EVALUATION OF IN VITRO AND IN VIVO EQUINE MICROBIAL POPULATION SHIFTS IN RESPONSE TO COLIC CONDITIONS

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ERIN VENABLE

Dr. Monty S. Kerley, Dissertation Supervisor

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EVALUATION OF IN VITRO AND IN VIVO EQUINE MICROBIAL POPULATION SHIFTS IN RESPONSE TO COLIC CONDITIONS

ERIN VENABLE

Dr. Monty Kerley, Dissertation Supervisor

ABSTRACT

Colic has been identified as the second leading cause of death in horses, led only by natural processes (Hines, 2010). Although many factors have been identified as causative, (Hudson et al., 2001; Reeves et al., 1989, 1996; Kaneene et al., 1997; Cohen and Peloso 1996; Proudman 1991), there is great variation in the published reports regarding those factors.

Undigested starch entering the cecum is one of the aforementioned causative agents (Al Jassim, et. al., 2009). Once a colic episode has occurred, a common industry practice is to withhold feed and water. Little is known about the effects of this "starvation" upon the bacterial community in the cecum. The purpose of this research was to examine the effects of starch dosage and starvation upon equine fecal micro flora. To test these effects, we collected fecal material from six mature mares housed at the University of Missouri Equine Farm. The mares were 11.5 years (± 6.5 years), with a BCS of 5 (± 1) and weighed 1,210 lbs (± 110lbs). Fecal innoculum was used to dose 24 single flow effluent continuous culture fermenters with 1,460 mls of 4:1 buffer/feces

mixture (Slyter, 1990). Each independent fermenter was submerged in a 39◦C water bath. The fermenters were outfitted with ports such that carbon dioxide and buffer were continuously infused. The fermenters were designed with ports that allowed for the addition of treatment diets, to take liquid samples and to monitor pH. A liquid dilution rate of 4% was maintained throughout. Each run utilized four diets to investigate treatment effects on VFA and NH3 concentration, and pH.

To examinine effects of starvation (STRV), dietary treatments were, Purina Equine Sr. (Sr.), Sr. + FOS 0.1% (FOS), Sr. + MOS 0.1% (MOS), and Purina Well Gel (WG). Each diet was ground to 3 mm for uniform particle size and fermenters were fed 20 g every 12 hours. A liquid dilution rate of 4% was maintained. Fecal material and McDougall's buffer was used in a ratio of 4:1 to dose the fermenters (1,460 mls of inoculate). An acclimation period of six days was allowed, followed by 48 hours of starvation. Samples were then collected for the next 48 hours. Data were analyzed using the PROC MIXED procedure of SAS.

Total volatile fatty acid (VFA) concentrations were not affected by treatment ($P > 0.05$). However, isobutyric and isovaleric concentrations were significantly ($P < 0.05$) greater for the WG treatment. Additionally, butyric acid was significantly lower ($P < 0.05$) for treatments other than the basal diet, (Sr.). Ammonia concentration for the WG treatment was greater ($P < 0.05$) than those of the Sr., FOS, or MOS treatments. The pH levels for the WG diet were significantly higher (pH 6.36) than other treatments ($P < 0.05$) and therefore closer to neutral. This may be due to the lower total starch content of the WG treatment.

This research indicated that equine microflora are susceptible to shift following a period of starvation and that a specially designed therapeutic diet (i.e. WG) may provide increased levels of some branched chain amino acids and ammonia for the cecal microbial population.

To examine the effects of starch overload on the fermentation system (STRCH), dietary treatments were Purina Equine Sr. (Sr.), Sr. + FOS 0.2% (FOS), Sr. + FOS 0.1% +MOS 0.1% (FM), and Purina WG. Each diet was ground to 3 mm for uniform particle size and feedings of 20 g/fermenter occurred 12 hours apart each day. The same protocols were followed for dilution rate settings and inoculation of fermenters as described previously. Six days of acclimation were allowed for stabilization of the microbial community. On the morning of the seventh day, the 8 am feeding was replaced with 20 g of a 21.3% starch commercially available textured horse feed (Country Acres Horse Sweet 12, Purina Mills). The 8:00 pm feeding was withheld and the treatments were started 24 hours following the starch dosage and occurred immediately prior to sampling. Data were analyzed using the PROC MIXED procedure of SAS.

Total VFA concentrations were affected ($P < 0.05$) by diet. The Sr. and FM diets both resulted in higher total VFA concentrations than the WG diet. Isobutyric concentration was higher ($P < 0.05$) for the WG and FOS treatments, and isovaleric concentration was higher ($P < 0.05$) for the WG treatment. Again, we observed higher ammonia levels ($P < 0.05$) in the WG treatment group. Additionally, the previously observed higher pH values were repeated for the WG treatment as compared to other diets ($P < 0.05$).

This research indicated that following an abrupt change in the starch content of the diet, branched chain amino acid, and ammonia production may be affected by treatment. However, further research is needed, possibly using cecally cannulated horses, to determine if in vivo response can be achieved utilizing similar treatments and methods.

Little work has been done to identify changes in microbial populations during and following a colic episode. We performed 454 pyrosequencing (Roche Diagnostics, Indianapolis, IN) in order to phylogenetically characterize the shifts in the microbial populations observed in the feces of eight horses collected both during a colic episode and following a return to health.

Rectal grab samples were collected from eight horses admitted to veterinary schools for colic. Guidelines for admission to the study included large intestine, non-surgical colic cases. Follow up samples (i.e. "healthy") were collected via rectal grab by the attending/referring veterinarian 60 days (± 30) post discharge. Microbial DNA was extracted from the fecal samples and the V1, V2, and V3 regions of the 16S rDNA were amplified using primers suitable for pyrosequencing via emulsion PCR.

Our data set was comprised of 1, 241, 844 reads. Unassigned reads (no assigned operational taxonomic unit), were approximately 65% and were omitted from analysis. Reads were analyzed utilizing BLASTn 2.2.23+ (Basic Local Alignment Search Tool) and MEGAN (MEtaGenomics ANalyser) for identification and classification (Huson et al., 2007; Altschul et al. 1990).

More than 300 individual taxa were identified. Data were analyzed utilizing a Monte Carlo randomization approach and a Bonferroni correction to prevent undue numbers of false positives (R Core Development Team, 2008). Of these taxa, 19 were

unique to the colic state, and 10 were only present in the healthy state. The unique taxa identified in the healthy horses included representatives of the phyla Firmicutes (5), Bacteroidetes (3), Proteobacteria (1) and Fusobacteria (1). The unique taxa found in the diseased horses included microbes representing the phyla Firmicutes (11), Proteobacteria (6), Bacteroidetes (1), and Lentisphaerae (1).

It is interesting to note that across our samples some horses exhibited no significant difference within any of the identified taxa. Conversely, some horses had multiple significant taxa identified. When data was screened for microbes that were ubiquitous to both healthy and colic states, there were 22 that were identified. When those 22 were examined by ranking read numbers and pairing thusly, healthy > colic, 17 were significant ($P < 0.05$). Conversely, when ranked and paired colic > healthy, four were significant ($P < 0.05$). All sick horses had greater reads of Clostridium phytofermentans, an uncultured clostridiales bacterium, and Bacteroidetes as well as an uncultured bacteroidetes bacterium.

These data are the first to elucidate specific changes within the microbiome of the equine gastrointestinal tract during a colic episode. Further research is needed in order to understand how to prevent these dramatic population shifts, and facilitate a faster recovery upon occurrence of same.

CHAPTER 1

REVIEW OF LITERATURE

INTRODUCTION

The equine is an animal that enjoys a unique status within the world of animal science. Although legally classified as "livestock" (Missouri Revised Statutes, 2010), they are not produced for human consumption domestically, and thus tend to be lumped into the more general category of "companion animals". Millions of horses are owned in the U.S. purely for recreational purpose and serve no economic purpose for their owner. This is in sharp contrast to the production of cattle, swine, and dairy. These industries must employ management practices based on cost: benefit ratio if they are going to be successful.

Thus, it is not surprising that the body of knowledge surrounding other domestic animals is far more advanced with regard to nutrition than that of the equine. Since this is an animal that is primarily supported with disposable income, there is simply not the funding for research that is associated with food animals. However, advances are being made in equine nutrition and the NRC (2007) provides the latest comprehensive summary of nutritional research in equine.

Digestive Physiology

The digestive physiology of the equine would classify it as a non ruminant herbivore. As such, great care should be taken when feeding and managing this animal. Often times the value of the horse swings between the two extremes of family member

and competitive athlete. This makes nutritional recommendations very diverse and challenging. Much research has been focused on nutritional strategies and developments of feeding protocols to facilitate greater longevity for horses. Although many factors affect the nutritional requirements of the animal (age, lifestyle, reproductive status, genetic tendencies, etc), similar care should be given to all horses to minimize and avoid gastric upset or colic.

The stomach of the horse is relatively small, holding only about 8 L (Lawrence and Raub, 1995). The stomach of the horse is the primary site of protein digestion but it is also known that microbial colonization and fermentation can take place there as well (Yuki et al., 2000). However, the extent of the fermentation that takes place there is still unclear.

The small intestine in the horse is the site of primary macro nutrient digestion via enzymes. Fat, protein, as well as vitamins and minerals are all absorbed pre-cecally. Because of the location of the small intestine relative to the hindgut, the benefits of any crude protein generated via microbial fermentation are believed to be insignificant to individual amino acid requirements (Miller, 1997).

Feed processing and botanical origin of ingredients appears to greatly affect overall digestibility (Respondek et al., 2008; Hill, 2007; De Fombelle et al., 2003; Cuddeford, 1999). Additionally, digestibility is reduced when intake is increased (Potter et al., 1992, Kienzle, 1994). Therefore, feeding recommendations should always include multiple small meals in order to avoid large amounts of undigested starch arriving at the hindgut undigested. This has been shown to trigger an increase in lactic acid producing bacteria (Lawrence and Raub, 1995) and laminitis (Kronfeld and Harris, 2003,

Milinovich et al., 2006). Unfortunately, even under the best management practices, colic and gastric upset still occur.

The hindgut is the primary site of microbial fermentation in the horse. Its architecture is similar to that of the rumen in the steer, but its location relative to the small intestine means that the microbial protein that is produced via fermentation is not readily absorbed. Although the digestion and absorption of any significant amount of microbial protein are still being investigated, it is generally believed that any contribution by microbial protein to the animal's amino acid requirement is minor (Miller, 1997; Gibbs et al., 1988). Therefore, it is crucial to provide high quality amino acids to the horse in a form that is easily digestible pre-cecally.

Colic

Equine colic has been identified as the number one cause of emergency treatment and subsequent death of horses in the U.S. (Durham et al., 2009). While it is not a disease, per se, it is a condition that often requires medical or surgical intervention. It is defined simply as "abdominal pain of digestive origin" (Goncalves et al., 2002) believed to be caused by distention of the stomach or intestines (Kaneene et al., 1997). Reeves (1997) referred to colic as "the ultimate multi-factorial disease". Management of these horses is often confounded by the similarity of the conditions which may present the same symptoms.

Risk factors have been examined and identified including an overload of or treatment for parasites, impaction, geography, surgery, recent change in diet, starch overload, and recent change in forage (Cohen et al., 1999; Cohen, 2003; Goncalves et al., 2002; Shirazi-Beechey, 2008). Of these, a change in forage has been most often

associated with colic (Cohen et al., 1999). However, there are inconsistencies regarding risk factors in these published reports.

In 1998, the annual incidence of colic was identified as 4.2% of the total equine population(Hines, 2010), and it seems to be indifferent as it impacts all breeds, both genders, all ages, and all types of horses. Economic impacts assessed prior to 2000 were greater than $115 million annually in the U.S. alone (Durham et. al, 2009). Not only is the financial burden difficult to bear, but the necessary adjustments to management of the horse with colic often wreak havoc on training schedules or may be impossible for the recreational owner. Typical recommendations can include a return to a diet more like that of their feral ancestors, i.e. high fiber, low starch). Often, horses predisposed toward colic episodes must eat very small meals multiple times a day.

Colic may be classified into two groupings: gastrointestinal and nongastrointestinal (Abutarbush et al., 2005). Physical examination may provide evidence that can rule out non-gastrointestinal cases. Gastrointestinal cases are evaluated based on severity of pain, cardiovascular/systemic status, trans rectal palpation, nasogastric reflux, and abdominocentesis (Abutarbush et al., 2005). Only a small fraction of colic cases presented require surgical intervention (Hines, 2010; Abutarbush et al., 2005; Mair et al., 2007).

Fructooligosaccharides /Mannanoligosaccharides

Because of the variety of risk factors for colic, it seems an insurmountable task to try to find a way to prevent its occurrence. However, there is much work being done to facilitate a faster recovery for the colic prone horse. Currently little is known about the nutritional requirements of horses following a colic or gastric episode. Contradictory

estimates of energy requirements include lowered DE to approximately 70% maintenance because of the decrease in activity and increased DE because of the challenge to the immune system (Geor, 2007). It has been shown that energy requirements may be increased by as much as 30% in humans following gastrointestinal surgery (Heyland, 1998), but that too is contradictory to other reports (Sternberg et al., 2000).

Currently, there is much to consider by way of supplements or additives in the equine diet that may facilitate quicker recovery from colic. Prebiotics and probiotics are areas of research among many animal species today. Probiotics (also referred to as direct-fed microbials) are being investigated in other animal species, but have shown little evidence of consistent benefit to the horse (Swyers et al., 2008; Weese et al.; 2003 and 2004; Parraga et al., 1997). The subject of probiotics becomes more problematic when label claims and guarantees are shown to be false. Weese investigated 13 commercially available products marketed as probiotics. Of these 13, only two actually contained what the label described. Additionally, three of the products contained microorganisms that could have pathogenic effects (Weese, 2002).

Prebiotics are also being explored. Prebiotics are defined as "a nondigestible feed ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves gut health" (Gibson and Roberfroid, 1995). In essence, probiotics are the bugs themselves, while prebiotics are food for the bugs.

Prebiotics may include fructooligosaccharides (FOS), mannanoligosaccharides (MOS), and other short to medium length polysaccharides that are linked via a β bond. It is due to the presence of this β linkage that mammals are unable to digest these

saccharides. Digestion of this sort requires microbial fermentation (Gibson and Roberfroid, 1995). Dietary sources for these beneficial fibers may include soybeans, chicory root, inulin, whole (unrefined) oats, wheat, and barley, garlic, asparagus, and wheat bran.

The effects of FOS on gut health have been studied in many species (Gibson et al., 1995; Biggs et al., 2007; Swanson et al., 2002). Equine studies have shown potential for reduction in microbial shifts that occur following starch overload to the cecum (Respondek et al., 2008). The authors make the assumption that stimulation of growth by lactate utilizing bacteria may be the cause for the enhanced stabilization they saw due to the prevention of lactate buildup and subsequent production of butyrate. Other work has shown decreased fecal pH, and increased total VFA concentrations with FOS supplementation in horses (Berg et al., 2005). This work demonstrated that supplementation with 8 g/d of FOS caused a reduction in *lactobacilli*, however, 24 g/d showed no difference from the control (0 g/day). In contrast, work using FOS and MOS failed to show a difference in total VFA concentration or fecal pH (Gurbuz et al., 2010). However, it should be noted that the amount of supplementation that was fed in the Gurbuz study was 30 g/day. This is higher than the 24 g/d which failed to elicit a response in Berg's work. It seems clear that there is a maximum threshold that cannot be crossed if any efficacy is to be observed.

Metagenomics

Metagenomics research is a field that is utilizing new technology. This technology enables the study of microorganisms that are not easily cultured but that are environmentally accessible. Most often, the 16s rRNA gene is used in this research

because of its highly conserved nature (Janda and Abbott, 2007). Because of this, rRNA has been used to taxonomically identify microbial diversity from a common habitat or environment that would previously have been laborious and expensive using the Sanger method (Huson et al., 2007). Additionally, these new techniques give us access to the sequences of microbes that are not able to be cultured *in vitro*. This methodology (pyrosequencing) is referred to as "sequencing by synthesis" and is opening doors for microbial identification. The latest advances in this technology are owned by Roche Pharmaceuticals and is collectively referred to as 454 sequencing.

The sequencing process involves an initial isolation of DNA from an environmental sample (soil, water, feces, etc). MOGene LC (St. Louis, MO), has described this sequencing process in detail. The process begins with the confirmation that the DNA sample provided meets the specifications for purity and concentration (optical density 260/280, > 5 ng/ul) respectively. Following that PCR (polymerase chain reaction) is used to amplify the 16s region and primers are used to amplify the conserved regions. The hyper variable regions are sequenced relative to their position adjacent the conserved regions. The data are analyzed using known taxa in established databases (Huson et al., 2007). This approach utilizes PCR amplification that is emulsion based with a large number of DNA fragments. Because of this new and powerful tool, we are able to not only identify quantities of microbes that are present in a given sample, but also identify microbes that are as yet "unassigned" (without operational taxonomic units). This is useful because many microorganisms have been described as "unculturable" (Huson et al., 2007).

This type of sequencing could be beneficial in identifying the microbes present in the equine gut during a colic episode. Although it is generally assumed that there is a change in microbial population during the shift from a healthy to a sick state, there is no work to demonstrate those changes. However, some work has been done identifying bacterial diversity within the equine digestive tract in the healthy horse (Daly et al., 2001). They utilized 16s rRNA clones to identify diversity among the microflora and reported that only 5% of the sequences corresponded to known bacteria identified in public databases. Further, 89% had no correspondence to any publicly recorded sequence. It was concluded that equine gastrointestinal microflora are underrepresented in the taxonomic databases and suggests there may be novel species present.

This conclusion is supported by work done in Japan utilizing feces from healthy horses. Again, analysis of the 16s rRNA was useful in identifying a potentially novel strain of *lactobacilli* (Morotomi et al., 2002). This particular strain was seen in 10 of 20 horses and was present at six of six farms sampled. The name *lactobacillus equi* has been proposed.

As with any new technology, use of the 16s rRNA gene sequencing is not without its shortcomings. Although its benefits have been well documented (Janda and Abbott, 2007), its use is not without difficulty. The increase in recognized taxa is associated with the ease of use of the 16s rRNA gene for sequencing studies. However, a lack of definition for threshold value (required percentage of similarity) means there is no common agreement on conclusive identification at the rank of species. This may be troublesome for species with high degrees of sequence similarity, but low DNA homology. Janda and Abbott (2007) describe this issue in detail and use examples from

the genus Bacillus as examples. They cite *B. anthracis*, *B. cereus*, *B. globisporus,* and *B. psychrophilus* as problematic using 16s rRNA sequencing. The authors suggest DNA-DNA relatedness studies as an alternative for these issues. Additionally, the authors cite concerns over available databases and nomenclature issues. Although this new technology provides excellent opportunities, it must be used with caution.

CHAPTER 2

ASSESSMENT OF EFFECTS OF STARVATION AND STARCH OVERLOAD ON EQUINE FECAL INNOCULUM UTILIZING THE CONTINUOUS CULTURE SYSTEM

ABSTRACT

Starch overload is commonly considered to be a risk factor for equine colic. Once a colic episode has occurred, a common industry practice is to withhold feed and water. The purpose of our research was to test the effects of starch overload and starvation upon equine fecal microflora.

To test these effects, we inoculated 24 continuous culture fermenters with a 4:1 mixture of feces/buffer. Each run utilized four diets to investigate treatment effects on VFA and NH_3 concentration, and pH.

Treatments

Effects of starvation were examined using, Purina Equine Sr. (SR), SR + FOS 0.1% (FOS), SR + MOS 0.1% (MOS), and Purina Well Gel (WG). Each diet was ground to 3 mm for uniform particle size and fermenters were fed 20 g every 12 hours.

Effects of starch overload were examined using, SR (Purina Equine Sr.), FOS (SR + FOS 0.2%), FM (SR + 0.1% FOS and 0.1% MOS) and WG (Purina Well Gel).

An acclimation period of six days was allowed, followed by 48 hours of starvation. Samples were then collected for the next 48 hours. Data were analyzed using the PROC MIXED procedure of SAS.

Starvation

Total volatile fatty acid (VFA) concentrations were not affected by treatment. However, isobutyric and isovaleric concentrations were significantly greater for the WG treatment. Additionally, butyric acid was significantly lower for the WG treatment. Ammonia concentration for the WG treatment was greater than those of the SR, FOS, or MOS treatments. The pH levels for the WG diet were significantly higher (pH 6.36) than other treatments.

Starch Culture

On the morning of the seventh day, the 8 am feeding was replaced with 20 g of a 21.3% starch commercially available textured horse feed (Country Acres Horse Sweet 12, Purina Mills) and the 8:00 pm feeding was withheld. The SR and FM diets both resulted in higher total VFA concentrations than the FOS diet. Isobutyric concentration was higher for the WG and FOS treatments, and isovaleric concentration was higher for the WG treatment. Again, we observed higher ammonia levels in the WG treatment group. Additionally, the previously observed higher pH values were repeated for the WG treatment as compared to other diets.

This research indicated that following an abrupt change in the starch content of the diet, branched chain amino acid, and ammonia production may be affected by treatment.

KEYWORDS: Continuous culture, equine, volatile fatty acid, feces, ammonia, microbe

INTRODUCTION

Equine colic is a very serious and costly condition that threatens the lives of many horses and places serious economic hardships on their owners each year. Although change in forage is most commonly associated with equine colic (Cohen et al., 1999), starch overload is still a serious concern and one that can be quite serious for the colic prone horse. Additionally, it is a common industry practice to withhold all feed and water for the duration of the colic. This may be up to 48 hours or longer depending on the severity of the symptoms and the stoic nature of the horse that is diagnosed. It is unknown what effects this period of starvation may have upon the microbial processes taking place within the equine cecal community. Therefore, we designed two continuous culture experiments designed to investigate the effects of both starvation and starch overload on the equine cecal microbial community.

MATERIALS & METHODS

Experimental design

Two continuous culture experiments were conducted utilizing 24, single-flow fermenters of 2,000 mL liquid volume. These cultures were established as randomized complete block designs. The fermenters consisted of polycarbonate jars (Fisher Scientific, Pittsburgh, PA) with an outlet for overflow at approximately a 1,460 mL liquid volume. Each fermenter apparatus was maintained with constant stirring using magnetic stir plates and held constant at 39°C by immersion in a water bath using thermostatically controlled heaters (Model 730, Fisher Scientific, Pittsburgh, PA). McDougall's artificial saliva (Slyter, 1990) was introduced into each fermenter using peristaltic pumps (Masterflex, model 7520-10, Cole-Parmer Instrument Co., Chicago, IL). Dilution rates

for each fermeneor were held constant for all treatments at 4%/hr. Fermenters were randomly assigned to one of four treatments (n = 6).

Fecal material was collected from six mature mares housed at the University of Missouri Equine Farm. The mares were 11.5 years (± 6.5 years), with a BCS of 5 (± 1) and weighed 1,210 lbs (± 110 lbs). The mares were acclimated to a basal diet (Sr.) for approximately 10 days prior to each collection and were allowed free access to pasture and ad libitum water each day. Following collection material was brought back to the laboratory, composited, and mixed with buffer at a ratio of 4:1. Feces/buffer material was strained through two layers of cheese cloth and used to inoculate each fermenter up to a 1,460 ml liquid volume.

STARVATION CULTURE

Dietary treatments (Table 2.1) were SR (Purina Equine Sr) FOS (SR + FOS 0.1%), MOS (SR + MOS 0.1%) and WG (Purina Well Gel). The basal diet SR., is a commercially available complete horse feed that has been used extensively in equine-hospital settings. The addition of the FOS was to examine the effects of the prebiotic in conjunction with the basal diet. The MOS treatment was added as a different type of prebiotic to examine effects following starvation. The WG diet is a commercial product available only to veterinarians for use in clinical settings as an enteral diet or top dress. Each diet was ground to 3 mm for uniform particle size and fermenters were fed 20 g every 12 hours.

Sample collection

Fermenter contents were allowed multiple turnovers (6 days) prior to sampling to ensure stabilization of the microbial population (Meng et al., 1999). The 6 days of

acclimation were followed by a 48 hour period of sampling but no feeding (i.e. starvation). Following this starvation period, the treatment feedings began with 20 g/fermenter every 12 hours. Samples were then taken at 144, 148, 156, 168, 180, 192, 200, 204, 216, and 240 hours of fermentation. Effluent fluid was collected in ice-cooled reservoirs, measured, and composited prior to storage at 4°C prior to being frozen. A 20 ml aliquot of fermenter contents was taken immediately prior to feeding. The pH level was measured, followed with acidification using 6 N HCL and then frozen. Aliquots were composited for quantification of ammonia concentration (Broderick and Kang, 1980) and volatile fatty acid (VFA) concentration (Grigsby et al.,1992).

STARCH CULTURE

Dietary treatments (Table 2.10) were SR (Purina Equine Sr.), FOS (SR + FOS 0.2%) FM (SR + FOS 0.1% + MOS 0.1%), and WG (Purina Well Gel). Following completion of the starvation culture, the treatments were adjusted for the starch culture to examine higher levels of FOS on the culture following starch loading, and to assess the potential cumulative effects of the two prebiotics (FOS and MOS). The SR and WG diets did not change. Each diet was ground to 3 mm for uniform particle size and fermenters were fed 20 g every 12 hours.

Sample collection

Fermenter contents were allowed multiple turnovers (6 days) prior to sampling to ensure stabilization of the microbial population (Meng et al., 1999). Following the stabilization period, a "loading" dose of starch was administered (approximately 144 hours of fermentation). Samples were taken at 148, 156, 168, 192, 216, and 240 hours of fermentation. Effluent fluid was collected in ice-cooled reservoirs, measured, and

composited prior to storage at 4°C prior to being frozen. A 20 ml aliquot of fermenter contents was taken immediately prior to feeding. The pH level was measured, followed with acidification using 6 N HCL and then the sub-sample was frozen. Aliquots were composited for quantification of ammonia concentration (Broderick and Kang, 1980) and volatile fatty acid (VFA) concentration (Grigsby et al., 1992).

Analysis

At the conclusion of both cultures, fermenter contents were collected, blended, and strained through two layers of cheesecloth before being stored at 4°C until analyzed. Samples of the prepared fermenter contents, dietary treatments, and composited effluent were taken and analyzed for DM, OM (AOAC, 1984), and N content (Leco Model FP-248, Leco Co., St. Joseph, MI). Samples used for quantification of ammonia were thawed at room temperature and analyzed using the phenol-hypochlorite procedure (Broderick and Kang, 1980), with a DU-65 spectrophotometer (Beckman, Palo Alto, CA). Gas chromatography (Model 3400, Varian, Walnut Creek, CA) was used to determine VFA concentration of samples as previously described (Grigsby et al., 1992).

Statistical Analysis

Data were analyzed as a linear statistical model using PROC MIXED of SAS (SAS Inst. Inc., Cary, NC). Data were analyzed as a randomized complete block design with treatment as the main effect as previously described (Littell et al, 1998). Level of significance was set at $P < 0.05$.

Results

STARVATION CULTURE

Total VFA levels over time are shown in Figure 2.1. Measured levels of acetate, propionate, and butyrate over time are shown in Figures 2.2, 2.3, and 2.4, respectively, and mean values for VFA's following starvation are reported in Table 2.2. Values for branched chain volatile fatty acids (BCVFA) are higher for the WG treatment ($P < 0.05$) while SR, FOS, and MOS all had similar levels ($P > 0.05$). Total VFA levels, ratio of acetic:propionic, acetic, and valeric acid levels were not affected by treatment ($P > 0.05$). However, propionic levels for MOS were lower ($P < 0.05$) when compared against SR and FOS. Lactic acid concentration for FOS was higher than WG ($P < 0.05$). When acetic, propionic, and butyric acid values were expressed as a molar percentage of the total VFA production there was no difference ($P > 0.05$) between treatments. Ammonia concentrations increased with WG treatment ($P < 0.05$). Measured pH levels (Figure 2.6) were significant ($P < 0.05$) across treatments.

STARCH CULTURE

Total VFA production over time is shown in Figure 2.7. As expected, total production increased following the dosage of the starch to the fermenters. Values for acetate, propionate, and butyrate are shown in Figures 2.8, 2.9, and 2.10 respectively.

Measured levels for VFA mean concentrations following dosing with starch are reported in Table 2.4. Isobutyric levels were higher for the WG and FOS treatments ($P < 0.05$) as compared to FM and SR. However, isovaleric values were higher only for WG ($P < 0.05$). Butyric acid levels were lower for WG ($P < 0.05$) as compared to SR, FM, and FOS. Additionally, lactic acid levels were lower for WG ($P < 0.05$) as compared to

Sr, FM, and FOS. Total VFA levels were highest for SR, and FM and lowest for FOS ($P < 0.05$). The acetate:propionate was higher for WG as compared to FM ($P < 0.05$). When acetic acid was expressed as a molar percentage of the total, WG was higher than FOS ($P < 0.05$). Conversely, when butyric acid was expressed thusly, WG was lower than other treatments ($P < 0.05$). Ammonia concentrations (Figure 2.11) were similar across treatments FM, FOS, and SR ($P > 0.05$) and were all significantly lower than WG ($P < 0.05$). Measured pH values (Figure 2.12) were highest for WG and lowest for FM and Sr ($P < 0.05$).

Discussion

STARVATION CULTURE

As horse owners have become more educated, they have started to do a better job recognizing the signs/symptoms of a colic episode. Risk factors for and predisposing conditions that seem to facilitate a colic episode are being identified and caution taken to prevent such an occurrence. However, such a complex gastrointestinal tract will undoubtedly always provide some degree of complications. At such time, it falls upon the horse owner to be vigilant and recognize the signs and seek veterinary care. It is generally a universal practice that all feed and water are withheld until the colic symptoms have abated. It was this period of "starvation" that we were interested in.

The hypothesis of our study was that the addition of FOS or MOS would increase VFA production and cause in pH following starvation. There are currently no other published reports investigating these conditions and the differences that may result.

Total VFA levels over time are shown in Figure 2.1. As expected, the production was hindered substantially by the period of starvation. Future work should include more

examination of the specific microflora that expire first within the starvation period, so as to identify potential adjustments that should be made in recommendations surrounding the use of a starvation period.

The lower butyric acid values for the WG diet as compared to the SR diet suggest a negative associative effect for colonocyte formation and epithelial development following gastrointestinal distress. Butyrate response elements have been linked to control of gene expression and are thought to affect proliferation and development of the intestinal epithelia (Daly et al., 2001). Since butyric acid production is a function of acetic acid production, there may be other factors that inhibited the production of butyric acid that have been heretofore unexplained. Because of the preference of the colonic epithelials for butyrate, the MOS treatment may provide some benefit following starvation as related to total VFA production. Additionally, when time point is considered, the butyric acid production for the MOS treatment is significant at each time point starting at 200 hours of fermentation. It is also interesting that at 156 hours of fermentation there was an unexpected and unexplained spike in the FOS fermenters. When each time point was considered, acetate, butyrate, and total VFA production were approaching significance as fermentation progressed. This suggests an adjustment in our diets was warranted.

It is likely that the higher level of crude protein (CP) contained in the WG diet was responsible for some of the inherently higher ammonia levels.. However, the lower starch levels also may have reduced the scavenging of ammonia from starch digesting bacteria which would allow for more residual ammonia to be present in continuous

culture. Additionally, in a live model the amount of protein that would be digested pre-cecally would likely reduce this number.

Based on the information we gleaned from this continuous culture, recommendations regarding the colic horse must be reviewed. The starvation period that is so common should be examined further and when at all possible, the horse should be continuously fed very small amounts of a liquefied diet so as to minimize damage to the hindgut microbial population.

STARCH CULTURE

The hypothesis of our study was that the addition of higher levels of FOS or the combination of FM would change VFA production and pH. It is an unfortunately common mistake that equines are switched too rapidly from one feedstuff (grain or hay) to another. Although hay has been identified as the primary risk factor for colic episodes, failure to transition grain diets is often indicated anecdotally from equine veterinarians in relation to colic. It is not unheard of for equines to break out of their stalls and gorge on any available feed. The increased starch content of the textured feed should have provided ample substrate for amylolytic bacterial activity.

Total VFA levels over time show the anticipated spike and decline following the starch dosage. It is interesting to note that the SR, and WG diets showed no difference following starch administration, and that the FOS and FM diets were effectively following opposite trends at the culmination of the experiment. This would seem to indicate a separation in the production of VFA's as the fermentation progressed. This same separation was seen in measured acetate concentrations over time. Total VFA's have been previously measured in different segments of the equine gastrointestinal tract

(Al Jassim et. al., 2009; Mackie and Wilkins, 1988; Kern et al. 1974,). However, there are no published reports on the production of VFA's from equine fecal innoculum utilizing a continuous culture system. There is some agreement from the measured cecal concentrations referred to above, and the values reported here.

We observed higher values for the WG diet in isovaleric concentrations. This was observed in the starvation culture as well. Since the formation of the branched chain VFA's is carried out by cellulolytic bacteria and utilized by the non-cellulolytic bacteria it would seem that the WG diet facilitated this exchange very well.

The lower butyric acid levels for the WG treatment may be explained by the lower levels of fiber compared to other treatments. Thus, the fibrolytic bacteria were lacking in substrate. Although colic cellular health would be a concern, the diets design was such that it could be administered enterally and higher fiber content may make utilization of the feeding tube problematic.

Again, the higher levels of starch in the SR, FOS, and FM treatments may have afforded more opportunity for amylolytic bacteria to utilize available ammonia, while the WG treatment had lower starch levels and thus higher ammonia concentrations. As stated earlier, the amount of undigested protein arriving at the hindgut for fermentation would be significantly reduced in an *in vivo* model.

The differences in measured pH values may be due to a slight buffering from the excess ammonia in the WG cultures. The physiological significance of a pH difference this small is unknown but since much larger differences have been reported (de Fombelle et al., 2003) and suggested to only provide "an idea of the average composition", it is unlikely that any affect would be physiologically significant.

While much is known about the ruminant animal's ability to maximize microbial efficiency, there is still some argument whether or not the microbial protein synthesized in the cecum is of any value to the horse (Hintz and Cymbaluk, 1994). Therefore, more work is needed to establish evidence for technologies maximizing nutritional components, specifically protein and energy. The higher levels of ammonia for the WG diet that were seen in both cultures is undoubtedly due to the higher levels of CP. Additionally, it would be expected that the WG diet would have a higher pH level as lower levels of lactic acid production were expected due to very low levels of available carbohydrate.

The prebiotics that were utilized in our treatments provided mixed results. Despite the success of using FOS and MOS in other species (Swanson et al., 2002; Biggs et al., 2007) there is only one published paper supporting the benefits of FOS in the horse (Berg et al., 2005) while other reports differ (Gurbuz et al., 2010).

The goal of this research was to identify the effects of starvation and starch loading on the microbial population present in equine fecal material. The continuous culture system is limited by its design and cannot account for the digestion and absorption that occurs in the equine prior to cecal microbial activity. Moreover, the equine microbiome is largely unidentified. This may explain why there has been limited success with *in vitro* but *in vivo* studies utilizing probiotics have been unsuccessful.

Table 2.1 Treatment diets utilized in continuous culture with equine fecal innoculum following a starvation period of 48 hours.

Treatment diets for continuous culture measuring response to starvation

	DM basis												
Treatment	% CP	% starch	%WSC	%ESC	% DM	% OM	% NDF	% ADF	Fat	Ca	P	Zn	Cu
Sr	15.8	12.0	12.7	7.2	90.5	90.5	35.4	18.7	6.3	0.7	0.59	270	65
Sr + FOS (0.1%)	15.3	12.4	13.4	7.5	89.9	90.5	37.4	19.5	6.8	0.62	0.61	246	52
Sr + MOS (0.1%)	14.4	11.6	12.8	8.9	90.2	90.4	37.2	19.6	5.9	0.63	0.59	232	50
WG	33.9	2.8	10.0	6.3	90.5	90.5	21.8	14.0	6.2	1.15	0.64	225	118

WSC = simple sugars and fructans

ESC = simple sugars and some fructans

Sr = Equine Sr (Purina Mills, St. Louis MO)

Sr + FOS (0.1%) = Equine Sr with NutraFlora (Golden Technologies, Johnstown CO)

Sr + MOS (0.1%) = Equine Sr with Actigen (Alltech, Nicholasville, KY)

WG = WelGel (Purina Mills, St. Louis MO)

*values analyzed by Equi Analytical, Ithaca NY

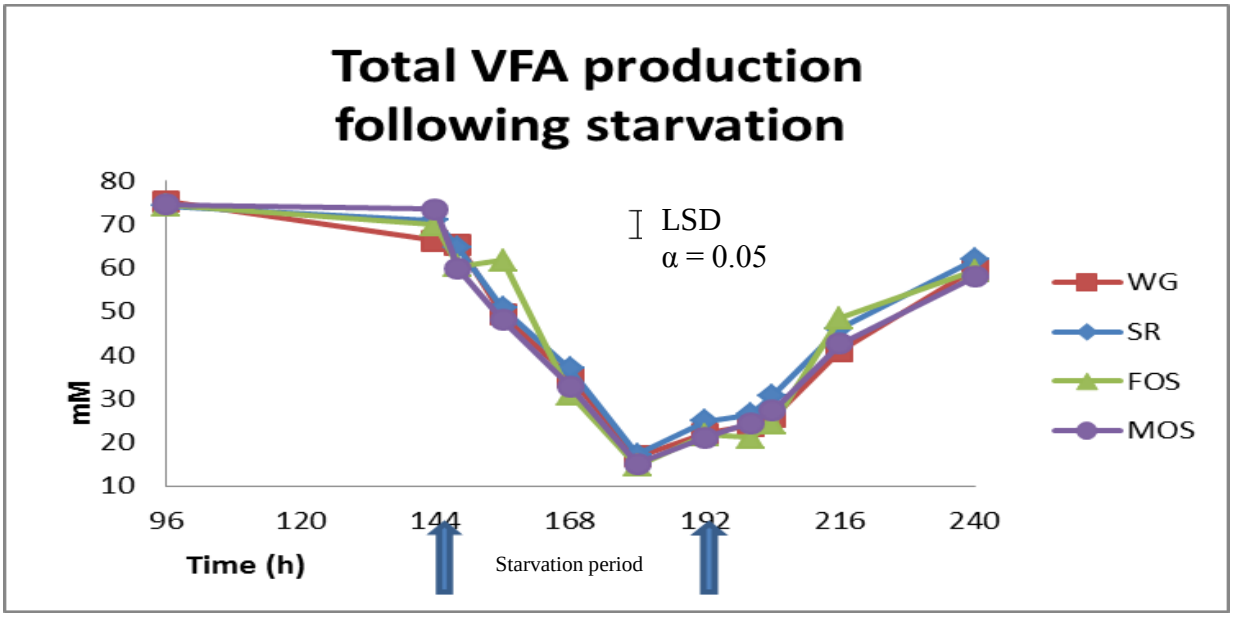

Figure 2.1. Effect of dietary treatment on total VFA production over time by equine fecal microflora following starvation in continuous culture.

Values reported utilizing LS means

*Samples were collected immediately prior to refeeding following starvation

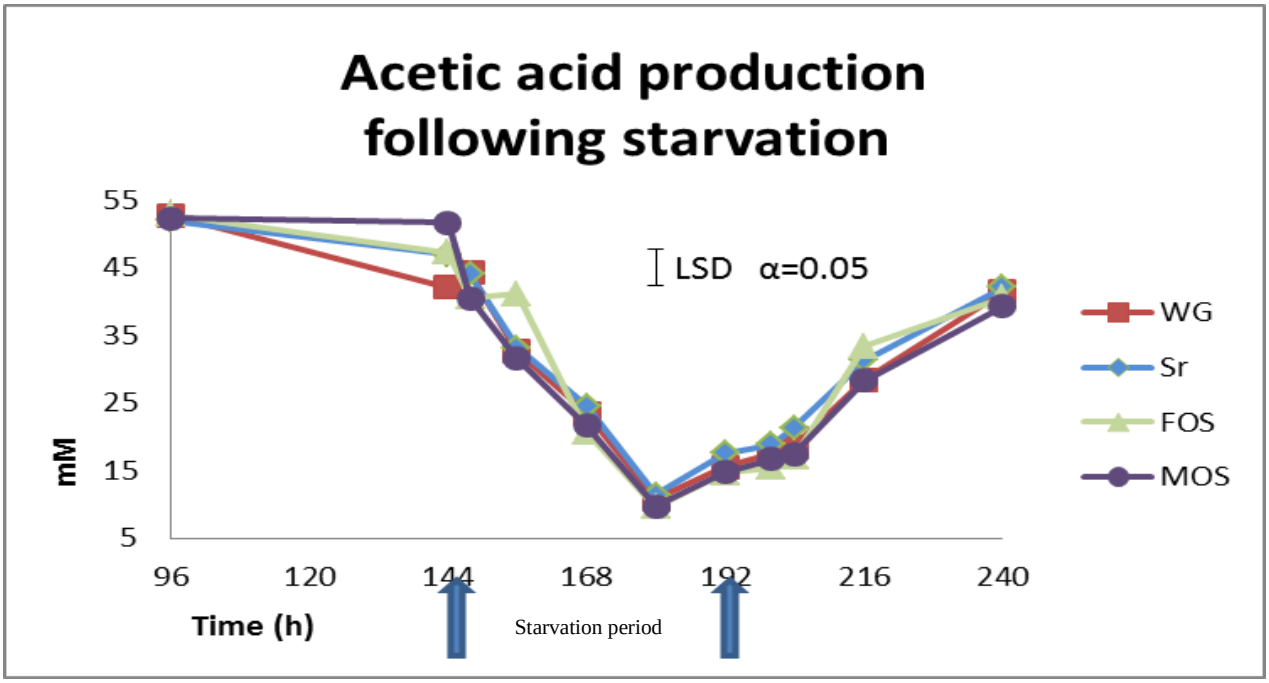

Figure 2.2 Acetic acid production (mM) following starvation in continuous culture utilizing equine fecal inoculum.

Treatment x time interactions reported using LS Means

*Samples were collected immediately prior to refeeding following starvation

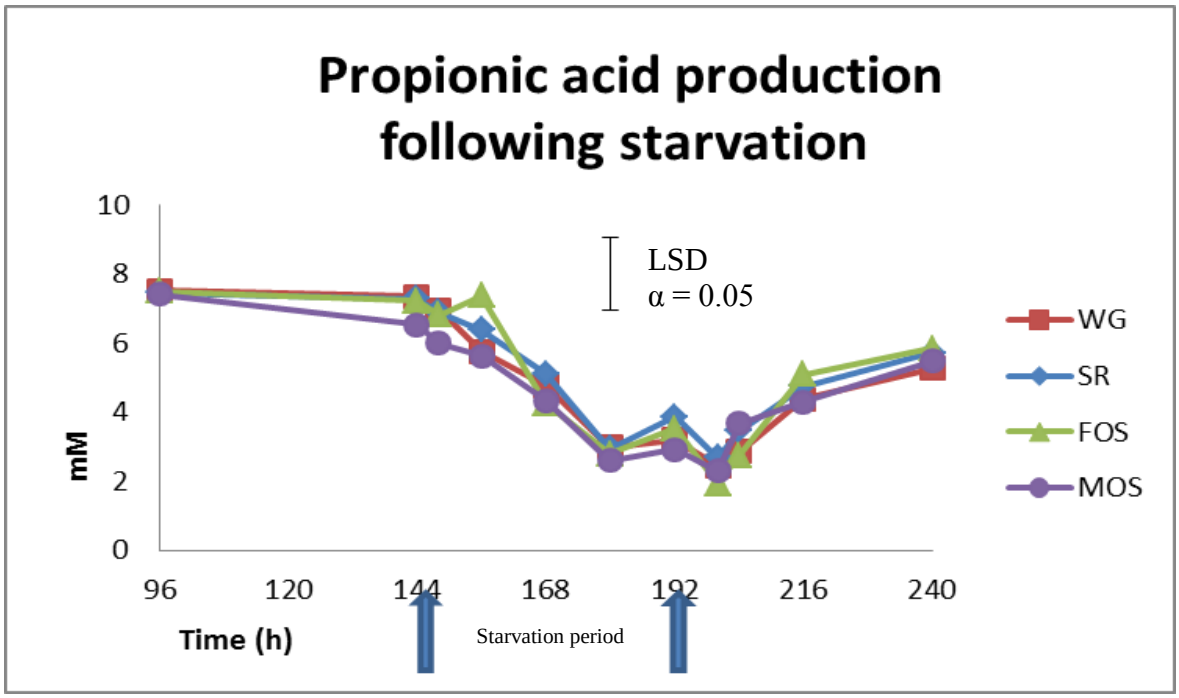

Figure 2.3 Propionic acid production (mM) following starvation in continuous culture utilizing equine fecal inoculum.

Treatment x time interactions reported using LS Means

*Samples were collected immediately prior to refeeding following starvation

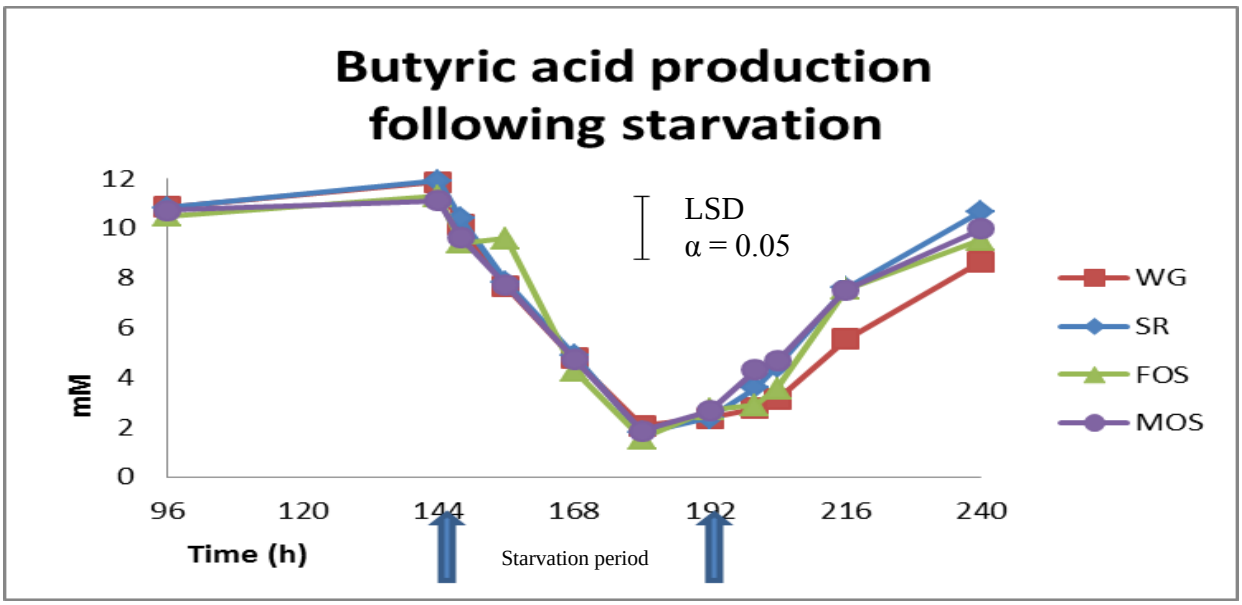

Figure 2.4 Butyric acid production (mM) following starvation in continuous culture utilizing equine fecal inoculum.

Treatment x time interactions reported using LS Means

*Samples were collected immediately prior to refeeding following starvation

Table 2.2. Total VFA levels measured in continuous culture utilizing equine fecal inoculum and following starvation.

	Treatment				
	Sr	FOS	MOS	WG	*SD*
Acetic mM	31.17	30.24	29.42	29.74	4.2131
Propionic mM	5.13[a]	5.0[a]	4.65[b]	4.86[ab]	0.8328
Butyric mM	6.93[a]	6.61[ab]	6.80[ab]	6.34[b]	0.8818
Isobutyric mM	0.02[b]	0.02[b]	0.01[b]	0.11[a]	0.0980
Valeric mM	2.34	2.17	2.22	2.19	0.3429
Isovaleric mM	0.24[b]	0.25[b]	0.23[b]	0.36[a]	0.0490
lactic mM	0.0009[b]	0.0014[a]	0.001[ab]	0.0004[b]	0.0034
TVFA mM	45.84	44.25	43.34	43.61	5.9278
Ace:Pro	5.96	5.97	6.25	6.06	0.4899
Acetic :TVFA	0.68	0.68	0.67	0.68	0.0196
Propionic :TVFA	0.12	0.12	0.12	0.12	0.0098
Butyric :TVFA	0.17	0.14	0.19	0.14	0.0147

LS Means with unlike superscripts within a row differ P <0.05

TVFA = Total VFA's

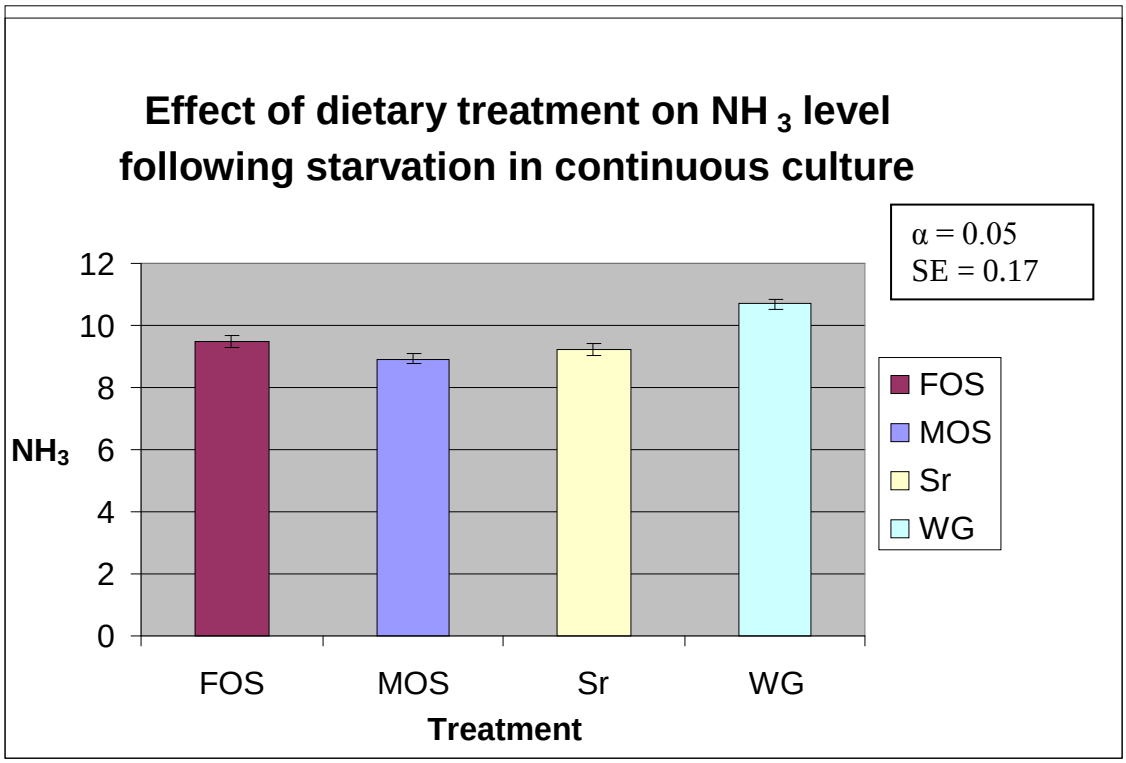

Figure 2.5 Effect of dietary treatment on ammonia level production by equine fecal micro flora following starvation in continuous culture.

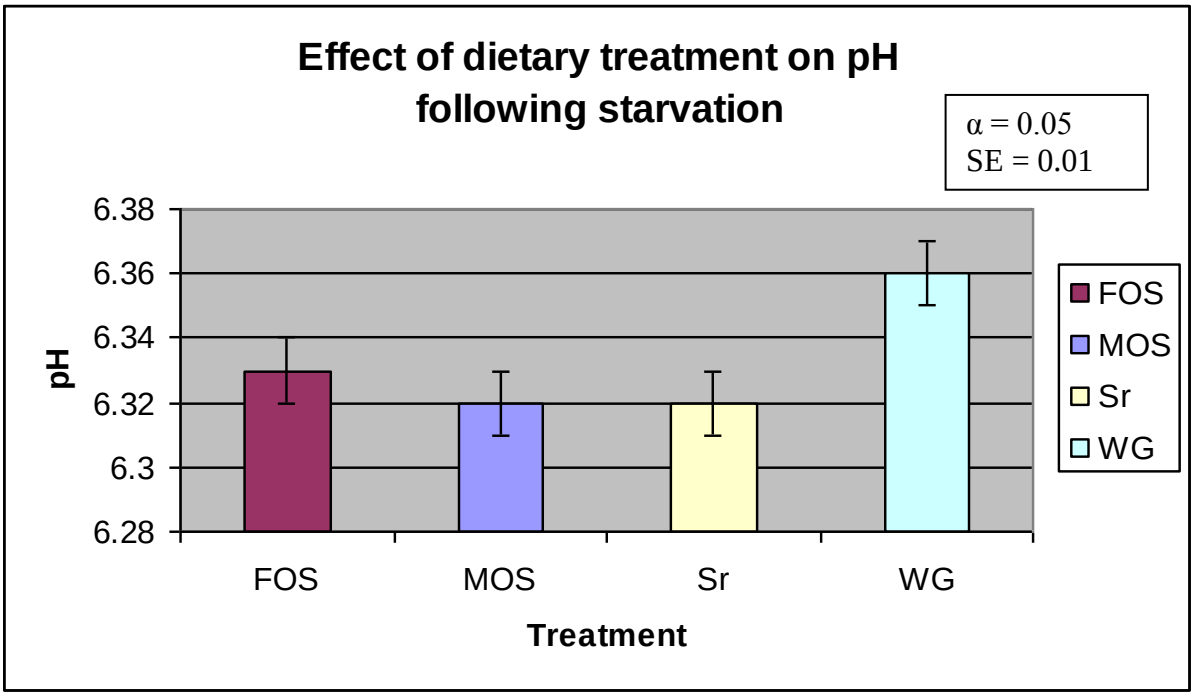

Figure 2.6 Effect of dietary treatment on pH of equine fecal microflora following starvation in continuous culture.

Table 2.3 Treatment diets utilized in continuous culture with equine fecal innoculum following a challenge with starch dosage.

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Treatment diets for continuous culture measuring response to starch overload													
	DM basis												
Treatment	% CP	% starch	%WSC	%ESC	% DM	% OM	% NDF	% ADF	Fat	Ca	P	Zn ppm	Cu ppm
Sr	15.8	12.0	12.7	7.2	90.5	90.5	35.4	18.7	6.3	0.7	0.59	270	65
Sr + FOS (0.2%)	14.3	11.6	12.2	7.3	89.6	90.4	36.7	19.5	5.7	0.71	0.59	267	55
Sr + FOS (0.1%) + MOS (0.1%)	14.7	11.7	12.3	8.3	88.8	90.4	37.5	19.9	6.8	0.66	0.59	259	57
WG	33.9	2.8	10.0	6.3	90.5	90.5	21.8	14.0	6.2	1.15	0.64	225	118
CA*	12.3	21.3	9.8	6.9	91.6	92.2	36.6	18.9	3.3	1.36	0.45	131	34

WSC = simple sugars and fructans
ESC = simple sugars and some fructans
CA* = Country Acres (starch dose) Purina Mills (St. Louis MO)

Sr = Equine Sr (Purina Mills, St. Louis MO)
Sr + FOS (0.2%) = Equine Sr with NutraFlora (Golden Technologies, Johnstown CO)
Sr + FOS (0.1%) + MOS (0.1%) = Equine Sr with NutraFlora and Actigen
WG = WelGel (Purina Mills, St. Louis MO)
*values analyzed by Equi Analytical, Ithaca NY

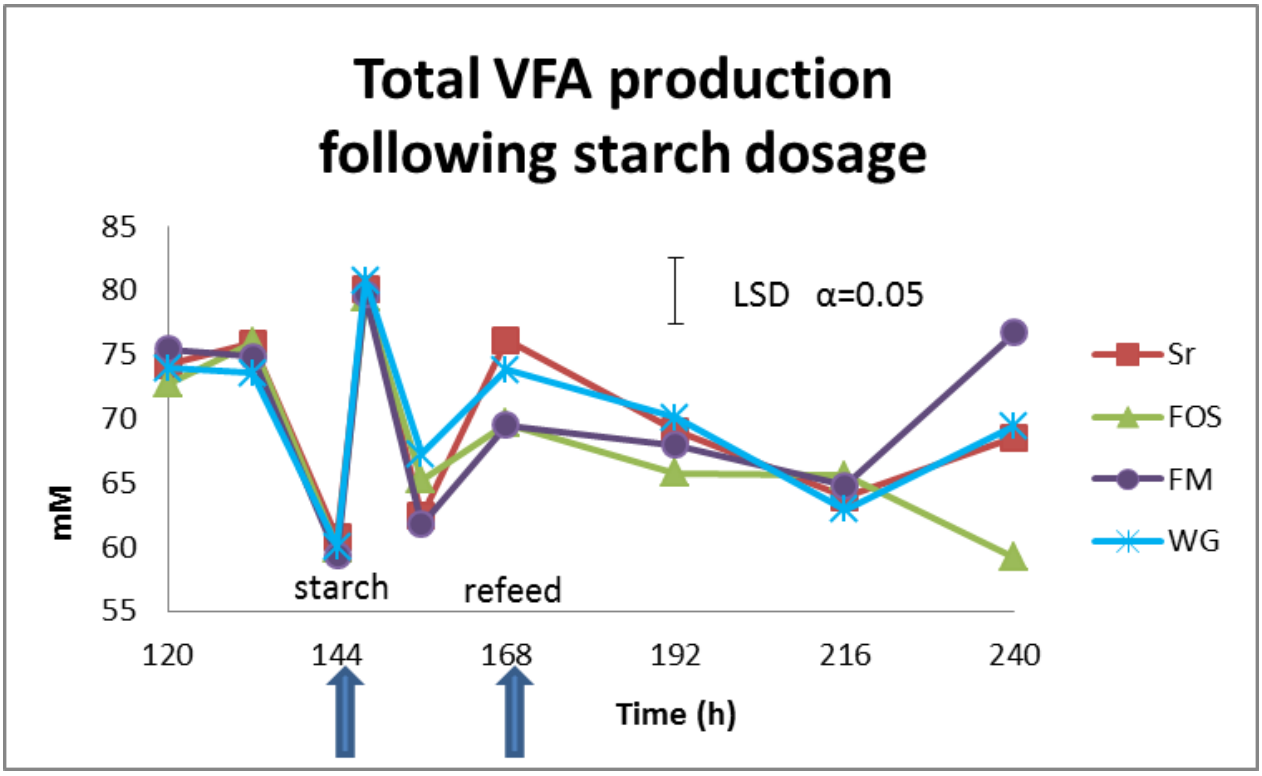

Figure 2.7 Effect of dietary treatment on total VFA production by equine fecal microflora over time following starch dosage.

*Samples were collected immediately prior to refeeding following starch dosage.

Values reported utilizing LS means

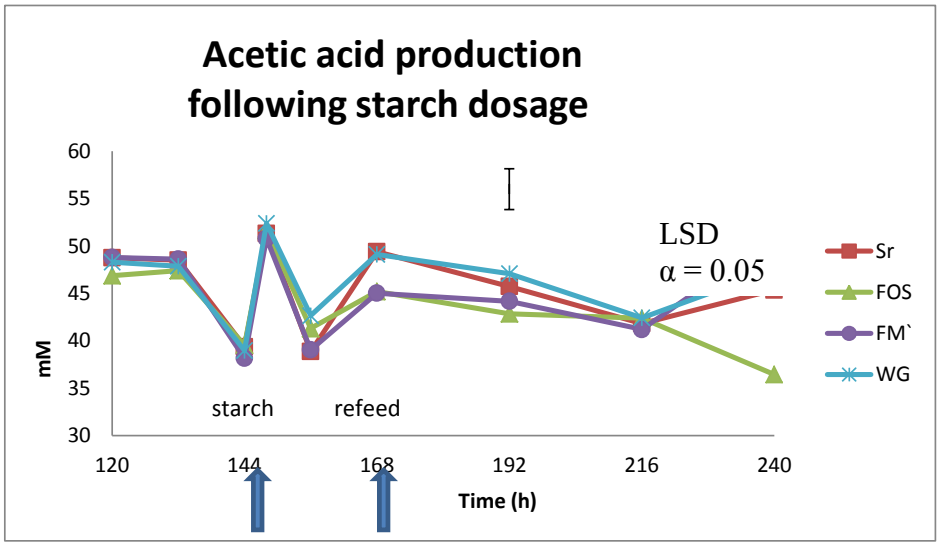

Figure 2.8 Acetic acid production (mM) following starch dosage in continuous culture utilizing equine fecal inoculum.

*Samples were collected immediately prior to refeeding following starch dosage.

Treatment x time interactions reported using LS Means

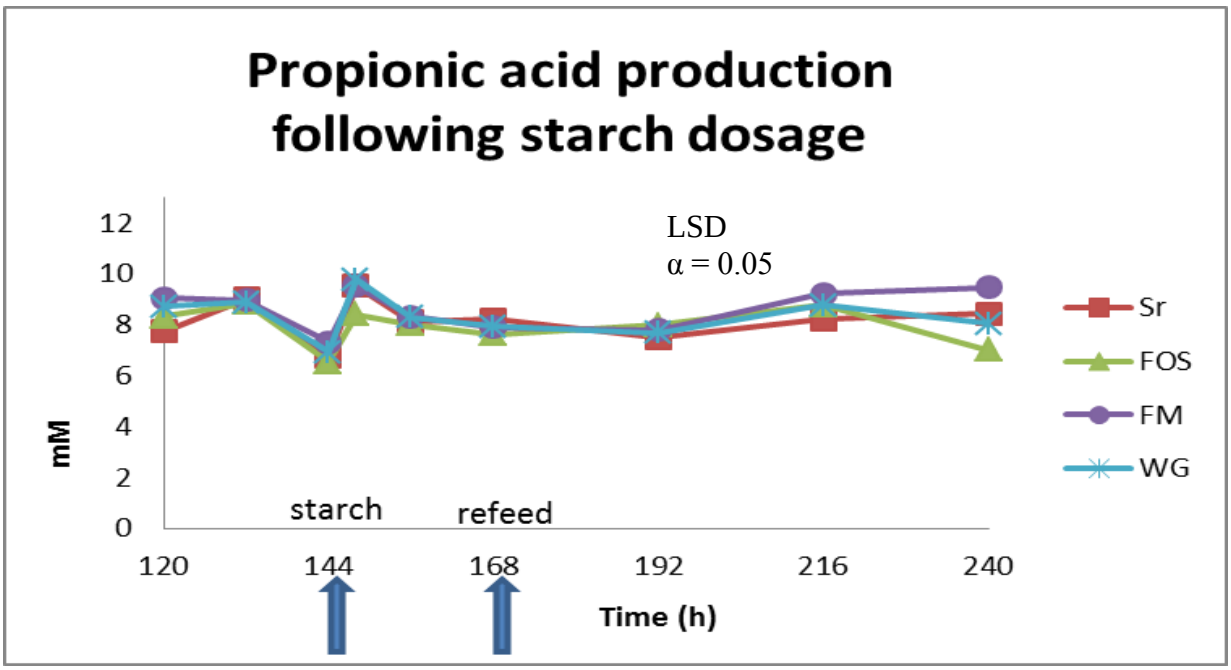

Figure 2.9 Propionic acid production (mM) following dosage in continuous culture utilizing equine fecal inoculum.

Treatment x time interactions reported using LS Means

*Samples were collected immediately prior to refeeding following starch dosage.

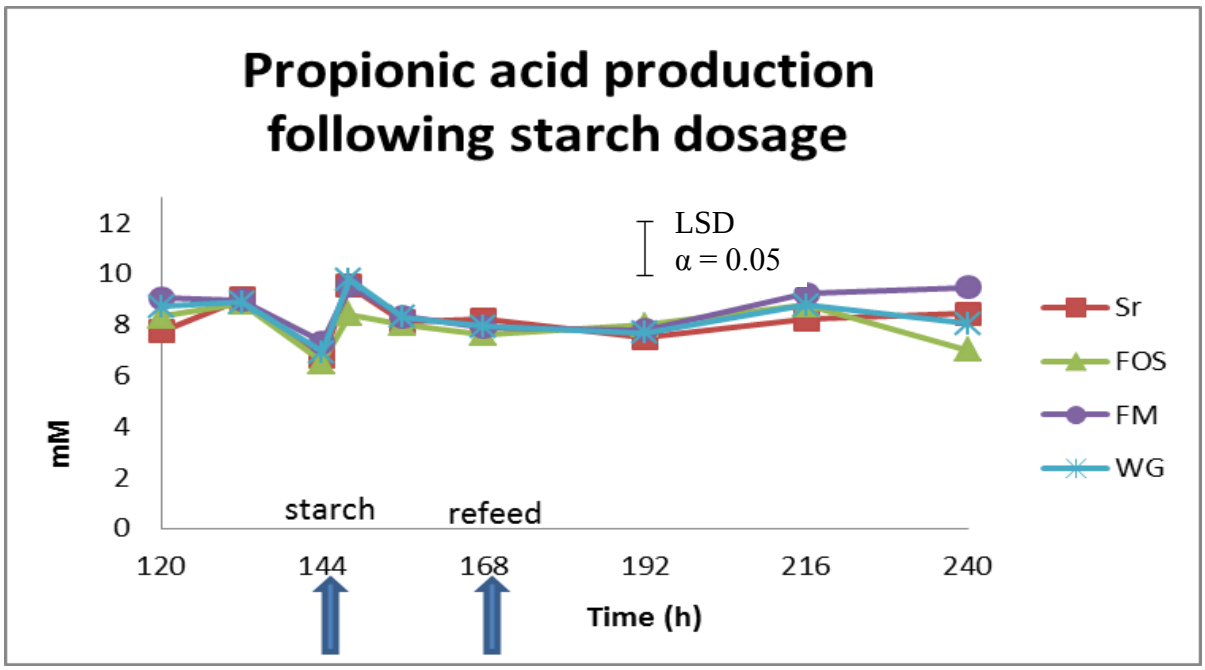

Figure 2.10 Butyric acid production (mM) following starch dosage in continuous culture utilizing equine fecal inoculum.

Treatment x time interactions reported using LS Means

*Samples were collected immediately prior to refeeding following starch dosage.

Table 2.4. Total VFA levels measured in continuous culture utilizing equine fecal inoculum and following starch overload.

	Treatment				
	Sr	FOS	FM	WG	*SD*
Acetic mM	45.17[a]	42.21[b]	44.7[ac]	45.56[a]	*4.2131*
Propionic mM	7.81[b]	7.71[b]	8.45[a]	8.01[b]	*1.0288*
Butyric mM	9.84[a]	9.53[a]	9.66[a]	8.55[b]	*0.9798*
Isobutyric mM	<0.00001[b]	0.2[a]	<0.00001[b]	0.43[a]	*0.0588*
Valeric mM	5.59[a]	5.37[a]	5.64[a]	4.94[b]	*0.0637*
Isovaleric mM	.48[b]	.44[b]	.47[b]	.88[a]	*0.0833*
lactic mM	.0010[a]	.0009[a]	.0010[a]	.0007[b]	*0.0004*
total VFA mM	68.88[a]	65.46[b]	68.93[a]	68.37[ab]	*5.5848*
Ace:Pro	5.90[a]	5.58[ab]	5.32[b]	5.98[a]	*0.9308*
Acetic:TVFA	.66[ab]	.64[b]	.65[ab]	.67[a]	*0.0343*
Propionic:TVFA	.11[b]	.12[a]	.12[a]	.12[a]	*0.0147*
Butyric :T VFA	.14[b]	.15[a]	.14[b]	.13[c]	*0.0147*

LS Means with unlike superscripts within a row differ P <0.05

TVFA = Total VFA's

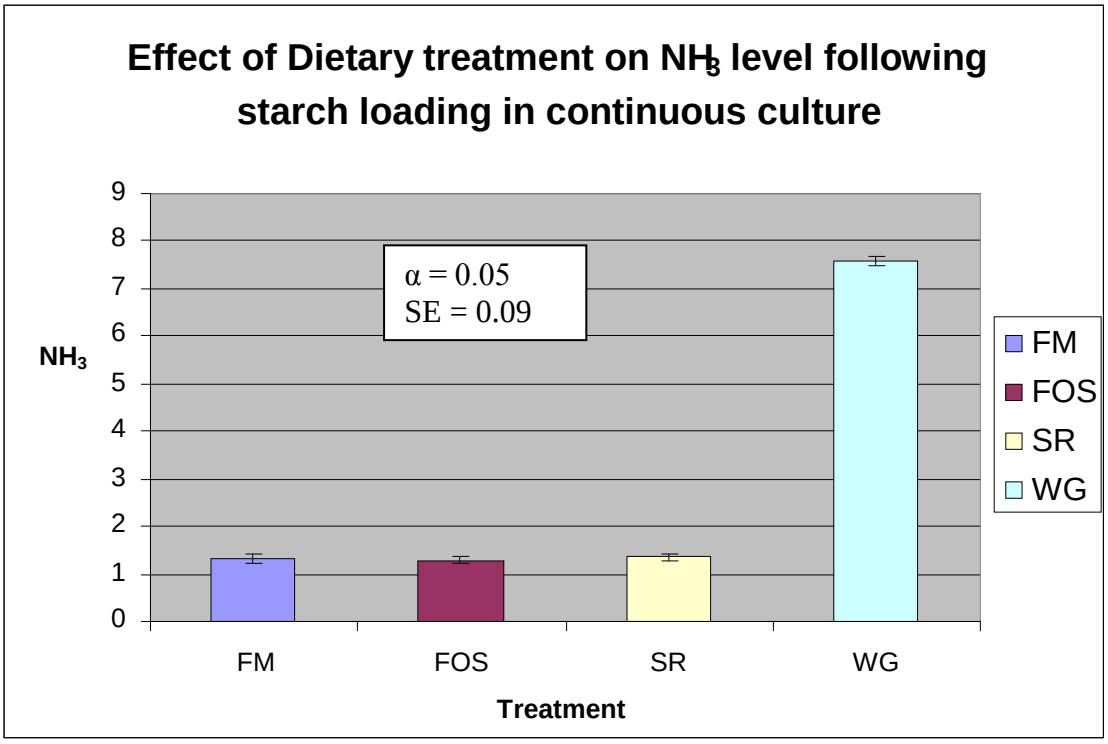

Figure 2.11 Effect of Dietary Treatment on ammonia level of equine fecal microflora following starch dosage into continuous culture.

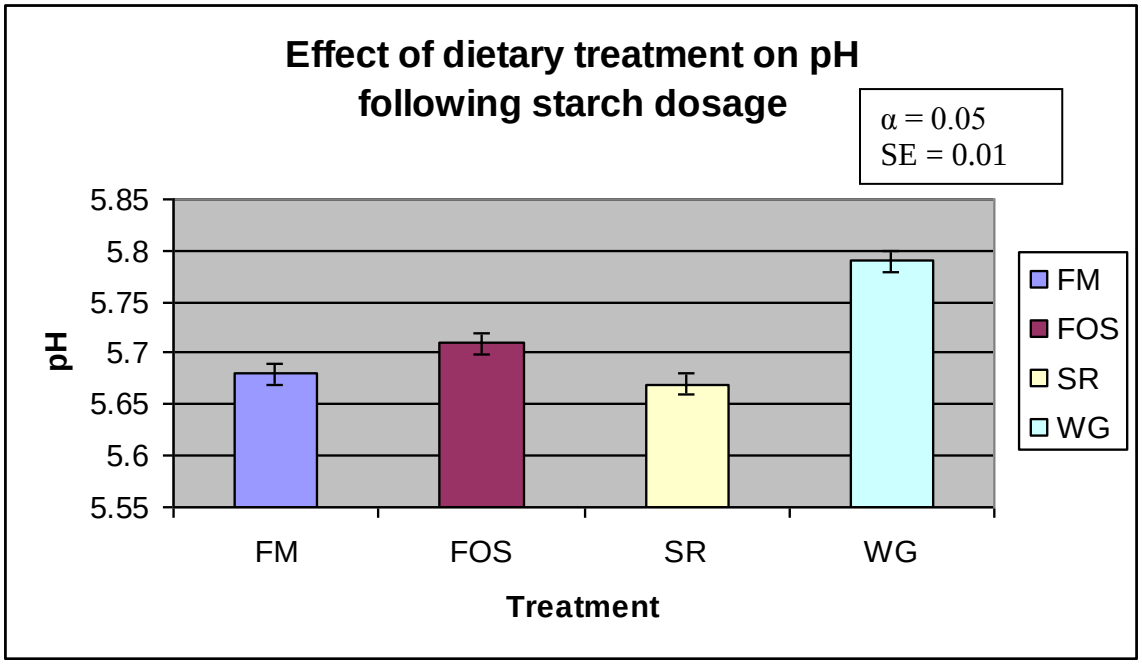

Figure 2.12 Effect of dietary treatment on pH of equine fecal microflora following starch dosage in continuous culture

CHAPTER 3

METAGENOMICS OF EQUINE COLIC

ABSTRACT

Colic in the equine has been called the "ultimate multi-factorial disease". We performed 454 pyrosequencing in order to phylogenetically characterize the shifts in the microbial populations observed in the feces of eight horses collected both during a colic episode and following a return to health.

Microbial DNA was extracted from the fecal samples and the V1, V2, and V3 regions of the 16S rDNA were amplified using primers suitable for pyrosequencing via emulsion PCR.

Our data set was comprised of 1, 241, 844 reads. Unassigned reads (no assigned operational taxonomic unit), were approximately 65% and were omitted from analysis. Reads were analyzed utilizing BLASTn 2.2.23+ (Basic Local Alignment Search Tool) and MEGAN (MEtaGenomics ANalyser) for identification and classification (Huson et al., 2007; Altschul et al. 1990).

Data were analyzed utilizing a Monte Carlo randomization approach and a Bonferroni correction to prevent undue numbers of false positives (R Core Development Team, 2008). Of these taxa, 19 were unique to the colic state, and 10 were only present in the healthy state. The unique taxa identified in the healthy horses included representatives of the phyla *Firmicutes* (5),

Bacteroidetes (3), *Proteobacteria* (1) and *Fusobacteria* (1). The unique taxa found in the diseased horses included microbes representing the phyla *Firmicutes* (11), *Proteobacteria* (6), *Bacteroidetes* (1), and *Lentisphaerae* (1).

There were 21 ubiquitous microbes identified. When examined by ranking read numbers and pairing thusly, healthy > colic, 17 were significant ($P < 0.05$). Conversely, when ranked and paired colic > healthy, four were significant ($P < 0.05$). All sick horses had greater reads of *Clostridium phytofermentans*, an uncultured *clostridiales* bacterium, and Bacteroidetes as well as an uncultured *bacteroidetes* bacterium.

These data are the first to elucidate specific changes within the microbiome of the equine gastrointestinal tract during a colic episode. Further research is needed in order to understand how to prevent these dramatic population shifts, and facilitate a faster recovery upon occurrence of same.

KEYWORDS: Equine, colic, metagenomics, microbe, DNA

INTRODUCTION

Colic has been an area of much research and focus for equine researchers and veterinarians in recent years. It is, therefore, surprising, that despite the importance of the equine microbiome to the animal's overall health and well-being, the microbial populations of the equine gastrointestinal tract are largely unknown. Much research has focused on risk factors for colic (Proudman, 1991; Abutarbush et al., 2005; Reeves et. al., 1989; 1995; and 1997) with less attention given to nutrition management for colic patients in recovery.

The microbes that colonize the gastrointestinal tract of the horse play a significant role in host biology. Notably, though the horse occupies an important part of the total agriculture sector, the study of its microbial ecosystem is far behind that of commercial cattle. Fortunately, because of the similarity between the rumen and the cecum, we can draw useful parallels with known patterns in ruminants.

Extensive fermentation of fibrous feedstuffs takes place in the large intestine of the equine. The volume of the cecum and colon accounts for more than two-thirds of the entire digestive tract. The fermentation of plant fiber within the cecum and colon releases volatile fatty acids (VFA's) that are subsequently absorbed into the bloodstream and provide as much as 80% of the energy required for the mature horse. Additionally, one of the by products of fermentation is the vitamin, biotin. Biotin has been associated with hoof health and is critical to the overall health of the animal.

Research currently taking place at the University of Kentucky (Lawrence, 2011) aims to address the question of how the original microbial colonization occurs in the foal. Young foals do not fully develop their cecal fermentative abilities until they are weaned. In this study,

researchers employed a metagenomics approach using denaturing-gradient-gel-electrophoresis along with amplification via PCR to identify changes in fecal microbial communities from foals and their dams. As expected, the foals had developed very similar microbial profiles as compared to their mothers within two weeks of birth, , suggesting that colonization appears immediately following birth.

Some work has been done to link changes in microbial populations to other health risks. Starch overload is the culprit most often identified with laminitis. Models utilizing oligofructose-induced laminitis have made progress toward better understanding the role specific microbes play in the balance of the gut as a whole. Additionally, this work has shown evidence relating microbial shifts to overall horse health (Milinovich et al., 2006).

Some progress has also been made (Shirazi-Beechey, 2008) towards elucidating the microbial impact on gut health and the populations contained therein. Thus, when bacterial diversity within the equine large intestine was examined, Daly et al., (2001) reported that 89% of the recovered sequences could not be matched to known sequences within public databases via operational taxonomic units (OTU's). These authors suggest that the equine microbiome may not be adequately represented in existing databases and that novel bacterial species may be present. This agrees with other reports (Morotomi et al., 2002).

It seems clear that additional knowledge of the bacterial species present within the equine gastrointestinal tract could both facilitate better understanding of the impacts of dietary change and help to ameliorate the effects of colic or other gastrointestinal disturbances. Toward this end, our research aimed to identify changes within the microbial population occurring in transition from the colic to the healthy state.

MATERIALS & METHODS

Sampling

Samples were collected from nine horses admitted to veterinary teaching hospitals for colic. Each was collected via rectal grab and was stored in a 50 ml sterile collection container at -70°C. Samples were frozen for a minimum of 24 hours, and were shipped on chemical ice pack via UPS overnight mail. Colic samples were considered for our study from horses that were admitted with large intestinal, non-surgical colic.

Follow-up samples were collected from the same nine horses 60 days (± 30) following discharge from the hospital. Samples were collected and shipped according to the same procedure outlined above. The follow-up sample from one horse was degraded and thus all data for that animal was omitted from analysis.

DNA extraction

DNA was extracted from each of the 16 fecal samples using the PowerSoil DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA). Samples were frozen for 24 hours prior to shipping via UPS overnight. Samples were shipped with chemical ice packs and their frozen state was confirmed immediately upon arrival at MOGene (St. Louis, MO).

Sequencing

Each fecal sample was analyzed for concentration and purity using Nanodrop (Thermo Fisher Scientific, Waltham, MA). Libraries of amplicons compatible with 454 Titanium sequencing chemistry were generated as outlined in Application Brief APP No. 001-2009 by Roche Diagnostics using loci a specific primer pair (27-forward AGRGTTTGATCMTGGCTCAG, 518 Reverse CGTATTACCGCGGCTGCTGG) that amplifies the V1, V2 and V3 hyper-variable regions of the 16S ribosomal RNA gene from a

variety of prokaryotes. The fusion primer design incorporates the Titanium Lib-A adaptor sequences and multiplex identifiers to create a poolable amplicon library. All purified amplicons were assessed on an Agilent chip (Agilent DNA 2100 Bioanalyzer, catalog # 5067-1506) to determine size. As expected, they were all identical. Subsequently, the purified amplicons were quantified with Picogreen (QuantiT Picogreen dsDNA Assay kit, catalog # P11496) to determine ng/μl concentration. Finally, emulsion PCR (polymerase chain reaction) was performed in order to obtain enriched beads for massively parallel pyrosequencing. High quality reads were separated based on Multiplex identifier (MID) and analyzed for phylogenetic origin by comparison to reference databases. Each MID generated upwards of 30,000 reads.

The massive data set that was generated (1, 241, 844 reads) contained only 64 miss-assigned reads. Miss-assigned reads were defined as experimental noise and were omitted from analysis. In order to provide phylogenetic rank at the lowest possible level, reads were analyzed utilizing BLASTn 2.2.23+ (Basic Local Alignment Search Tool) and MEGAN (MEtaGenomics ANalyser) for identification and classification (Huson et al., 2007; Altschul et al. 1990). Reads of < 100 bp were disallowed by MEGAN. Unassigned reads (having no assigned operational taxonomic unit), were approximately 65% and were omitted from analysis

Additionally, there was approximately a 3X spread between the highest and lowest numbers of reads per sample. This indicates the absence of emulsion PCR bias. Because PCR doubles the number of copies with each successive cycle, and it runs for 50 cycles, one would expect to see 50-100-1000x differences should bias be occurring. Based on the number of reads for each MID, all samples amplified equivalently in the emulsion PCR and thus, accurate representation of each sample occurred.

Statistical Analysis

To detect significant differences in microbial taxa abundance between paired samples from the healthy and sick animals, we adopted a randomization approach (Conant, 2011 available upon written request) as described below.

First, n_i was defined as the number of times the i^{th} microbial taxa was observed in the sample when the horse was sick. Second, m_i was the corresponding count from the healthy sample. We omitted all unidentified microbial individuals (missing OTU's) from analysis. Next we defined *s:*

$$s = \sum_i (n_i + m_i) \qquad (1)$$

as the total number of identifiable microbial individuals in that animal, and *n* as

$$n = \sum_i n_i \qquad (2)$$

i.e., the number of microbial individuals sampled during sickness, with *m* analogously defined for the healthy animals. Define *r=n/(n+m)*, i.e., the ratio of microbes observed in the sick sample relative to the total number of individuals seen. Finally, define *x*:

$$x = \left| n_i \Big/ (n_i + m_i) - r \right| \qquad (3)$$

as our statistic of interest. Under the simple null hypothesis, the number of individuals of the i^{th} taxa from the sick sample should be the total number of individuals from that taxa multiplied by *r*. Thus, *x* represents the deviation from the expected distribution of taxa *i* in the sick animal.

To ascertain if *x* is significantly larger than would be expected by chance, we created new pseudo samples by drawing *n* uniformly distributed random numbers between 1 and *s* to create a new "pseudo" sick animal and analogously *m* numbers to define the pseudo healthy sample. For

each of the *i* taxa, we defined $\overline{n_i}$ as the number of individuals of the i^{th} taxa observed in the random sample and analogously $\overline{m_i}$. We then compute $\bar{x}$:

$$\bar{x} = \left| \overline{n_i} \Big/ \left(\overline{n_i} + \overline{m_i}\right) - r \right| \qquad (4)$$

which is completely analogous to *x*. We created 100,000 pseudo individuals and counted the number of instances where $\bar{x}$ was greater than *x*. This count over 100,000 gave us our *P*-value: we then used the statistics package R (R Core Development Team 2008) to compute a Bonferroni correction to that value.

RESULTS

The results of the sequencing yielded 1, 241, 844 reads. As shown in Table 3.1, there were 21 microbes identified that were ubiquitous (present in all samples and both states). Approximately 28% of reads generated could be assigned no classification more distinct than "Bacteria"..

Table 3.2 demonstrates the shifts in total populations from the colic to healthy state. Values are expressed as a percentage of the total number of reads of healthy to colic. As shown, horses 2, 5, and 8 each displayed marked increases in the total number of reads that were sequenced in the healthy state as opposed to the colic state. In contrast, horses 1, 3, 4, and 7 showed much more similar percentages while horse 6 had markedly increased reads in the colic state over the healthy state.

Tables 3.3 and 3.4 show both the taxonomic rank and number of reads sequenced in the healthy and colic states (respectively). It is interesting to note that the number of unique microbes in the colic state is almost double that in the healthy state. In Table 3.5 we can see the difference in the total number of reads sequenced in both states for the ubiquitous microbes.

These data demonstrate that greater than 76% of the organisms sequenced appeared more frequently in the healthy state as opposed to the colic state. Additionally, nine of the organisms classified in the healthy state fall within the *Firmicutes* phyla. Moreover, when we rank the data according to state, there are four organisms that appear consistently higher in the colic state (Table 3.7) and seventeen that appear consistently higher in the healthy state (Table 3.8).

Table 3.9 gives the demographic data on each of the sample horses. The breeds represented here are a good representative of the total equine population (Quarter Horse = 4, Arabian = 2, Tennessee Walking Horse = 1, Thoroughbred = 1). All horses led very active lifestyles (i.e. performance, rodeo, show, etc) with the exception of 1, and 8 who were both retired. All horses were treated by a referring veterinarian for pain prior to presentation at the veterinary hospital for colic treatment.

DISCUSSION

The horse is an animal that evolved to continue grazing for long periods of time each day and as such are classified as "trickle feeders". Perturbation of this natural cycle has resulted from domestication and the large meals that most stalled equines are given twice daily. It is this feeding behavior that makes the arrival of a large starch bolus into the cecum problematic. The hindgut fermentation patterns require sufficient digestible and indigestible fiber in order to prevent gastric upset and or colic. Since it is unlikely that most equines will be able to return to a more feral diet, it is necessary to have a better understanding of the microbial populations that reside in the hindgut.

My intent in designing this experiment was to investigate the possibility that there are different species present during a colic episode as opposed to the healthy state. To my knowledge, there are currently no other published reports investigating these differences.

Although we failed to identify a sentinel microbe, the data suggest microbial diversity is key to the healthy condition.

The total number of reads that were reported showed only 64 that were miss-assigned. This is indicative of the absence of PCR bias and reflects an accurate portrayal of the bacterial species that were contained in our samples. Phylogenetic characterization of the sequenced reads was challenging. Only 35% could be assigned to known taxonomic groupings using public databases. This scarcity of information on gut microbiota has been identified by other authors (Golomidova et al., 2007; Dowd et al., 2008; Daly et al., 2001). However, work is currently being done to remedy this situation so that public database will ultimately provide better resources for microbial species identification (Sun et al., 2010).

The higher taxonomic ranks encompassed greater numbers of reads. As expected, the phyla *Firmicutes* (which contains the class *Clostridia*) represented almost half of the reads for these microbes in both states. This agrees with previous work (Daly et al., 2001). It is interesting to note that the total number of reads sequenced for each state (colic 178,936 : healthy 235,911) seem to provide further evidence that the diversity of the microbial profile in a healthy state facilitates growth across a variety of organisms. Although there are some specific organisms within the class *Clostridia* that have been associated with colic and diarrhea (Donaldson and Palmer, 1999) these taxa were not present within the sequences we observed. It is also of note to point out that greater than 28% of the ubiquitous reads were ranked no lower than *Bacteria*. This is another example of the paucity of publicly available sequence information.

With the exception of horses 6 and 7, each horse had larger sums of total reads in the healthy state as opposed to the colic. This suggests that competition during times gastrointestinal distress would result in the overpoplulation of microbes better suited to those conditions.

Unique microbes present only in one of the states are shown in Tables 3.3 and 3.4 Again, there are organisms from *Clostridium spp* present in each table that do not appear in the paired sample. This agrees with previously published reports (Dowd et al., 2008) ascribing both positive and negative effects to this particular genus and noting specific species may be linked to results regarding the overall health of the animal as well as the gastrointestinal tract. Additionally, the significant numbers of two different Lactobacillus organisms in the healthy state would agree with current trends regarding benefits to the digestive tract and its potential as a probiotic (Weese, 2002).

The numerical representation of the ubiquitous microbes in Table 3.5 shows their ability to adjust to the complex microbial ecosystem within the cecum. Additionally, it suggests that differences in the two states are not associated with one specific microorganism (or even two) but rather a dynamic shift in an entire population that is substrate dependent. Although there were specific microbes identified that were only present in the colic samples, it is plausible that their presence depends on their ability to survive rather than their intrinsic "colic causing" characteristic.

Despite the common assumption within the equine community that grain is the primary culprit for a colic episode, only two of the horses presented were reported to have any grain in their diets and no owners indicated a recent change in diets (Table 3.6). This disagrees with previously published reports (Cohen et al., 1999) that attribute the primary risk factor of colic to a recent change in diet, with a change in hay (forage) being the primary causative factor.

However, the completion of the forms and the memory of the owners may be suspect. It is not uncommon to discover that owners omit details when completing admission forms. This is undoubtedly not due to any intent to deceive, but rather a desire to complete the forms quickly and the stress of the situation itself.

The most remarkable data here is that we have identified specific microbes that are consistently present during a colic episode yet are absent from the healthy state. Although it is difficult to make generalizations regarding the equine population at large, it appears that we have identified the beginning of where future research must go in order to more fully understand the microbial processes at work during colic.

The variety of the microflora represented in the data is also interesting. Published studies have demonstrated the remarkable diversity of the equine hindgut (Daly et al., 2001). However, these authors cite multiple studies that seem to indicate PCR bias in certain species which may lead to underrepresentation and may explain the contradiction between PCR and traditional hybridization sequences.

Despite the importance of the gut micro floral population to the horse, we know little about its complex interactions. This is the first work done elucidating the differences in gut micro biota between the colic and the healthy state. Although it is acknowledged that these findings may be limited due to the high percentage of unknown OTUs , this is the first step in filling that void. As reported previously, the number of unique taxa in publicly available databases is listed as approximately 1.5 million while the actual number of 'species' is believed to be closer to 10 million (Blaxter et al., 2005).

It seems clear that the sequencing methods we utilized, as well as others currently in development, will help to generate the data needed for a more complete picture of the gut

population and its shifts during illness, a diet change, etc. Work in mice suggests that a microbial transplant can alter the hosts ability to utilize dietary fat (Turnbaugh et al., 2009). In this work, gnotobiotic mice were used to test theories on adiposity and the effect of the micro biome on weight loss. Researchers were able to alter weight gain by a transfer of microbes in the mice. It is interesting to note that the relative abundance of *Bacteroidetes* measured within the cecum and feces in this study was lower in mice fed a diet rich in fat and higher in mice fed a diet low in fiber but rich in plant polysaccharides. Turnbaugh et al. (2009) suggest that this difference may be due to the propensity of *Bacteroidetes* to forage for mucus glycans, a readily available substrate. This observation may offer insight as to why this population was prevalent in all of the colic horses. The ability to confer potential weight gain (or loss) with a simple microbial transplantation could affect huge changes in the way nutrition as a whole is approached. Much work is still needed to identify differences between age groups, geographic regions, breeds, reproductive status, and many other factors that may play a part in influencing the gut.

Other researchers have utilized pyrosequencing to identify microbial profiles in dogs fed different fiber levels (Middelbos, et al., 2010). In this work, the authors fed adult dogs a control diet without supplemental fiber and a fiber supplement diet that contained 7.5% beet pulp. Only 129 OTU's were identified and *Firmicutes* and *Bacteroidetes* were two of the three dominant phyla. The authors reported that the structure of the gut microbiome was affected by treatment and suggested that a small amount of dietary fiber could affect appreciable change in the canine hindgut. Their work was designed to provide a basis for investigation of "dietary interventions" for gastrointestinal issues in the canine related to microbial profile.

Work in dairy cattle (Dowd et al., 2008) has revealed several non-harmful species were ubiquitous as well as several pathogenic species. It is likely that as the technology develops, there may be more time sensitive methods that may facilitate intervention in the event of identification of pathogenic bacteria. Such methods and technologies could potentially prevent food recalls and foodborne illnesses occurring in the U.S. each year. Additionally, a better understanding of the complete micro biome of commercial livestock will help nutritionists to develop more efficacious methods of feeding and production.

Because colic is the single greatest contributor to equine mortality (other than natural causes), it is critical to further investigate these effects on the equine gut. Some work suggests a "top down" model that links the host's genetic factors more closely than other external environmental factors (Benson et al., 2010). These data suggest that external perturbation may be less significant than host genetic control. This may explain why probiotics have thus far been unable to demonstrate consistent results *in vivo* utilizing the horse as a model (Weese, 2004). This would make sense considering our lack of identifiable sequences related to the equine micro biome.

The data presented here provide an important first step in discovering the microbial processes that may facilitate recovery from or prevent colic episodes altogether. Further research should include sequencing equines from specific age groups, geographic regions, controlled diets, and following administration of antibiotics. All of these factors impact microbial populations. Additionally, as public databases are brought current with newly identified microbes, the complex interrelationship between host, diet, and microbial composition will be better understood.

Table 3.1 Taxonomic names for ubiquitous microbes sequenced from both colic and healthy states in the equine.

Table 3.1 Ubiquitous microbes for both colic and healthy states				
	Colic State		Healthy State	
Taxon Name	Sum of reads for each microbe	% total ubiquitous reads	Sum of reads for each microbe	% total ubiquitous reads
Bacteria	44925	25.11	66736	28.29
uncultured Firmicutes bacterium	37251	20.82	58892	24.96
Bacteroidetes	33918	18.96	12579	5.33
Firmicutes	18211	10.18	44241	18.75
uncultured Clostridiales bacterium	11984	6.70	3083	1.31
uncultured Bacteroidetes bacterium	11850	6.62	10709	4.54
uncultured Ruminococcaceae bacterium	4968	2.78	5210	2.21
uncultured Gram-positive bacterium	3405	1.90	3692	1.56
Bacteroides	2319	1.30	10019	4.25
Clostridiales	1888	1.06	5687	2.41
Bacteroidales	1163	0.65	1556	0.66
uncultured Clostridia bacterium	1095	0.61	1518	0.64
Lachnospiraceae	854	0.48	1739	0.74
uncultured Lachnospiraceae bacterium	752	0.42	1571	0.67
butyrate-producing bacterium L2-7	730	0.41	1189	0.50
Coriobacteriaceae	707	0.40	1403	0.59
uncultured planctomycete	690	0.39	1603	0.68
Clostridia	672	0.38	1911	0.81
Clostridium phytofermentans	650	0.36	392	0.17
Clostridium	462	0.26	1473	0.62
uncultured Clostridiaceae bacterium	442	0.25	708	0.30
The taxonomic name is ordered by the most abundant sequence.				

Table 3.2. Population shifts in microbial species as determined total read counts via pyrosequencing in the colic and healthy equine.

Population shifts per horse in both colic and healthy states

	horse 1		horse 2		horse 3		horse 4		horse 5		horse 6		horse 7		horse 8	
	C	H	C	H	C	H	C	H	C	H	C	H	C	H	C	H
Total reads	12,646	17,887	11,640	30,220	30,350	35,716	16,158	28,063	6,649	23,521	59,435	25,448	18,064	17,544	23,994	57,512
% total reads	7.07	7.58	6.51	12.81	16.96	15.14	9.03	11.90	3.72	9.97	33.22	10.79	10.10	7.44	13.41	24.38
healthy:colic	1.41		2.60		1.18		1.74		3.54		0.43		0.97		2.40	

C = Colic state
H = Healthy state

% total reads = Total number of reads per horse divided by total reads for all horses

Table 3.3 Unique microbes identified within equine fecal material in the healthy horse following a colic episode.

Unique microbes significant in the healthy horse

		# of reads per horse							
rank	**Taxon Name**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
family	Pasteurellaceae	0	0	0	0	0	0	0	225[a]
genus	Fusobacterium	0	0	0	0	0	0	0	695[a]
family	Lactobacillaceae	0	22	12	85[a]	0	0	0	7
species	Clostridium glycolicum	0	0	0	0	0	0	0	92[a]
species	unidentified rumen bacterium 12-56	0	0	0	0	7	0	50[a]	0
species	unidentified rumen bacterium 30-25	0	0	0	54[a]	0	0	10	0
species	Prevotella sp. oral clone ID019	0	0	0	0	0	0	0	156[a]
species	Lactobacillus hayakitensis	0	72[a]	69[a]	291[a]	0	0	0	21
species	Clostridium sp. CM-C52	0	0	0	0	0	0	0	69[a]
genus	Blautia	0	0	0	0	0	0	0	66[a]

a = p value < 0.05 utilizing Bonferroni correction

Table 3.4 Unique microbes identified within equine fecal material in the colic horse following admission to a veterinary teaching hospital.

Unique microbes significant in the colic horse

		# of reads per horse							
rank	**Taxon Name**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
genus	Helicobacter	76[a]	0	0	0	0	0	0	0
genus	Acetobacter	0	29[a]	0	0	0	0	0	0
species	Streptococcus equi	694[a]	107[a]	0	0	0	0	0	63[a]
order	Bacillales	0	119[a]	0	0	0	0	3051[a]	0
genus	Bacillus	0	125[a]	0	6	0	0	12	0
species	Bacillus anthracis	0	34[a]	0	0	0	0	183[a]	0
species	Bacillus cohnii	0	29[a]	0	0	0	0	0	0
species	unidentified rumen bacterium 12-103	0	68[a]	0	0	0	0	0	0
species	Solibacillus silvestris	0	479[a]	0	15	12[a]	0	0	0
genus	Candidatus Odyssella	0	0	4	10	0	0	0	16[a]
species	uncultured beta proteobacterium	0	19	5	0	0	0	5	13[a]
species	uncultured gamma proteobacterium	7	0	0	0	0	0	0	13[a]
species	Clostridium xylanovorans	0	0	0	0	12[a]	0	0	0
species	Acetobacter estunensis	0	49[a]	0	0	0	0	0	0
species	uncultured Veillonellaceae bacterium	18	0	57[a]	0	0	0	32	0
species	uncultured Lentisphaerae bacterium	0	0	10	0	0	0	15	20[a]
species	Bacillales bacterium CCUG 49712	0	80[a]	0	0	0	0	0	0
species	Bacillus sp. ge12	0	44[a]	0	0	0	0	0	0
species	Clostridiales bacterium oral taxon 093	0	0	4	0	0	0	0	18[a]

a = p value < 0.05 utilizing Bonferroni correction

Table 3.5 Ubiquitous microbes identified within the equine during both colic and healthy states as determined utilizing pyrosequencing.

Number of reads sequenced for ubiquitous microbes		
Taxon Name	**Colic**	**Healthy**
Bacteria	44925	66736
uncultured Firmicutes bacterium	37251	58892
Bacteroidetes	33918	12579
Firmicutes	18211	44241
uncultured Clostridiales bacterium	11984	3083
uncultured Bacteroidetes bacterium	11850	10709
uncultured Ruminococcaceae bacterium	4968	5210
uncultured Gram-positive bacterium	3405	3692
Bacteroides	2319	10019
Clostridiales	1888	5687
Bacteroidales	1163	1556
uncultured Clostridia bacterium	1095	1518
Lachnospiraceae	854	1739
uncultured Lachnospiraceae bacterium	752	1571
butyrate-producing bacterium L2-7	730	1189
Coriobacteriaceae	707	1403
uncultured planctomycete	690	1603
Clostridia	672	1911
Clostridium phytofermentans	650	392
Clostridium	462	1473
uncultured Clostridiaceae bacterium	442	708

Denotes higher number of reads per state

Table 3.6 Ubiquitous microbes identified within the equine during both colic and healthy states as determined by pyrosequencing and numerically ranked colic > healthy.

Ubiquitous microbes in ranked pairs (sick > healthy)								
Taxon Name	horse 1	horse 2	horse 3	horse 4	horse 5	horse 6	horse 7	horse 8
Clostridium phytofermentans	7	462				13	7	70
uncultured Bacteroidetes bacterium	915		1914	3830	578	375	1268	1483
uncultured Clostridiales bacterium	92	92	307		18	11022	68	
Bacteroidetes	388	778	1056		524	27092	1103	1508

Read numbers represent P value < 0.05 utilizing Bonferroni correction

Table 3.7 Ubiquitous microbes identified within the equine during both colic and healthy states as determined by pyrosequencing and numerically ranked healthy > colic.

Ubiquitous microbes in ranked pairs (healthy > sick)								
Taxon Name	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5	Horse 6	Horse 7	Horse 8
Bacteria	4439	9003	10209	5646	5849	5041		23003
uncultured Firmicutes bacterium	5026	9060	12546	10828	7183	8966	4057	1226
Firmicutes	1955	3975		3002	3999	3702	2226	19743
uncultured Ruminococcaceae bacterium	488	1651	698	498	687	356	626	206
uncultured Gram-positive bacterium	263	734	768	698		327		222
Bacteroides	867		562	1846	1379	3094	1688	245
Clostridiales	182	263	802	323	527	519	234	2837
Bacteroidales	71	462	20	121		100		331
uncultured Clostridia bacterium	141	369		161		115	153	52
Lachnospiraceae	86	112		138	291	313	229	48
uncultured Lachnospiraceae bacterium	125	142			166	284	124	
butyrate-producing bacterium L2-7	78	112	440	171		198	70	59
Coriobacteriaceae				302		287		128
uncultured planctomycete	269			789	120	301	16	17
Clostridia	83				80			1139
Clostridium				206	46			960
uncultured Clostridiaceae bacterium				210		54		33

Read numbers represent P value < 0.05 utilizing Bonferroni correction

Table 3.8 Demographic data on horse presented to veterinary teaching hospitals for colic episodes and utilized for fecal sample collection to determine microbial pattern changes.

Demographic data on horses utilized for sample collection													
ID	School	% reads	Age (yrs)	Breed	Gender	BW (lbs)	Use	pain mds	NGT	Forage	History	Antibiotics	Other
1a	MU	7.39	16-20	TW	G	900-1100	Retired	x	x	grass	No	No	No
1b		6.34											
2a	MU	5.14	<2	QH	F	1100-1300	Pleasure	x	x	legume	No	No	No
2b		7.23											
3a	Iowa	10.22	11-15	QH	G	700-900	Work	x		grass	Surg colic	Yes	No
3b		9.04											
4a	MU	6.91	5-10	QH	G	900-1100	Rodeo	x		grass		Yes	
4b		6.00											
5a	Iowa	5.19	5-10	QH	G	1100-1300	Work	x	x	legume/grass mix*	Surg colic	Yes	No
5b		5.85											
6a	MU	3.78	5-10	ARB	F	900-1100	Work	x	x	grass	lacerations	Yes	ulcer
6b		2.92											
7a	MU	8.13	11-15	TH	G	1100-1300	Work		x	grass	No	No	No
7b		4.29											
8a	MU	4.63	>20	ARB	F	900-1100	Retired	x	x	grass*	NS Colic/lam	No	choke
		6.92											

* denotes grain mixed with hay
MU denotes University of Missouri Veterinary Teaching Hospital
Iowa denotes Iowa State University Veterinary School
G = Gelding F = Female
NGT denotes use of nasogastric tube

QH = American Quarter Horse
TW = Tennessee Walking Horse
ARB = Arabian
TH = Thoroughbred

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VITA

Erin Beth Venable was born in Cape Girardeau, MO in 1972 and grew up in Southeast Missouri. She is the daughter of Jim and Johnne Griffin. She has two brothers, Jim and Jared Griffin and one sister Gina Koch, MSPN. In November of 1999, Erin married Christopher Ray Venable. Chris graduated with his Bachelor of Science in Criminal Justice and went on to get his Master's in Criminal Justice from Southeast Missouri State University. Chris works for the city of Cape Girardeau as a Master Firefighter. Erin and Chris have two sons, Chase and Gavin

Erin Graduated from Southeast Missouri State University in December of 2001 with a Bachelor of Science in Animal Science. She completed her Master's Degree in December of 2004 and went on to teach at Southeast Missouri State University for a year before joining the team at Purina Mills as an Equine Specialist.

Erin began her doctoral program at University of Missouri – Columbia under the guidance of Dr. Monty S. Kerley and will receive her Ph.D. in Animal Science with a emphasis in Equine Nutrition in May 2011.

Erin is a member of a Federal Urban Search Team and is a Canine Handler for the Federal Emergency Management Agency within the Missouri Task Force One team. She is a member of the American Society of Animal Scientists, the Morris Animal Foundation and Delta Tau Alpha and has served as past National President.