fecal samples differed significantly over study days Dm28-D28.

4.4 Results

Metronidazole administration began as planned on Day 0 after sample collection. By the evening treatment time on Day 3, all horses had become inappetent and had developed significant skin scalding associated with the canula site. Metronidazole was discontinued after a total of 5 doses had been administered (Day 3 of administration) due to the investigators' concern of impending colitis. An extra fecal sample was collected on Day 3, while cecal samples were not obtained at this time point due to the horses' level of discomfort with canula manipulation. The skin scalding was treated with local wound care including gentle washing of the skin and application of an emollient ointment around the

canula site. All horses were normal on physical examination and their appetites had recovered within 48 hours of discontinuing metronidazole administration. Skin scalding resolved by Day 7. Horse 3 developed mild signs of colic (i.e., flank watching, pawing) on Day 13 and was moved from the research facility to the hospital for further diagnostics and monitoring. The horse's vital parameters (i.e., temperature, heart rate, respiratory rate, mucous membrane color) remained within normal limits and it was producing normal feces. Palpation per rectum, passage of a nasogastric tube and preliminary bloodwork to assess hydration status (i.e., packed cell volume, total protein and lactate) were performed. No abnormalities were detected. Due to the mild nature of the colic, Horse 3 received 7 L of oral fluids with electrolyte supplementation and one dose of flunixin meglumine (1.1 mg/kg iv). The horse remained hospitalized for 24 hours during which time his vital parameters remained within normal limits, he was passing normal manure and the displayed no symptoms of colic. The horse was returned to the research facility in the afternoon of Day 14. After scheduled sample collection, the horse was found deceased 4 hours later. The Institutional Animal Care and Use Committee was notified, and a necropsy was performed. The cause of death was determined to be due to typhylocolitis. Due to the death of this subject, data from Horse 3 is missing from the Day 28 and 52 time point data sets. The remaining 4 horses completed the study with no other adverse events.

4.4.1 Sequencing results

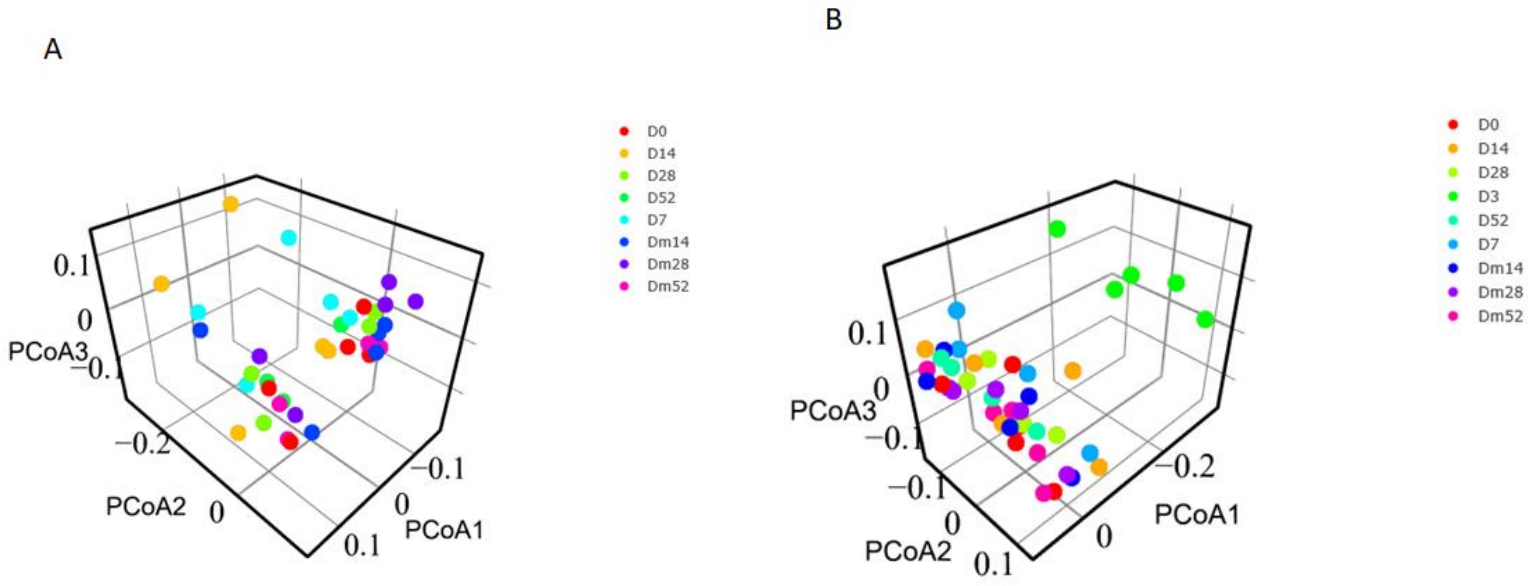
The total sequence analysis yielded 6,288,464 quality sequences for all analyzed samples (n=81, mean ± SD = 77,635 ± 15, 531). For cecal content samples (n=38), the mean quality sequences and standard deviation was 77,054 ± 18841. For fecal samples

(n=43), the mean quality sequences and standard deviation was 78,212 ± 12202. Samples were rarefied to an even depth of 40,005 reads per sample. The sequences were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the accession number SRP119693.

4.4.1.1 Beta diversity (between sample)

Beta diversity (Fig 4.1), as measured by Unifrac distances showed no significant differences for cecal samples (unweighted, R= 0.072, p=0.108; weighted, R=0.159, p=0.001). Fecal samples (Fig 12B) showed visible clustering on Day 3 (ANOSIM, R=0.152, p=0.004), which was confirmed by pairwise comparisons.

Figure 4.1 Principal coordinate analysis plots (PCoA) of unweighted cecal (A) and fecal (B) samples over study days.



Beta diversity on Day 3 was significantly different from all other time points for both weighted and unweighted Unifrac distances in the fecal samples (Table 4.2).

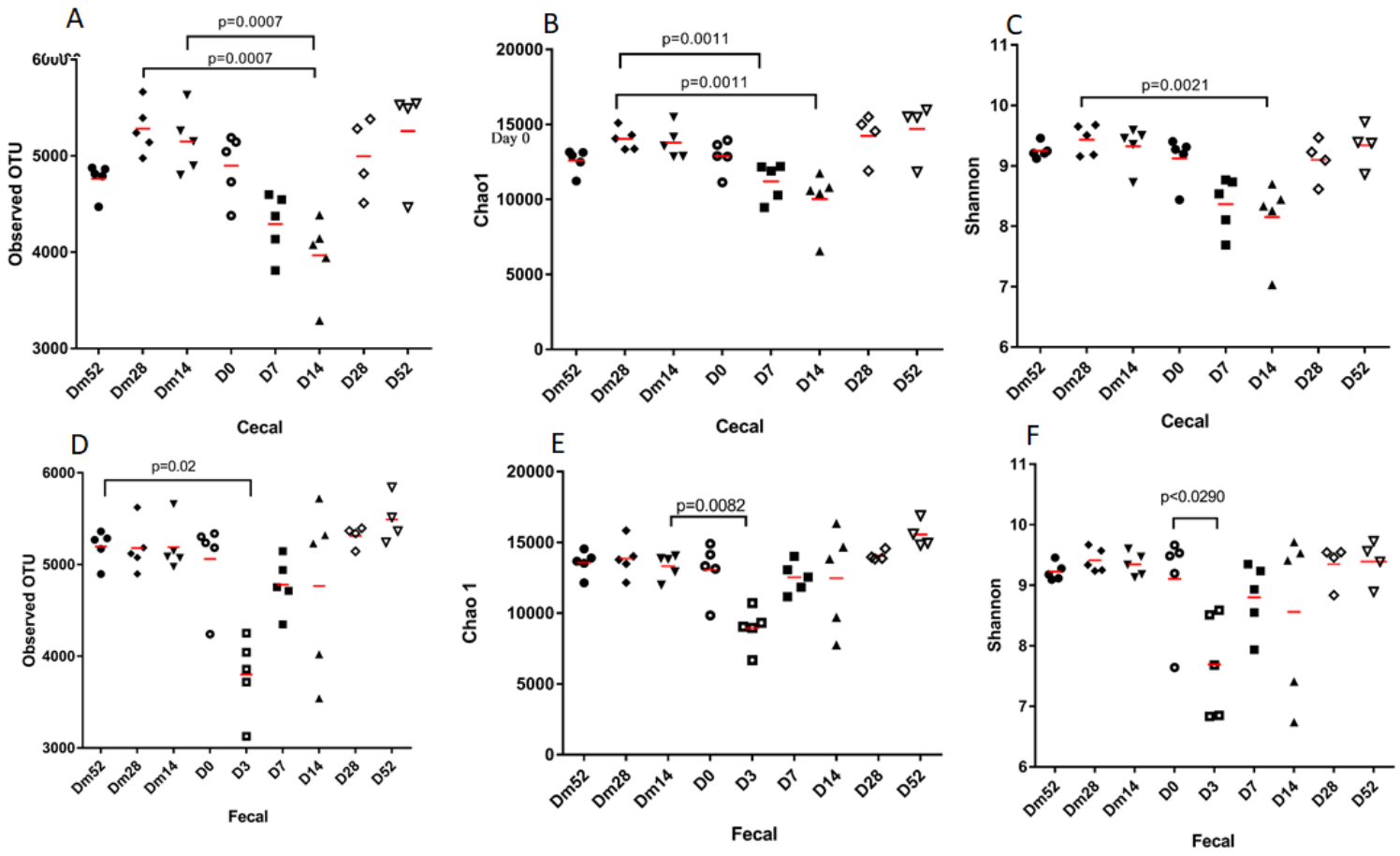
Table 4.2. The results of pairwise ANOSIM testing of unweighted and weighted Unifrac distances for fecal samples between Day 3 and all other study days.

Unifrac Distance	**Unweighted**		**Weighted**	
Study Days	**R statistic**	**P value**	**R statistic**	**P value**
D3, Dm52	0.92	0.008	0.744	0.008
D3, Dm28	0.892	0.008	0.752	0.008
D3, Dm14	0.896	0.008	0.716	0.008
D3, D0	0.884	0.008	0.764	0.008
D3, D7	0.868	0.008	0.716	0.008
D3, D14	0.64	0.008	0.572	0.032
D3, D28	0.838	0.008	0.575	0.024
D3, D52	0.913	0.008	0.638	0.024

4.4.8.2 Alpha diversity (within sample)

Alpha diversity indices (observed OTUs, Chao1, and Shannon) for cecal and fecal samples are reported in Figure 4.2. Due to the death of Horse 3 on Day 14, analysis by Friedman's test on Days 28 and 52 could not be completed, but the data for the remaining 4 horses is provided. For cecal samples, the following significant differences were noted: Observed OTUs between Dm28 and D14 (p=0.0007), Dm14 and D14 (p=0.0007); Chao 1 between Dm28 and D14 (p=0.001) and Dm28 and D7 (p=0.001); and Shannon for Dm28 and D14 (p=0.0021). For fecal samples, the following differences were noted: observed OTUs Dm52 and D3 (p=0.015), Chao 1 between Dm14 and D3 (p=0.008), and Shannon between D0 and D3 (p=0.0209).

Figure 4.2 Alpha diversity measures by study day. (A) Observed OTUs of cecal samples. (B) Chao1 of cecal samples. (C) Shannon of cecal samples. (D) Observed OTUs of fecal samples. (E) Chao1 of fecal samples. (F) Shannon of fecal samples.



4.4.8.3 Taxonomy

In the cecal samples, only the phyla Firmicutes (increased in abundance from Dm52 to Dm28; p=0026, q=0.0208) and Tenericutes (decreased in abundance from Dm52 to D14; p=0026, q=0.0208) were significantly different between study days after adjustments for multiple comparisons had been made. Other phyla such as Fibrobacteres (p=0.0154, q=0.0778) and Planctomycetes (p=0.0357, q=0.1428) had significantly different p values, but did not achieve significance after adjustments for multiple comparisons were made (Fig 14). At the family level, there were 27 taxa that were significantly different on a Friedman's test, but not after adjustment for multiple comparisons.

Figure 4.3 The median abundance (%) of significantly different cecal phyla across study days. (A) Fibrobacteres. (B) Firmicutes. (C) Planctomycetes. (D) Tenericutes.

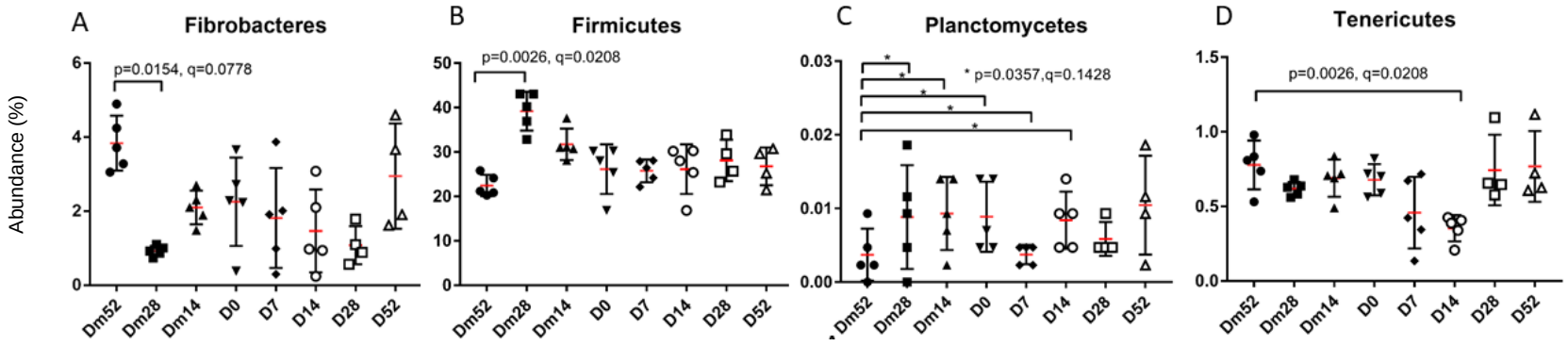


Table 4.3 Distribution of cecal phyla across study days.

Phyla	**Dm52**	**Dm28**	**Dm14**	**D0**	**D7**	**D14**	**D28**	**D52**		
	Median	**Median**	**Median**	**Median**	**Median**	**Median**	**Median**	**Median**	**P value**	**Q value**
Unassigned;Other	2.907[b]	1.635[a]	2.295[a,b]	1.928[a,b]	1.937[a,b]	1.949[a,b]	2.095	2.202	**0.0162**	0.0778
Euryarchaeota	0.444	0.072	0.309	0.477	0.172	0.095	0.336	0.365	0.0507	0.1445
Other	0.002	0.007	0.005	0.002	0.002	0.007	0.006	0.003	0.3936	0.4294
Actinobacteria	0.13	0.191	0.112	0.133	0.191	0.167	0.185	0.235	0.1397	0.2579
Armatimonadetes	0.002	0	0	0	0	0.002	0	0	0.1643	0.2629
Bacteroidetes	52.837	43.914	46.786	51.37	52.133	54.505	51.591	52.071	0.0961	0.2097
Deferribacteres	0	0	0	0.002	0	0.005	0.003	0	0.1617	0.2629
Elusimicrobia	0.007	0.009	0.007	0.009	0.007	0.009	0.013	0.017	0.8051	0.8051
Fibrobacteres	3.716[b]	0.921[a]	2.109[a,b]	2.286[a,b]	1.914[a,b]	0.979[a,b]	0.992	2.79	**0.0154**	0.0778
Firmicutes	21.202[b]	40.202[a]	31.037[a,b]	28.002[a,b]	26.458[a,b]	23.684[a,b]	27.648	27.417	**0.0026**	0.0208
Fusobacteria	0.023	0.035	0.042	0.042	0.037	0.091	0.043	0.119	0.3277	0.4106
GN02	0	0	0	0	0	0	0	0	0.1195	0.2390
Lentisphaerae	0.021[a]	0.03[a]	0.026[a]	0.035[a]	0.016[a]	0.016[a]	0.021	0.026	**0.0432**	0.1445
Planctomycetes	0.002[b]	0.009[a]	0.009[a]	0.007[a]	0.005[a]	0.009[a]	0.005	0.01	**0.0357**	0.1428
Proteobacteria	3.235	2.763	3.588	2.893	3.926	5.837	5.578	2.976	0.0786	0.1886
SR1	0.081	0.049	0.051	0.053	0.014	0.007	0.01	0.023	0.0542	0.1445
Spirochaetes	9.458	6.333	6.098	6.605	5.851	7.347	4.459	6.737	0.3262	0.4106
Synergistetes	0.016	0.044	0.037	0.028	0.03	0.035	0.04	0.033	0.261	0.3685
TM7	0.002	0.007	0.007	0.002	0.005	0	0.003	0.001	0.5182	0.5407
Tenericutes	0.809[b]	0.628[a,b]	0.707[a,b]	0.702[a,b]	0.423[a,b]	0.388[a]	0.651	0.674	**0.0026**	0.0208
Verrucomicrobia	2.928	1.64	4.056	5.014	1.916	3.433	3.363	2.037	0.2082	0.3123

In fecal samples, Lentisphaerae (p=0.0035, q=0.0438) and Spirochaetes (p=0.0060, q=0.05) were decreased on D3. Elusimicrobia (p=0.0159, q=0.0795) was significantly decreased on D14 compared to Dm28, but this difference was not evident once adjusted for multiple comparisons. Actinobacteria (p=0.0117, q=0.0731), Fibrobacteres (p=0.0440, q=0.1000), SR1 (p=0.0212, q=0.0850), Synergistetes (p=0.0266, q=0.0850), and Verrucomicrobia (p=0.0387, q=0.1000) were only significantly different on the Friedman's, but not the Dunn's post-test (Figure 4.4 and Table 4.4).

Figure 4.4 The median abundance (%) of significantly different fecal phyla across study days. (A)Actinobacteria. (B) Elusimicrobia. (C) Lentisphearae. (D) Spirochaetes. (E) Verrucomicrobmia.

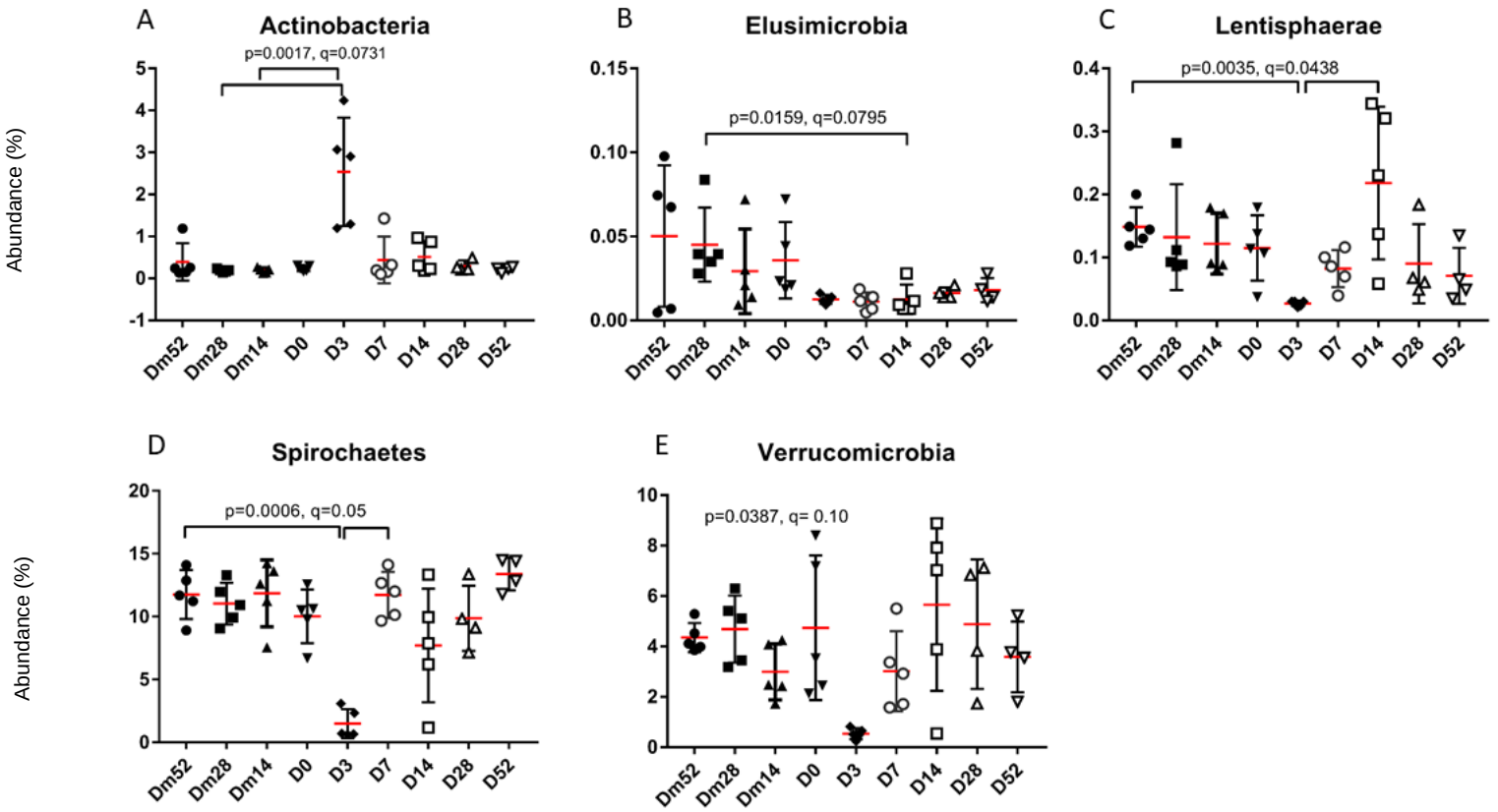


Table 4.4 The median abundance of fecal phyla across study days.

Phyla	Dm52	Dm28	Dm14	D0	D3	D7	D14	D28	D52	Dm52 vs Dm28 vs Dm14 vs D0 vs D3 vs D3 vs D14	
	Median	Median	Median	Median	Median	Median	Median	Median	Median	Pvalue	Qvalue
Unassigned;Other	2.256[a]	2.112[a]	2.333[a]	2.44[a]	2.228[a]	1.444[a]	1.96[a]	2.077	2.57	**0.0272**	0.0850
Euryarchaeota	2.04[b]	3.098[a,b]	1.688[a,b]	1.837[a,b]	0.056[a]	1.286[a,b]	2.228[a,b]	1.333	1.401	**0.0426**	0.1000
Other	0.005	0.005	0.005	0.007	0.002	0.007	0.009	0.006	0.012	0.2108	0.2635
_Actinobacteria	0.244[a,b]	0.191[b]	0.212[b]	0.228[a,b]	2.902[a]	0.193[a,b]	0.3[a,b]	0.238	0.234	**0.0117**	0.0731
_Armatimonadetes	0.014	0.007	0.012	0.009	0	0.002	0.005	0.008	0.01	0.1878	0.2635
_Bacteroidetes	42.342	39.872	39.002	38.17	40.688	41.64	37.807	38.347	38.257	0.1509	0.2358
_Deferribacteres	0	0	0	0	0	0	0.002	0.001	0	0.0572	0.1008
_Elusimicrobia	0.067[a,b]	0.04[b]	0.021[a,b]	0.023[a,b]	0.012[a,b]	0.012[a,b]	0.009[a]	0.015	0.016	**0.0159**	0.0795
_Fibrobacteres	4.009[a]	5.098[a]	6.481[a]	4.293[a]	0.405[a]	2.36[a]	3.295[a]	1.44	7.22	**0.044**	0.1000
Firmicutes	28.533	29.637	30.481	29.077	27.279	28.207	24.812	38.826	30.836	0.8088	0.3882
Fusobacteria	0.021	0.012	0.023	0.012	0.026	0.009	0.021	0.016	0.015	0.2019	0.2635
Lentisphaerae	0.144[a]	0.093[a,b]	0.091[a,b]	0.114[a,b]	0.03[b]	0.086[a,b]	0.23[a]	0.064	0.057	**0.0035**	0.0438
Planctomycetes	0.019	0.016	0.019	0.03	0.009	0.021	0.016	0.037	0.021	0.0521	0.1008

Table 4.4 Continued.

Phyla	Dm52	Dm28	Dm14	D0	D3	D7	D14	D28	D52	Dm52 vs Dm28 vs Dm14 vs D0 vs D3 vs D3 vs D14	
	Median	Median	Median	Median	Median	Median	Median	Median	Median	Pvalue	Qvalue
Planctomycetes	0.019	0.016	0.019	0.03	0.009	0.021	0.016	0.037	0.021	0.0521	0.1008
Proteobacteria	2.96	2.137	2.307	1.86	25.107	1.805	4.414	2.981	1.503	0.0547	0.1008
SR1	0.028[a]	0.03[a]	0.053[a]	0.049[a]	0.028[a]	0.007[a]	0.005[a]	0.009	0.013	**0.0212**	0.0850
Spirochaetes	11.686[b]	10.919[a,b]	12.6[a,b]	10.512[a,b]	0.714[a]	12.002[b]	7.877[a,b]	9.473	13.641	**0.006**	0.0500
Synergistetes	0.035[a]	0.03[a]	0.037[a]	0.058[a]	0.012[a]	0.009[a]	0.07[a]	0.069	0.036	**0.0266**	0.0850
TM7	0.009	0.005	0.012	0.014	0.002	0.002	0.005	0.009	0.006	0.3963	0.3882
Tenericutes	0.677	0.886	1.012	0.981	0.826	0.844	0.802	0.476	0.965	0.6151	0.3882
Verrucomicrobia	4.116[a]	5.112[a]	2.479[a]	3.53[a]	0.509[a]	2.921[a]	7.028[a]	5.331	3.662	**0.0387**	0.1000
WPS-2	0	0.005	0.002	0	0	0	0	0.001	0.003	0.202	0.2635

4.4.8.4 Linear discriminant analysis effects size

Linear discriminant analysis effect size (LEfSe) was used to elucidate taxa associated with study day. A linear discriminant analysis (LDA) score > 3.5 was considered significant. In the cecal content samples, only 2 phyla (i.e., Firmicutes and Fibrobacteres) met this threshold. At the family level in cecal samples, 5 taxa (i.e., *Fibrobacteriaceae, Unclassified Y2, Ruminococcaceae, Unclassified Clostridiales,* and *Lachnospiraceae*) were more abundantly expressed prior to metronidazole administration, whereas 3 taxa (i.e., *Porphyromonadaceae*, *Veillonellaceae,* and *Succinivibrionaceae*) were more abundantly expressed after metronidazole administration. Table 4.4 contains complete information regarding the taxa, study day, and LDA scores from cecal samples.

Table 4.5 Linear discriminant analysis of bacterial taxa in cecal samples and their associations with study day. Only LDA scores of >3.5 are shown.

Taxa	**LDA**	**Time point**
Phylum		
Firmicutes	4.92	Dm28
Fibrobacteres	4.16	Dm52
Family		
Fibrobacteraceae	4.15	Dm52
Unclassified_YS2	3.6	Dm52
Ruminococcaceae	4.37	Dm28
Unclassified_Clostridiales	4.39	Dm28
Lachnospiraceae	4.57	Dm28
Porphyromonadaceae	3.52	D14
Veillonellaceae	4.32	D14
Succinivibrionaceae	4.4	D14

In fecal samples, 2 taxa at the phylum level (i.e., Elusimicrobia and Euryarchea) and 3 taxa at the family level (i.e., *Clostridicaceae*, *Methanocorpusculaceae*, and *Ruminococcaceae*) were associated with an LDA score of greater than 3.5 at baseline. Fecal taxa at the phylum level significantly associated study days following metronidazole administration included: D3: Actinobacteria and Protobacteria; D14: Verrucomicrobia and Lentisphaerae; and D28: Planctommycetes and Synergistetes. At the family level, *Alcaligenaceae*, *Corynebacteriaceae*, *Neisseriaceae*, *Actinomycetaceae*, *Porphyomonadaceae*, *Tisserellaceae*, *Enterobacteriaceae*, *Pastuerellaceae*, *Streptococcaceae*, *Aerococcaceae*, *Lacobacillaeceae*, and *Aeromondaceae* were more abundant on D3, whereas *Methanobacteriaceae* and *RFP12* were more abundant on D14. *Bacillaceae* and *Planococcaceae* were associated with D28. Table 4.5 displays the results of LEfSe analysis for fecal samples at both the phylum and family levels.

Table 4.6 Linear discriminant analysis of bacterial taxa in fecal samples and their associations with study day. Only LDA scores of >3.5 are shown.

Taxa	LDA	Time point
Phylum		
Elusimicrobia	3.92	Dm52
Euryarchaeota	4.12	Dm28
Actinobacteria	4.10	D3
Proteobacteria	5.06	D3
Verrucomicrobia	4.43	D14
Lentisphaerae	3.53	D14
Planctomycetes	3.72	D28
Synergistetes	3.66	D28
Family		
Clostridiaceae	3.76	Dm52
Methanocorpusculaceae	4.04	Dm28
Ruminococcaceae	4.37	Dm28
Alcaligenaceae	3.61	D3
Corynebacteriaceae	3.62	D3
Neisseriaceae	3.64	D3
Actinomycetaceae	3.90	D3
Porphyromonadaceae	3.93	D3
Tissierellaceae	4.00	D3
Enterobacteriaceae	4.12	D3
Pasteurellaceae	4.18	D3
Streptococcaceae	4.34	D3
Aerococcaceae	4.35	D3
Lactobacillaceae	4.60	D3
Aeromonadaceae	4.68	D3
Methanobacteriaceae	3.77	D14
RFP12	4.45	D14
Bacillaceae	4.01	D28
Planococcaceae	4.47	D28

4.4.8.5 Testing for enteric pathogens

All horses were negative for *Salmonella* PCR in fecal samples across all time points. Four horses (i.e., horses 1, 2, 4, and 5) were positive for *Salmonella* by PCR testing in cecal content samples on Day 7. Horses 1, 2, and 4 remained positive on Day 14, but tested negative on Day 28. Horse 5 remained positive at D52, the final time point of the study. Horse 3 was positive on Day 14, and subsequently died later that day. Serotyping revealed *Salmonella enterica* serotype Newport in one horse (horse 1), *Salmonella enterica* serotype Anatum in 3 horses (horses 2, 3, and 4) and multiple serotypes in one horse (horse 4). All horses were negative for *Clostridium perfringens* and *C. difficile* toxins in both cecal and fecal samples at all time points.

4.4.2 Metabolomic results

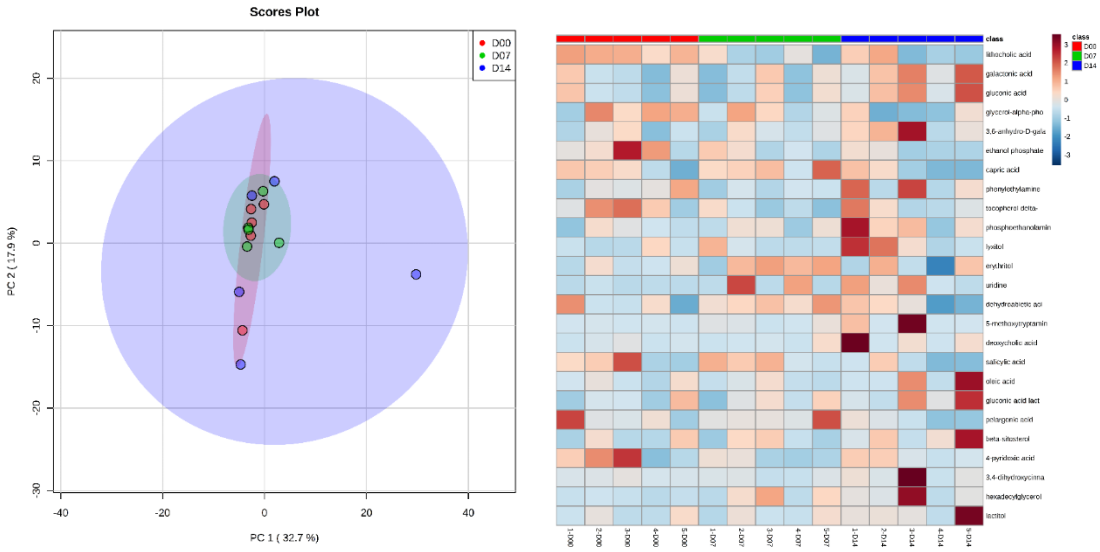
Using an untargeted approach, a total of 554 unique metabolites were detected, 223 of which were named. Metabolomic data has been submitted to metabolomicsworkbench.org under the submission ST001248.

Only named metabolites were included in the analysis. Metabolites were examined in the cecum on Days m28, m14, 0, 7, 14 and 28. Metabolites were examined in the feces on Days m28, m14, 0, 3, 7, 14 and 28. Samples were not collected on Day m52 and 52 for metabolomic analysis, and on Day m28 Horse 5 did not have enough sample for analysis. Metabolites were analyzed using PCA score plots and heat maps. In the cecal samples, the PCA plot of all named metabolites (Figure 4.5) indicated that samples on D14 were different from other study days. This is due to the presence of an outlier, Horse 3, whose metabolite profiles were presumably altered by the presence of advanced gastrointestinal

disease and subsequent death. No cecal metabolites were significantly different by study day after adjustments for multiple comparisons were made (Figure 4.5).

Figure 4.5 PCA scores plots and heatmap of cecal samples. (A) PCA scores plot by study day (D-28-red, D-14-green, D0-blue, D7-teal, D14-pink, D28-yellow) and (B) heatmap by study day (D0-red, D7-green, D14 blue) (color intensity indicates intensity the increase, red or the decrease, blue in metabolite concentration).

A. B.

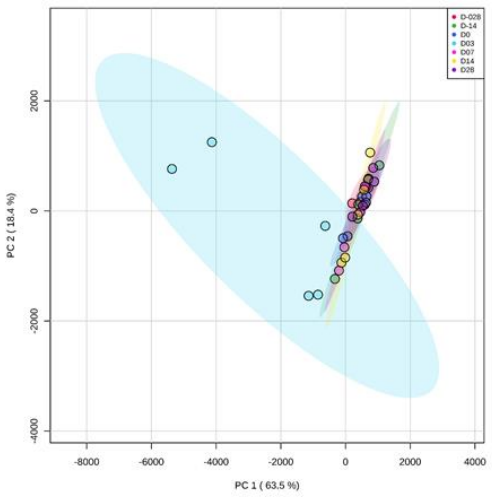


In fecal samples, Day 3 samples clustered distinctly from all other study days in the PCA scores plot (Figure 4.6). Mulitvariate analysis of all the named metabolites found 21 fecal metabolites that were significantly different across study days following adjustment for multiple comparisons. A heatmap of the significantly different named fecal metabolites (Figure 4.6) indicated a visible change in concentration relative to study day. Metabolites related to nucleic acid metabolism (thymine, uracil, xanthine), amino acid

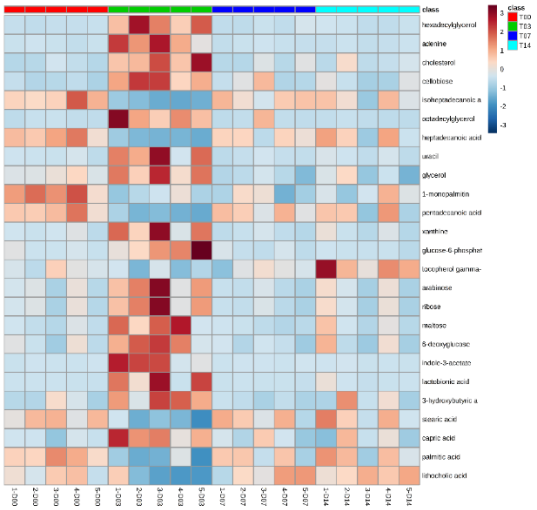
metabolism (putrescine, salicylic acid, serine, threonine, tryptophan, tyramine, valine), lipid metabolism (phosphoenolamine, ribonic acid) and carbohydrate metabolism (ribose) all had significantly elevated levels on Day 3. Other metabolites related to lipid metabolism (phytanic acid, stearic acid) and the metabolism of cofactors and vitamins (δ-tocopherol, γ-tocopherol) were decreased on Day 3 in relation to other study days.

Figure 4.6 PCA scores plots and heatmap of fecal samples. (A) PCA scores plot by study day (D-28-red, D-14-green, D0-blue, D3-teal, D7-pink, D14-yellow, D14-purple) and (B) heatmap by study day (D0-red, D3-green, D7 blue, D15-teal) (color intensity indicates intensity the increase, red or the decrease, blue in metabolite concentration).

A.



B.



Information regarding the significant fecal metabolites and their respective KEGG pathways is provided in Table 4.6. Information regarding the changes in significant fecal metabolites by study day is provided in Figures 4.5-4.10.

Table 4.7 Significantly different fecal metabolites by study day.

Metabolite	f.value	p.value	log10(p)	FDR	Fisher's LSD	KEGG	KEGG Pathway	KEGG Pathway
putrescine	3.166	0.018	1.756	0.048	D03 vs D-028, D-14, D0, D07, D14, D28	C00134	Arginine and proline metabolism, glutathione metabolism	Amino acid metabolism
salicylic acid	5.089	0.001	2.883	0.008	D03 vs D-028, D-14, D0, D07, D14; D28	C00805	Pheylalanime metabolism	Amino acid metabolism
serine	4.717	0.002	2.678	0.011	D03 vs D-028, D-14, D0, D07, D14, D28	C00065	Glycine, serine and threonine metabolism	Amino acid metabolism
threonine	4.488	0.003	2.549	0.014	D03 vs D-028, D-14, D0, D07, D14, D28	C00188	Glycine, serine and threonine metabolism, monobactam biosynthesis	Amino acid metabolism
tryptophan	3.264	0.015	1.818	0.046	D03 vs D-028, D-14, D0, D07, D14, D28	C00078	Glycine, serine and threonine metabolsim	Amino acid metabolism
tyramine	3.182	0.017	1.766	0.048	D03 vs D-028, D-14, D0, D07, D14, D28	C00483	Tyrosine metabolism	Amino acid metabolism
valine	3.876	0.006	2.192	0.022	D03 vs D-028, D-14, D0, D07, D14, D28	C00183	Valine, leucine and isoleucine degradation and biosynthesis	Amino acid metabolism
xanthine	7.089	0.000	3.884	0.002	D03 vs D-028, D-14, D0, D07, D14, D28	C00385	Purine metabolism	Amino acid metabolism
phytanic acid	5.394	0.001	3.046	0.007	D03 vs D-028, D-14, D0, D07, D14, D28		Pentose phosphate pathways, metabolic pathways	Carbohydrate metabolism
pinitol	4.190	0.004	2.378	0.016	D03 vs D-028, D-14, D0, D07, D14, D28		Pentose and glucuronate interconversions	Carbohydrate metabolism
ribose	6.214	0.000	3.465	0.004	D03 vs D-028, D-14, D0, D07, D14, D28	C00121	Pentose phosphate pathway	Carbohydrate metabolism
tagatose	5.451	0.001	3.076	0.007	D03 vs D-028, D-14, D0, D07, D14, D28	C00795	Galactose metabolism	Carbohydrate metabolism

Table 4.6 Continued

xylonic acid	7.435	0.000	4.043	0.002	D03 vs D-028, D-14, D0, D07, D14, D28	C05411	Pentose and glucoronate metabolism	Carbohydrate metabolism
ribonic acid	8.418	0.000	4.472	0.001	D03 vs D-028, D-14, D0, D07, D14, D28	C01685	Biosynthesis of unsaturated fatty acids, biosynthe of secondary metabolites	Lipid metabolism
stearic acid	5.912	0.000	3.314	0.005	D03 vs D-028, D-14, D0, D07, D14, D28	C01530	Fatty acid biosynthesis	Lipid metabolism
tocopherol delta- NIST	3.736	0.008	2.108	0.025	D03 vs D-028, D-14, D0, D07, D14, D28	C14151	Ubiquinone and terpernoid-quione metabolites	Metabolism of cofactors and vitamins
tocopherol gamma-	4.132	0.005	2.343	0.016	D03 vs D-028, D-14, D0, D07, D14, D28	C02483	Ubiquinone and terpernoid-quione metabolites	Metabolism of cofactors and vitamins
phosphoethar amine	4.231	0.004	2.401	0.016	D03 vs D-028, D-14, D0, D07, D14, D28		Glycerophospholipid metabolism	Nucleic acid metabolism
piperidone	5.047	0.001	2.860	0.008	D03 vs D-028, D-14, D0, D07, D14, D28		Purine metabolism	Nucleic acid metabolism
thymine	4.192	0.004	2.379	0.016	D03 vs D-028, D-14, D0, D07, D14, D28	C00178	Pyrimidine metabolism	Nucleic acid metabolism
uracil	10.390	0.000	5.252	0.000	D03 vs D-028, D-14, D0, D07, D14, D28	C00106	Pyrimidine metabolism	Nucleic acid metabolism

Figure 4.7 Fecal metabolites from pathways of amino acid metabolism.

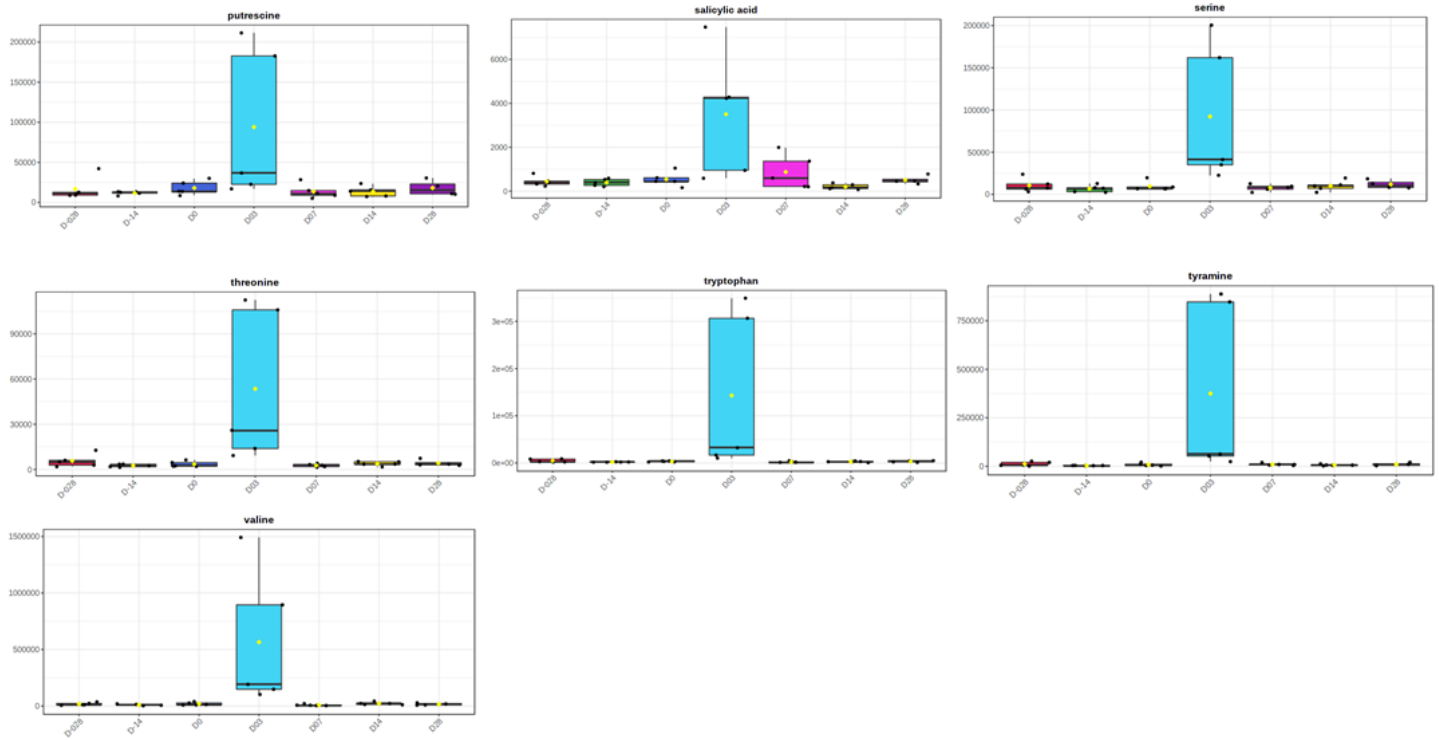


Figure 4.8 Fecal metabolites from pathways of amino acid metabolism.

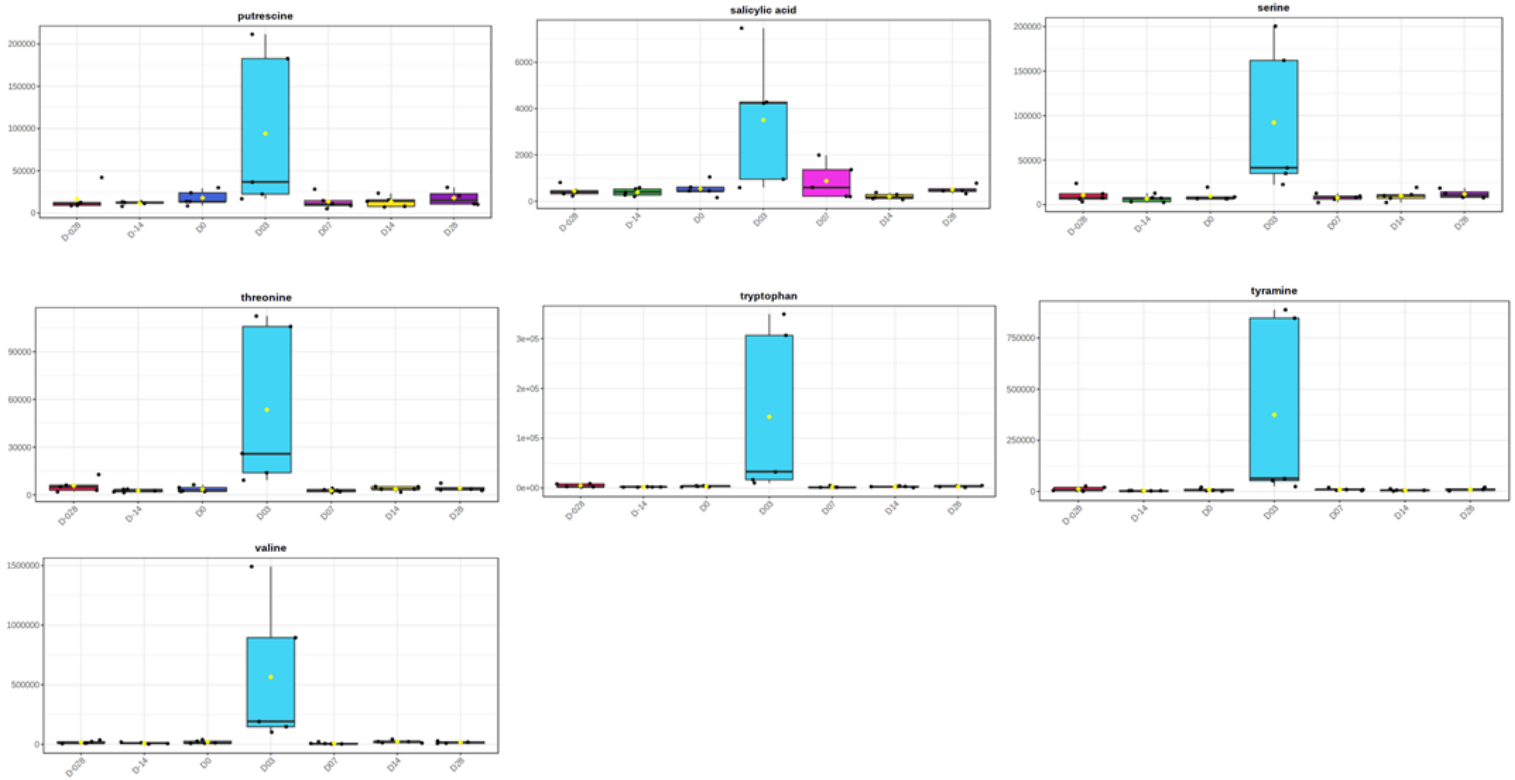


Figure 4.9 Fecal metabolites from pathways of nucleic acid metabolism.

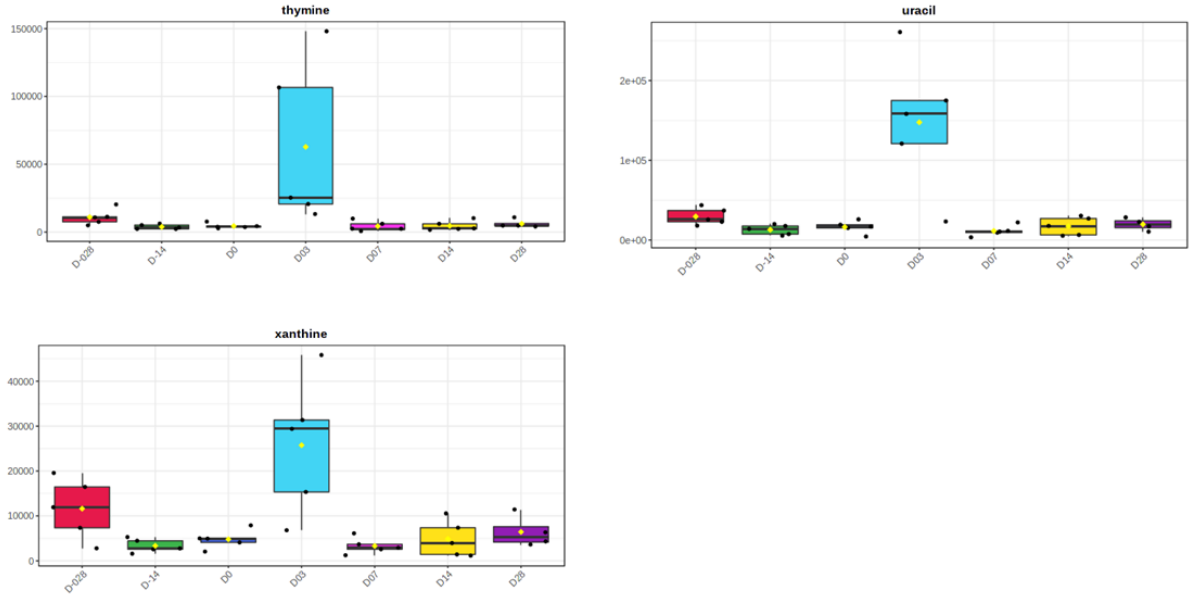


Figure 4.10 Fecal metabolites from pathways of carbohydrate metabolism.

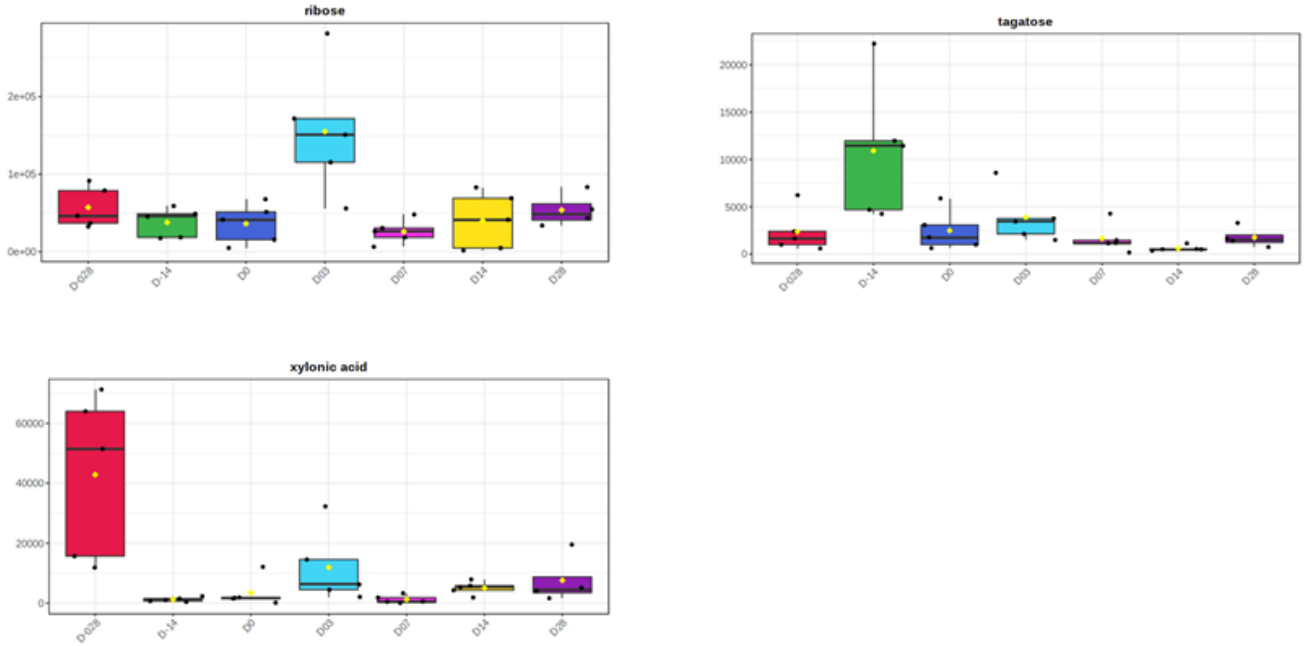


Figure 4.11 Fecal metabolites from pathways of lipid metabolism.

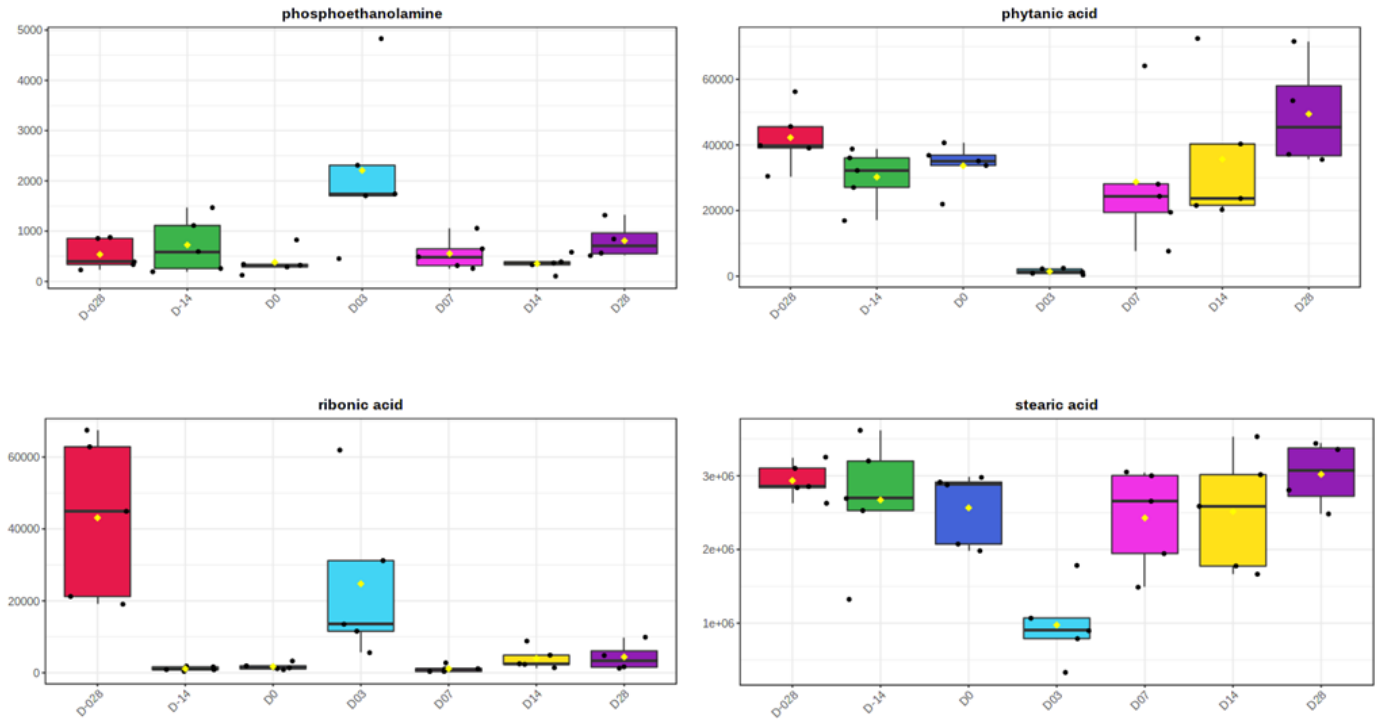
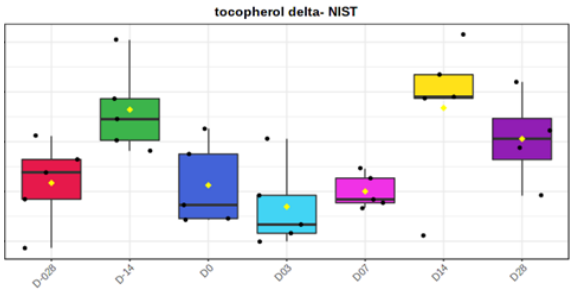
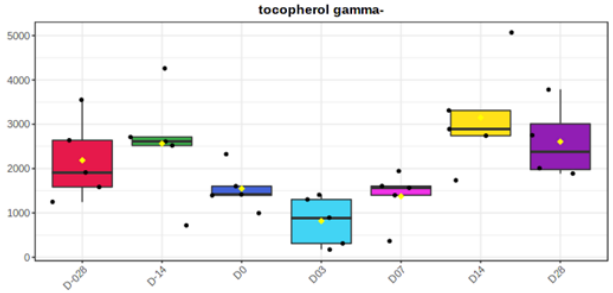


Figure 4.12 Fecal metabolites from pathways of cofactor and vitamin metabolism.





4.5 Discussion

This study utilized a herd of horses with cecal cannulas to investigate the effects of metronidazole on the equine cecal content and fecal microbiome and metabolome. The horses had been cannulated approximately 10 years earlier, and had experienced no disruption in environment, feed, housing, or exposure to new horses for 6 months prior to and during the study period. While the use of cannulated animals is commonplace in nutritional studies, it is somewhat novel in microbiome and metabolome studies. The presence of a cannula in the cecum does allow entry of oxygen into the cecum during sampling periods. However, in other species it has been demonstrated that anaerobic conditions are quickly restored following resealing (79). In ruminant species such as goats and cattle, the presence of the cannula does not significantly altered diversity indices or taxonomy of major phyla (80-82). Because horses were sampled every 7-14 days, the short-term introduction of oxygen likely had a minimal impact. Although unlikely to significantly alter the results, the use of cannulated animals in this study should be interpreted with some degree of caution.

The horses experienced adverse effects at two time points in the study, Days 3 and 14. The first adverse event occurred on Day 3 in all 5 horses and was manifested as inappetence and skin scalding at the canula site. Inappetence can be an early manifestation of gastrointestinal disease, and the skin scalding was presumably due to a change in the pH of the cecal fluid. Previously studies using these same 5 cannulated horses for grain overload studies indicated that decreasing the pH of the cecal fluid could result in fluid leaking from the canula and subsequent skin scalding (50). Out of caution, the authors

elected to suspend metronidazole administration after only 5 of the 14 doses had been given. All the horses returned to an apparently healthy state within 48 hours, and none of the horses showed symptoms of gastrointestinal disease until later in the study. The second adverse event occurred on Day 13 when Horse 3 experienced an episode of colic and died 24 hours later. Post-mortem examination indicated colitis as the cause of death. As the study had been suspended 11 days earlier and the remaining horses appeared healthy, the investigators chose to continue sampling from the remaining horses and use the data from the deceased horse. Due to the loss of Horse 3, samples from Days 28 and 52 only included horses 1, 2, 4, and 5. Friedman's analysis for these two time points was not possible, although the data from these 4 horses is presented.

Similar to reports in dogs, metronidazole decreased the diversity of the microbiome in the horses evaluated, but at different time points for cecal content and fecal samples ((75, 83). The OTU, Shannon, and Chao 1 metrics were consistently reduced in cecal samples on Day 14, whereas fecal samples had the lowest alpha diversity measures on Day 3. Recovery of alpha diversity indices occurred by Day 28 in both sample types. Beta diversity was unaffected by metronidazole administration in the cecal samples. However, fecal samples were distinctly clustered on Day 3 compared to the other time points in the weighted and unweighted PCoA plots. Metronidazole had the greatest effects on alpha and beta diversity on Days 3 and 14, which is consistent with the clinical appearance of gastrointestinal disease in the study subjects.

At baseline, both cecal and fecal samples appeared to have a similar microbial community composition. Bacteroidetes and Firmicutes, important phyla for fiber

degradation and affected by the presence of gastrointestinal disease in other studies (68-71), constituted the majority of the phyla. Spirochaetes, Fibrobacteres, Proteobacteria, Tenericutes, and Verrucomicrobia comprised approximately 1% or more of the total bacteria. The cecum had a greater percentage of Bacteroidetes, while the feces contained more Fibrobacteres and Spirochaetes. This is consistent with other reports comparing the anatomic compartments of the GIT in normal horses (31, 84) and also in previous reports of cannulated horses (85).

Over time, the bacterial community composition was altered by metronidazole. In this study, the abundance of Bacteroidetes remained unchanged in either sample type. In the cecum, the phyla Firmicutes, Fibrobacteres, Planctomycetes, and Tenericutes were affected. There was a significant change in the abundance of these phyla from Dm52 to Dm28, which is likely related to normal variation in the microbiota over time, ambient temperature, and season (86-88). The magnitude of this change likely overshadowed the downward trend in abundance of Fibrobacteres, Firmicutes, Planctomycetes, and Tenericutes on Days 7 and 14 due to metronidazole administration. The alterations in the Firmicutes occurred within the class Clostridia and the families Other, Unknown, Christensenellaceae, Dehalobacteriaceae, Lachnospiraceae, Ruminococcaceae, and Veillonellaceae. A similar effect was noted with the phylum Fibrobacteres and the family Fibrobacteraceae prior to metronidazole. These changes did not achieve significance after adjustments for multiple comparisons were made at the family level. This finding was reinforced by the LEfSe analysis. These changes may have reached significance if the

number of horses used in the study would have been greater or if metronidazole administration had not been terminated early on Day 3.

In the feces, the phyla Actinobacteria, Elusimicrobia, Lentispherae, Spirochaetes, and Verrucomicrobia were affected by metronidazole administration. All of these phyla, except Actinobacteria, decreased in abundance on either Days 3, 7, or 14. The abundance of Actinobacteria was significantly elevated on Day 3 in the feces compared to other study days, with the family Bifidobacteriaceae accounting for majority of this increase. Actinobacteria is a gram-positive facultative anaerobe organism that accounts for a relatively small percentage of the total number of bacteria. However, a similar increase was reported after metronidazole use in dogs and rats (75, 89). The significance of the decrease in the abundance Elusimicrobia, Lentisphereae, and Spirochaetes at various time points after metronidazole administration is unknown. Lentisphereae and Spirochaetes are anaerobes or facultative anaerobes, which were likely affected by metronidazole. Lentispherae is commonly found in the gut of mammals but comprises less than 1% of all taxa. Spirochaetes, however, represent 6.5 % and 11.3% of the cecal content and fecal samples, respectively. Metronidazole caused a significant decrease in the Spirochaetes at both the phylum and family level on D3 but quickly recovered by the last sampling points. The functional role of these three phyla and their response to metronidazole are yet to be fully elucidated.

Univariate analysis and LEfSe indicated that Actinobacteria and Verrucomicrobia were affected on Day 3 in the feces. Verrucomicrobia showed a marked decline in both cecal content and fecal samples on Days 3 and 7, although this result did not reach

significance with the Dunn's post-test (q=0.0717). Verrucomicrobia is a phylum of strict anaerobes that maintains the mucus layer between the gut lumen and the enterocytes. In some studies of healthy horses, Verrucomicrobia have been reported to account for up to 40% of the fecal microbiota (70). In this study, Verrucomicrobia accounted for a much smaller percentage (i.e., 2.6-5.4%) of the total fecal microbiota at the four baseline sampling points, but even further decreased on Day 3 in fecal samples. This trend to decline after antibiotic use has been previously noted in both dogs and humans, and is thought to play a role in the loss of the intestinal barrier function in colitis.

All five of the horses in this study were PCR positive for *Salmonella* in their cecal content, but not in fecal samples, after metronidazole administration. Although not routinely tested for *Salmonella* before this study, the horses had never previously displayed symptoms of GIT disease. Identification of multiple serovars of *Salmonella* from the horses (Newport and Anatum) lends evidence against a herd outbreak of infectious *Salmonella*, which would typically include one serovar. All horses, except one, reverted to negative PCR status after the discontinuation of the metronidazole by the final study time point. Also, all horses were PCR negative at the 4 pre-treatment time points in both sample locations and remained negative in the fecal samples. Thus, the authors suggest that the metronidazole induced a degree of dysbiosis, which resulted in expansion of this enteric pathogen in the cecum, but not the entire the distal hindgut.

This study employed GC-MS methods to identify and quantitate changes in the end products of metabolism in the cecal and fecal samples before and after metronidazole administration. Of these 2 sample types, only the fecal metabolites were significantly

altered from baseline after adjustments for multiple comparisons were made. The fecal metabolites represented diverse metabolic pathways, such as nucleic acid, amino acid, carbohydrate, lipid and cofactor and vitamin metabolism. All significant metabolites belonging to the amino acid group were increased on Day 3. This could result from alterations in the commensal bacteria and their role in the absorption or synthesis of these amino acids. Similar trends have been reported in dogs and humans with inflammatory bowel disease (90, 91). Ribose, a metabolite of the pentose phosphate pathway in carbohydrate metabolism, was significantly elevated on Day 3, consistent with an oxidative stress response noted in the metabolomic profiles dogs with gastrointestinal disease (29). Similarly, decreases in tocopherols, analogs of the anti-oxidant vitamin E, have been reported in horses suffering from obesity(92).

Although metronidazole is reportedly safe to use in equine patients, the horses in this study appeared to develop early indications of colitis despite having normal feces. AAD is poorly defined in the veterinary literature in regards to the number or character of abnormal stools, but is generally regarded as a temporal association with the initiation or discontinuation of an antimicrobial agent and the development of diarrhea (93). While diarrhea is characteristically considered pathognomonic for colitis, most horses exhibit prodromal symptoms associated with the gastrointestinal tract before diarrhea is clinically manifested. These symptoms often include inappetance, malaise, fever, and behavioral expressions of abdominal pain that precede the development of diarrhea. The clinical impression is that AAD is often acute in onset, occurring rapidly after the initiation of antimicrobial therapy. In horses, one study reported that the average time for development

of diarrhea was 3.4 days after antibiotic administration (range: 1-11days) (94). In humans, there is also evidence for the development of diarrhea associated with antibiotic discontinuation. This may have played a role in the death of Horse 3 on Day 14.

In this study, metronidazole decreased the diversity and altered the bacterial composition of cecal content and fecal samples. Subsequent functional alterations of the microbiome were reflected in the metabolite profile of the fecal samples. The timing of these changes coincides with the development of symptoms of GIT disease in these horses. Antibiotic administration, including metronidazole, is recognized as a risk factor for the development of diarrhea in species, such as humans, dogs, cats, and horses (27, 29, 33, 70, 95).

4.6 Conclusion

Metronidazole administration decreased microbial richness and evenness indices in both cecal and fecal samples by Day 3. Dysbiosis of the fecal microbiome on Study Day 3 resulted in changes in the abundance of Actinobacteria, Spirochaetes, Lentisphaerae, and Verrucomicrobia. Metronidazole did not alter the cecal metabolome, but did affect the metabolism of amino acids, carbohydrates, lipids, nucleic acids and cofactors and vitamins in the feces with the greatest effect observed on Day 3.

5. THE FECAL MICROBIOME OF HORSES THAT DEVELOPED DIARRHEA ON TREATMENT WITH ANTIMICROBIALS COMPARED TO THOSE THAT MAINTAINED NORMAL FECAL CONSISTENCY AND HEALHTY CONTROLS

5.1 Overview

This study investigated the effects of antimicrobials on the equine fecal microbiome. Horses on antimicrobials that developed diarrhea were matched to two control groups: horses on antibiotics that did not develop diarrhea and healthy horses not on antibiotics. Horses on antibiotics were matched for specific type of antibiotic, route and number of days of administration. All horses were matched by diet.

Antimicrobial agents induced a severe dysbiosis of the fecal microbiome of horses, regardless of whether they developed diarrhea or not. Horses under the influence of antimicrobial agents show a reduction across alpha diversity indices compared to control horses and an altered taxonomic composition.

5.2 Introduction

Diarrhea is a common adverse effect of antimicrobial administration across species, including horses (93). Because AAD is not well defined in the veterinary literature in regards to stool character, frequency, temporal association to administration of antibiotics, or degree of the resulting illness, the true incidence of AAD in the equine population is difficult to assess. In veterinary referral centers, AAD in adult horses has been reported to range from 22-94% (31, 65, 93, 96-100), with significant associated mortality rates of 15-50% (65, 96, 99). One retrospective study reported that horses with

AAD were 4.5 times more likely to die compared to horses with other types of colitis (65). These statistics reflect a need to better understand the pathogenesis of equine AAD, prevent disease occurrence, and improve survival rates.

The role of intestinal dysbiosis is likely a key factor in the pathogenesis of AAD. Antibiotics reduce the diversity and alter the bacterial composition of the fecal microbiome in many species, even when animals remain healthy and maintain a normal fecal character. This effect has been demonstrated in healthy horses that were treated with commonly used antimicrobials, such as penicillin, trimethoprim sulfa, ceftiofur, and metronidazole (69, 70, 101-103). Although all antibiotics have the potential to cause diarrhea, some antimicrobial agents have been associated with an increased risk due to high concentrations in the intestinal lumen due to a low oral absorption, biliary excretion, or enterohepatic recycling (104). Alterations in the microbiota may confer functional metabolic changes, which ultimately cause diarrhea. In humans, AAD is known to reduce concentrations of short chain fatty acids in the intestinal lumen, resulting in the accumulation of carbohydrates and bile acids. This ultimately inhibits water absorption from the gut, causing osmotic diarrhea (105). Furthermore, a decrease in the abundance of commensal bacteria may lead to overgrowth and colonization by enteric pathogens (106). To date, tetracyclines, macrolides, cephalosporins, fluoroquinolones, trimethoprim-sulfonamides, aminoglycosides, chloramphenicol, β-lactams, and metronidazole have all been reported to cause diarrhea in horses (74, 103).

While antimicrobials cause a dysbiosis in the fecal microbiome of healthy horses and that horses with colitis suffer from dysbiosis, it is unknown if horses with AAD

develop a more severe form of dysbiosis that can lead to colitis. The purpose of this study was to compare the fecal microbiome of hoses with diarrhea as a result of antibiotic administration (AAD) to two populations of control horses, those on antibiotics that did not develop diarrhea (ABX) and healthy horses not on antibiotics (CON).

5.3 Materials and methods

5.3.1 Subjects

Fecal samples were collected from hospitalized horses one year of age or older that were prescribed antimicrobials as prophylactically prior to elective surgery or to treat a suspected or known infection. Horses that developed diarrhea as a result of antibiotic treatment (AAD) were matched to 2 control populations: hospitalized horses on the same antibiotic therapy that did not develop diarrhea (ABX) and healthy horses not on antimicrobial therapy (CON). AAD horses were classified by the clinician as having diarrhea due only to antimicrobial administration. ABX horses were matched to AAD horses by the specific antimicrobial and the duration of antibiotic therapy (i.e., less than or greater than 5 days). Horses in the ABX group maintained normal fecal consistency and developed no symptoms of gastrointestinal illness during the period of antimicrobial administration. Inclusion criteria for horses in the CON group consisted of the following: residence on a farm (non-hospital environment), no antibiotic or non-steroidal anti-inflammatory administration within 6 months, and a normal physical examination performed by a veterinarian on the day of sample collection. Horses in all 3 groups had no previous history of gastrointestinal disease prior to inclusion in the study for the previous 6 months. Horses in each group were also matched by diet (i.e., forage, amount of

concentrate fed, and percentage of fiber in the concentrate). Fecal samples were collected after natural elimination and stored at -80°C until processed in the laboratory.

5.3.2 DNA extraction

One hundred mg of feces was aliquoted into a sterile 1.7 ml tube (Microtube, Sarstedt AG & Co, Numbrecht, Germany) containing 150 µl of 0.1 mm zirconia-silica beads and 100 µl of 0.5 mm zirconia-silica beads (BioSpec Products Inc., Bartlesville, OK, USA). Samples were then homogenized (FastPrep-24, MP Biomedicals, Irvine, CA, USA) for a duration of 1 minute at a speed of 4 m/s. DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) following the manufacturer's instructions.

5.3.3 Sequencing of 16S rRNA genes

Sequencing of the V4 region of the 16S rRNA gene was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq platform (Illumina Inc., San Diego, CA). Following the manufacturer's guidelines, 2x300 paired-end reads were produced using 515F (5'-GTG YCA GCM GCC GCG GTA A-3') and 806R (5-GGA CTA CNV GGG TWT CTA AT-3') primers (53, 54). The PCR reaction was performed in a single-step 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 28 cycles (5 cycles used on PCR products) of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. Using Illumina TruSeq DNA's protocol, a DNA library was set up and Illumina MiSeq was used for sequencing according the manufacturer's guidelines.

5.3.4 Analysis of Sequences

A total of 63 samples were analyzed, which generated a total of 11,281,494 quality sequences. Sequences were analyzed using a QIIME 2 (Quantitative Insights into Microbial Ecology) (55) v.2019.7 pipeline as described previously (56, 57). Briefly, barcodes, and primers were removed and short (<150 bp), ambiguous, homopolymeric sequences were depleted from the dataset. DADA2 was used to identify and remove chimeric sequences (58). The amplicon sequence variant (ASV) table was created using DADA2 (59), and rarefied to 88,730 sequences per sample based on the lowest read depth in all samples for even depth of analysis. Sequences determined to be from mitochondria, chloroplasts, unassigned, or those belonging to the phylum cyanobacteria were excluded from further analysis.

5.3.5 Statistical analysis

As the data did not show normal distribution based on a Shapiro-Wilk test (JMP Pro 14, SAS, Marlow, Buckinghamshire), non-parametric measures were used throughout the study. Statistical analysis of alpha diversity (Amplicon Sequence Variant (ASV), Chao 1, and Shannon) was performed using Kruskal-Wallis test with a Dunn's multiple comparison post-test in the software package PRISM (PRISM 7, GraphPad Software Inc., San Diego, CA). Beta diversity (bacterial community composition) was evaluated with weighted and unweighted UniFrac metrics and visualized for clustering with Principle Coordinate Analysis (PCoA) plots. An Analysis of Similarity test (ANOSIM) within the PRIMER 6 (PRIMER-E Ltd. Luton, UK) software package was performed on the beta diversity distance matrices to assess the significance of the differences in the bacterial

community composition. Analysis of the bacterial taxa in the fecal samples was evaluated using a Kruskal-Wallis test (PRISM 7, GraphPad Software Inc., San Diego, CA) followed by a Dunn's multiple comparison post-test.

5.4 Results

5.4.1 Study participants

A total of 63 horses were enrolled in the study, including 17 horses in the AAD group, 15 horses in the ABX group, and 31 horses in the CON group. Age, gender, and breed of the enrolled horse are detailed in Appendix C. Antimicrobials given to horses in the AAD and ABX groups included: ceftiofur crystalline (AAD, n=4; ABX, n=2); doxycycline (AAD, n=4; ABX, n=4); penicillin and gentamycin (AAD, n=4; ABX, n=4); penicillin, gentamycin and metronidazole (AAD, n=2; ABX, n=2); penicillin, gentamycin and doxycycline (AAD, n=2; ABX, n=2); and trimethoprim sulfa (AAD, n=1; ABX, n=1).

5.4.2 Beta diversity (between sample)

Bacterial community composition (beta diversity) was affected by antibiotic use, with clustering of AAD and ABX horses compared to CON horses (R=0.391, p=0.001) based on weighted Unifrac distances (Figure 5.1). AAD horses showed a stronger separation from CON horses (R=0.560, p=0.0001) compared to ABX horses (R=0.3, p=0.0012) in a pairwise comparison. There was a significant but weak separation between AAD and ABX horses (R=0.121, p=0.0012) (Table 5.1).

Figure 5.1 PCoA plot showing clustering of AAD (red) and ABX (blue) horses healthy control horses (yellow).

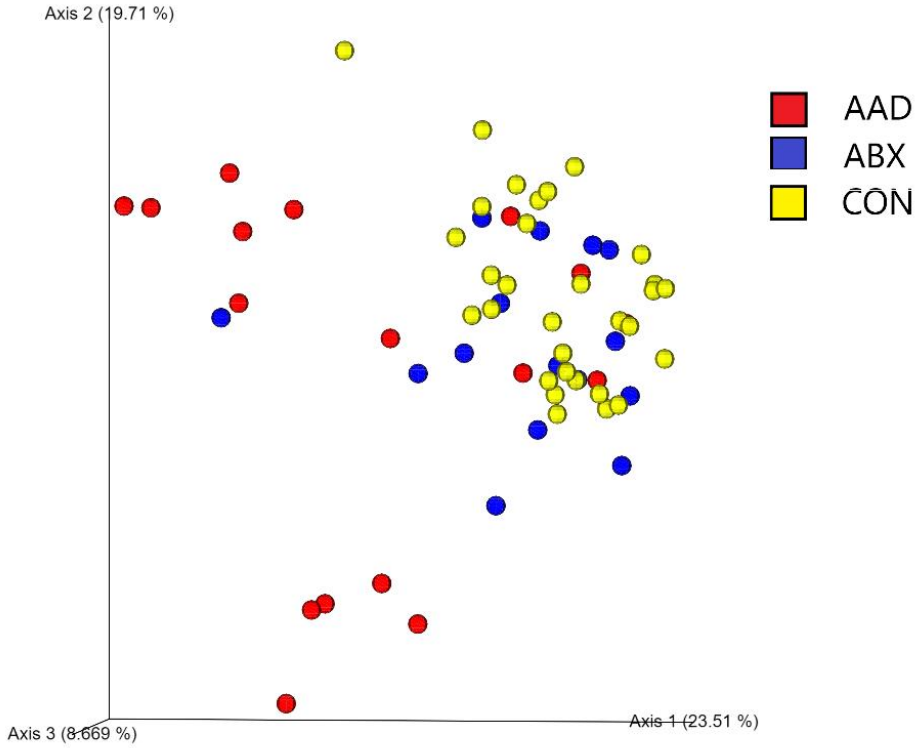


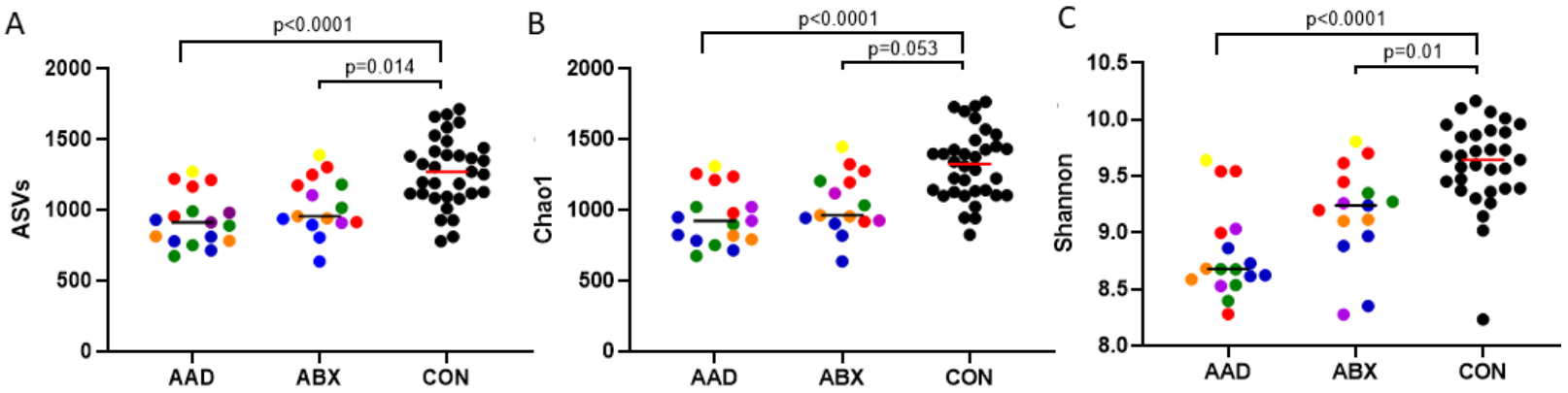
Table 5.1 Unweighted and weighted Unifrac distances of AAD, ABX, and CON horses.

	Unweighted		Weighted	
Pair	**R statistic**	**P value**	**R statistic**	**P value**
Overall	0.398	0.0001	0.391	0.0001
AAD vs CON	0.547	0.001	0.569	0.0001
AAD vs ABX	0.063	0.001	0.121	0.0012
ABX vs CON	0.37	0.001	0.3	0.0001

5.4.3 Alpha diversity (within sample)

Metrics for richness and evenness (alpha diversity) were significantly decreased in both the AAD and the ABX groups, compared to the CON group, but not between each other (Figure 5.2). For species richness, horses with AAD and ABX had significantly decreased ASVs compared to control horses ($p<0.0001$ and $p=0.014$, respectively). This relationship was also true for the Chao 1 ($p<0.0001$, $p=0.053$) and the Shannon ($p<0.0001$, $q=0.01$) indices.

Figure 5.2 Alpha diversity indices of for AAD, ABX, and CON horses. AAD and ABX horses show a decreased richness and evenness compared to CON horses, but showed no significant difference between each other. Antibiotic use is denoted by color: doxycycline (blue), ceftiofur (green), procaine penicillin G/gentamycin (red), procaine penicillin G/gentamycin/doxycycline (orange), procaine penicillin G/gentamycin/metronidazole (purple), trimethoprim sulfonamide (yellow), and none (black). A) ASV B) Chao1 C) Shannon



5.4.4 Taxonomy

Analysis of bacterial taxa indicated that 13 of 19 phyla identified in the fecal samples showed significant differences in abundance between groups (Table 5.2). Many significant differences occurred between horses in the AAD and the CON horses. The abundance of Bacteroidetes was increased in the AAD group compared to the CON group ($p=0.0001$, $q=0.0005$). For the remaining 6 phyla, the horses of the AAD group showed decreased abundances compared to the CON group. This included Actinobacteria ($p=0.0115$, $q=0.0192$), Armatimonadetes ($p=0.0032$, q=0.0.008) Fibrobacteres ($p=0.0492$, $q=0.0656$), Spirochaetes ($p=0.0147$, $q=0.0211$), Synergistetes ($p=0.0114$, $q=0.1912$), and Tenericutes ($p=0.0004$, $q=0.0013$). The abundance of TM7 was increased in CON horses only compared to AAD horses ($p=0.0075$, $q=0.0167$). The abundances of Elusimicrobia ($p=0.001$, $q=0.0005$), Planctomycetes ($p=0.0002$, $q=0.0008$), and SR1 ($p=0.001$, $q=0.005$) were decreased in horses on antimicrobials (AAD and ABX) relative to CON horses. The abundances of Fusobacteria ($p=0.0214$, q=0.0.0306) and WPS-2 ($p=0.0109$, $q=0.0192$) were increased in ABX horses when compared to CON horses. The abundance of Verrucomicrobia ($p=0.0001$, $q=0.0005$) was decreased in AAD horses compared to those on antibiotics with normal fecal consistency (ABX) or healthy controls (CON).

Table 5.2. Median abundances of taxa in AAD, ABX, and CON horses. Superscripts denote significant differences between groups.

Phyla	AAD		ABX		CON		AAD vs ABX vs CON	
	Median	Range	Median	Range	Median	Range	P value	Q value
Euryarchaeota	0.98	0.1-2.12	0.84	0.07-1.98	0.78	0.02-1.43	0.4535	0.4535
Unknown	0	0-0.04	0.01	0-0.03	0	0-0.03	0.25	0.2632
Actinobacteria	0.36^{a}	0.08-1.94	$0.66^{a,b}$	0.18-1.57	0.9^{b}	0.25-4.6	0.0115	0.0192
Armatimonadetes	0^{a}	0-0.02	$0^{a,b}$	0-0.07	0.01^{b}	0-0.13	0.0032	0.0080
Bacteroidetes	43.88^{a}	24.16-66.11	$40.43^{a,b}$	31.59-48.07	36.46^{b}	9.29-47.6	0.0001	0.0005
Elusimicrobia	0^{a}	0-0.09	0^{a}	0-0.14	0.02^{b}	0-0.14	0.0001	0.0005
Fibrobacteres	0.38^{a}	0.06-4.38	$0.89^{a,b}$	0.1-6.65	1.51^{b}	0.18-8.75	0.0492	0.0656
Firmicutes	36.45	21.78-54.83	40.8	28.59-58.12	41.68	29.81-68.78	0.2058	0.2287
Fusobacteria	$0.01^{a,b}$	0-8.36	0.05^{a}	0-0.1	0^{b}	0-0.08	0.0214	0.0306
Lentisphaerae	0.02	0-0.37	0.07	0.01-0.31	0.05	0-0.61	0.1373	0.1615
Planctomycetes	0.02^{a}	0-0.07	0.04^{a}	0-0.39	0.09^{b}	0.02-0.41	0.0002	0.0008
Proteobacteria	2.93	0.88-14.32	1.97	0.28-25.99	1.59	0.37-10	0.0695	0.0869
SR1	0^{a}	0-0.02	0^{a}	0-0.02	0.03^{b}	0-0.38	0.0001	0.0005
Spirochaetes	4.07^{a}	0.77-9.96	$4.41^{a,b}$	0.72-10.46	6.03^{b}	2.44-10.39	0.0137	0.0211
Synergistetes	0.02^{a}	0-0.17	$0.03^{a,b}$	0-0.29	0.04^{b}	0.02-0.17	0.0114	0.0192
TM7	0^{a}	0-0.01	$0^{a,b}$	0-0.05	0.02^{b}	0-0.14	0.0075	0.0167
Tenericutes	$1.5^{a,b}$	0.18-9.72	1.11^{a}	0.34-2.52	2.44^{b}	1.24-5.7	0.0004	0.0013
Verrucomicrobia	1.15^{a}	0.31-6.93	5.23^{b}	0.24-14.55	7.78^{b}	2.9-14.02	0.0001	0.0005
WPS-2	$0^{a,b}$	0-0.01	0^{a}	0-0.02	0.01^{b}	0-0.31	0.0109	0.0192

Within the phylum Actinobacteria and the class Actinobacteria, the abundance of the family *Norcardiaceae* was decreased in AAD compared to CON horses (p=0.0171, q=0.0519). Within the order Bifidobacteriales, abundance of the family *Bificobacteriaceae* in AAD was increased compared to those in the ABX or the CON group (p=0.0082, q=0.0276). In the class Coriobacteriia and the order Coriobacteriales, the abundance of the family *Coriobacterieiacea* was decreased in AAD horses compared to ABX or CON horses (p=0.0024, q=0.0107).

In the phyla Bacteroidetes, class Bacteroidia and order Bacteriales, the abundance of *Bacteroidaceae* was increased in AAD compared to ABX and CON (p=0.0001, q=0.001175) whereas the abundance of *Porphyromonadaceae* was increased in AAD compared to CON only (p=0.0035, q=0.0137).

Within the phylum Elusimicrobia, class Elusimicrobia and order Elusimicrobiales, the abundance of the family *Elusimicrobiaceae* was increased in CON compared to AAD and ABX horses (p=0.0034, q=0.0137). Within the same phylum but class Endomicrobia and an unknown order, the abundance of an unknown family was increased in CON compared to AAD and ABX horses (p=0.0002, q=0.0015).

In the phyla Fibrobacter, class Fibrobacteres, order Fibrobacterales, the abundance of the family *Fibrobacteriaceae* decreased in AAD horses compared to ABX and CON horses (p=0.00492, q=0.1101).

Within Firmicutes, the class Bacilli and order Lactobacillales, the abundance of the family *Aerococcaceae* was decreased in AAD compared to ABX and CON horses (p=0.0065, q=0.0235). The abundance of the family *Enterococcaceae* was increased in

AAD compared to ABX and CON horses (p=0.0177, q=0.052). Within the class Clostridia, the order Clostridiales, the abundance of an unknown taxa (p=0.0039, Q=0.0417) and an unknown family (p=0.0345, q=0.0645) were decreased in AAD compared to CON. The abundance of the family *Clostridiaceae* was decreased in AAD and ABX compared to CON (p=0.0002, q=0.0016). The abundances of the families *EtOH8* (p=0.0001, q=0.0012) and *Eubacteriaceae* (P=0.0001, Q=0.0012) were decreased in AAD compared to CON. The abundance of the family *Peptostreptococcaceae* was increased in CON compared to ABX horses (p=0.0001, q=0.0012). The abundance of the family [*Mogibacteriaceae*] was decreased in AAD horses compared to ABX and CON horses (p=0.0002, q=0.0016).

Within the phyla Fusobacteria, the class Fusobacteriia and order Fusobacteriales, the abundance of the family *Fusobacteriaceae* was increased in ABX horses compared to AAD and CON horses (p=0.0214, q=0.0591).

In the phyla Lentispherae, class [Lentisphaeria], order Z20, the abundance of the family *R4-45B* was increased in AAD compared to ABX and CON horses (p=0.0247, q=0.0644).

In Planctomycetes, the class Planctomycetia and order Pirellulales, the abundance of the family *Pirellulaceae* was decreased in AAD and ABX compared to CON horses (p=0.0001, q=0.0012). Within the class vadin Ha49, the abundances of order PeHg47 and an unknown family were decreased in AAD horses compared to ABX and CON horses (p=0.0008, q=0.0044)

In the phyla Proteobacteria, class Alphaproteobacteria and an unknown order, the abundance of an unknown family was decreased in AAD compared to CON hoses (p=0.0008, q=0.0044) Within the order RF32, the abundance of an unknown family was decreased in AAD compared to CON horses (p=0.0006, q=0.004). Within the class Deltaproteobacteria, order Desulfovibrionales, the abundance of the family *Desulfovibrionaceae* increased in AAD compared to CON (p=0.0191, q=0.0544). In the order GMD14H09, the abundance of an unknown family was decreased in AAD and ABX compared to CON horses (p=0.0007, q=0.0044). In the class Gammaproteobacteria and order Enterobacteriales, the abundance of the family *Enterobacteriaceae* increased in AAD and ABX compared to CON horses (p=0.0016, q=0.0084).

In the phyla SR1, an unknown class, order and family was decreased in abundance in AAD and ABX compared to CON horses (p=0.0001, q=0.001175)

In the phyla Spirochaetes, class Spirochaetes and order Spirochaetales, the family *Spirochaetaceae* was decreased in AAD compared to CON (p=0.0143, q=0.04481).

In the phyla Synergistetes, the class Synergistia, order Synergistales, family *Synergistaceae* decreased in AAD compared to CON (p=0.0002, q=0.0016).

Within the phyla TM7, class TM7-3, order CW040, the family *F16* was decreased in AAD compared to CON horses (p=0.0074, q=0.0258).

Within the phyla Tenericutes, class Mollicutes, order RF39, unknown family was decreased in AAD and ABX compared to CON horses (almost) (p=0.0001, q=0.0012). Within the order Acholeplasmateales, the family *Acholeplasmataceae* was increased in AAD compared to ABX and CON horses (p=0.0306, q=0.0754)

In the phyla Verrucomicrobia, the class Verruco-5 and order WCHB1-41, an unknown taxa was decreased in AAD and ABX compared to CON horses (p=0.0018, q=0.0089). The family *RFP-12* decreased in AAD compared to ABX and CON horses (p=0.0001, q=0.00112). The family WCHB1-25 was decreased in AAD compared to CON horses (p=0.0022, q=0.00103).

In the phyla WPS-2 an unknown class, order and family was decreased in ABX compared to CON horses (p=0.0109, q=0.03533). The results of taxa analysis at the family level are described in Table 5.3 below.

Scatter plots of the abundance of phyla, class, order and families that distinguish AAD from ABX and CON horses are listed below in Figure 5.3.

Figure 5.3 Abundances of taxa that are significantly different in the fecal microbiota between horses with antimicrobial associated diarrhea (AAD), antibiotic-treated control horses (ABX), healthy control horses not treated with an antibiotics (CON). A) The abundance of Bifidobacteriales is increased in horses with AAD compared to ABX and CON horses (p=0.039, p=0.012). B) The abundance of *Bifidobacteriaceae* is increased in horses with AAD compared to ABX and CON horses (p=0.039, q=0.011). C) The abundance of *Bacteriodaceae* is increased in horses with AAD compared to ABX and CON horses (p=0.006, p<0.0001). D) The abundance of *Prevotellaceae* is increased in AAD horses compared to ABX horses (p=0.028).

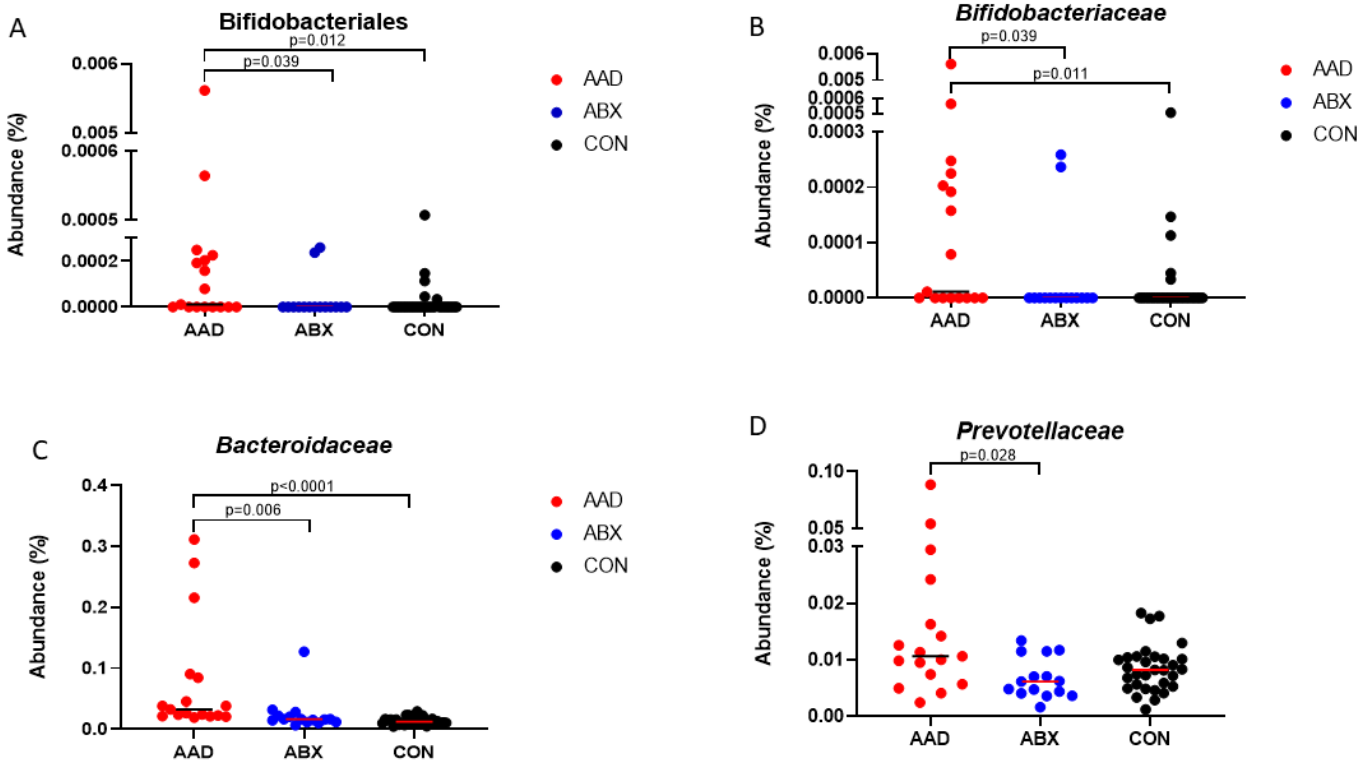


Figure 5.3 Continued. E) The abundance of *Enterococcocaceae* is significantly increased in horses with AAD vs. ABX and CON horses ($p<0.0001$, $p=0.034$). H) The abundance of [*Mogibacteriaceae*] is significantly decreased in AAD horses compared to ABX and CON horses ($p=0.034$, $p<0.0001$).

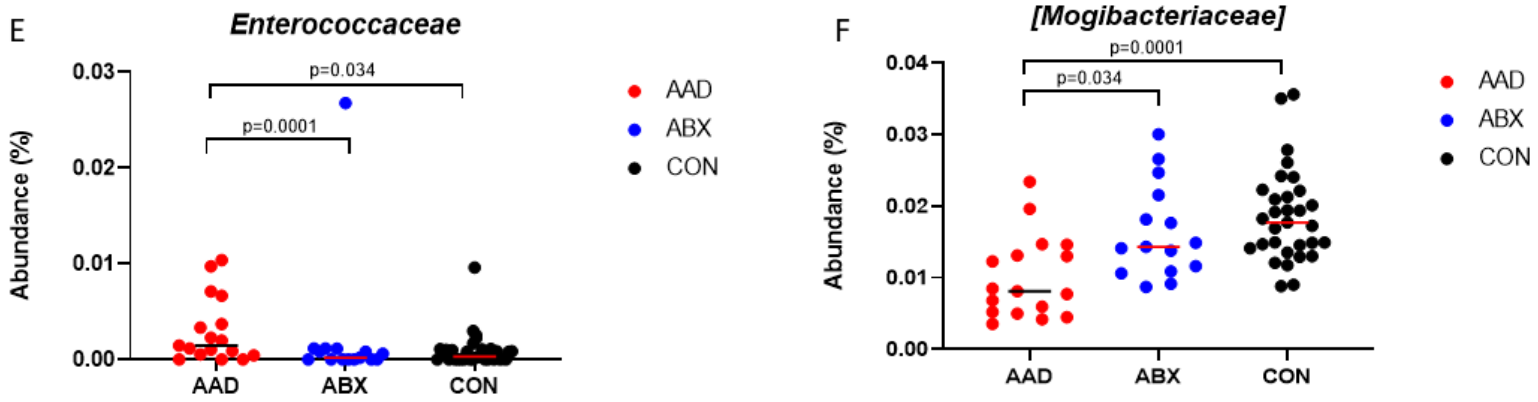


Figure 5.3 Continued G) The abundance of Verrucomicrobia is significantly decreased in horses with AAD vs. ABX and CON horses (p=0.014, p<0.0001). Antibiotic use is denoted by color: doxycycline (blue), ceftiofur (green), procaine penicillin G/gentamycin (red), procaine penicillin G/gentamycin/doxycycline (orange), procaine penicillin G/gentamycin/metronidazole (purple), trimethoprim sulfonamide (yellow) and none (black). H) The abundance of Verruco-5 is significantly decreased in AAD horses compared to ABX and CON horses (p=0.174, p<0.01) I) The abundance of WCHB1-41 is significantly decreased in AAD horses compared to ABX and CON horses (p=0.174, p<0.0001) J) The abundance of RFP12 significantly decreased in AAD horses compared to ABX and CON horses (p=0.016, p<0.0001)

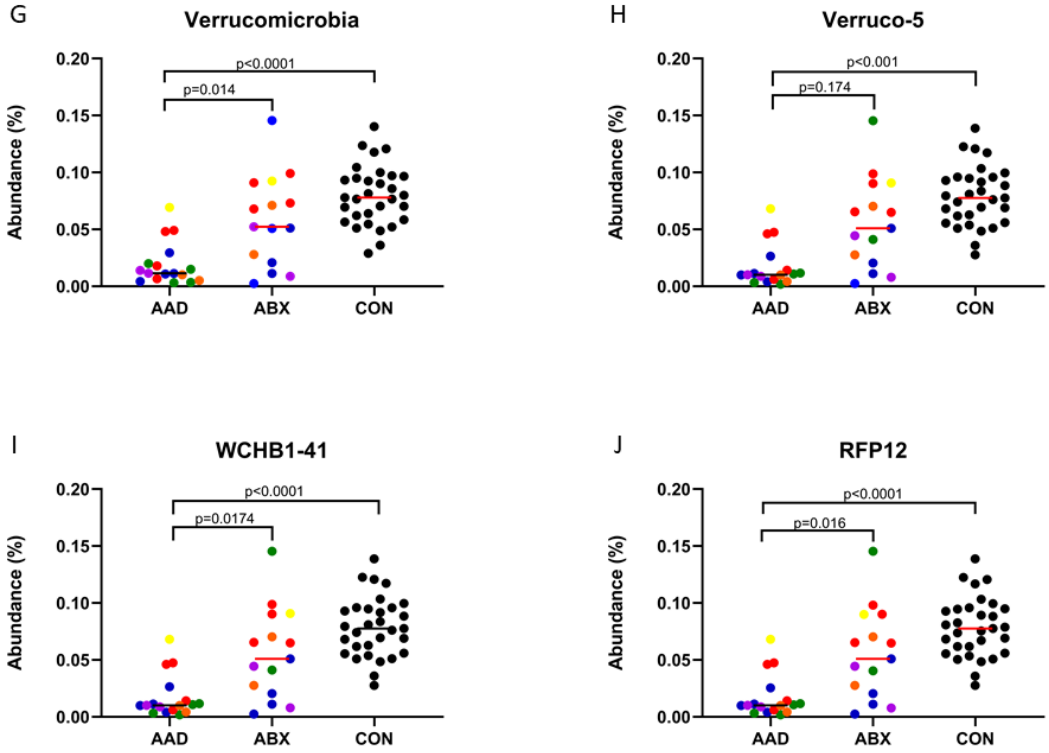


Table 5.3 Taxa that showed significantly different abundance in the fecal microbiota of horses with antimicrobial associated diarrhea (AAD) and antibiotic control horses (ABX) or healthy control horses (CON).

Taxa	AAD		ABX		CON		AAD vs ABX vs CON	
	Median	Range	Median	Range	Median	Range	Pvalue	Qvalue
p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales	0[a]	0-0.56	0[b]	0-0.03	0[b]	0-0.05	0.008	0.026
p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae	0[a]	0-0.56	0[b]	0-0.03	0[b]	0-0.05	0.008	0.028
p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae	3.25[a]	1.92-31.14	1.64[b]	0.66-12.7	1.22[b]	0.44-2.91	0.0001	0.001
p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	1.06[a]	0.24-8.86	0.62[b]	0.16-1.34	0.82[a,b]	0.12-1.83	0.031	0.075
p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae	0.15[a]	0-1.04	0.02[b]	0-2.68	0.03[b]	0-0.96	0.018	0.052
p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae]	0.81[a]	0.35-2.34	1.43[b]	0.87-3.01	1.77[b]	0.88-3.56	0.000	0.002
p__Verrucomicrobia	1.15[a]	0.31-6.93	5.23[b]	0.24-14.55	7.78[b]	2.9-14.02	0.0001	0.001
p__Verrucomicrobia;c__Verruco-5	1.01[a]	0.18-6.81	5.1[b]	0.24-14.55	7.75[b]	2.76-13.88	0.0001	0.001
p__Verrucomicrobia;c__Verruco-5;o__WCHB1-41	1.01[a]	0.18-6.81	5.1[b]	0.24-14.55	7.75[b]	2.76-13.88	0.0001	0.001
p__Verrucomicrobia;c__Verruco-5;o__WCHB1-41;f__RFP12	1.01[a]	0.18-6.8	5.09[b]	0.24-14.55	7.75[b]	2.75-13.88	0.000	0.001

Table 5.4 Taxa that showed significantly different abundances between horses with antimicrobial associated diarrhea (AAD) and control horses, those on antibiotics (ABX) and healthy horses (CON).

Taxa	AAD		ABX		CON		AAD vs ABX vs CONy	
	Median	Range	Median	Range	Median	Range	Pvalue	Qvalue
p_ Actinobacteria	0.36[a]	0.08-1.94	0.66[a,b]	0.18-1.57	0.9[b]	0.25-4.6	0.012	0.019
p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Nocardiaceae	0[a]	0-0.01	0[a,b]	0-0.01	0[b]	0-0.42	0.017	0.052
p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales	0[a]	0-0.56	0[b]	0-0.03	0[b]	0-0.05	0.008	0.026
p__Actinobacteria;c__Actinobacteria;o__Bifidobacteriales;f__Bifidobacteriaceae	0[a]	0-0.56	0[b]	0-0.03	0[b]	0-0.05	0.008	0.028
p__Actinobacteria;c__Coriobacteriia	0.26[a]	0.03-1.92	0.45[a,b]	0.18-1.39	0.8[b]	0.22-3.67	0.002	0.008
p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales	0.26[a]	0.03-1.92	0.45[a,b]	0.18-1.39	0.8[b]	0.22-3.67	0.002	0.011
p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae	0.26[a]	0.03-1.92	0.45[a,b]	0.18-1.39	0.8[b]	0.22-3.67	0.002	0.011
p__Actinobacteria	0.36[a]	0.08-1.94	0.66[a,b]	0.18-1.57	0.9[b]	0.25-4.6	0.012	0.019
p__Armatimonadetes	0[a]	0-0.02	0[a,b]	0-0.07	0.01[b]	0-0.13	0.003	0.008
p__Armatimonadetes;c__SJA-176	0[a]	0-0.02	0[a,b]	0-0.07	0.01[b]	0-0.13	0.003	0.010
p__Armatimonadetes;c__SJA-176;o__RB046	0[a]	0-0.02	0[a,b]	0-0.07	0.01[b]	0-0.13	0.003	0.013
p__Armatimonadetes;c__SJA-176;o__RB046;f__	0[a]	0-0.02	0[a,b]	0-0.07	0.01[b]	0-0.13	0.003	0.014
p__Bacteroidetes	43.88[a]	24.16-66.11	40.43[a,b]	31.59-48.07	36.46[b]	9.29-47.6	0.000	0.001
p__Bacteroidetes;c__Bacteroidia	43.88[a]	24.16-66.1	40.43[a,b]	31.59-48.07	36.46[b]	9.29-47.51	0.000	0.001
p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;__	0.58	0.19-1.65	0.64	0.39-3.82	0.71	0.2-1.62	0.397	0.511
p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__	24.43	2.28-44.45	26.17	10.98-40.42	22.86	6.06-31.15	0.320	0.436

Table 5.4 Continued

Taxa	AAD		ABX		CON		AAD vs ABX vs CONy	
	Median	Range	Median	Range	Median	Range	Pvalue	Qvalue
p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Bacteroidaceae	3.25[a]	1.92-31.14	1.64[b]	0.66-12.7	1.22[b]	0.44-2.91	0.000	0.001
p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae	1.32[a]	0.22-6.28	0.78[a,b]	0.05-4.39	0.44[b]	0.03-2.54	0.004	0.014
p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae	1.06[a]	0.24-8.86	0.62[b]	0.16-1.34	0.82[a,b]	0.12-1.83	0.031	0.075
p__Elusimicrobia	0[a]	0-0.09	0[a]	0-0.14	0.02[b]	0-0.14	0.000	0.001
p__Elusimicrobia;c__Elusimicrobia	0[a]	0-0.07	0[a]	0-0.02	0.01[b]	0-0.13	0.003	0.010
p__Elusimicrobia;c__Elusimicrobia;o__Elusimicrobiales	0[a]	0-0.07	0[a]	0-0.02	0.01[b]	0-0.13	0.003	0.013
p__Elusimicrobia;c__Elusimicrobia;o__Elusimicrobiales;f__Elusimicrobiaceae	0[a]	0-0.07	0[a]	0-0.02	0.01[b]	0-0.13	0.003	0.014
p__Elusimicrobia;c__Endomicrobia	0[a]	0-0.01	0[a]	0-0.12	0.01[b]	0-0.05	0.000	0.001
p__Elusimicrobia;c__Endomicrobia;o__	0[a]	0-0.01	0[a]	0-0.12	0.01[b]	0-0.05	0.000	0.002
p__Elusimicrobia;c__Endomicrobia;o__;f__	0[a]	0-0.01	0[a]	0-0.12	0.01[b]	0-0.05	0.000	0.002
p__Elusimicrobia	0[a]	0-0.09	0[a]	0-0.14	0.02[b]	0-0.14	0.000	0.001
p__Elusimicrobia;c__Elusimicrobia	0[a]	0-0.07	0[a]	0-0.02	0.01[b]	0-0.13	0.003	0.010
p__Fibrobacteres	0.38[a]	0.06-4.38	0.89[a,b]	0.1-6.65	1.51[b]	0.18-8.75	0.049	0.066
p__Fibrobacteres;c__Fibrobacteria	0.38[a]	0.06-4.38	0.89[a,b]	0.1-6.65	1.51[b]	0.18-8.75	0.049	0.091
p__Fibrobacteres;c__Fibrobacteria;o__Fibrobacterales	0.38[a]	0.06-4.38	0.89[a,b]	0.1-6.65	1.51[b]	0.18-8.75	0.049	0.100
p__Fibrobacteres;c__Fibrobacteria;o__Fibrobacterales;f__Fibrobacteraceae	0.38[a]	0.06-4.38	0.89[a,b]	0.1-6.65	1.51[b]	0.18-8.75	0.049	0.110
p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae	0[a]	0-0.01	0[a,b]	0-0.01	0[b]	0-0.22	0.007	0.024
p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Enterococcaceae	0.15[a]	0-1.04	0.02[b]	0-2.68	0.03[b]	0-0.96	0.018	0.052
p__Firmicutes;c__Clostridia;o__Clostridiales;__	0.61[a]	0.14-1.56	0.76[a,b]	0.33-1.65	1.02[b]	0.42-2.56	0.004	0.015
p__Firmicutes;c__Clostridia;o__Clostridiales;f__	4.58[a]	1.56-11.89	6.15[a,b]	3.29-9.79	6.62[b]	3.91-12.93	0.025	0.064

Table 5.4 Continued

Taxa	AAD		ABX		CON		AAD vs ABX vs CONy	
	Median	**Range**	**Median**	**Range**	**Median**	**Range**	**Pvalue**	**Qvalue**
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae	0.74[a]	0.27-2.22	1.07[a]	0.45-1.95	1.63[b]	0.72-3.21	0.000	0.002
p__Fusobacteria;c__Fusobacteriia	0.01[a,b]	0-8.36	0.05[a]	0-0.1	0[b]	0-0.08	0.021	0.044
p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales	0.01[a,b]	0-8.36	0.05[a]	0-0.1	0[b]	0-0.08	0.021	0.053
p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae	0.01[a,b]	0-8.36	0.05[a]	0-0.1	0[b]	0-0.08	0.021	0.059
p__Firmicutes;c__Clostridia;o__Clostridiales;f__EtOH8	0[a]	0-0.01	0.01[a,b]	0-0.03	0.01[b]	0-0.04	0.000	0.001
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Eubacteriaceae	0.05[a]	0.02-0.21	0.11[a,b]	0.02-0.43	0.17[b]	0.05-1.03	0.000	0.001
p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae	0.03[a,b]	0-0.34	0[a]	0-0.03	0.05[b]	0-0.4	0.000	0.001
p__Firmicutes;c__Clostridia;o__Clostridiales;f__[Mogibacteriaceae]	0.81[a]	0.35-2.34	1.43[b]	0.87-3.01	1.77[b]	0.88-3.56	0.000	0.002
p__Lentisphaerae;c__[Lentisphaeria];o__Z20	0.01[a]	0-0.1	0.01[a,b]	0-0.17	0.02[b]	0-0.23	0.025	0.059
p__Lentisphaerae;c__[Lentisphaeria];o__Z20;f__R4-45B	0.01[a]	0-0.1	0.01[a,b]	0-0.17	0.02[b]	0-0.23	0.025	0.064
p__Planctomycetes	0.02[a]	0-0.07	0.04[a]	0-0.39	0.09[b]	0.02-0.41	0.000	0.001
p__Planctomycetes;c__Planctomycetia	0.01[a]	0-0.07	0.02[a]	0-0.05	0.06[a,b]	0-0.39	0.000	0.001
p__Planctomycetes;c__Planctomycetia;o__Pirellulales	0.01[a]	0-0.07	0.02[a]	0-0.05	0.06[b]	0-0.39	0.000	0.001
p__Planctomycetes;c__Planctomycetia;o__Pirellulales;f__Pirellulaceae	0.01[a]	0-0.07	0.02[a]	0-0.05	0.06[b]	0-0.39	0.000	0.001
p__Planctomycetes;c__vadinHA49	0[a]	0-0.05	0.01[a,b]	0-0.34	0.02[b]	0-0.14	0.001	0.004
p__Planctomycetes;c__vadinHA49;o__PeHg47	0[a]	0-0.05	0.01[a,b]	0-0.34	0.02[b]	0-0.14	0.001	0.004
p__Planctomycetes;c__vadinHA49;o__PeHg47;f__	0[a]	0-0.05	0.01[a,b]	0-0.34	0.02[b]	0-0.14	0.001	0.004
p__Proteobacteria;c__Alphaproteobacteria	0.07[a]	0.03-1.43	0.12[a]	0.02-0.87	0.28[b]	0.02-1.01	0.001	0.004
p__Proteobacteria;c__Alphaproteobacteria;o__	0.02[a]	0-0.11	0.03[a,b]	0-0.45	0.09[b]	0-0.55	0.001	0.004
p__Proteobacteria;c__Alphaproteobacteria;o__;f__	0.02[a]	0-0.11	0.03[a,b]	0-0.45	0.09[b]	0-0.55	0.001	0.004
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__RF32	0.02[a]	0-0.54	0.03[a,b]	0-0.28	0.08[b]	0-0.29	0.001	0.004
p__Proteobacteria;c__Alphaproteobacteria;o__RF32;f__	0.02[a]	0-0.54	0.03[a,b]	0-0.28	0.08[b]	0-0.29	0.001	0.004
p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__	0[a]	0-0.01	0[a]	0-0.01	0[a]	0-0.13	0.039	0.089

Table 5.4 Continued

Taxa	**AAD**		ABX		CON		**AAD vs ABX vs CONy**	
	Median	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**
p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfovibrionaceae	0.36[a]	0.1-1.97	0.22[a,b]	0.08-0.74	0.16[b]	0.02-0.89	0.019	0.054
p__Proteobacteria;c__Deltaproteobacteria;o__GMD14H09;f__	0.02[a]	0-0.07	0.02[a]	0-0.11	0.06[b]	0-0.55	0.001	0.004
p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__	0.01[a]	0-0.02	0.01[a]	0-0.68	0.02[a]	0-0.73	0.030	0.075
p__Proteobacteria;c__Gammaproteobacteria	1.04[a]	0.25-11.46	0.82[a,b]	0.01-25.37	0.64[b]	0.13-7.22	0.036	0.07
p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae	0.53[a]	0-4.8	0.38[a]	0.01-24	0.23[b]	0-2.27	0.002	0.008
p__SR1	0[a]	0-0.02	0[a]	0-0.02	0.03[b]	0-0.38	0.000	0.001
p__SR1;c__	0[a]	0-0.02	0[a]	0-0.02	0.03[b]	0-0.38	0.000	0.001
p__SR1;c__;o__	0[a]	0-0.02	0[a]	0-0.02	0.03[b]	0-0.38	0.000	0.001
p__SR1;c__;o__;f__	0[a]	0-0.02	0[a]	0-0.02	0.03[b]	0-0.38	0.000	0.001
p__Spirochaetes	4.07[a]	0.77-9.96	4.41[a,b]	0.72-10.46	6.03[b]	2.44-10.39	0.014	0.021
p__Spirochaetes;c__Spirochaetes	4.03[a]	0.77-9.94	4.39[a,b]	0.69-9.93	5.93[b]	2.38-10.25	0.014	0.030
p__Spirochaetes;c__Spirochaetes;o__Spirochaetales	4.03[a]	0.76-9.72	4.38[a,b]	0.69-9.91	5.93[b]	2.38-10.25	0.014	0.039
p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae	4.03[a]	0.76-9.72	4.38[a,b]	0.69-9.91	5.93[b]	2.38-10.25	0.014	0.045
p__TM7;c__TM7-3	0[a]	0-0.01	0[a,b]	0-0.05	0.02[b]	0-0.14	0.007	0.020
p__TM7;c__TM7-3;o__CW040	0[a]	0-0.01	0[a,b]	0-0.05	0.02[b]	0-0.14	0.007	0.025
p__TM7;c__TM7-3;o__CW040;f__F16	0[a]	0-0.01	0[a,b]	0-0.05	0.02[b]	0-0.14	0.007	0.026

Table 5.4 Continued

Taxa	AAD		ABX		CON		AAD vs ABX vs CONy	
	Median	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**
p__Tenericutes	1.5[a,b]	0.18-9.72	1.11[a]	0.34-2.52	2.44[b]	1.24-5.7	0.000	0.001
p__Tenericutes;c__Mollicutes	1.26[a,b]	0.17-9.69	1.08[a]	0.34-2.5	2.35[b]	1.22-5.68	0.001	0.004
p__Tenericutes;c__Mollicutes;o__Anaeroplasmatales	0.55[a]	0.03-8.46	0.12[b]	0-0.88	0.14[a,b]	0.05-0.57	0.031	0.067
p__Tenericutes;c__Mollicutes;o__Anaeroplasmatales;f__Anaeroplasmataceae	0.55[a]	0.03-8.46	0.12[b]	0-0.88	0.14[a,b]	0.05-0.57	0.031	0.075
p__Tenericutes;c__Mollicutes;o__Mycoplasmatales	0.07[a]	0.02-1.24	0.06[a]	0.03-1.47	0.24[a]	0-1.01	0.035	0.075
p__Tenericutes;c__Mollicutes;o__Mycoplasmatales;f__Mycoplasmataceae	0.07[a]	0.02-1.24	0.06[a]	0.03-1.47	0.24[a]	0-1.01	0.035	0.083
p__Tenericutes;c__Mollicutes;o__RF39	0.28[a]	0.09-5.75	0.63[a]	0.12-1.5	1.56[b]	0.59-5.42	0.0001	0.001
p__Tenericutes;c__Mollicutes;o__RF39;f__	0.28[a]	0.09-5.75	0.63[a]	0.12-1.5	1.56[b]	0.59-5.42	0.0001	0.001
p__Verrucomicrobia	1.15[a]	0.31-6.93	5.23[b]	0.24-14.55	7.78[b]	2.9-14.02	0.0001	0.001
p__Verrucomicrobia;c__Verruco-5	1.01[a]	0.18-6.81	5.1[b]	0.24-14.55	7.75[b]	2.76-13.88	0.0001	0.001
p__Verrucomicrobia;c__Verruco-5;o__WCHB1-41	1.01[a]	0.18-6.81	5.1[b]	0.24-14.55	7.75[b]	2.76-13.88	0.0001	0.001
p__Verrucomicrobia;c__Verruco-5;o__WCHB1-41;__	0[a]	0-0	0[a]	0-0.01	0[b]	0-0.09	0.002	0.009
p__Verrucomicrobia;c__Verruco-5;o__WCHB1-41;f__RFP12	1.01[a]	0.18-6.8	5.09[b]	0.24-14.55	7.75[b]	2.75-13.88	0.000	0.001
p__Verrucomicrobia;c__Verruco-5;o__WCHB1-41;f__WCHB1-25	0[a]	0-0.11	0.01[a,b]	0-0.09	0.01[b]	0-0.22	0.002	0.010
p__Verrucomicrobia	1.15[a]	0.31-6.93	5.23[b]	0.24-14.55	7.78[b]	2.9-14.02	0.0001	0.001
p__Verrucomicrobia;c__Verruco-5	1.01[a]	0.18-6.81	5.1[b]	0.24-14.55	7.75[b]	2.76-13.88	0.0001	0.001

Family	AAD		ABX		CON		AAD vs ABX vs CONy	
	Median	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**
p__WPS-2	0[a,b]	0-0.01	0[a]	0-0.02	0.01[b]	0-0.31	0.011	0.019
p__WPS-2;c__	0[a,b]	0-0.01	0[a]	0-0.02	0.01[b]	0-0.31	0.011	0.027
p__WPS-2;c__;o__	0[a,b]	0-0.01	0[a]	0-0.02	0.01[b]	0-0.31	0.011	0.032
p__WPS-2;c__;o__;f__	0[a,b]	0-0.01	0[a]	0-0.02	0.01[b]	0-0.31	0.011	0.035
p__WPS-2	0[a,b]	0-0.01	0[a]	0-0.02	0.01[b]	0-0.31	0.011	0.019

The results of linear discriminant effect size analysis indicated that the abundances of Bacteroidetes, Tenericutes, and Fusobacteria are higher in AAD horses whereas those of Verrucomicrobia, Spirochaetes, SR1, Armatimonadetes, WPS2, TM7, Elusimicrobia, Actinobacteria, and Planctomycetes were higher in healthy horses. The effects of LEfSe analysis are shown in Figure 5.4.

Figure 5.4 Results of linear discriminant effects size analysis in horses with antimicrobial-associated diarrhea (AAD, red), those on antimicrobials that did not develop diarrhea (ABX, gray), and healthy control horses (CON, blue). A) Phylum level.

A

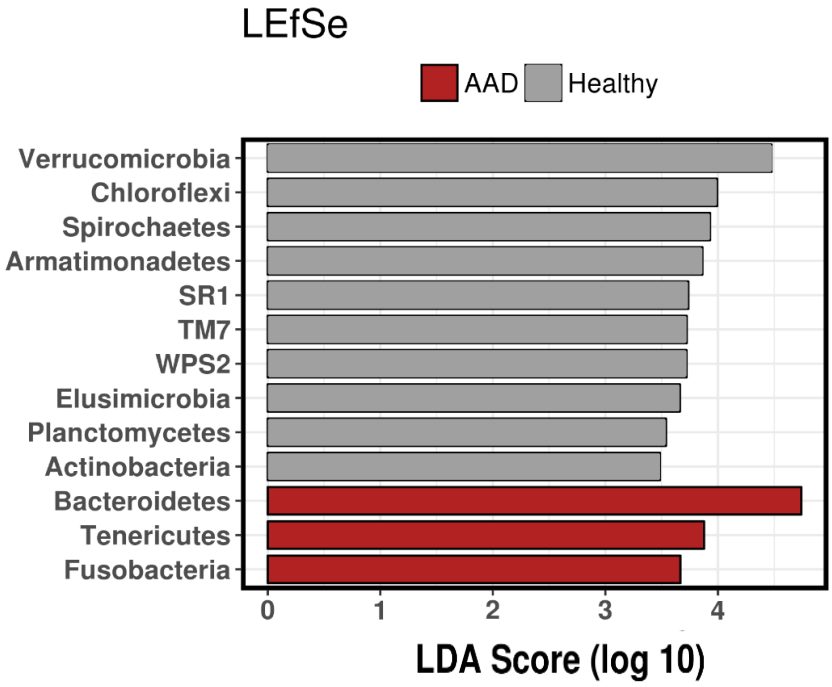
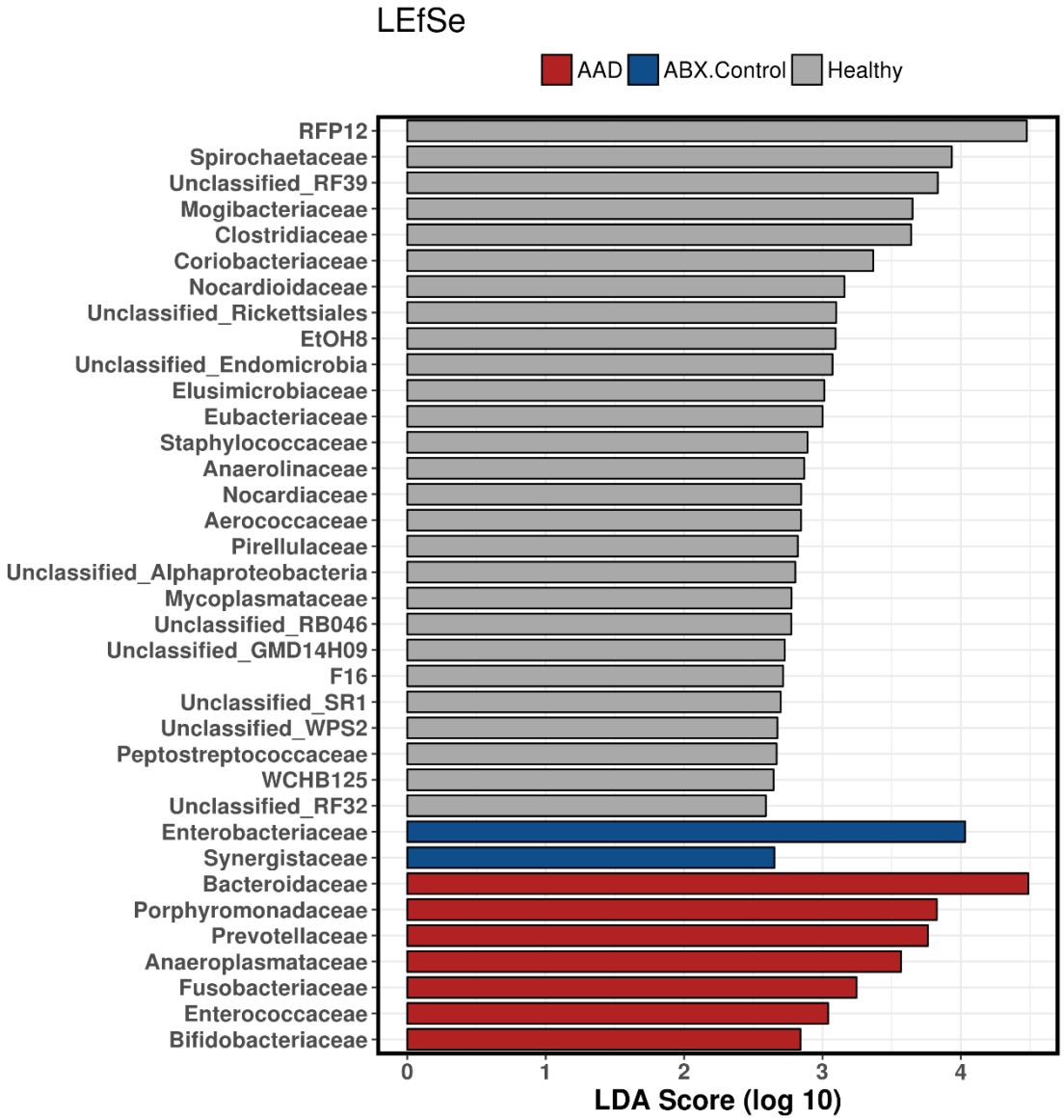


Figure 5.4 Continued. B) Family level.

B



The median abundances of statistically significant phyla as determined by LEfSe analysis are displayed in scatter plots in Figure 5.5-5.7.

Figure 5.5 The median abundances (%) of significantly altered phyla according to linear discriminant effects size analysis. Horses with antimicrobial associated diarrhea (AAD, red spheres), antibiotic control horses (ABX, blue spheres), and control horses (CON, black spheres) horses are displayed. A) Actinobacteria is more abundant in CON horses than AAD horses (P=0.012). B) The abundance of Armatimonadetes is reduced in AAD horses compared to CON horses (p=0.003). C) The abundance of Planctomycetes is reduced in AAD horses compared to CON horses (p=0.02). D) The abundance of SR1is reduced in AAD horses compared to CON horses (p=0.001).

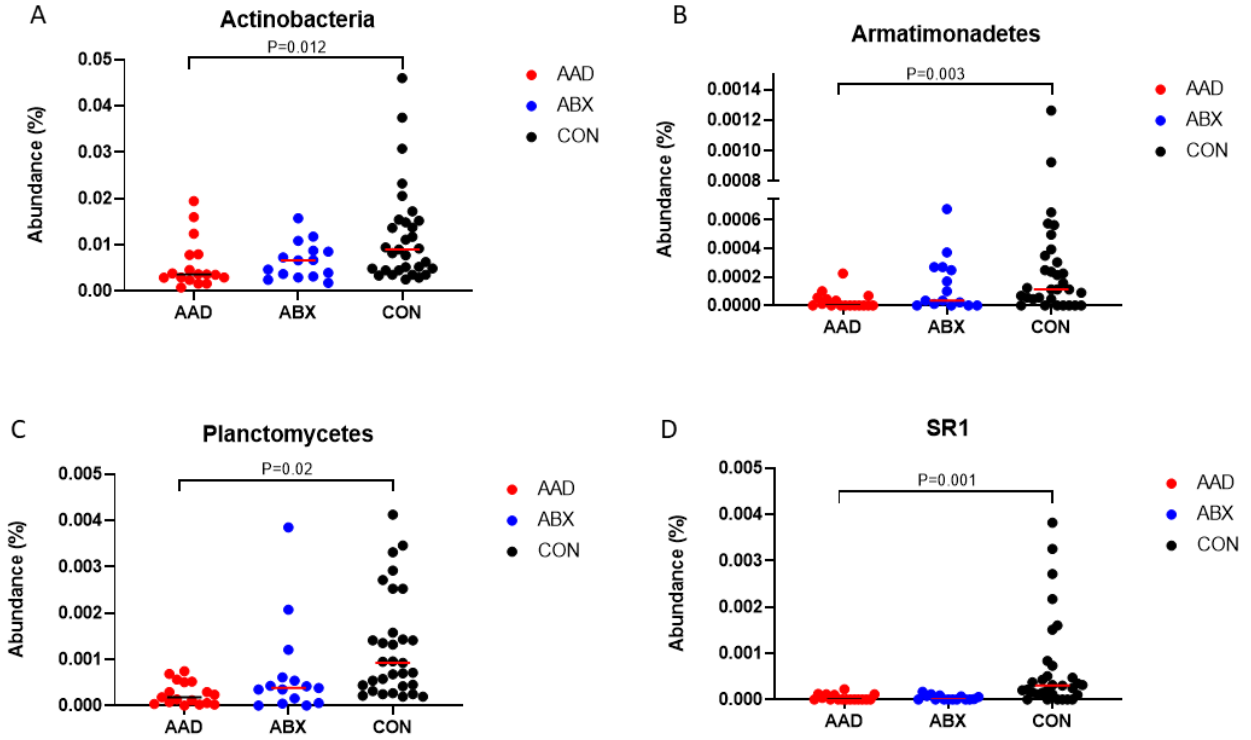


Figure 5.5 Continued. E) The abundance of SR1 is reduced in AAD horses compared to CON horses (p=0.001) F) The abundance of Spirochaetes is reduced in AAD horses compared to CON horses (p=0.014). G) The abundance of TM7 is reduced in AAD horses compared to CON horses (p=0.017). H) The abundance of Verrucomicrobia is reduced in AAD compared to CON horses (p=0.001).

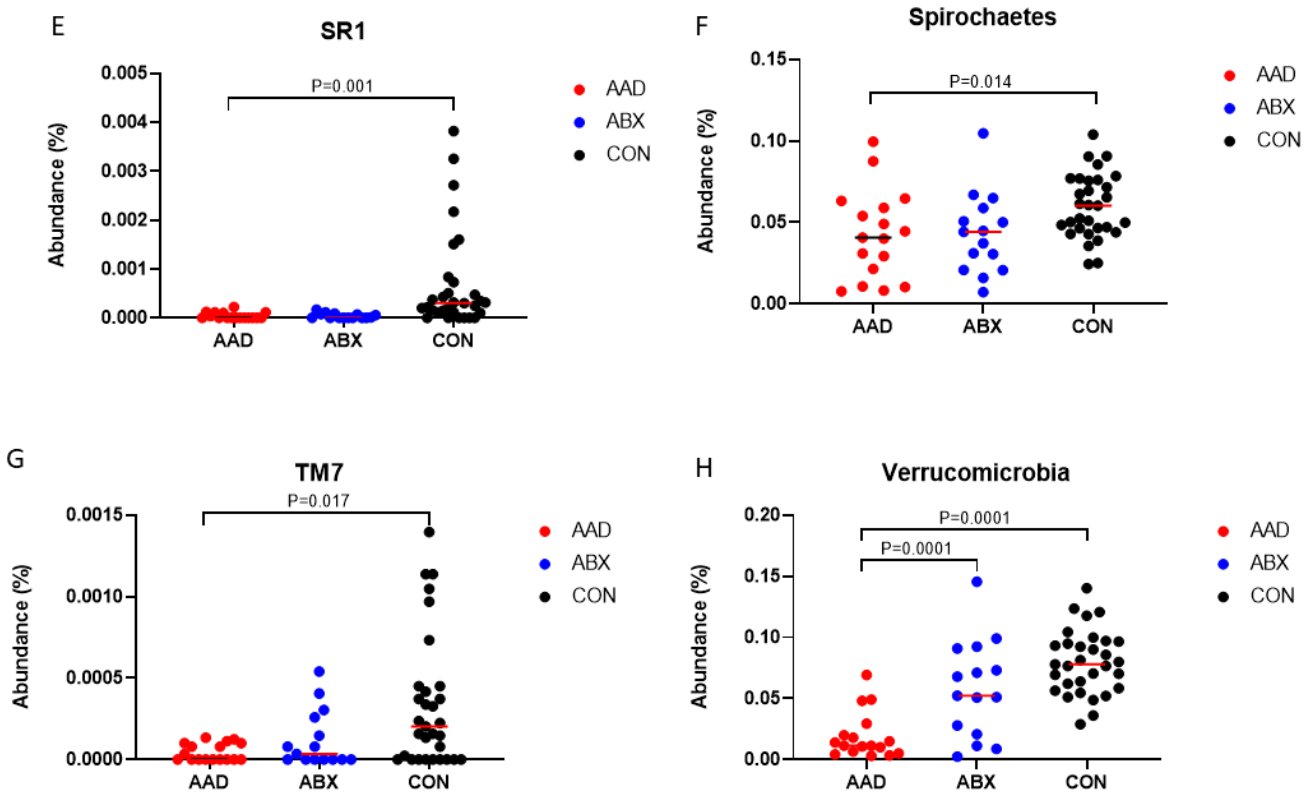


Figure 5.5 Continued. I) The abundance of WPS02 is reduced in ABX horses compared to CON horses (p=0.012).

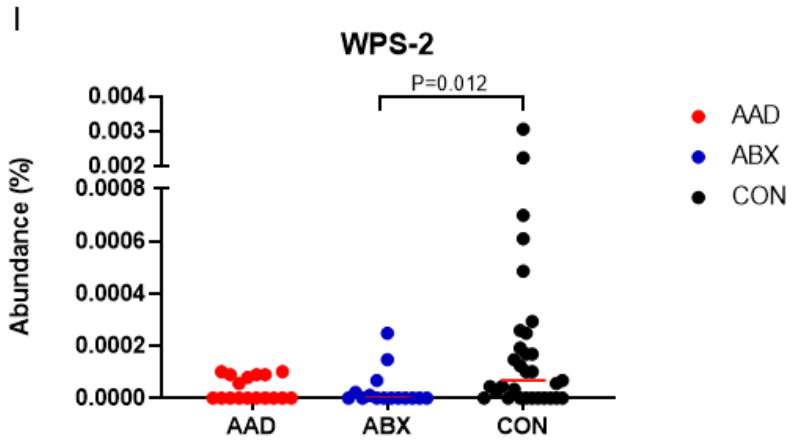
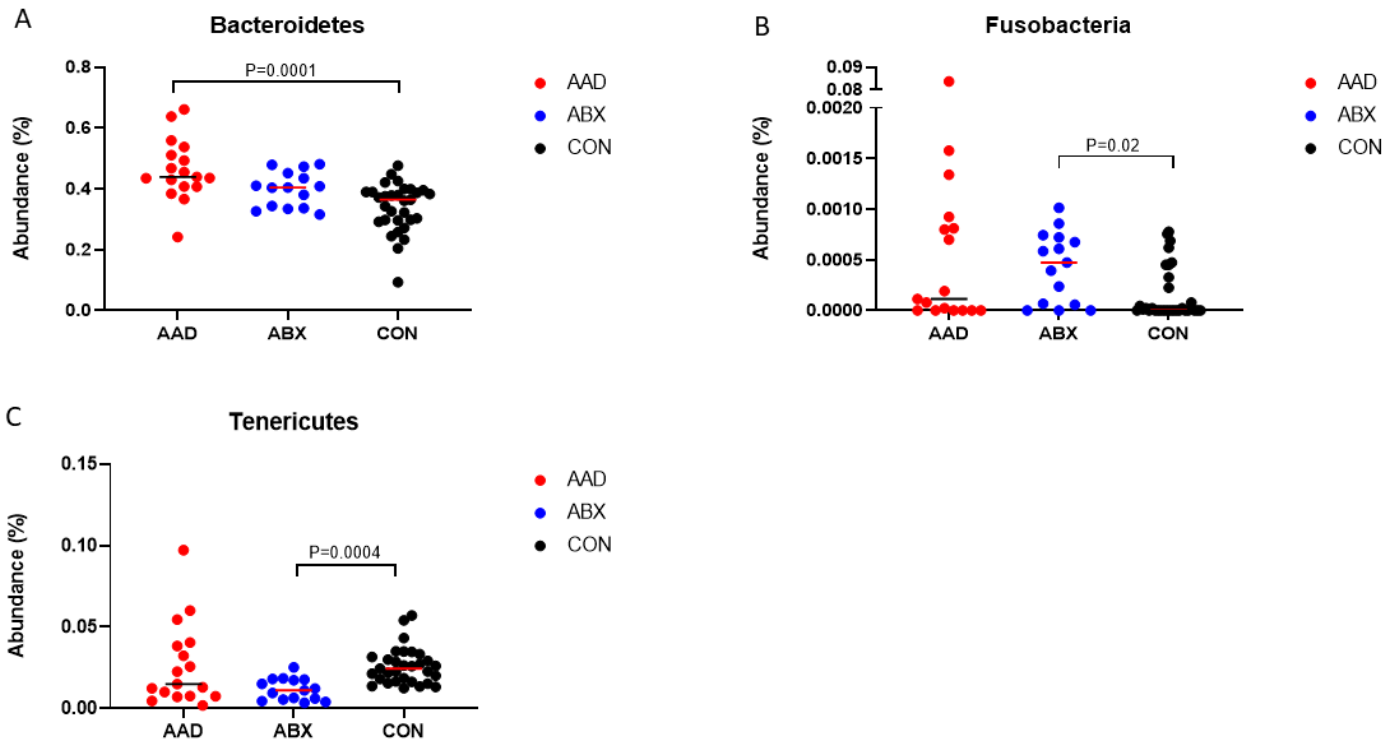


Figure 5.6 The median abundance (%) of significantly altered phyla in the fecal microbiome of horses with antimicrobial associated diarrhea (AAD) according to linear discriminant effects size analysis. Horses with antimicrobial associated diarrhea (AAD, red spheres), antibiotic control horses (ABX, blue spheres), and control horses (CON, black spheres) horses are displayed. A) The abundance of Bacteroidetes is significantly higher in AAD horses than in CON horses (p=0.0001) B) The abundance of Fusobacteria is significantly reduced in CON horses compared to ABX horses (p=0.02). C) The abundance of Tenericutes is reduced in AAD compared to CON horses (p=0.0004).



5.5 Discussion

Antibiotics have been used to treat bacterial infections since the early 1900's. While antibiotics have saved millions of human and animal lives from sepsis, the negative side effects associated with their use seemed inconsequential in comparison and were often overlooked. With the discovery of the gut microbiome and its relationship to overall human health, there is a growing awareness that antibiotics can have a harmful effect on the intestinal microbiome and subsequently affect host health.

Antimicrobial agents have been known to induce diarrhea in humans, with clinical symptoms that range from mild and self-limiting to those that are severe and life-threatening. *Clostridial difficile* diarrhea in aged, hospitalized, or immunocompromised patients represents an extreme but not uncommon scenario on this spectrum (107). The diarrhea caused by antimicrobial agents is likely mediated through the dysbiosis of the gut microbiome via the depletion of commensal bacteria. The resulting functional differences can induce an osmotic diarrhea or allow for colonization by enteric pathogens, effects that may last for weeks to years (108, 109).

As with humans, horses appear to be susceptible to adverse effects of antimicrobial agents. They are a known risk factor for the development of colitis and horses experience a similar range of clinical symptoms from mild to life-limiting. Recent investigation into factors affecting the equine microbiome have indicated that antibiotics reduce bacterial diversity in horses, with subsequent functional changes, resulting in diarrhea.

This study investigated differences in the fecal microbiome between 3 groups of horses: those on antibiotics that developed diarrhea, horses on antibiotics that maintained

a normal fecal consistency and health status, and healthy control horses from non-hospital environments without the influence of antimicrobials. Horses on antimicrobial therapy (AAD and ABX) were matched by antibiotic agent and length of exposure. Horses from all three groups (AAD, ABX and CON) were matched by diet. Ideally, horses would have also been matched for other variables, such as age (37, 38), breed (36), and gender. While these factors can have a minor impact, practicality necessitated that the authors chose to control for factors with the greatest influence on the fecal microbiome, such as antimicrobial agent (70), days of antimicrobial exposure (70), and diet (110).

Antimicrobial administration caused a marked reduction in diversity in all horses, regardless of their diarrhea status. This occurred due to the loss of both richness and evenness. While AAD horses had the lowest score on each metric, they were not statistically different from the antibiotic control horses. This result is similar to studies in which antimicrobials were administered to healthy horses (70, 101-103) or to clinical patients that subsequently developed diarrhea (Section 2). Because the reduction in diversity of the microbiome is a persistent feature of both antibiotic use and of multiple types of colitis, the subsequent functional changes may share common metabolic pathways.

While alpha diversity metrics were equally impacted by antibiotic use, horses with AAD displayed significant changes within their microbial communities compared to antibiotic control horses and non-antibiotic control horses. AAD horses clustered significantly and distinctly from CON horses on the PCoA plot. There was less, but still significant separation between ABX and CON horses. AAD and ABX horses exhibited

the smallest degree of clustering for pairwise comparisons, indicating fewer, but still significant differences in microbial community composition between these groups.

Univariate analysis of the bacterial taxa found that AAD horses showed significant differences in bacterial community composition from non-antibiotic control horses. These changes occurred in 6 phyla, including many that constitute a significant percentage of the fecal microbiome of healthy horses. AAD horses showed an increased abundance of Bacteroidetes, and decreased abundances of Actinobacteria, Armatimonadetes, Fibrobacteres, Spirochaetes, and Synergistetes compared to CON horses. Non-antibiotic-treated control horses showed an increased abundance of Elusimicrobia, Planctomycetes, and SR1 compared to AAD and ABX horses. CON horses had significantly different abundances of Fusobacteria, Tenericutes, and WPS-2 than antibiotic control horses (ABX). Antimicrobial use appears to deplete the commensal bacteria of the equine microbiome, which may allow for expansion of phyla such as Bacteroidetes and Fusobacteria.

While the abundance of many phyla were significantly different between AAD and CON horses, only a select number of taxa were able to distinguish between horses that developed colitis (AAD) and those that maintained a normal health status and fecal character on antibiotics (ABX). From the phylum Actinobacteria, the abundance of the order Bifidobacteriales and the family *Bifidobacteriaceae* were increased in AAD horses relative to ABX and CON horses. From the phyla Bacteroidetes, the abundances of the families *Bacteroidaceae* and *Prevotellaceae* were increased in AAD horses, compared to ABX and CON horses. From the phylum Firmicutes, the abundance of the family

Enterococcaceae was increased in AAD horses relative to ABX and CON horses, while that of the family *Mogibacteriaceae* was decreased. Finally, in the phylum Verrucomicrobia, the abundances of class Verruco-5, order WCHB1-41, and family RPF-12 were all dramatically decreased compared to ABX and CON horses.

The functional significance of these changes in taxa between AAD and ABX horses is inferred at this point in time. The phyla Bacteroidetes and Firmicutes account for the majority of the bacteria in the equine fecal microbiome and play important roles in fiber digestion. Changes in their levels of abundance have been associated with GIT disease, such as colic and colitis (33, 68). In particular, the class Clostridia within the phyla Firmicutes is recognized as an important symbiotic bacterial phylum for gut health whereas others such as *Enterococcaceae* are considered pathogenic (111).

The abundance of the phylum Verrucomicrobia was dramatically decreased in horses with AAD, compared to horses on antibiotics that maintained health status and normal fecal consistency as well as non-antibiotic control horses. This effect was significant from the level of the phylum to the family, with RFP-12 accounting for a large percentage of this change. CON and ABX horses had approximately 7 and 5 times the abundance of RFP12 compared to those with AAD. Verrucomicrobia plays an important role in gut barrier function by maintaining the mucus layer at the epithelial cell- lumen interface of the large intestine. This may be an important mechanism by which antimicrobials induce colitis in the horse (112). Verrucomicrobia is adversely affected by antibiotic administration in humans and horses (70), and once depleted by antimicrobials,

it may take weeks to replenish. RFP-12 may serve as a biomarker for AAD or as a potential target for therapeutic intervention.

This study examined the effect of 8 different antimicrobials, several of which were used in combination with each other on the fecal microbiome in horses. The limited number of animals per group precluded analysis of each individual antibiotic or combination of antibiotics. However, the scatter plots of the diversity indices (Figures 5.2) and the abundances of Verrucomicrobia (Figure 5.4 G-J) provide information regarding the antibiotic given to each horse in the AAD and ABX groups. From these plots, it appears that doxycycline, ceftiofur, and the combination of procaine penicillin, gentamycin, and metronidazole had the largest impact on diversity and the abundances of RFP-12, WCHB1-41, Verruco-5, and Verrucomicrobia. Horses given trimethoprim sulfa and penicillin in combination with gentamycin appeared to be the least affected. Given the differences in the mechanisms of action and target bacteria (i.e., gram positive versus negative, aerobic versus anaerobic), this result is worthy of further focused investigation.

5.6 Conclusions

Antimicrobial agents induce a severe dysbiosis of the fecal microbiome of horses, regardless of whether they develop diarrhea or not. Horses under the influence of antimicrobial agents show a reduction across alpha diversity indices compared to control horses and an altered taxonomic composition. The abundance of the family RPF12 within the phylum Verrucomicrobia was markedly diminished in horses that develop AAD compared to ABX and CON horses.

6. CONCLUSION

Culture independent technologies have enabled the discovery of bacterial communities that occupy niches in both the environment around us and within us. The diversity of these bacterial groups far exceeds previous estimations of the number of species residing in these ecosystems. The symbiotic relationship between the microbiome and the host has provided insights into the metabolic contributions of these bacterial communities. Across multiple species and disease states, alterations in the number and abundances of these species results in functional differences in metabolism. The term dysbiosis has been used to describe the changes in the bacterial communities and subsequent alterations in metabolic function that occur in disease states.

The concept of dysbiosis is particularly relevant to the GIT. This highly complex bacterial community plays critical roles in digestion, metabolism, and immunity. The GIT microbiome is now considered an organ, an essential component to health similar to other body systems. No longer reliant upon culture-based techniques to identify the diverse, typically anaerobic bacteria, recent advances in sequencing technologies have enabled rapid, more affordable, and accurate identification of the bacteria in the GIT. Extensive use of 16S rRNA sequencing has characterized the bacterial populations of the GIT in many species. By comparing the bacterial communities in health and disease, differences in taxa may indicate potential associations between specific bacterial groups and function. Data from microbiome analysis can then be combined with other molecular methods, such as metabolomics to investigate functionality. Using this approach, advances have been made in our understanding of the pathogenesis, diagnosis, and treatment of human GIT diseases

such as IBD, ulcerative colitis, colon cancer, obesity, diabetes mellitus, and metabolic diseases.

Researchers are employing similar investigative strategies regarding the equine microbiome and its relevance to gastrointestinal diseases of the horse. Preliminary studies indicate that dysbiosis is present patients with gastrointestinal disease, but progress has been hindered by 4 factors: the classification of multiple equine GIT diseases into one broad category (colic), a lack of standardization regarding sample collection, processing, and sequencing methodologies, the incomplete characterization of the normal fecal microbiome, and a lack of knowledge regarding the impact of normal variants and diseases on the fecal microbiome.

The work presented in this thesis characterized the healthy equine fecal microbiome and described the dysbiosis induced by diet, antimicrobial use, and colitis. The fecal microbiome of a large, diverse population of healthy horses was described and compared to those of horses with two variants of acute inflammatory GIT disease, antimicrobial-associated diarrhea and colitis due to infection with *Salmonella*. The effects of a specific antimicrobial agent, metronidazole, on both the cecal and fecal microbiome and metabolome were also described. Finally, the degree of dysbiosis caused by antibiotic therapy in horses that developed diarrhea and those that did not develop diarrhea were compared.

This work will serve as the foundation for further investigation into equine GIT disease. Future work will focus on molecular tools to better assess and manipulate the microbiome to treat dysbiosis-induced disease. The author's immediate short-term goal is

the creation of an assay to rapidly evaluate the fecal microbiome in clinical patients, a dysbiosis index or DI. The DI is a series of qPCR assays that can rapidly assess specific bacterial groups whose abundances are related to states of health or disease. A canine DI index (29) is currently in use and a feline DI is in the process of validation. This instrument has proven useful in identifying dogs with clinical disease and measuring response to treatment.

An equine DI index will be developed by examining the sequencing data from horses with colitis (work performed in Sections 3 and 5). Bacterial taxa with significant differences between healthy horses and those with colitis will be chosen. Using the same sample set as the sequencing data, PCR reactions for these bacterial groups will then be run using equine-specific primers. Mathematical modeling will be used to find which combinations of the qPCR reactions have the highest discriminatory power to distinguish between healthy horses and those with colitis. An algorithm will be developed that combines the results from the individual qPCR tests into a single numeric value, with ranges established for normal and disease states. The index will then be validated using a second sample set collected from clinical patients. The author has chosen to develop an equine DI using horses with colitis, as opposed to obstructive or ischemic lesions due to colic. Colitis was selected as dysbiosis is a central feature in this disease, and samples from over 100 patients have been collected that can be used during the validation phase. Within this group, there are four subsets of colitis that can be further explored: antibiotic induced colitis, primary *Salmonella* colitis, IBD, and undifferentiated.

The DI will allow to assess the fecal microbiome of clinical patients with colitis, the ability to identify patients at risk for colitis, and to measure the response of the microbiome after recovery from disease and or therapeutic intervention.

Another future endeavor is the manipulation of the microbiome for the purpose of treating dysbiosis. Currently, there are 2 methods used to change the microbiome that might be useful in horses, fecal microbiota transplantation and probiotic/prebiotic administration. FMT involves the transfer of feces from a healthy donor to a colitis patient in order to replenish the microbiome. FMT is well-described in the human literature for the treatment of C. *difficile* diarrhea, a form of colitis often induced by antimicrobial use (113, 114). Preliminary studies in dogs with chronic treatment-refractory diarrhea indicate a positive outcome for dogs treated with FMT as well (115). In horses, FMT has been used for years in clinical practice, with only anecdotal evidence as to its efficacy. Recent work has indicated that FMT may be helpful in the treatment of equine colitis (38, 116). A pilot study conducted by this author found that the fecal microbiome of 8 horses with colitis was positively affected by twice daily transfaunation for 3 days. While this small study did improve fecal consistency and shifted the recipients' microbiome, the results may have been affected by the chronic nature of the horses enrolled. FMT may be an optimal treatment for horses with acute *Salmonella* infection, a disease caused by overgrowth of enteric pathogens following depletion of the normal enteric bacteria.

Finally, horses with antibiotic-induced dysbiosis may benefit from therapy designed to restore the mucin layer of the epithelial-lumen interface. Horses are profoundly sensitive to endotoxin, and frequently succumb to SIRS (systemic inflammatory response

syndrome) as a result of colitis. Antibiotics may diminish the mucin protective layer at the epithelial cell-lumen interface, allowing for absorption of endotoxin. The abundances of family RPF12, order WCHB1-41, class Verruc-5 and phyla Verrucomicrobia are all significantly depleted in horses with antimicrobial associated diarrhea compared to antibiotic-treated and non-antibiotic treated control horses. These taxa may serve as potential markers for disease severity in colitis patients and identify horses at risk of colitis. These taxa could also be investigated for possible use as a probiotic agent. A study using *Akkermansia mucinophilia*, a related organism, to treat dogs following antibiotic administration indicated a positive effect on fecal consistency and possible effect at the GIT epithelium (117). Studies of these taxa could help identify the potential mechanism by which antimicrobials can induce diarrhea in horses.

The work represented by this thesis is a small step forward for further exploration into the relationship between the GIT microbiome and equine GIT disease. Future work will likely focus on the functional role of the microbiota, development of a dysbiosis index, and therapeutic interventions.

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APPENDIX A

SIGNALMENT, BODY WEIGHT, DIET, STATE OF RESIDENCE, ZONE, AND SEASON OF SAMPLING IN HORSES

INCLUDED IN SECTION 2.

Horse ID	Breed	Sex	Age	Body weight	% Fiber in grain	Grain at %BW	Hay	Pasture Exposure	State	Grass Zone	Season	Diet
1	Quarter Horse	Mare	22	507	NA	NA	None	Continuous	TX	W	Sp	A
2	Quarter Horse	Mare	7	432	NA	NA	None	Continuous	TX	W	Sp	A
3	Paint	Gelding	10	500	NA	NA	None	Continuous	TX	W	Sp	A
4	Quarter Horse	Gelding	18	500	NA	NA	None	Continuous	MT	C	Sp	A
5	Draft	Gelding	7	636	NA	NA	None	Continuous	MT	C	Sp	A
6	Appoloosa	Gelding	16	454	NA	NA	None	Continuous	KS	T	Su	A
7	Quarter Horse	Mare	15	454	NA	NA	None	Continuous	KS	T	Su	A
8	Draft Cross	Gelding	14	590	NA	NA	None	Continuous	GA	W	Sp	A
9	Quarter Horse	Mare	12	500	NA	NA	None	Continuous	CA	T	Sp	A
10	Warmblood	Stallion	10	408	NA	NA	None	Continuous	TX	W	Sp	A
11	Quarter Horse	Mare	3	454	NA	NA	Orchard Grass	Continuous	WA	C	Sp	A
12	Quarter Horse	Mare	8	450	NA	NA	Alfalfa,Wheat	Continuous	CA	T	Sp	A
13	Warmblood	Gelding	23	550	NA	NA	Alfalfa,Grass Hay	Some	VA	T	Sp	A
14	Warmblood	Gelding	22	550	NA	NA	Alfalfa,Grass Hay	Some	VA	T	Sp	A
15	Quarter Horse	Stallion	21	450	NA	NA	Alfalfa	Continuous	NV	T	Su	A
16	Quarter Horse	Gelding	4	450	NA	NA	Alfalfa	Continuous	NV	T	Su	A
17	Warmblood	Gelding	14	622	NA	NA	Coastal	No	TX	W	Sp	A

18	Quarter Horse	Gelding	17	500	NA	NA	Alfalfa	No	TX	W	Sp	A
19	Mule	Gelding	4	454	NA	NA	Grass Hay	No	MT	C	Sp	A
20	Arab	Mare	12	432	NA	NA	Alfalfa,Orchard Grass,Timothy	No	WA	C	Sp	A
21	Fox Trotter	Gelding	9	454	5.5	0.005	Prarie Hay	No	KS	T	Su	B
22	Arab	Mare	5	900	5.5	0.005	Alfalfa	Some	MO	T	Su	B
23	Quarter Horse	Mare	16	485	6	0.005	Alfalfa,Orchard Grass	Some	TX	W	W	B
24	Thoroughbred	Mare	4	590	6.5	0.005	Clover	Some	KY	T	Sp	B
25	Quarter Horse	Mare	12	500	7.5	0.005	Prarie Hay	Some	KS	T	Su	B
26	Quarter Horse	Stallion	19	500	5.5	0.50%	Alfalfa, Grass	No	NV	T	Su	B
27	Warmblood	Gelding	10	545	6	0.50%	Alfalfa,Grass Hay	No	CA	T	Sp	B
28	Thoroughbred	Mare	4	500	6	0.50%	None	Continuous	KY	T	Sp	B
29	Paint	Mare	20	600	7	0.50%	Alfalfa,Coastal	Some	TX	W	Sp	B
30	Quarter Horse	Mare	13	537	6	0.50%	Alfalfa,Orchard Grass	Some	TX	W	W	B
31	Pony	Mare	21	500	5.5	0.50%	Brome	Some	MO	T	Su	B
32	Thoroughbred	Mare	16	590	6.5	0.50%	Clover	Some	KY	T	Sp	B
33	Warmblood	Gelding	12	545	6	0.50%	Grass Hay	No	CA	T	Sp	B
34	Quarter Horse	Mare	15	500	5.5	0.50%	Brome, Alfalfa	Some	MO	T	Su	B
35	Arab	Mare	12	500	10	0.50%	Alfalfa,Coastal	Continuous	TX	W	Sp	C
36	Quarter Horse	Gelding	12	500	12.5	0.50%	Fescue	Some	GA	W	Sp	C
37	Quarter Horse	Gelding	17	454	12.5	0.50%	Fescue	Some	GA	W	Sp	C
38	Warmblood	Gelding	2	454	13	0.50%	None	Continuous	FL	W	Sp	C
39	Standardbred	Mare	5	590	11	0.50%	Timothy	Some	KY	T	Sp	C
40	Quarter Horse	Gelding	8	500	12	0.50%	Alfalfa, Orchard Grass	Some	VA	T	Sp	C

41	Appoloosa	Gelding	8	454	12	0.005	Alfalfa,Fescue	Continuous	NC	T	Sp	C
42	Warmblood	Gelding	6	545	12.5	0.50%	Alfalfa,Grass Hay	No	CA	T	Sp	C
43	Quarter Horse	Mare	2	454	13	0.50%	Alfalfa	Continuous	NV	T	Su	C
44	Quarter Horse	Gelding	20	450	10	0.50%	Wheat	Continuous	NC	T	Sp	C
45	Warmblood	Gelding	7	545	12.5	0.50%	Alfalfa	Continuous	MT	C	Sp	C
46	Quarter Horse	Gelding	2	432	13	0.50%	Alfalfa	Continuous	NV	C	Su	C
47	Quarter Horse	Mare	18	500	13	0.50%	Alfalfa, Grass	No	NV	C	Su	C
48	Draft	Gelding	7	550	11	0.50%	Coastal	Some	KY	T	Sp	C
49	Quarter Horse	Gelding	12	545	23	0.50%	Brome, Alfalfa	Some	MO	T	Su	D
50	Tennessee Walker	Gelding	17	410	18	0.50%	Alfalfa,Orchard Grass	Continuous	FL	W	Sp	D
51	Warmblood	Mare	12	590	18	0.50%	Alfalfa	None	NV	T	Su	D
52	Quarter Horse	Mare	2	500	18	0.50%	Grass Hay	No	MT	C	Sp	D
53	Quarter Horse	Mare	14	461	18.5	0.50%	Alfalfa,Coastal	Some	TX	W	Sp	D
54	Paint	Gelding	11	454	18.5	0.50%	Timothy	Some	GA	W	Sp	D
55	Warmblood	Mare	8	590	18.5	0.50%	Grass Hay	No	CA	T	Sp	D
56	Quarter Horse	Gelding	19	454	18.5	0.50%	Brome	Some	KS	T	Su	D
57	Arab	Gelding	14	900	20	0.50%	Timothy	No	MI	C	Sp	D
58	Tennessee Walker	Mare	3	450	20	0.50%	Coastal	Continuous	NC	T	Sp	D
59	Thoroughbred	Mare	12	545	20	0.50%	Timothy	No	MI	C	Sp	D
60	Thoroughbred	Mare	6	500	29	0.50%	Alfalfa,Grass Hay	Some	VA	T	Sp	D
61	Quarter Horse	Stallion	3	545	33	0.50%	Alfalfa,Grass Hay	No	MT	C	Sp	D
62	Appoloosa	Gelding	21	454	20	0.50%	Timothy	No	MI	C	Sp	D

63	Thoroughbred	Gelding	24	545	18	0.50%	Alfalfa,Orchard Grass	Continuous	FL	W	Sp	D
64	Thoroughbred	Mare	2	500	10	1.1%	Timothy mixed	No	NY	C	W	E
65	Thoroughbred	Mare	4	500	10	1.1%	Timothy mixed	No	NY	C	W	E
66	Thoroughbred	Mare	3	477	10	0.012	Timothy mixed	No	NY	C	W	E
67	Thoroughbred	Mare	3	477	10	1.2%	Timothy mixed	No	NY	C	W	E
68	Thoroughbred	Mare	3	477	10	1.2%	Timothy mixed	No	NY	C	W	E
69	Thoroughbred	Gelding	4	500	10	1.4%	Grass/alfalfa	No	KY	T	W	E
70	Thoroughbred	Gelding	4	454	10	1.5%	Grass/alfalfa	No	KY	T	W	E
71	Thoroughbred	Gelding	4	454	10	1.5%	Grass/alfalfa	No	KY	T	W	E
72	Thoroughbred	Gelding	5	545	14	1.7%	Timothy/alfalfa	No	FL	W	W	E
73	Thoroughbred	Mare	3	454	10	1.8%	Timothy/alfalfa	No	KY	T	W	E
74	Thoroughbred	Gelding	2	454	10	1.8%	Timothy/alfalfa	No	KY	T	W	E
75	Thoroughbred	Stallion	5	500	14	1.8%	Timothy/alfalfa	No	FL	W	W	E
76	Thoroughbred	Mare	3	477	14	2.0%	Timothy/alfalfa	No	FL	W	W	E
77	Thoroughbred	Gelding	4	454	14	2.0%	Timothy/alfalfa	No	FL	W	W	E
78	Thoroughbred	Mare	2	409	10	2.1%	Timothy/alfalfa	No	FL	W	W	E
79	Thoroughbred	Gelding	4	409	14	2.3%	Timothy/alfalfa	No	FL	W	W	E
80	Thoroughbred	Mare	2	409	14	2.3%	Timothy/alfalfa	No	FL	W	W	E

APPENDIX B

SIGNALMENT, BODY WEIGHT, DIET, STATE OF RESIDENCE AND SEASON OF SAMPLING HORSES WITH COLITIS IN SECTION 3

Horse ID	Breed	Sex	Age	Weight	% Fiber in Grain	Grain at %BW	Hay	Pasture Exposure	State	Zone	Season	Diet	Health Status	Antibiotic
Horse 111	Paint	Mare	11	544	18	0.50%	Coastal	Some	TX	W	W	D	AAD	PPG, Gentamycin
Horse 138	Paint	Stallion	2	390	8	0.50%	Coastal	Continous	TX	W	F	B	AAD	Excede
Horse 102	Quarter Horse	Gelding	9	377	15	0.50%	Coastal	Continuous	TX	W	Sp	C	AAD	Excede
Horse 103	Quarter Horse	Stallion	1	217	18	0.50%	Alfalfa	Some	TX	W	F	D	AAD	PPG/Gentamycin /Doxycycline
Horse 104	Quarter Horse	Mare	1	300	18	0.50%	Alfalfa	Some	TX	W	W	D	AAD	Doxycycline
Horse 106	Quarter Horse	Stallion	5	501	15	0.50%	Alfalfa	Some	TX	W	Sp	C	AAD	PPG/Gentamycin
Horse 108	Quarter Horse	Stallion	1	300	12	0.50%	Coastal	Some	TX	W	Su	C	AAD	Excede
Horse 113	Quarter Horse	Mare	12	472	6	0.50%	Alfalfa,Coastal	Some	TX	W	Sp	B	AAD	PPG/Gentamycin /Metronidazole
Horse 107	Thoroughbred	Mare	25	374	18.5	0.50%	Alfalfa,Coastal	Some	TX	W	Su	D	AAD	PPG/Gentamycin /Metronidazole
Horse 115	Thoroughbred	Mare	12	443	18	0.50%	Alfalfa, Coastal	None	TX	W	SP	D	AAD	Chloramphenicol
Horse 105	Warmblood	Gelding	12	610	15	0.50%	Coastal	None	TX	W	Sp	C	AAD	PPG/ Gentamycin /Doxycycline
Horse 109	Warmblood	Gelding	6	479	15	0.50%	Alfalfa,Coastal	Some	TX	W	W	C	AAD	Metronidazole
Horse 137	Quarter Horse	Mare	14	523	0	0.00%	None	Continous	TX	W	Su	A	Salmonella	None

Horse 136	Draft Cross	Gelding	17	636	18	0.50%	Alfalfa	Some	TX	W	Sp	D	Salmonella	None
Horse 143	Irish Draught	Mare	7	654	18	0.50%	Alfalfa, coastal	Some	TX	W	F	D	Salmonella	None
Horse 133	Paint	Mare	10	450	18	0.50%	Alfalfa	Some	TX	W	F	D	Salmonella	None
Horse 140	Paint	Gelding	19	470	15	0.50%	Coastal	Continuous	TX	W	W	C	Salmonella	None
Horse 142	Paint	Mare	8	388	18	0.50%	Alfalfa, coastal	Some	TX	W	Su	D	Salmonella	None
Horse 128	Quarter Horse	Mare	13	450	8	0.50%	Alfalfa/coastal	Continuous	TX	W	F	B	Salmonella	None
Horse 130	Quarter Horse	Gelding	10	400	18	0.50%	Alfalfa, Coastal	Continuous	TX	W	SP	D	Salmonella	None
Horse 131	Quarter Horse	Mare	13	572	18	0.50%	Alfalfa, Coastal	Continuous	TX	W	F	D	Salmonella	None
Horse 135	Quarter Horse	Mare	12	454	18	0.50%	Coastal	Some	TX	W	W	D	Salmonella	None
Horse 132	Warmblood	Gelding	15	550	18	0.50%	Alfalfa	Some	TX	W	SP	D	Salmonella	None
Horse 139	Warmblood	Gelding	8	609	15	0.50%	Coastal	Some	TX	W	W	C	Salmonella	None

APPENDIX C

SIGNALMENT, DIET, HEALTH, STATE OF RESIDENCE, AND ANTIBIOTIC STATUS OF HORSES INCLUDED IN SECTION 5

Horse ID	Breed	Sex	Age	Diet	State	Group	Antibiotic	Days	Reason ABX
Horse.144	Quarter Horse	Gelding	20	D	TX	AAD	Doxycycline	5	Surgery
Horse.151	Quarter Horse	Gelding	6	C	TX	AAD	Doxycycline	3	Surgery
Horse.153	Miniature Donkey	Stallion	1	C	TX	AAD	Excede	7	Respiratory disease
Horse.108	Quarter Horse	Stallion	1	C	TX	AAD	Excede	7	Trauma
Horse.155	Paint	Stallion	1	D	TX	AAD	Excede	2	Respiratory disease
Horse.102	Quarter Horse	Gelding	9	C	TX	AAD	Excede	3	Respiratory disease
Horse.111	Paint	Mare	11	D	TX	AAD	PPG Gentamycin	8	Surgery
Horse.106	Quarter Horse	Stallion	5	C	TX	AAD	PPG Gentamycin	4	Trauma
Horse.164	Warmblood	Gelding	1	C	TX	AAD	PPG Gentamycin	1	Surgery
Horse.103	Quarter Horse	Stallion	1	D	TX	AAD	PPG Gentamycin	3	Surgery
Horse.116	Mixed	Gelding	1	B	TX	AAD	PPG Gentamycin Doxycycline	7	Respiratory disease
Horse.105	Warmblood	Gelding	12	C	TX	AAD	PPG Gentamycin Doxycycline	7	Surgery
Horse.104	Quarter Horse	Mare	1	D	TX	AAD	Doxycycline	5	Trauma
Horse.113	Quarter Horse	Mare	12	B	TX	AAD	PPG Gentamycin Metronidazole	3	Respiratory disease
Horse.107	Thoroughbred	Mare	25	D	TX	AAD	PPG Gentamycin Metronidazole	4	Respiratory disease
Horse.176	Miniature Horse	Gelding	26	D	TX	AAD	TMS	7	Dental/sinus disease

APPENDIX C CONTINUED

Horse ID	Breed	Sex	Age	Diet	State	Group	Antibiotic	Days	Reason ABX
Horse.148	Quarter Horse	Gelding	9	C	TX	AAD	Doxycycline	7	Surgery
Horse.149	Quarter Horse	Gelding	12	C	TX	ABX	Doxycycline	8	Surgery
Horse.152	Quarter Horse	Gelding	9	C	TX	ABX	Doxycycline	2	Surgery
Horse.154	Thoroughbred	Gelding	12	C	TX	ABX	Excede	5	Dental sinus disease
Horse.145	Quarter Horse	Gelding	13	D	TX	ABX	Doxycycline	5	Foot abscess
Horse.156	Quarter Horse	Mare	19	D	TX	ABX	Excede	4	Surgery
Horse.159	Quarter Horse	Mare	8	D	TX	ABX	PPG Gentamycin	3	Surgery
Horse.161	Warmblood	Mare	12	C	TX	ABX	PPG Gentamycin	4	Surgery
Horse.165	Quarter Horse	Gelding	2	C	TX	ABX	PPG Gentamycin	1	Surgery
Horse.167	Quarter Horse	Stallion	1	D	TX	ABX	PPG Gentamycin	3	Surgery
Horse.169	Saddlebred	Stallion	1	B	TX	ABX	PPG Gentamycin Doxycycline	7	Surgery
Horse.172	Paint	Gelding	10	C	TX	ABX	PPG Gentamycin Doxycycline	7	Surgery
Horse.173	Warmblod	Gelding	19	C	TX	ABX	PPG Gentamycin Metronidazole	4	Respiratory disease
Horse.174	Criollo	Mare	19	D	TX	ABX	PPG Gentamycin Metronidazole	4	Trauma
Horse.147	Quarter Horse	Mare	1	D	TX	ABX	Doxycycline	2	Surgery
Horse.177	Miniature Horse	Gelding	23	D	TX	ABX	TMS	14	Dental/sinus disease
Horse.150	Mix	Gelding	8	C	KY	CON	None	0	None
Horse.45	Warmblood	Gelding	7	C	MT	CON	None	0	None
Horse.36	Quarter Horse	Gelding	12	C	GA	CON	None	0	None
Horse.48	Mix	Gelding	7	C	KY	CON	None	0	None
Horse.42	Warmblood	Gelding	6	C	CA	CON	None	0	None
Horse.46	Quarter Horse	Gelding	2	C	NV	CON	None	0	None
Horse.157	Tennessee Walking horse	Stallion	8	D	NC	CON	None	0	None
Horse.158	Draft cross	Gelding	9	C	VA	CON	None	0	None
Horse.55	Warmblood	Mare	8	D	CA	CON	None	0	None
Horse.50	Tennessee Walker	Gelding	17	D	FL	CON	None	0	None

Horse ID	Breed	Sex	Age	Diet	State	Group	Antibiotic	Days	Reason ABX
Horse.160	Quarter Horse	Mare	14	D	TX	CON	None	0	None
Horse.162	Quarter Horse	Stallion	3	C	TX	CON	None	0	None
Horse.163	Quarter Horse	Stallion	6	C	TX	CON	None	0	None
Horse.166	Quarter Horse	Gelding	1	C	TX	CON	None	0	None
Horse.38	Warmblood	Gelding	2	C	Fl	CON	None	0	None
Horse.146	Quarter Horse	Gelding	18	D	NC	CON	None	0	None
Horse.61	Quarter Horse	Stallion	3	D	MT	CON	None	0	None
Horse.168	Draft	Stallion	1	D	TX	CON	None	0	None
Horse.170	Quarter Horse	Gelding	7	B	MO	CON	None	0	None
Horse.171	Tennessee Walker	Stallion	6	B	NC	CON	None	0	None
Horse.40	Quarter Horse	Gelding	8	C	VA	CON	None	0	None
Horse.178	Warmblood	Gelding	16	C	TX	CON	None	0	None
Horse.30	Quarter Horse	Mare	13	B	TX	CON	None	0	None
Horse.25	Quarter Horse	Mare	12	B	KS	CON	None	0	None
Horse.175	Quarter Horse	Mare	24	D	MO	CON	None	0	None
Horse.62	Appaloosa	Gelding	21	D	MI	CON	None	0	None
Horse.56.	Quarter Horse	Gelding	19	D	KS	CON	None	0	None
Horse.63	Thoroughbred	Gelding	24	D	FL	CON	None	0	None
Horse.58.	Tennessee Walker	Mare	3	D	NC	CON	None	0	None
Horse.52	Quarter Horse	Mare	2	D	MT	CON	None	0	None
Horse.59	Thoroughbred	Mare	12	D	MI	CON	None	0	None