Alterations in the Fecal Microbiome of Healthy Horses in Response to Antibiotic Treatment

Thesis

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By

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Abstract

Acute colitis is the most common and devastating complication of antibiotic therapy in horses. Fecal culture often fails to identify etiologic agents and has low sensitivity in detecting organisms that are difficult to cultivate. Metagenomic studies utilize 16S ribosomal RNA (rRNA) sequence for bacterial identification and classification and can produce profiles of genetic diversity from microbial communities. There are limited data to explain how antimicrobials affect the fecal microbiome of horses over time. The purpose of this study was to provide a comprehensive description of bacterial phylum structures within feces of normal horses in central Ohio during the winter and summer, to determine how commonly used antibiotics affect these communities over time and if these changes persisted over longer time periods.

Healthy horses were acclimated to the same diet, environment and husbandry and were treated intravenously with different classes of antibiotics (ceftiofur sodium, enrofloxacin and oxytetracycline) or saline for 3 or 5 days consecutively and fecal microbiota evaluated. Fecal samples were collected and frozen, bacterial DNA was extracted and PCR amplified, and analyzed by 454 pyrosequencing of the 16S rRNA gene from baseline (before treatment), and at various time points.
We demonstrated that the fecal microbiome of healthy horses in central Ohio was highly diverse both within and between subjects before and after treatment. Major phyla present in greatest abundance in the feces of all subjects included *Firmicutes* and *Bacteroidetes*, while minor phyla included *Proteobacteria, Spirochaetes, Tenericutes* and *Verrucomicrobia*. Enrofloxacin and ceftiofur lead to the greatest shifts in the fecal microbiome over time, however none of the treated horses developed diarrhea. Many of these changes persisted over longer time periods, while some returned to baseline. These variations were specific to the antibiotic used and may represent repeatable trends. Additionally, 3 and 5 days of treatment were sufficient to identify alterations in the fecal microbiome induced by antibiotic treatment.
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**Fields of Study**

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Chapter 1: Introduction and Literature Review

1.1 Antibiotic associated diarrhea

Antibiotic associated diarrhea (AAD) is the most common and devastating complication of antibiotic therapy in horses. Defining this term is difficult, as there are varying case definitions in the literature in several species. Diagnosis is typically based upon the occurrence of diarrhea that is otherwise unexplained except that it is associated in time with administration or cessation of antibiotics. It is nearly impossible to make a direct causal link between the implicated antibiotic and occurrence of AAD. It can begin as soon as 24 hours after starting antibiotics or up to 8 weeks after stopping them and has been shown to prolong hospital stays, increase cost of treatment and is associated with high mortality\(^1\). Weese found that on average, 5.7 days from the start of treatment is most common\(^1,2\) while Wilson and colleagues found an average of 3.4 days was enough\(^3\).

Depending on the study, the incidence of AAD in horses varies from around 22-94% of mature horses with diarrhea. Foals may also be affected, but their disease course is typically milder. Also important to note is the frequency of use of the implicated antibiotic in the population being studied. In humans, vancomycin and clindamycin have
been linked more with this complication, however more cases are treated with cephalosporins and ampicillin, and thus more reports of AAD are associated with these medications\textsuperscript{1,3}. Classically, reports of AAD in horses were associated with use of macrolides for pneumonic foals of mature horses. Båverud found that dams often developed diarrhea when stalled with their foals undergoing combination erythromycin/rifampin treatment for pneumonia\textsuperscript{4}. Foals treated with erythromycin have also been reported to develop diarrhea, with less frequency than adult horses\textsuperscript{5}. However, as noted above, it seems that any antibiotic may be associated with the development of AAD, a problem that is likely multifactorial.

Antibiotics commonly associated with equine AAD include enrofloxacin, trimethoprim-sulfonamides, oxytetracycline, penicillin, ceftiofur, gentamicin, doxycycline, erythromycin, neomycin, lincomycin, clindamycin, rifampin, moxifloxacin, florfenicol and tylosin\textsuperscript{6-8}. Often, combinations of these medications are reported to cause AAD, and multiple studies have been published on individual and combination therapy underlying disease\textsuperscript{3,5,7,9}. Doses, frequencies and methods of administration vary among studies, which makes comparing outcomes quite difficult. Cephalosporins, particularly ceftiofur, either intramuscular or intravenous, are anecdotally associated with AAD, however outcomes of studies vary and definitive evidence linking this medication to development of diarrhea is lacking\textsuperscript{6,10}. Tetracyclines, both orally and intravenously, are historically associated with frequent episodes of AAD. In older studies citing this class of antibiotics as a cause of AAD,
even single doses or very high doses of oxytetracycline were implicated in the development of severe AAD. Even with the limited oral bioavailability of doxycycline in horses, AAD has been reported. Fluoroquinolones were historically considered to pose a low risk in development of AAD due to their poor efficacy against anaerobic bacteria, however Weese reported a series of acute colitis cases in horses administered oral ciprofloxacin. Also, moxifloxacin, a newer generation fluoroquinolone with an extended anaerobic spectrum, was experimentally associated with development of AAD. Enrofloxacin, although historically considered lower risk, was one of the major antibiotics associated with AAD in the study by Barr and colleagues. Thus, although the spectrum of activity may be important, it must be one of many determinants of development of AAD in the horse.

Also important to consider are the routes of excretion and metabolism of a given antibiotic and its resultant concentration within the GI tract. Those that undergo enterohepatic circulation or excretion into the GI tract are able to reach higher concentrations in the large intestine, which may lead to more disturbances in microfloral populations. Oxytetracycline, macrolides, rifampin and some cephalosporins are incompletely absorbed from the gastrointestinal (GI) tract or are excreted from the liver in their active form. However, once there it is not clear if they are enzymatically inactivated or if they bind to organic material, which would alter their exposure to resident microbes.
Additional factors for consideration when studying AAD in horses include diet or abrupt dietary change, geographic location, concurrent disease, hospitalization, immunologic and physiologic status and other treatments\textsuperscript{1,7}. The equine intestinal microflora is complex and highly dominated by anaerobic bacteria\textsuperscript{13,14}. Thus, the spectrum of action of a given antibiotic should be considered in the context of AAD and logically, it would seem that classes that do not affect anaerobes should not be commonly associated with development of diarrhea\textsuperscript{3,6}. Dr. Barr and colleagues performed a multicenter retrospective study evaluating the occurrence of AAD in equine referral practices. Several antibiotic classes were implicated in causing AAD, with penicillin, gentamicin combination therapy, enrofloxacin and doxycycline being most frequently associated. Out of 5,251 horses treated with antibiotics for non-gastrointestinal reasons, only 32 were diagnosed with probable AAD although 18.8\% of those died\textsuperscript{12}. Aminoglycosides and fluoroquinolones have poorer efficacy against anaerobes and should be less likely to cause AAD, however this fact is unsupported by several studies\textsuperscript{6,12}. Also, several reports have been published that implicate potentiated sulfonamides as a cause of AAD, another class of antibiotics with poor efficacy against anaerobes\textsuperscript{3,6,9}. Overall it is clear that AAD as a disease is poorly understood and it is assumed that a change in GI microflora in response to antibiotic treatment is underlying the development of diarrhea. It is therefore essential for the scientific community to better define this disease and to discover the “normal” flora of the equine GI tract and contrast that to the “abnormal” flora that flourishes and may persist in the presence of antibiotics or after cessation of treatment.
The intestinal microflora serve several critical functions; a major one is acting as a barrier to colonization of pathogens—either exogenous or opportunists already present. This is known as colonization resistance. This term was coined in the 1970s to indicate that the indigenous microflora had an inherent property of being resistant to colonization of potentially pathogenic microorganisms. When overcome, pathogens are able to colonize and proliferate within the GI tract. Colonization resistance is composed of several anatomic and physiologic factors including an intact mucosa, saliva and swallowing, secretion of immunoglobulin A, gastric acid production, normal GI motility and mucous membrane desquamation and turnover. The mechanisms of protection offered by this population of microbes include competition for receptors and resources, production of antibacterial products and decreasing the GI pH by volatile fatty acid production. With antibiotic treatment, disruption of colonization resistance is inevitable. The extent of alterations in microbiota induced by antibiotic therapy depends on several factors including the bacterial spectrum, dose and duration of treatment, route of administration and pharmacology of the agent used. Antibiotics can affect both commensal populations of bacteria as well as the target bacterial population. Therefore, treatment may also lead to selection and proliferation of potential pathogens or microbes resistant to the antibiotic used. Alternatively, if the pathogen(s) responsible for disease are inhibited during treatment but not killed, then they may proliferate after cessation of treatment, further disrupting return of the normal population of microbes.
There are still several unknowns regarding AAD and this is largely due to the paucity of information about the composition of the equine GI microflora and how antibiotics affect it. Also, there is little published information about the duration of treatment and development of AAD or when in the course of treatment AAD might develop. As previously mentioned, equine AAD may be seen early on in the treatment course or begin after cessation of treatment. Weese found that 20% of horses developed AAD 1-7 days after stopping treatment but that on average, most cases occurred within approximately 5 days of starting treatment\(^2\). In humans, onset of AAD is defined as early or delayed: early occurring during antibiotic treatment and delayed, from 2-8 weeks post-treatment. Increased duration of treatment has been cited as a risk factor for development of AAD in people, with the risk increasing as duration increases from 3 to 7 days. However, risk was not further increased when antibiotics were administered for more than 7 days\(^1\). This suggests that the microfloral changes in the human GI tract occur soon after starting treatment. It is still unknown which risk factors predispose equids to developing this condition and most studies focus on the presence of specific pathogens in cases with AAD, especially Clostridial species\(^{13,17-19}\).

Specific pathogens have long been associated with AAD. These most commonly include *Clostridium difficile*, *Clostridium perfringens* and *Salmonella* species. After intestinal colonization, these pathogens overgrow, releasing toxins that cause mucosal
damage, inflammation and alterations in normal absorptive and secretory functions of the GI tract\textsuperscript{1,2,7,20}. Approximately 40\% of human cases of AAD are caused by overgrowth of specific pathogens, while the etiology of the remainder of cases is unknown. A similar proportion of equine cases of AAD can be attributed to specific pathogens\textsuperscript{1,7}. \textit{Clostridium difficile} is most commonly associated with AAD in humans and most often implicated in equine AAD. Gustaffson reported that antibiotic therapy can increase the frequency of isolation of \textit{C. difficile} from healthy horses\textsuperscript{7}. \textit{C. difficile} was also isolated from mares with AAD being housed with their foals undergoing treatment with combination erythromycin/rifampin for pneumonia\textsuperscript{4}. Highly resistant spores are ingested from the environment and within the host GI tract, become vegetative, multiply and colonize the intestine. They elaborate toxins that damage the GI tract leading to AAD and other systemic complications. Horses may ingest the vegetative form from an infective, shedding horse, from personnel or from the environment. If the GI microflora is disturbed in an asymptomatic horse harboring even a small population of \textit{C. difficile}, they may also develop disease\textsuperscript{2,7,17-19}. Humans treated with some third generation cephalosporins tend to have a higher risk of developing AAD with overgrowth of \textit{C. difficile}\textsuperscript{2}. Magdesian and colleagues reported that 75\% of horses with diarrhea and positive fecal culture for \textit{C. difficile} were previously treated with antibiotics\textsuperscript{20}. The consequences associated with colonization of the GI tract with \textit{C. difficile} are largely dependent on the strain and toxins involved. Colonization with nontoxigenic \textit{C. difficile} has been documented and may
be protective against toxigenic *C. difficile* colonization. This also holds true for other potential GI pathogens¹.

Other pathogens less commonly implicated in AAD of horses include *Clostridium perfringens* and *Salmonella* species. *Clostridium perfringens* is associated with development of AAD in horses, humans and other species, just less commonly than *C. difficile*. More recently, the beta-2 toxigenic form of *C. perfringens* was linked with the syndrome colitis X, which causes severe, often deadly enterocolitis⁷. *Salmonella* is frequently implicated in AAD in horses, however, several other factors have been shown to be associated with shedding of this organism including transportation, surgery, hospitalization, nasogastric intubation, colic and laminitis²,⁷. It is also notable that all of these pathogens can be isolated by culture and other molecular techniques from horses in the absence of outward disease, again making clinical significance and causation difficult to prove.
1.2 Methods to study antibiotic associated diarrhea

Culture has been the classic method used to isolate pathogens in horses and other species with AAD. Dr. Mackie and colleagues sampled multiple compartments of the healthy equine GI tract and cultured for anaerobes in an attempt to estimate the number and type of organisms within the different segments. They found a large population of microbes, with numbers and composition of organisms varying along the intestinal tract. A large percentage of proteolytic bacteria were identified among all cultivable organisms\textsuperscript{14}. This was one of the first studies to try to enumerate the bacteria in the GI tract of healthy horses to identify a normal baseline in hopes of trying to parse out the etiology of colic.

Once \textit{C. difficile} began to be identified in association with AAD, especially in human hospitals, several studies sought out to try to culture this organism in horses with diarrhea. In people, about 25% of AAD cases are associated with \textit{C. difficile}, while 75% are due to an unknown etiology\textsuperscript{4}. In the human literature, it has been found that when exposed to pathogenic strains of \textit{C. difficile} without prior antibiotic treatment, the normal, stable GI flora is able to prevent disease. However, both experimentally in hamsters and in people pre-treated with antibiotics, diarrhea developed when exposed to pathogenic \textit{C. difficile}\textsuperscript{21}. Several horses in a veterinary teaching hospital with acute onset diarrhea had fecal cultures and toxin assays performed for \textit{C. difficile}. Nine of ten affected horses cultured positive for this organism compared to
only 1/23 hospitalized without diarrhea. All of the sampled animals were receiving one or more antibiotics and were hospitalized simultaneously. The outbreak occurred over a 2-day period when 23 other horses without diarrhea were also hospitalized. Six different strains of toxigenic *C. difficile* were identified and horses were housed in four separate areas of the hospital. This study was one of the first to correlate hospitalization, antibiotic treatment and diarrhea with *C. difficile* colonization of the GI tract\(^9\). A study in 1997 correlated use of antibiotics and subsequent development of AAD with toxigenic *C. difficile* colonization of the equine GI tract. Forty percent of adult horses that developed acute onset colitis and were treated with antibiotics for disorders other than diarrhea were positive on fecal culture for the organism\(^9\). Weese and colleagues then performed a prospective study culturing feces of adult horses and foals with diarrhea or normal feces for *C. difficile*, *C. perfringens* and ran associated toxin assays. They found that 12.7% of adult horses with colitis and 35.5% of foals with colitis cultured positive while the horses with normal feces were mostly culture negative. Horses with colitis that cultured positive for *C. difficile* and had the toxigenic variant had higher mortality rates\(^18\). *C. difficile* was also found to be associated with the acute, severe colitis that mares develop when their foals are treated with combination erythromycin and rifampin therapy for pneumonia. The affected mares were housed with foals with high fecal concentrations of erythromycin and several of the foals were considered reservoirs of *C. difficile*, as it was identified in their feces\(^4\). Although these studies found correlations between AAD or acute onset
colitis and the presence of toxigenic *C. difficile*, culture-based studies remain limited in their ability to isolate more than one organism underlying development of diarrhea.

Another important point to consider is if cultured organisms are truly causal to diarrhea or if a change in the ecological balance of the intestinal tract is responsible. As a scientific community, this question is just beginning to be investigated by evaluating microbial populations of the GI tract at the community level rather than by selecting to screen for individual organisms. Our traditional view of disease as being caused by a single pathogen has been recently challenged, and a more global view of microbial populations as complex ecosystems has been adopted. Culture is still valuable in identifying specific pathogens, however several of the organisms recognized in more recent molecular based studies are fastidious or at this time, uncultivable. It has been estimated that about 80% of the GI and 70% of oral microbiome are unculturable.\(^{22}\)
1.3 Molecular assessment of gastrointestinal microbiota

Studying the microbiota has developed rapidly over the past decade and recently, different profiles or alterations of microbiota have been found to underlie major diseases in humans\textsuperscript{22-25}. These investigations are in their infancy in veterinary medicine, although studies in several species have been undertaken\textsuperscript{22,26-28}. The microbiota is a collection of different organisms that colonize the multicellular host. A multitude of combinations of organisms inhabit individual sites of the host and contribute to many functions including immunity, metabolism and pathogen resistance. Methods to study the microbiome include targeted approaches such as 16S rRNA gene next-generation sequencing and larger scale approaches including shotgun sequencing and metatranscriptomics\textsuperscript{22,25}. The former tells mostly about microbiome membership, while approaches such as metagenomics can detect functional potential of those members and metatranscriptomics describe active gene expression by those microbes\textsuperscript{25}.

There are multiple methods of next-generation sequencing (NGS) used today for these studies. NGS allows for sequencing of thousands of DNA molecules in parallel at a reduced cost with less manpower compared to the traditional method of Sanger sequencing. Sanger sequencing was first introduced in the late 1970s and was used as the main sequencing method until the early 2000s. It uses a method known as chain termination and produces long, individual sequences. Using a DNA polymerase,
nucleotides and a single-stranded DNA template, four separate sequencing reactions are undertaken adding individual nucleotides: adenine (A), thymine (T), cytosine (C) and guanine (G). The resulting DNA fragments are denatured and separated by gel electrophoresis into four lanes corresponding to each nucleotide (A, T, C, or G). The resultant bands are visualized by UV light and the DNA sequence is read directly from the image. Development of NGS, a more efficient method of sequencing, was driven by the human genome project, as Sanger was the method used for this endeavor in 2001. Several companies were launched shortly thereafter, around 2005, when 454, Illumina and Sequencing by Oligo Ligation Detection (SOLiD) were created. These technologies have evolved and become the three main platforms used in NGS.

Roche’s 454 system was one of the first successful NGS systems that uses pyrosequencing technology to detect nucleotide incorporation. Briefly, DNA is tagged with 454 specific adapters or barcodes to identify the sequences to a specific sample. The DNA is denatured and captured as single strands by amplification beads (one DNA fragment per bead) by emulsion PCR in an oil and water mixture. The beads are loaded onto a picotiter plate and as nucleotides are added to the single stranded genetic material, in the presence of sulfurylase, luciferase, luciferin, DNA 5’ phosphosulfate and DNA polymerase, pyrophosphate is released. The ATP generated from release of pyrophosphate causes luciferin to be transformed to oxyluciferin and generates a light signal. This signal is recorded onto a flowgram, which is then
incorporated into the final readout file. 454 technologies have improved since their introduction in 2005 and now can achieve some of the longest read lengths available in NGS. Read lengths can reach about 700 base pairs with high accuracy and a sequencing run can be completed in one day. Also, several of the steps are now automated, which decreases manpower involved. Disadvantages are cost and decreased number of reads compared to some of the other technologies\textsuperscript{29,30}.

Illumina, previously known as Genome Analyzer (GA) was launched in 2006. This sequencing system uses methodology known as sequencing by synthesis. Briefly, the DNA library is tagged with adaptors to identify the sequences to their specified sample. The DNA is then denatured to single strands and these strands are bound to specific primers present in flowcells on a slide. The genetic material is then amplified via bridge amplification with a polymerase to create clonal libraries or “DNA clusters,” which are then sequenced. Reverse terminator bases with fluorescent labels are added and a camera takes images of the fluorescently labeled nucleotides as they are added in parallel, so thousands of sequences can be read simultaneously. This technology has also improved since its introduction and can sequence thousands of samples at once. It is touted as producing the most sequences for the lowest cost and the company has even developed benchtop sequencers (MiSeq), which offer a more convenient alternative for researchers. Compared to the other technologies, Illumina produces more sequences with shorter read lengths and currently, it can take several days to run samples\textsuperscript{29,30}. 
SOLiD is the third main method of NGS used today that generates thousands of small sequences in parallel. It uses technology known as two-base sequencing and sequencing by ligation. The DNA is fragmented and adapters attached to the fragments to identify them to a given sample. The DNA is amplified by emulsion PCR and the beads with single stranded DNA are deposited on a slide. On a flowcell, DNA libraries are sequenced by 8-base probe ligation. A pool of 8 base nucleotide sequences is labeled according to sequencing position: they contain a ligation site (the 1st base), a cleavage site (the 5th base) and four different fluorescent dyes (linked to the last base). As the probes are ligated onto the sequences, the system reads the color signal the round is repeated. Each sequencing step is composed of five rounds with each round about 5-7 cycles in length with periodic primer resets offset by one base. A fluorescent signal is generated as DNA probes are added and a complete reaction sequences about 25 base pairs. This methodology is highly accurate and sequencing is relatively inexpensive but it also produces shorter reads and takes several days.$^{29,30}$

Despite the methodology chosen for sequencing studies, it has become apparent that consistency is key and there is not yet a gold standard of sequencing or analysis, especially when using fecal material as the DNA source. Changes in integrity of the samples can be introduced at several steps of the process and depending on the chosen methodology, different results may be obtained from the same samples. For example, when collecting fecal samples, it is critical to handle them all in the same
way, making an effort to freeze them as immediately as possible and avoiding multiple freeze thaw cycles\textsuperscript{25}. Although major changes in abundance of taxa were not observed after storing fecal samples at temperatures between -80°C and -20°C over two weeks\textsuperscript{25}, freeze thaw cycles affected overall community structure with different relative abundances of taxa compared to those analyzed immediately after freezing\textsuperscript{31}. DNA extraction protocols, the choice of primers and PCR annealing times may also have effects on the quality of samples and overall diversity profiles. Using widely employed commercial kits for DNA extraction from fecal samples is likely the best way to avoid inconsistencies. Choice of primers depends on the region of interest, although since the phylogenetic information on the 16S gene varies along its length and there is no described gold standard of the region best suited for study of the GI tract, it is again important to stay consistent. The choice of primers targeting a specific region appears to be more important than the length of the sequence obtained\textsuperscript{25}. One study investigating the use of different primers to target disparate regions of the 16S gene found no significant differences in community fingerprints when sequencing V1-V3 versus V7-V9 regions. However, they suggested that these two regions may be the best to target for deep sequencing studies\textsuperscript{32}. Also, longer annealing times and use of error-correcting polymerases in PCR may be important to reduce chimera formation and PCR error and improve quality of samples overall\textsuperscript{25,31}.

As previously mentioned, culture-based studies can demonstrate global shifts in large populations of organisms within different GI compartments, but are not able to detect
the complex changes induced by antibiotics. Newer studies employing NGS methods have identified alterations that were previously undetected by culture-based studies. A good example of this in the human literature involved the investigation of effects of ciprofloxacin treatment on the fecal bacterial community using different methods. First, a culture-based study by Nord and colleagues found that the number of enterobacteria decreased in response to treatment. Another study using denaturing gradient gel electrophoresis in evaluation of the fecal microbiome of hospitalized patients treated with ciprofloxacin found that overall, little change was observed in the fecal microbiome. However, when fecal microbiota of patients treated with a short course of ciprofloxacin (twice daily for five days) was later evaluated by 16S rRNA gene sequencing over a four-week period, major changes were identified. These studies demonstrate the shortcomings of using culture-based studies or targeted sequencing studies to evaluate the microbiota when compared to newer NGS technologies.
1.4 The human gastrointestinal microbiome: effects of antibiotics

In humans, the question of whether antibiotics alter the GI microbiome has been investigated in several prospective, longitudinal studies and several authors have assessed the temporal effects of antibiotic therapy as well. A comprehensive review by Keeney and colleagues highlighted many important findings from multiple studies\textsuperscript{22}. As previously mentioned, studies by Dethlefsen and colleagues showed that both a single course and repeated courses of ciprofloxacin treatment in humans led to significant shifts in the GI microbiome. Although large shifts in composition occurred, the microbiome mostly returned to the pretreatment state four weeks after a single course of treatment, while some taxa failed to return even six months later. Also with significant interindividual variation in GI microbiome composition, the overall effect of antibiotics varied among the treatment group; antibiotics had more significant effects on diversity in two of the three subjects\textsuperscript{35}. In a follow up study by the same group, repeated treatment with ciprofloxacin (two short course treatments twice daily for 5 days) over a ten month period led to even larger shifts in the composition of the GI microbiota, both short and longer term\textsuperscript{36}. There is also evidence that even short-term antibiotic therapy can lead to stabilization of resistant intestinal bacterial populations for years\textsuperscript{16}.

The impact of beta-lactam antibiotics on the GI microbiome has also been investigated. Several studies using either culture based or targeted sequencing
methodologies demonstrated that treatment with amoxicillin with or without clavulanic acid led to an increase in resistant enterobacteria and decrease in aerobic Gram-positive cocci. In general, the overall effects were mild to moderate with populations normalizing to pre-administration levels one week after treatment. Another study using temperature gradient gel electrophoresis to investigate the impact of a five day course of amoxicillin on the fecal microbiome of healthy adults found that most bacterial profiles returned to near normal within 60 days of administration\textsuperscript{16}. Combination antibiotic therapy with clindamycin and metronidazole for treatment of gastric ulceration and \textit{Helicobacter pylori} infection also caused perturbations in the fecal microbiota, some of which persisted for around four years. This study also evaluated the throat microbiota and found that this population was more resilient than the fecal microbiota\textsuperscript{16,37}. As demonstrated above, multiple antibiotics can affect the GI microbiome in different ways even when this is not the target bacterial population.
1.5 The equine gastrointestinal microbiome: effects of disease and antibiotics

The equine fecal microbiome has only just begun to be investigated. It has been shown that adult horses have significant individual variations in their fecal microbiome even when maintained on the same high fiber diet\textsuperscript{38}. However it is clear that they are highly susceptible to GI problems when sudden changes in diet or other factors are introduced. Dougal and colleagues evaluated the bacterial communities of several GI compartments to try to determine if a core microbiome existed, or one that is present in the majority if not all individuals within a population. They found that in proximal compartments like the ileum, fewer taxa dominated in greater relative abundance, while more distally in the large colon, more taxa were present in lower relative abundance. Diversity increased down the length of the large colon from cecum to right ventral colon then declined from there. They suggested that this highly diverse population in the distal GI tract may be more unstable and easily altered leading to multiple disease states, which may explain why horses are so sensitive to small changes in environment and diet and are uniquely susceptible to the development of AAD\textsuperscript{39,40}.

Most equine studies using NGS methods have focused on determining a baseline microbiome and how this bacterial community is altered in multiple disease states such as laminitis, colitis and post-partum colic\textsuperscript{27,41-46}. To the author’s knowledge, only one other equine study to date has evaluated the response of the normal equine fecal
microbiome to antibiotic administration using NGS\textsuperscript{28}. Several studies have reported a baseline fecal microbiome with mostly \textit{Firmicutes} (between 43-69\%) dominating. Abundance of \textit{Bacteroidetes}, \textit{Proteobacteria}, and \textit{Verrucomicrobia} are more variable. Some studies report \textit{Bacteroidetes} as a dominant phylum while others \textit{Verrucomicrobia}\textsuperscript{27,44-47}. In healthy Thoroughbred racehorses in Ireland fed different diets, the phyla \textit{Firmicutes} and \textit{Bacteroidetes} dominated with a ratio of >2:1 in all subjects and accounted for between 73-85\% of all sequences. Nineteen phyla were identified, 12 of which were present in all horses and only five of those were present in relative abundance greater than 0.5\%\textsuperscript{41}. These findings were similar to those of other studies of baseline fecal microbiota\textsuperscript{27,44,46,48,49}. However, it is difficult to generalize the baseline microbiome to various horse populations since these investigations use horses from several regions of the world with differing climates, diets and management practices\textsuperscript{43}.

In most mammalian GI tracts, a higher \textit{Bacteroidetes} to \textit{Firmicutes} ratio is linked to disease states such as obesity and inflammatory bowel disease\textsuperscript{39}. In horses with colitis, healthy individuals had higher proportions of \textit{Firmicutes} while \textit{Bacteroidetes} predominated in colitis cases\textsuperscript{27}. \textit{Verrucomicrobia} increased in horses with chronic laminitis, as did diversity overall\textsuperscript{44}. Costa and colleagues reported a predominance of \textit{Firmicutes} (68\%) in healthy horses with \textit{Bacteroidetes} comprising 14\% and \textit{Proteobacteria} 10\% of the microbiome. However, in horses with colitis, this balance was shifted and the \textit{Bacteroidetes} phylum (40\%) dominated with \textit{Firmicutes} (30\%).
and *Proteobacteria* (18%) following. Healthy horses also harbored more *Actinobacteria* and *Spirochaetes* while horses with colitis had more *Fusobacteria*. Members of the class *Clostridia* were most abundant among healthy horses and this was one of the first publications to provide data to suggest that colitis may be caused by a dysbiosis of the microbiome rather than overgrowth of individual pathogens\(^2^7\). A recent investigation of mares with post-partum colic by Weese and colleagues found that mares that developed colic had lower abundance of *Firmicutes*, *Bacteroidetes* and *Tenericutes* but higher *Proteobacteria*. An increased *Firmicutes* to *Proteobacteria* ratio was also associated significantly with odds of colic, with decreased odds of colic linked to increases in this ratio. The decreased relative abundance of *Firmicutes* and *Bacteroidetes* and increased *Proteobacteria* found in this study associated with colic largely agreed with findings from horses with acute colitis\(^2^7,4^5\). Steelman and colleagues investigated the fecal microbiome of horses in central Texas with chronic laminitis compared to normal horses. *Firmicutes* (69% in controls, and 57% in laminitis) and *Verrucomicrobia* (18% in controls, and 28% in laminitis) dominated in this population of horses while *Bacteroidetes*, *Proteobacteria* and *Spirochaetes* comprised the more minor phyla. Bacterial diversity was highest in horses with chronic laminitis compared to controls and there was large interindividual variation between subjects.

A recent study by Costa and colleagues investigated the effects of systemic antibiotics on the fecal microbiome of healthy horses in Ontario, Canada. Horses were given
three classes of antibiotics by different routes of administration: procaine penicillin intramuscularly, ceftiofur sodium intramuscularly, and trimethoprim sulfadiazine orally for five days. Feces were collected for analysis before and after treatment and at 14 and 30 days of the trial. Oral trimethoprim sulfadiazine caused the most changes in the fecal microbiome, decreasing both richness and overall diversity, with the phylum *Verrucomicrobia* decreasing most significantly. At the 30-day sampling point, the bacterial communities had mostly returned to near baseline. All antibiotics caused alterations in the fecal microbiome. This study was the first to investigate the fecal microbiome of healthy horses in response to antibiotic administration using NGS methods\textsuperscript{28}.
1.6 Investigating the effects of intravenous antibiotics on the equine fecal microbiome

Although a recent study investigated the effects of antibiotics on the healthy horse fecal microbiome, several different classes of antibiotics and routes of administration were used in this study. Additionally, horses were sampled during different seasons and in a more northern climate\textsuperscript{28}. There is still little published about the effects of antibiotics on the equine fecal microbiome, and the subsequent development of antibiotic associated diarrhea. Also, limited data is available regarding if these changes persist over time in horses and how long it takes to identify changes in the microbiome caused by antibiotic treatment. To the author’s knowledge, there is no study reporting the effects of various classes of intravenous antibiotics on the equine fecal microbiome over time.

The purpose of our first study (Chapter 2) was to investigate the effects of two commonly used intravenous antibiotics (ceftiofur and enrofloxacin) on the fecal microbiome of healthy horses compared to saline treatment. We sought to evaluate if changes in the microbiome were induced with three days of treatment and to characterize the baseline microbiota of healthy horses during the winter in central Ohio. We hypothesized that the equine fecal microbiome was complex with a unique structure and that three days of intravenous treatment with standard doses of a cephalosporin and fluoroquinolone antibiotics would cause fecal microbiome alterations.
The purpose of our second study (Chapter 3) was to investigate the effects of three commonly used intravenous antibiotics (ceftiofur, enrofloxacin and oxytetracycline) on the fecal microbiome of healthy horses compared to saline treatment. We sought to compare these results with our first study, to characterize the baseline fecal microbiome of healthy horses in the summer in central Ohio and to determine if five days of treatment induced alterations in the fecal microbiome. We also evaluated if changes persisted over one month after stopping treatment. We proposed that the diversity of the equine fecal microbiome would be reduced by antibiotics compared to saline treatment, that treatment with fluoroquinolone, tetracycline and cephalosporin antibiotics for five days would cause alterations in the fecal microbiome that would persist over 30 days post-treatment.
Chapter 2:

Effects of intravenous fluoroquinolone and cephalosporin antibiotics on the equine fecal microbiome

2.1 Materials and Methods:

Animals

Six healthy castrated male horses (one Appaloosa, three Thoroughbreds, and two Quarter Horses) with a median age of 17 years (range 10-25 years) and a median weight of 535 kg (range 493-579 kg) were included in this study. All horses were housed in standardized conditions and fed the same diet of grass hay for two weeks prior to study inclusion. Horses were considered healthy based on physical examination, hematology, serum chemistry, and fibrinogen concentrations. Horses had no evidence of endoparasitism based on examination of fecal samples. All horses were free of known GI disease, had no history of antimicrobial administration prior to the study, and were up to date on vaccinations and deworming. The Ohio State University Institutional Animal Care and Use Committee approved this study.
Horses were randomly assigned into three groups: group 1 (enrofloxacin, n=2); group 2 (ceftiofur sodium, n=2) and group 3 (0.9% saline solution, control, n=2).

Experiment

A 14 gauge 5½” polyurethane catheter (Mila International, Erlanger, KY) was aseptically inserted in the jugular vein of each horse for antibiotic administration. Horses were administered enrofloxacin (Baytril, Bayer Animal Health, Shawnee, KS; 7.5 mg/kg, IV, q24h [AM] and 30 ml of 0.9% NaCl, IV, q24h [PM]), ceftiofur sodium (Naxcel; Zoetis, Florham Park, NJ; 2.2 mg/kg, IV, q12h), and physiologic saline solution (30 ml of 0.9% NaCl, IV, q12h) for three days. Physical examinations and evaluation of fecal output and consistency were performed twice daily. Antibiotics were given after fecal samples were obtained. Fecal samples were collected from the rectum of each horse via a sterile rectal sleeve every morning, frozen in liquid nitrogen and subsequently stored at -80°C until processing.

DNA Extraction, PCR Amplification and Sequencing

Bacterial DNA was isolated from fecal samples using a commercial kit (QIAamp DNA Stool Mini Kit, QIAGEN, Valencia, CA). DNA quantity and quality (260/280 ratio) was determined by spectrophotometry (NanoDrop, Thermo Scientific, Wilmington, DE). Samples from baseline (time 0, prior to treatment), one day post-
treatment (time 1) and three days post-treatment (time 3) were analyzed by means of pyrosequencing.

Bacterial DNA was amplified using specific primers to the hypervariable region V1-V3 of the 16S rRNA gene using a 454 FLX-Titanium pyrosequencer (Roche, Branford, CT) with modifications as described by one of the co-authors. An approximately 500 bp fragment of the 16S rRNA gene was amplified (HotStart Master Mix Kit, QIAGEN) using 100 ng of DNA and Eubacterial primers specific for most GI bacteria and numbered in relation to Escherichia coli 16S rRNA gene (28F = 5'- GAGTTTGATCNTGGCTCAG - 3'; 518R = 5'-GTNTTACNGCGGCKGCTG - 3'). The forward primer carried the A pyrosequencing adaptor and a multiplex identifier (MID) sequence, while the reverse primer carried the B pyrosequencing adaptor. The following cycling conditions were used: Denaturation at 94°C for three min, followed by 32 cycles of 94°C for 30 seconds, annealing at 60°C for 40 seconds and 72°C for one min; and a final elongation step at 72°C for five min. A secondary PCR was performed to incorporate linker tags as described for multiplexed 454 FLX amplicon pyrosequencing (Roche). Amplified PCR products were purified using Ampure beads (Beckman Coulter, Indianapolis, IN).

**Data Analysis**

Genus-level operational taxonomic unit (OTU) assignments (97% similarity) were
made after adaptor and MID removal, nucleotide trimming and discarding fragments of <200 bp. Sequences with multiple ambiguous base calls were excluded from the analysis. Sequences were analyzed for formation of chimeras by the UChime program \(^51\). Potential chimeras were also excluded from further analysis. Assignments were made to the genus level by alignment (allowing 3% divergence) against a phylogenetically diverse collection of 16S rRNA gene sequences in the SILVA database and classified against the GreenGenes database (http://greengenes.lbl.gov) using MOTHUR \(^52\).

Alpha and beta diversity of each sample were measured using the reciprocal Simpson index and the Yue and Clayton Index of Dissimilarity (\(\theta_{YC}\)), respectively \(^52\). Alpha diversity estimates species richness and evenness and higher values correlate with greater diversity. Beta diversity (\(\theta_{YC}\)) estimates how the composition of fecal microbiota between individuals changes over the treatment period. Non-metric multidimensional scaling (NMDS) was used to compare beta diversity of individual samples. Calculated values are subtracted from 1, with values ranging from 0-1, 0 meaning samples are identical and 1 completely dissimilar. Good’s coverage was calculated to estimate the percentage of total species represented in a sample \(^53\), with a range from 0-1, 1 being 100% representation of species in the sample. This helps relate our sampling effort to the entire population.

Mixed linear models were used to assess how the subjects’ fecal microbiota changed
over time and how this varied by treatment, with treatment type as a fixed categorical effect and horses as subjects in a repeated measurements analysis. The R project software (www.r-project.org/) and SigmaStat 3.5 (Systat Software Inc., San Jose, CA) were used for data analysis. Figures were generated by use of Excel (Microsoft, Bellevue, WA), SigmaPlot 11 (Systat Software, San Jose, CA), and the R project. Results are expressed as means, medians, ranges, and interquartile ranges (IQR). A P value <0.05 was considered significant.
2.2 Results:

*Response to treatment*

There were no changes in physical examination parameters, behavior, appetite, fecal output or consistency throughout the study in any horse, and none of the horses developed clinical evidence of diarrhea.

*Parenteral treatment with enrofloxacin or ceftiofur alters the diversity of fecal microbiota in healthy horses*

The microbiota between individuals was not statistically different at time zero and no statistically significant differences of alpha diversity between or within subjects were identified (data not shown). Although antibiotic treatment did not induce diarrhea, the core phylum composition of the fecal microbiome was altered three days after treatment with antibiotics (Figure 2.1). These effects were most evident in the enrofloxacin treated subjects.

Since the spectrum of bacteria affected by enrofloxacin is different of that targeted by ceftiofur, we compared the effects of each antibiotic on bacterial diversity. Alpha diversity estimates at time points zero and three indicated that there were minimal changes in samples from saline and ceftiofur treated horses at the phylum level.
However, diversity at day three was most altered in horses treated with enrofloxacin (Figure 2.1). *Verrucomicrobia*, *Tenericutes*, and *Proteobacteria* percentages decreased to almost undetectable limits after three days of treatment. On the other hand, *Bacteroidetes* percentage increased after three days of treatment with enrofloxacin in both horses. *Spirochaetes* increased in the enrofloxacin group but decreased in the ceftiofur group. The percentage of the microbiome composed of *Firmicutes* stayed relatively constant (Figure 2.1).

At the 97% similarity level, all samplings had more than 97.7% coverage, which suggests that the majority of diversity was captured by our sampling method (Table 2.1a). This is also reflected by rarefaction curves (Figure 2.2). Beta diversity estimates showed that communities of subjects treated with enrofloxacin were less similar after treatment than control or ceftiofur treated subjects. The microbiota of controls and ceftiofur treated subjects were similar over time (Table 2.1b).

*Changes in microbial OTUs after parenteral treatment with enrofloxacin or ceftiofur*

The average distributions of phyla before and after treatment are shown in Figure 2.1. Thirteen bacterial phyla were found amongst six samples for time zero, with four phyla having abundance of >0.5% and several sequences categorized as unclassified. The median abundances and interquartile ranges were calculated for each time point. At time zero, abundances of the four phyla were: *Bacteroidetes* (29.73%, IQR 25.45,
36.70%), *Firmicutes* (26.85%, IQR 23.42, 32.05%), *Spirochaetes* (0.91%, IQR 0.67, 1.10%) and *Tenericutes* (2.52%, IQR 1.72, 3.48%). After three days of treatment median and interquartile ranges of the top four phyla were as follows: *Bacteroidetes* (41.78%, IQR 35.16, 48.83%), *Firmicutes* (25.0%, IQR 21.73, 26.98%), * Spirochaetes* (2.11%, IQR 1.83, 3.67%) and *Tenericutes* (1.97%, IQR 0.24, 2.67%). The greatest changes were seen in the two major phyla, *Firmicutes* and *Bacteroidetes*

Rarefaction curves demonstrated adequate sampling depth. Samples from enrofloxacin treated horses showed decreased diversity at day 3 in both subjects (Figure 2.2).

Based on NMDS plots (stress = 0.12 in 2 dimensions with an $R^2 = 0.97$; Figure 2.3), samples from the control and ceftiofur groups clustered closely together during treatment, while enrofloxacin treatment caused more divergence from baseline. This indicates greater changes in population diversity in response to enrofloxacin compared to control and ceftiofur samples.
2.3 Discussion:

In this preliminary study, we found that enrofloxacin had the greatest effect on changing the overall diversity of the fecal microbiome over a treatment period of three days in horses in central Ohio. Enrofloxacin is a fluoroquinolone antibiotic effective against a variety of Gram-negative bacteria including enteric pathogens with limited Gram-positive coverage and little activity against anaerobes. It is commonly used in equine practice for various diseases. The relevance of these findings is unknown; however, the magnitude of these shifts was greater in the enrofloxacin treatment group than either the ceftiofur or saline control groups over the treatment period. Three days of treatment was sufficient to demonstrate changes in fecal microbiota and perhaps, longer treatment duration would allow identification of clearer trends.

Several equine studies have concluded that hospitalized horses treated with antibiotics, regardless of reason, are at a higher risk of developing AAD, with *Salmonella enterica, Clostridium difficile* and *Clostridium perfringens* commonly implicated. However, in cases of antibiotic associated colitis, the association with antibiotic therapy is often made presumptively. It is unclear, however, whether these pathogens are the true etiologic agents of colitis or if their isolation represents overgrowth due to dysbiosis and they were previous inhabitants of the GI tract. The “dysbiosis hypothesis” suggests that alterations in microbial community structure and
stability can result in changes in health and be a contributor to disease \(^{55}\). Changes in the GI microbiome that allow pathogens to overcome colonization resistance predispose to the development of severe disease. Several large scale and ongoing human studies have addressed the question of what comprises the baseline community in healthy individuals and how that community changes under the influence of varying disease states \(^{41,52,56}\). Studies have shown that members of the phylum *Proteobacteria* increase in abundance in the GI tracts of clinically ill animals. These organisms are most commonly implicated in clinical cases of AAD. This phylum was a minor component of our study subjects’ fecal microbiota, which is consistent with these horses being free of GI disease, however, subjects treated with ceftiofur had higher numbers of *Proteobacteria* post-treatment.

In agreement with the results of our study, *Bacteriodetes* and *Firmicutes* were the dominant phyla represented in recent equine studies \(^{27,44,48}\). It has been proposed that members of the phylum *Firmicutes* are vital contributors to the ecological community in both herbivores and omnivores, since they are present in the feces of various species in high proportions \(^{23,41,57-59}\). In a study by Costa et al, comparing fecal microbiota of healthy horses and patients with colitis, *Firmicutes* dominated the microbiome of the healthy horses while *Bacteroidetes* was the most abundant phylum in horses with colitis \(^{27}\). Steelman and colleagues found that overall fecal microbiome diversity of horses with chronic laminitis was greater than that of control animals \(^{44}\). Concerns among clinicians about overgrowth of *Clostridia*, a major contributing
member to the *Firmicutes* phylum, are the reason horses with suspected antibiotic-associated colitis are often treated with metronidazole. The *Firmicutes* phylum is composed of several fermenter organisms as well as *Clostridia* and known pathogens. An emerging theory of the importance of *Clostridia* to GI health is gaining credibility with evidence from multiple species that the fecal microbiota is highly dominated by members of the phylum *Firmicutes* in the absence of disease \(^{43,57}\). Our results support this theory, as *Clostridia* remained relatively constant (data not shown) within the fecal microbiome throughout the study period in healthy horses, none of which developed diarrhea. Mice treated enterally with a third generation cephalosporin (cefoperazone) developed severe colitis or were moribund after challenge with oral *Clostridium difficile*, while control mice or those treated with a different antibiotic did not \(^{57}\). This study demonstrated the effect of a pathogenic strain like *C. difficile* on a compromised GI community, which is likely similar to situations in hospitalized patients. It is unclear however, if a baseline concentration of *Clostridia* may be protective to GI health despite its presence in all of our study subjects. Additionally, it seems that most Clostridial species are nonpathogenic.

Our study showed that when antibiotic selection pressures were placed on the sampled flora, changes occurred both within major and minor phyla, after a short course of treatment. It remains unknown if changes in major or minor phyla are the true determinants of disease manifestation. The duration of treatment necessary to cause changes in GI microbiota is also unknown. In equine practice, three days is a
typical minimum treatment course for most antibiotics or disease states, although
treatment interval is often longer. However, it is important to note that changes
demonstrated by our work were evident after only three days of treatment. This is
relevant to equine veterinarians that may see antibiotic associated colitis as early as
one day after instituting antibiotic treatment. Additional studies with longer durations
of treatment and larger sample sizes will provide further and more detailed
information about changes that may occur in equine dysbiosis.

One of the limitations of fecal microbiome studies is the paucity of data comparing
fecal microbiota to that in other segments of the GI tract. A recent study found
enormous variability between horses despite similar dietary and husbandry
management and concluded that fecal samples were not highly representative of the
small or large intestine and that intestinal microbiota were unique to each subject\textsuperscript{60}. However other studies have shown that samples from the right dorsal colon were
similar to those obtained from feces but differed substantially from the right ventral
colon, cecum and ileum\textsuperscript{39,40}. Obtaining samples from other GI compartments comes
with inherent risk, unless performed during an exploratory celiotomy or in terminal
studies. A study by Daly et al in 2001 reported that bacterial composition of luminal
contents and mucosal surfaces were not significantly different. This group used a
different sequencing methodology, and fewer sequences had been identified at the
time of this study, which makes these results difficult to compare \textsuperscript{48}. Therefore,
despite these conclusions, it is feasible that luminal samples alone may provide an
incomplete picture of the GI microbiome

This preliminary study identified changes over a wide taxonomic range in response to antimicrobial administration in healthy horses, with enrofloxacin causing the most alterations in fecal microbiota. This study provided a description of the bacterial phylum structure of fecal samples from normal horses in central Ohio. A more comprehensive understanding of the composition of normal equine GI flora and how antibiotics affect this population will be helpful to clinicians in preventing and treating colitis and intestinal dysbiosis, and perhaps metabolic disorders. In addition, our results could be used to identify susceptible bacterial populations (affected most by antimicrobials) and help to design future clinical studies regarding strategies to avoid overgrowth of pathogenic bacteria. Data presented in this study provide further insight into the fecal microbiota of healthy horses and their response to antibiotic therapy.
Figure 2.1: Stacked bar graphs showing the average percentage of major phyla in the fecal microbiome over time. Day of sampling represented on X-axis next to treatment designation (ie. Ceftiofur0 = Ceftiofur day 0) TM7= candidate division TM7, SR1= candidate division SR1.
Figure 2.2: Rarefaction curves (non-normalized data) displaying species richness in each sample by time period at a) time 0, b) time 1, and c) time 3. Each subject is represented individually. Flattening of the curves indicate that sampling depth was adequate.

(Continued)
Figure 2.2 continued
Figure 2.2 continued
Figure 2.3: NMDS plot of all samples. Pink = ceftiofur treatment, blue = enrofloxacin treatment, black = saline controls. Note that samples from control animals cluster together, while enrofloxacin treatment caused divergence.
<table>
<thead>
<tr>
<th>Subject</th>
<th>Good’s Coverage</th>
<th>Reciprocal Simpson’s Index</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftiofur 1-0</td>
<td>0.988</td>
<td>4.345</td>
<td>3.955 - 4.821</td>
</tr>
<tr>
<td>Ceftiofur 1-3</td>
<td>0.994</td>
<td>4.766</td>
<td>4.316 - 5.321</td>
</tr>
<tr>
<td>Ceftiofur 2-0</td>
<td>0.982</td>
<td>4.655</td>
<td>4.175 - 5.261</td>
</tr>
<tr>
<td>Ceftiofur 2-3</td>
<td>0.994</td>
<td>4.081</td>
<td>3.605 - 4.700</td>
</tr>
<tr>
<td>Enrofloxacin 1-0</td>
<td>0.988</td>
<td>4.081</td>
<td>3.600 - 4.711</td>
</tr>
<tr>
<td>Enrofloxacin 1-3</td>
<td>0.993</td>
<td>2.879</td>
<td>2.573 - 3.276</td>
</tr>
<tr>
<td>Enrofloxacin 2-0</td>
<td>0.988</td>
<td>4.226</td>
<td>3.826 - 4.720</td>
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<td>Enrofloxacin 2-3</td>
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<td>5.114 - 6.268</td>
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<tr>
<td>Saline Control 1-0</td>
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<td>4.271</td>
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<td>Saline Control 1-3</td>
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<td>4.424</td>
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<tr>
<td>Saline Control 2-0</td>
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<td>2.892 - 3.660</td>
</tr>
<tr>
<td>Saline Control 2-3</td>
<td>0.985</td>
<td>3.555</td>
<td>3.237 - 3.941</td>
</tr>
</tbody>
</table>

Table 2.1: Reciprocal Simpson’s Indices (alpha diversity estimates) and Good’s coverage values of all subjects at time points 0 and 3. 1-0 = subject 1, time 0.
Table 2.2: Yue and Clayton values (beta diversity estimates) comparing times 0 and 3 for each treatment group. 95% confidence interval is shown. 1-0 = subject 1, time 0.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Θ YC values</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftiofur 1-0</td>
<td>Ceftiofur 1-3</td>
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<tr>
<td>Ceftiofur 2-0</td>
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<td>Enrofloxacin 2-0</td>
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<tr>
<td>Saline Control 2-0</td>
<td>Saline Control 2-3</td>
<td>0.079</td>
</tr>
</tbody>
</table>
Chapter 3:

Longitudinal effects of antibiotic treatment on the equine fecal microbiome

3.1 Materials and Methods

Animals

Sixteen horses were used for this study with a mean and median age of 13.9 and 14 years old, respectively (standard deviation 4.2 years, range 8-24 years old). Five mares and eleven geldings were used, and breeds represented included Appaloosa (n=1), Warmbloods (n=4), Thoroughbreds (n=5), Standardbreds (n=3), Quarter horses (n=3) and Saddlebred (n=1).

All horses were housed in standardized conditions and fed the same diet of grass hay for a minimum of three weeks prior to study inclusion. Horses were considered healthy based upon physical examination, hematology, serum chemistry and fibrinogen concentrations. Horses had no evidence of endoparasitism based on examination of fecal samples. All horses were free of known GI disease, had no history of antimicrobial administration prior to the study, and were up to date on core vaccinations and deworming. The Ohio State University Institutional Animal Care and Use Committee approved this study.
Horses were randomly assigned into four treatment groups: group 1 (enrofloxacin, n=4); group 2 (ceftiofur sodium, n=4); group 3 (oxytetracycline, n=4); group 4 (0.9% saline solution, control, n=4). All treatments and sampling were performed at the same time of year in the same conditions by two investigators.

**Experiment**

A 14 gauge 5½” polyurethane catheter (Mila International, Erlanger, KY) was aseptically inserted in the jugular vein of each horse for antibiotic administration. Blood was drawn from each horse for complete blood count and serum chemistry analysis before treatment and at the end of the treatment period. Horses were administered enrofloxacin (Baytril, Bayer Animal Health, Shawnee, KS; 7.5 mg/kg, IV, q24h [AM] and 30 ml of 0.9% NaCl, IV, q24h [PM]), ceftiofur sodium (Naxcel; Zoetis, Florham Park, NJ; 2.2 mg/kg, IV, q12h), oxytetracycline (Oxytetracycline Injection 200, Norbrook Inc. USA, Lanexa, KS; 6.6 mg/kg, IV, q24h) and physiologic saline solution (0.9% sodium chloride solution, Baxter, Deerfield, IL; 20 ml, IV, q12h) for five days. Physical examinations and evaluation of fecal output and consistency were performed twice daily. Antibiotics were given after fecal samples were obtained. Fecal samples were collected from the rectum of each horse via a sterile rectal sleeve every morning, frozen in liquid nitrogen and subsequently stored at -80 °C until processing.
DNA Extraction, PCR Amplification and Sequencing

Samples from baseline (time 0, prior to treatment), one, three, five and 30 days post-treatment (times 1, 3, 5 and 30, respectively) were analyzed. Bacterial DNA was isolated from fecal samples using a commercial kit (QIAamp DNA Stool Mini Kit, QIAGEN, Valencia, CA). DNA quantity and quality (260/280 ratio) was determined by spectrophotometry (NanoDrop, Thermo Scientific, Wilmington, DE).

Bacterial DNA was amplified using specific primers to the hypervariable region V1-V3 of the 16S rRNA gene using a 454 FLX-Titanium pyrosequencer (Roche, Branford, CT) with modifications as described by Sun et al."50. An approximately 500 bp fragment of the 16S rRNA gene was amplified (HotStart Master Mix Kit, QIAGEN) using 100 ng of DNA and Eubacterial primers specific for most GI bacteria and numbered in relation to Escherichia coli 16S rRNA gene (28F = 5’- GAGTTTGATCCTGAGGCTCAG-3’; 518R = 5’-GTNTTACNGCGGCGTGGCCTG -3’)50. The forward primer carried the A pyrosequencing adaptor and a multiplex identifier (MID) sequence, while the reverse primer carried the B pyrosequencing adaptor. The following cycling conditions were used: denaturation at 94°C for three minutes, followed by 32 cycles of 94°C for 30 seconds, annealing at 60°C for 40 seconds and 72°C for one minute; and a final elongation step at 72°C for five minutes. A secondary PCR was performed to incorporate linker tags as described for multiplexed 454 FLX amplicon pyrosequencing (Roche, Branford, CT). Amplified PCR products were purified using Ampure beads (Beckman
Coulter, Indianapolis, IN).

**Data Analysis**

Genus-level operational taxonomic unit (OTU) assignments (97% similarity) were made after adaptor and MID removal, nucleotide trimming and discarding fragments of <200 base pairs. Sequences with multiple ambiguous calls were excluded from the analysis. Sequences were analyzed for formation of chimeras by the UChime program. Potential chimeras were also excluded from further analysis. Assignments were made to the genus level by alignment (allowing 3% divergence) against a phylogenetically diverse collection of 16S rRNA gene sequences in the SILVA database (http://www.arb-silva.de) and classified against the GreenGenes database (http://greengenes.lbl.gov) using MOTHUR (http://www.mothur.org).

In an attempt to decrease bias introduced by differences in sequence depth and number from samples, a subsample from the dataset was used to calculate diversity indices. Alpha diversity calculations estimate the richness and relative abundance of taxa in an individuals’ fecal microbiome and higher values correlate with greater diversity (values ranging from 0-1, 1 indicating maximum diversity of a sample). Beta diversity estimates bacterial diversity among different samples and how the composition of the fecal microbiome changes between individuals over the treatment period. Alpha and beta diversity of each sample were calculated using Simpson’s Index of Diversity and the Yue and Clayton Index of Dissimilarity ($\theta_{YC}$), respectively. Calculated values of $\theta_{YC}$ are
subtracted from 1, with values ranging from 0-1, 0 meaning samples are identical and 1 completely dissimilar (data not shown). Dendrograms were generated using $\theta_{YC}$ values in FigTree (version 1.4.2, http://tree.bio.ed.ac.uk/software/figtree/). Good’s coverage was calculated to ensure adequate representation of subsamples and to estimate the percentage of total species represented in a sample, with a range from 0-1, 1 being 100% representation of species in the sample. Rarefaction curves were constructed from non-normalized data. Richness was estimated using the Chao 1 index, which estimates the number of rare OTUs found in a sample. These calculations helped relate our sampling effort to the entire population (Table 1).

The relative proportion of four major taxa were transformed using the arcsine square root, and used as independent variables in four separate generalized mixed models (PROC MIXED, SAS, v. 9.4, Cary, NC). The dependent variables for time, treatment and their interaction were forced into each model, and the model residuals subjectively assessed for normality. To account for repeated sampling of the same horses over time, horse was included as a repeated statement. Pairwise comparisons of the least squared means of the transformed data were used to determine the statistical significance of changes in the relative proportion of the four major phyla over time. Analysis of molecular variance (AMOVA) was used to detect differences between groups by antibiotic treatment and time. The R project software (http://www.r-project.org/), Excel (Microsoft, Bellevue, WA) and MOTHUR (http://www.mothur.org) were used for data analysis and figure
generation. Results are expressed as medians and ranges. A P value <0.05 was considered significant.
3.2 Results

Response to treatment

There were no changes in physical examination parameters, behavior, appetite or fecal output or consistency throughout the study in any horse, and none of the horses developed clinical evidence of diarrhea. There was normal variation in character of feces daily and no consistent patterns of change were noted. No significant endoparasitism was detected. Baseline complete blood count and serum chemistry profiles were within normal limits.

Phylum composition of fecal microbiota before, during and after treatment

Sixty-four samples were analyzed, with four samples from each subject submitted for analysis. Sequences were assigned to 18 phyla with an average of 19.9% of sequences remaining unclassified at the phylum level. Good’s coverage estimates (mean 97%, standard deviation 0.5%) and rarefaction curves after subsampling verified adequate coverage of diversity in all samples. The core microbiome\textsuperscript{27,39} was composed of 9 phyla and several unclassified phyla. These phyla included \textit{Actinobacteria}, \textit{Bacteroidetes}, \textit{Cyanobacteria}, \textit{Firmicutes}, \textit{Plantomyces}, \textit{Proteobacteria}, \textit{Spirochaetes}, \textit{Tenericutes} and \textit{Verrucomicrobia}. Other phyla were present inconsistently and in fewer samples in small proportions. There was no significant effect of treatment or time on overall diversity.
(alpha and beta diversity) over the treatment interval in the studied population of healthy horses. Relative abundances of bacteria at the phylum level are represented in figure 3.1. The major phyla represented in our samples included Bacteroidetes, Firmicutes, and unclassified. Minor phyla present included Proteobacteria, Verrucomicrobia and Spirochaetes. Similar to a pilot study (unpublished) treatment with enrofloxacin lead to decreases in Verrucomicrobia, Tenericutes and Proteobacteria while Spirochaetes decreased in response to ceftiofur treatment.

There were statistically significant changes in community structure at the phylum level over time (figure 3.2). No significant differences in diversity were detected between samples at the start of the study (baseline) although each sample had an individual profile of diversity that was independent from other samples. All samples analyzed were highly diverse (Simpson’s Index of Diversity >0.85). Proteobacteria decreased significantly after treatment with ceftiofur, oxytetracycline and enrofloxacin, and no significant decreases were identified in the control group. The major phyla, Firmicutes and Bacteroidetes, varied most after ceftiofur treatment, and significant changes were identified in Firmicutes alone after enrofloxacin treatment. Abundance of Verrucomicrobia, a minor phylum in our population, changed significantly in response to oxytetracycline and enrofloxacin treatment from the beginning to end of treatment and remained different from baseline at time 30 (figure 3.2). Notably, several changes were identifiable after five days of treatment. The Chao 1 estimates of richness demonstrated
that richness decreased in general with antibiotic treatment but mostly returned to baseline after cessation of antibiotics (table 3.1).

Beta diversity varied over time with some treatments associated with persistent alterations 30 days post treatment. There were trends in how the population structure changed as visualized on the phylogenetic tree (figure 3.3). Several subjects’ microbiota were more related after 30 days of treatment, while some treatment groups clustered together after three or five days of treatment. For example, after three and five days of treatment, samples from horses treated with enrofloxacin were more related than before treatment. Similar trends were noted after treatment with ceftiofur. Using AMOVA to compare groups based on Yue and Clayton values by treatment, population structures from groups treated with ceftiofur and oxytetracycline were significantly different from the placebo group. All antibiotic treated groups had significantly different population structures from one another. Also, all populations were significantly different from one another at all time points except between times 3 and 5 post-treatment.
3.3 Discussion

Fecal microbiota of horses in central Ohio was diverse and as expected, changes occurred in all groups in response to antibiotic treatment. Five days of treatment was sufficient to identify these changes and some phyla remained affected 30 days after treatment began, while others returned to near baseline levels. Overall, diversity of the fecal microbiome was affected by all antibiotics as compared to saline treatment in various ways. Both major and minor phyla were altered with most significant changes observed after ceftiofur and enrofloxacin treatment. Although changes in microbiota were expected in response to antibiotic treatment, the differences and persistence of these variations were notable.

The fecal microbiota of healthy horses in our population was similar to that noted in other studies, with *Firmicutes* and *Bacteroidetes* dominating\(^{27,40,42,44,47,48}\). *Verrucomicrobia* was a major phylum in several equine fecal microbiome studies\(^{28,44,45}\), however, this phylum was a minor component in our population of horses. This could be due to variation in the time of the year when samples were taken or as a result of dietary or regional differences. *Proteobacteria* was also a minor phylum in our study but varied significantly in all antibiotic treatment groups. This phylum is of particular importance since it contains several bacterial pathogens often associated with GI disease such as *Salmonella, Escherichia coli* and *Helicobacter*. 
The most significant antimicrobial effects were noted after treatment with ceftiofur and enrofloxacin, although changes were also seen with oxytetracycline. Both major and minor phyla were affected in various ways. The phyla most significantly affected by antibiotics were *Proteobacteria*, *Verrucomicrobia*, *Firmicutes* and *Bacteroidetes* (Figure 3.2). Several changes, especially in these phyla, were still present 30 days after the study began. A recent study in horses and studies in humans also showed that several changes in fecal microbiota persist after antibiotic treatment\(^{22,28}\). Human studies have shown that a single treatment with ciprofloxacin, a commonly used fluoroquinolone antibiotic, lead to major shifts in fecal bacterial composition and although the microbiota mostly reverted to baseline after four weeks, some OTUs remained absent longer term\(^{35,36}\). Repeated administration of this antibiotic caused even greater changes, however, as observed, both in our study and a recent study by Costa and colleagues using healthy horses. Despite these changes, diarrhea did not develop\(^{28,36}\). This emphasizes the importance of the bacterial microbiome as part of a larger system that can have normal basic functions and resilience even in the face of changing microorganisms\(^{62}\).

It remains unclear however, which changes are necessary to lead to the development of AAD. Also, without deeper sequencing and studying the function of these microorganisms (ie. metabolomics, transcriptomics) we can only make inferences about how and why diarrhea develops and population dynamics go awry. Loss of *Clostridial* species from the fecal microbiome has been implicated as a cause of diarrhea in people\(^{22}\) while infection with pathogenic *Clostridia* has been shown to cause diarrhea and even
death in mouse models\textsuperscript{57}. It is clear that colonization resistance is important in preventing these alterations from causing disease, and understanding the baseline microbiome in healthy subjects may help us deduce how this delicate balance can be overcome leading to disease\textsuperscript{15,63}.

Several studies have analyzed the equine fecal microbiome in disease states. Steelman and colleagues found that diversity of the fecal microbiome was increased in horses with chronic laminitis compared to controls\textsuperscript{44}. Costa and colleagues studied hospitalized horses with colitis and found that \textit{Bacteroidetes} dominated the fecal microbiome of horses with disease, while abundance of \textit{Firmicutes} was greater in healthy horses\textsuperscript{27}. Relative abundance of \textit{Proteobacteria} was increased in post-partum mares with large colon volvulus compared to controls\textsuperscript{45}. Recent human studies have shown that broad-spectrum antibiotics can result in significant decreases in \textit{Bacteroidetes} with concomitant increases in \textit{Firmicutes} \textsuperscript{64} and that multiple diseases are a result of a significant reduction in bacterial diversity\textsuperscript{22}. In our study, the trend of decreasing \textit{Bacteroidetes} and increasing \textit{Firmicutes} was evident during the treatment period with ceftiofur, however this was not true for the other treatments and none of our subjects developed signs of disease.

The antibiotics, doses and frequencies of administration used in this study were chosen based upon common equine practitioner use in the field and anecdotal association of these medications with the development of AAD. A multi-institutional retrospective study of horses with AAD showed that monotherapy with enrofloxacin was the most
common treatment associated with development of diarrhea however, tetracycline and cephalosporin antibiotics were also implicated in other cases of AAD\textsuperscript{12}. All antibiotics in our study were given intravenously and diets, as well as environment and management factors were controlled. All horses were sampled during the same time of year in the same environment. These efforts were made in an attempt to control for confounders that may influence our data throughout sampling and to try to minimize pre-sampling inter-individual variations. Tetracycline and fluoroquinolone antibiotics are excreted into the GI tract and reach high concentrations in this compartment. Although ceftiofur is not specifically excreted into the GI tract (GIT), it has been shown to induce changes in luminal bacteria populations\textsuperscript{65}. Differences in population structure were calculated using Yue and Clayton’s index of dissimilarity, which was visually represented as a phylogenetic tree (figure 3). Several of the samples at baseline were closely related and after treatment, both individual and group differences could be seen. For example, it is interesting to note that after three and five days of treatment, several of the ceftiofur treated samples were more related than before treatment. Also notable were the number of samples clustering closely together at baseline (eight total) and that many were closely related 30 days post-treatment. This may reflect a reversion back to near baseline despite treatment. It would be interesting to investigate if this relatedness increased after more time post-treatment.
The observed significant changes in relative abundances of phyla in the ceftiofur and enrofloxacin treatment group emphasize the potential for these antibiotics to precipitate disease states. Intriguingly, after five days of treatment with ceftiofur *Proteobacteria* reduced from 5.5% to 0.93% while *Verrucomicrobia* increased from 0.6% to 4.3%. Functionally the implications of these changes are unclear, although intuitively it would seem that reducing a major pathogen-containing phylum and increasing numbers of other phyla in its place would be advantageous to the host, however the function of *Verrucomicrobia* is mostly unknown. The reduction in some phyla allow for proliferation of other microorganisms and vice versa. In the human literature, even treatment with the same antibiotic at an identical dose and frequency can lead to conflicting results.\(^6\) Thus, it remains difficult to predict how antibiotics will alter the microbial population of the GIT, due to a paucity of data, vast differences in methodology, and the rapidly advancing knowledge in this field. Also, individual and regional differences in microbiota make it difficult to compare results across studies.

Limitations of this study include the relatively small number of subjects and thus smaller sample size than ideal. This may influence our ability to detect statistical trends with more certainty due to limited statistical power. Despite this, some statistical differences were detected and other trends identified. Additional sampling time points would have been useful to more precisely detect when populations began to revert back to baseline and to see if following the subjects further after treatment allowed more subjects’ microbiota to return to baseline. Also, as noted in most studies of GIT microbiota,
sampling of other sites in the GIT would be useful to detect where the greatest shifts in composition are occurring. Although fecal samples are most convenient and least invasive for sampling, they may only reflect changes in the distal intestinal tract.\textsuperscript{39,40,60}

In conclusion, enrofloxacin and ceftiofur treatment were associated with alterations in the fecal microbiome, some of which persisted weeks after treatment cessation. The microbiota was diverse and changes were evident after three and five days of treatment. Compared to placebo treatment, antibiotics caused more changes in the microbiome over time. Larger scale studies with more frequent sampling may lend more insight into these trends and if populations return to baseline over longer time periods.
Figure 3.1: Relative abundances of phyla at each time point by treatment, saline the control. Day 0 is baseline (before treatment) and day 5, the final day of treatment.
Figure 3.2: Bar graphs of median percentages of major phyla by treatment group (with 95% confidence interval). Brackets with asterisks represent significant differences (P<0.05)
Figure 3.3: Phylogenetic tree showing relatedness between populations based upon Yue and Clayton analysis. Cef= Ceftiofur (red), Enro= Enrofloxacin (green), Oxy= Oxytetracycline (brown), Sal= Saline (control, blue). The number following the name represents the subject (1-4) and the second number represents the sampling time (ie. time 0, 3, 5 or 30).
<table>
<thead>
<tr>
<th>Time point (days)</th>
<th>Treatment</th>
<th>Simpson's Index of Diversity (1-D)</th>
<th>Good's coverage</th>
<th>Chao 1 Index</th>
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<tr>
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<td>44.77</td>
</tr>
<tr>
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<td>0.98</td>
<td>40.15</td>
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<td>0.98</td>
<td>44.59</td>
</tr>
<tr>
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<td>0.97</td>
<td>48.94</td>
</tr>
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<td>Saline</td>
<td>0.89</td>
<td>0.98</td>
<td>40.38</td>
</tr>
</tbody>
</table>

Table 3.1: Average alpha diversity indices and Good’s coverage values by treatment and time.
Chapter 4: Summary

The fecal microbiome of horses in central Ohio was diverse despite controlling for diet, husbandry and season during which the studies were performed. Even with treatment with antibiotics commonly implicated in the development of AAD, all of our subjects remained healthy and none developed diarrhea. However, treatment with antibiotics caused shifts in major and minor phyla within fecal samples over short and longer time periods. In both studies, enrofloxacin caused the most changes in the microbiome, and *Proteobacteria* was reduced in response to treatment with all antibiotic classes. These studies were the first to demonstrate changes in the equine fecal microbiome of healthy horses in response to intravenous antibiotic therapy.

Although several changes in the composition of the equine fecal microbiome were identified, the functional implications of such changes are still unknown. Since none of our subjects developed diarrhea, we still have much to learn about which changes underlie the development of AAD. Previous studies in horses and other species have identified a shift in the *Firmicutes* to *Bacteroidetes* ratio as underlying diarrhea, with *Firmicutes* dominating in healthy animals and *Bacteroidetes* dominating in diarrheic horses\textsuperscript{27}. *Proteobacteria* was higher in post-partum mares with colic\textsuperscript{45}. In our studies,
horses treated with enrofloxacin showed trends of decreasing *Firmicutes* and increasing *Bacteroidetes*, although none developed disease. With more longitudinal studies, more sampling time points and further study of diarrheic horses, we hope to determine if dysbiosis truly underlies the development of AAD and how we might prevent and treat such a devastating complication.
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