THESIS

CHARACTERIZING THE MICROBIOTA AND PROFILING SMALL NON-CODING RNAS IN THE COMPARTMENTS OF THE EQUINE HINDGUT

Submitted by

Kailee Janelle Reed

Department of Animal Sciences

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Master's Committee:

Advisor: Stephen J. Coleman

Jason Bruemmer
Philip Turk
Gerrit Bouma

ABSTRACT

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Gastrointestinal homeostasis is a complex relationship that encompasses the host's immune response, physiology, gut structure and the microbes residing within the host. Each one of these has pathways of communication in order to keep the host in a 'healthy state' or homeostasis. While each category has been extensively researched independently, interactions that occur between host and microbe are largely still unknown, especially within the equine species. Because horses are extremely prone to various gastrointestinal diseases, understanding the microbial populations and how the horse might communicate with those populations will provide more insight on equine gut homeostasis. The main objectives were to delineate the microbial structures residing within compartment of the hindgut and to begin to profile gene expression patterns of small RNAs within the same areas.

Two different populations of animal subjects were used for the two projects in this thesis: a herd from the University of Kentucky (n=6) and a herd from Colorado State University (n=3). The herd from Kentucky was used for the microbiota data set in order to determine the microbial population structure within the cecum, right ventral colon, right dorsal colon and feces. First, we characterized microbial communities present in each of these anatomical sites and then completed a multivariate model to determine similarities of compartments and compared those to the fecal sample. The population of microorganisms observed in the proximal hindgut appeared similar between cecum and ventral colon, while the dorsal colon and fecal samples appeared to be more alike. Interestingly, there is an anatomical structure separating ventral and dorsal portions of the

colon called the pelvic flexure. This could possibly be an indication of the host's contribution of determining the microbial communities in each anatomical region. We also demonstrated that while some microbial signatures from the proximal gut were identified in the feces, the distal gut seemed to be more represented in the fecal sample.

The herd from Colorado was used to produce the gene expression data for the second project and the main focus was to profile microRNA (miRNA) expression along the hindgut. These small non-coding RNAs have been identified to be involved in gastrointestinal homeostasis within the intestinal epithelium and are host derived molecules. We demonstrated that each tissue (n=8 for each horse) had unique miRNA expression profiles and these miRNAs identified were used to complete a target pathway analysis which shows possible pathways that could be associated with the biological function of each intestinal site.

While each project had different objectives, they are both key players of gastrointestinal homeostasis. For future research, we plan to combine these two areas of study by knowing which miRNA could target specific bacteria residing in the gut, which may further the knowledge of how the host contributes to the population structure of the microbes within their gastrointestinal tracts.

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CHAPTER I: REVIEW OF LITERATURE

INTRODUCTION

The equine hindgut is a host to a vast complex community of commensal, symbiotic, and occasionally pathogenetic microorganisms (Costa & Weese, 2012). Microbes residing in the gastrointestinal (GI) tract of the horse (and most mammals) contribute significantly to many physiological processes that take place within the gut. One of several interactions that occur in the GI involve the luminal surface of the epithelium tissue, and the microbes within the lumen of the intestinal compartments (Al Jassim & Andrews, 2009). Through various complex interactions, this host-microbe interplay has recently been associated with maintaining gastrointestinal homeostasis, which is vital for a healthy GI tract to carry out many physiological functions like digestion and aiding in absorption of nutrients (Belcheva, 2017). Although there are many other factors influencing GI homeostasis (Figure 1), the host-microbe communication pathway is poorly understood in most mammals. The objective of the studies within this thesis is to enhance understanding of the microbial populations residing in the hindgut of the horse and to begin profiling gene expression along the equine hindgut.

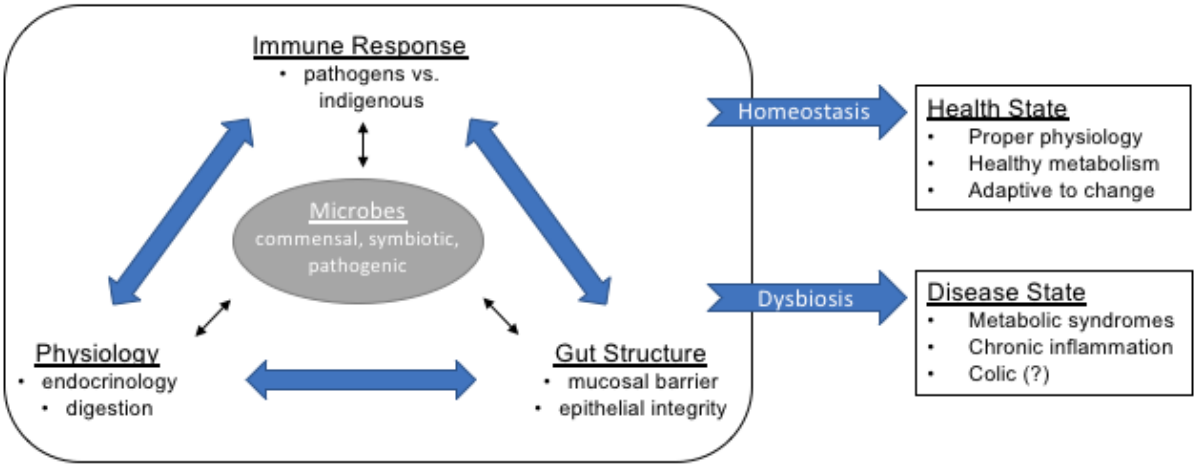

Figure 1: Model displaying factors influencing gastrointestinal homeostasis. Model adapted from the Buchon Lab at Cornell University (www.buchonlab.com/intestinal-homeostasis.html)

EQUINE GASTROINTESTINAL ANATOMY

The equine GI tract is a multifaceted system, and being that these animals are hindgut fermenters, they rely heavily on the cecum and large colon for fermentation (Costa & Weese, 2012). Volatile fatty acids are produced during fermentation and then utilized for energy (accounts for 65% of energy production) (Al Jassim & Andrews, 2009; Costa & Weese, 2012). Acetate, butyrate and propionate are produced during this process and are sources of energy for tissue restoration and regeneration, gut epithelia and precursors for gluconeogenesis, respectively (Hoffman, 2001; Milinovich et al., 2010; Costa & Weese, 2012). Hindgut fermenters possess a vast and complex microbial population that supports digestion, health, and even synthesizing vitamins (NRC, 2007; Debelius et al., 2016). The main digestive mechanisms, enzymatic and microbial degrading properties, in addition to the highly evolved cecum morphology (Sneddon & Argenzio, 1998), all combine to construct the particularly distinct digestive system of hindgut fermenters.

The GI tract of the horse can be divided into different anatomical areas (Figure 2) that have characteristic jobs depending on the compartment which the digesta is traveling through. Although the start of digestion occurs in the mouth by chewing and the breaking down of foodstuffs with the aid of saliva (Clauss et al., 2014; Dicks et al., 2014), this review focuses on basic functionality of the foregut and the hindgut of the equine GI tract. The foregut consists of the stomach (glandular and non-glandular regions) and small intestine (duodenum, jejunum and ileum), which are the main portions of the GI tract (beyond saliva) where enzymatic digestion and absorption of nutrients occur (Moore et al., 2001; Pilliner, 1993). While the stomach is mainly utilized for storing and mixing feed, there are also digestive acids produced by the glandular region to help further breakdown of feed (Moore et al., 2001; Dicks et al., 2014). The majority of enzymatic digestion begins once the digesta moves into the small intestine, which is the main site for digestion and absorption of carbohydrates, fats and proteins (Kararli, 1995; Pagan, 1998). The luminal surface of the small intestine is covered in villi that also extend into microvilli, that permits the organ to have an enormous surface area for absorption of nutrients (Kararli, 1995). Several tissue types, such as epithelial and connective tissue comprise diverse cell types and enterocytes that give support to barrier function, secreting immunological intermediaries, delivering bacterial symbiont or antigens and many other physiological processes (Garrett et al., 2010; Wong et al., 2001; Okumura & Takeda, 2017).

After digesta has passed through the ileum (last portion of small intestine), it is deposited into the hindgut, consisting of the cecum, large colon and small colon. The large colon has several sections and flexures (right/left ventral colon, left/right dorsal colon) and are separated by the sternal, pelvic and diaphragmatic flexures. Accounting for about 62% of entire GI tract and about 75% of total GI volume (Table 1), the hindgut accounts for a significant proportion of the

gastrointestinal system size and function (Popesko & Getty, 1971; Sneddon & Argenzio, 1998). The cecum and large colon are where the majority of microbial populations exist, these populations contribute to fermentation process to produce volatile fatty acids, one of the main sources of energy production for this animal (Al Jassim & Andrews, 2009; Costa & Weese, 2012). Starting at the cecum and throughout the hindgut, microbial digestion occurs by fermenting complex carbohydrates (like starch) and synthesizing essential amino acids (Dicks et al., 2014). Tissues of the hindgut are different compared to small intestine tissue structure. Villi are not present on the luminal surface, but they are divided into areas by diagonal undulations that are not as packed together as the small intestine tissues (Taylor & Anderson, 1972; Kararli, 1995), in order to promote GI motility and further breakdown of feed.

Table 1: Equine foregut and hindgut capacities, lengths and total percentages of digestive tract (adapted from Ontario Ministry of Agriculture, Food and Rural Affairs website: http://www.omafra.gov.on.ca/)

Digestion Type	**Foregut**	**Capacity**	**% of GI**
Enzymatic	Stomach	8-15 liters	8%
	Small Intestine (21 meters)	68 liters	30%
Digestion Type	**Hindgut**	**Capacity**	**% of GI**
Microbial	Cecum (1.2 meters)	28-36 liters	15%
	Large Colon (3.6 meters)	86 liters	38%
	Small Colon (3-3.6 meters)	16 liters	9%

The importance of microorganisms reaching and continuing a homeostatic relationship with its host is crucial for an animal that relies heavily on the fermentation process for digestion and absorption of nutrients. Further understanding of a ‘healthy’ equine microbiota in the hindgut

can lead to understanding the functionality of diseases that can occur there. Some diseases that can occur in the equine hindgut include impactions, strangulations, diseases from bacteria that cause diarrhea, colitis leading to colic, etc. (Waguespack, 2006). Colitis and colic are the leading causes of morbidity in horses (USDA, 2001) and has a large impact on the equine industry. This demonstrates why the equine community would benefit from advancing knowledge of the equine hindgut from a molecular view.

THE GASTROINTESTINAL MICROBIOTA

The GI microbiota has been recognized as an essential ecosystem of microbial communities within the gut of mammals, which are intricate populations that consist of commensal, symbiotic and sometimes pathogenetic microorganisms. These communities also the main interaction with the host's environment (nutrients, treatments, toxins, bacteria, pathogens etc.), making it a vital organ for all mammals. Since discovering that the microbes present outnumber the cells of the host and contribute their genetic information (Debelius et al., 2016), they have emerged as important regulators of physiological processes and overall health or disease (Hooper et al., 2010). This rise of meaningful significance was made possible by the application of next generation sequencing (NGS) technologies, which supported identifying non-culturable bacteria and specific taxonomic communities that exist in niche environments (Turnbaugh et al., 2007). While there has been extensive research prior to the application of NGS using culture-based methods, this type of sequencing propelled microbiome research forward in many species.

In model organisms such as mice and humans, microbial populations have been shown to influence digestive physiology (D'Argenio & Salvatore, 2015), behavior (Vuong et al., 2017), host metabolism (Vijay-Kumar et al., 2010; Koren et al., 2012), immune system development and function (Chung et al., 2012), endurance and athletic ability (Mach et al., 2017), and the

metabolites secreted by the GI microbiota influence gene expression of the host (Daly & Shirazi-Beechey, 2006; Belcheva, 2017). While there are different communities throughout the GI tract that harbor vibrant microbial communities, such as the stomach (Perkins et al., 2012; Turnbaugh et al., 2007), the GI tract possesses the largest microbial populations (Debelius et al., 2016). An imbalance or a dysbiosis of these communities has been associated with several diseases like metabolic syndrome (Wen et al., 2008; Elzinga et al., 2016), colitis (Garrett et al., 2007; Costa et al., 2012; Michail et al., 2012), inflammatory bowel disease (Willing et al., 2010) and many other GI disturbances. These influential impacts display the need of continuing to characterize these communities and understand their biological roles within the gastrointestinal ecosystem.

Several factors, exogenous or endogenous, affect the microbial composition of the gut, and these factors are all important for conducting microbiota research (Goodrich et al., 2014). Some of the greatest variables affecting the community composition include diet (Xu & Knight, 2015), sex (Bolnick et al., 2014), age (Yatsunenko et al., 2012) and the most influential being host genotype (Benson et al., 2010; Goodrich et al., 2014; Turnbaugh et al., 2009). These factors are continuously being studied in order to better understand why and how they affect the community structure, but this also demonstrates the importance of having a balanced and consistent population when sampling or conducting a microbiota study.

THE EQUINE GASTROINTESTINAL MICROBIOTA

Since fecal samples were identified as good indicators of the GI microbial community structures in humans and other model organisms (Turnbaugh et al., 2007), it was assumed the same for other mammals including horses and cattle. The use of fecal samples also created a non-invasive, inexpensive ‘biomarker’ for researchers to begin to use for horses; however, minimal research has been conducted in order to understand how closely the microbial populations of fecal

samples actually characterize the equine large intestine (Hastie et al., 2008; Sadet-Bourgeteau et al., 2010; Dougal et al., 2012), especially utilizing NGS technologies.

Some studies that have characterized the various compartments of the equine GI tract demonstrate that microbial communities shift throughout the system in terms of abundance and similarity, but do not go into great depth of statistical comparisons that indicate similar signatures between compartments or comparisons to the matter (Dougal et al., 2012; Costa et al., 2015; Ericsson et al., 2016; Julliand et al., 2016). Another factor that had a large impact on all of the compartment studies is the researchers had a variety of animals that were not consistent in terms of breed, age, housing situations, diets or where the animals originated, making it fairly challenging to remove environmental factors or individual variation that could alter the composition of the microorganisms inhabiting the GI sections and feces.

To be able to state with confidence if a fecal sample can be a predictive model of what is occurring microbially in the GI tract, we need to characterize compartments within the equine gut and compare the microbial populations back to that of the fecal microbiota. By implementing various multivariate statistical analyses (such as indicator species analysis), we can begin to understand which microbial families can indicate a particular compartment, and then observe if any of those specific families appear in the fecal matter (Carignan & Villard, 2002). Understanding which groups appear in fecal samples could possibly lead equine researchers and owners to a better management strategy for the health of their animals by displaying the microbes residing within the GI with a simple tool such as a fecal sample.

EPITHELIAL TISSUES & SMALL NON-CODING RNAS IN GASTROINTESTINAL HOMEOSTASIS

While there is a wide variety of non-coding RNAs involved in many physiological processes, there has been an emergence of a significant category of non-coding RNAs called microRNAs (miRNA) (Bartel, 2004). First being reported in the early 90's from the *lin*-4 RNA gene and its regulation of *lin*-14 (Lee et al., 1993), this class of small non-coding RNAs have been identified as regulators of post-transcriptional gene expression (Wightman & Ruvkun, 1993). Subsequently, miRNAs have been well described in hundreds of studies that further the support of their regulation of gene expression, specifically post-transcriptional regulation of abundance and usage of protein-coding genes (Shimoni et al., 2007; Jackson & Standart, 2007; Suarez & Sessa, 2009; Dykes & Emanueli, 2017). MiRNAs are classified as 'small' RNAs due to the fact they are single-stranded molecules about 18 – 22 nucleotides long (Jackson & Standart, 2007; Suarez & Sessa, 2009; Gulyaeya & Kushlinskiy, 2016); however, there are several types and sizes of miRNA as they progress through their biogenesis to mature transcripts. Mature miRNA transcripts are extremely powerful tools of gene regulation due to the fact that a single miRNA can have multiple mRNA targets (Graves & Zeng, 2012) and are predicted to regulate around 60% of protein-coding genes in humans (Friedman et al., 2009; Duval et al., 2017).

Some regions in which miRNA can be expressed are monocistronic intergenic loci, polycistronic intergenic loci or within the introns of transcribed messenger RNA (mRNA) (Craig et al., 2014; Bartel, 2004; Kim & Kim, 2007). Biogenesis starts in the cell nucleus and is transcribed by RNA Polymerase II as primary or pri-miRNA (Lee et al., 2003; Bartel, 2004; Bartel, 2009). Further processing occurs when Drosha, a RNAse III endonuclease, trims the terminal ends of pri-miRNA into 70-90 basepairs, creating pre-miRNA transcripts that have hairpin like

assemblies (Lee et al., 2003). Pre-miRNA are then exported into the cytoplasm by exportin 5 to continue on the journey of becoming mature transcripts (Craig et al., 2014). Once in the cytoplasm, another RNAse III endonuclease, Dicer, further processes pre-miRNA by cleaving the turn of the hairpin structure (Kuehbacher et al., 2007). Cleavage from Dicer leaves a miRNA: miRNA duplex, and these interact with AGO (argonaute) and RISC (RNA induced silencing complex) complexes that guide the mature miRNA transcripts to targets within mRNA sequences (Gregory et al., 2005; Wang et al., 2008).

MiRNAs have been recognized as important regulators in numerous physiological roles such as developmental timing (Lee et al., 1993; Reinhart et al., 2000), cell death and proliferation (Brennecke et al., 2003), neuronal cell fate (Johnston & Hobert, 2003) and many others. However, within the last few years, miRNAs are emerging as having roles associated with maintenance of gut homeostasis in model organisms such as mice (Runtsch et al., 2014; Belcheva, 2017). MiRNAs are activated within intestinal epithelial cells that aid in regulation of processes associated with proliferation and differentiation of epithelium (McKenna et al., 2010; Peck et al., 2016), apoptosis (Hanahan & Weinberg, 2011), inflammation (Wu et al., 2008) and can also be packaged into vesicles and secreted into the intestinal lumen (Belcheva, 2017; Choi et al., 2017). Knowing that host-derived miRNAs can be produced in intestinal epithelium and be delivered into the lumen through many pathways, adds another dimension of the host possibly contributing to the composition of microbial communities in the gut. Recent studies demonstrated that different microbial community composition correlated with expression levels of specific host-derived miRNAs (Liu et al., 2016) and that small RNAs secreted by bacteria in the GI influence expression of both mRNA and miRNA in epithelium (Filip et al., 2016; Choi et al., 2017). The interaction

between host-derived molecules and microbiota present adds another dimension that could possibly help explain the regulatory processes of GI homeostasis.

There has been a great deal of work in the horse to annotate miRNA transcripts and try to determine expression profiles in different biological systems (Kim et al., 2014; Desjardin et al., 2014; Pacholewska et al., 2016). MiRNAs identified to be horse-specific are now being investigated in terms of their specific roles in equine physiology such as reproduction (Klohonatz et al., 2015; Loux et al., 2017; Twenter et al., 2017; da Silveira et al., 2018), tissue specific or breed-related miRNAs (Pacholewska et al., 2016; Kim et al., 2014), and potential use of miRNAs in equine medicine for possible therapeutic tools (van der Kolk et al., 2015). A little amount has been done in terms of the equine GI system and which miRNAs are present throughout the hindgut. While a small amount of research has showed miRNA expression in the equine colon (Kim et al., 2014; Pacholewska et al., 2016), there has not been a study conducted that begins to profile miRNA expression from tissues starting at the cecum and ending at the small colon. By providing more tissue samples from different areas of hindgut, it can perhaps provide insight on if the horse (host) has a contribution of the microbial populations present within each compartment. In order to do this, understanding which miRNAs are present in each compartment is needed.

CONCLUSION

Equine gastrointestinal homeostasis is a delicate and complex process with many interactions occurring. While some of the components (Figure 1) have been well described, host-microbe communication path is poorly understood and has been shown to be more intricate than previously thought. Further investigation into the equine GI microbiota and gene expression within this vast organ can begin to demonstrate how this pathway interacts. The goal of these two studies

presented are to evaluate microbial compositions in each major gastrointestinal compartment and to profile mature miRNA transcripts present in the equine hindgut.

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CHAPTER II: CHARACTERIZATION OF SHARED MICROBIAL COMMUNITY STRUCTURE IN COMPARTMENTS OF THE EQUINE HINDGUT

SUMMARY

The large intestine encompasses the largest population of microbes in the gastrointestinal tract. Interactions between these microbial populations and host are critical for maintaining gut homeostasis, which plays an important role in proper digestion, disease processes and management. Characterizing populations along the large intestine could be valuable in diagnosing, treating and managing gastrointestinal issues in the horse. It is not feasible to sample these communities directly. Fecal samples are relatively easy to obtain and could provide a non-invasive approach to monitor microbial populations of the equine hindgut. The current project seeks to determine if aspects of fecal microbiota can serve a predictive role in understanding other microbial communities in the large intestine. Digesta from the cecum, ventral colon, dorsal colon and feces were collected from 6 mixed-breed pony yearlings (3 males & 3 females) [horses were maintained under identical conditions prior to collection]. Microbial DNA was isolated from each sample and 16S rRNA gene sequence was targeted by amplification of the V4 region using 515-806 primer set. Amplicons were pooled, barcoded and 2x150 paired-end reads were sequenced on an Illumina MiSeq platform. Diversity metrics were analyzed with Qiime2 (2017.11 distribution) and sequences were aligned to GreenGenes (v13.8) reference database for taxonomic classification. A principal coordinate analysis plot of the Bray Curtis dissimilarity matrix between each sample revealed that the cecal microbial population is distinct compared to those observed in the dorsal colon and feces. In contrast, microbial populations of the cecum and ventral colon were similar. This clustering before and after the pelvic flexure was significant ($p=0.0001$; $F=5.2393$).

An indicator species analysis was also applied to these data. Demonstrating that fecal samples may be good indicators of microbial populations of the distal hindgut of horses. The proximal gut is not fully embodied. Fecal samples can be used as estimators of the gut, but not standing as a full representation of what is occurring throughout the hindgut.

INTRODUCTION

The equine gastrointestinal tract is a large multifaceted organ that can be divided into two main sections that have specific purposes. The foregut consists of the stomach (glandular and non-glandular regions) and small intestine (duodenum, jejunum and ileum), which are the main portions of the GI tract where enzymatic digestion and absorption of nutrients occur (Moore et al., 2001; Pilliner, 1993). While the stomach is mainly utilized for storing and mixing feed, there are also digestive acids produced by the glandular region to help further breakdown of feed (Moore et al., 2001; Dicks et al., 2014). Enzymatic digestion begins once the digesta moves into the small intestine, which is the main site for digestion and absorption of carbohydrates, fats and proteins (Kararli, 1995; Pagan, 1998). The luminal surface of the small intestine is covered in villi that also extend into microvilli, that permits the organ to have an enormous surface area for the absorption of nutrients discussed previously (Kararli, 1995). There are several tissue types, such as epithelial and connective tissue, that comprise numerous diverse cell types and enterocytes that gives support to barrier function, secreting immunological intermediaries, delivering bacterial symbiont or antigens and many other physiological processes (Garrett et al., 2010; Wong et al., 2001; Okumura & Takeda, 2017). After the digesta has passed through the ileum (last portion of small intestine), it is then deposited into the hindgut, consisting of the cecum and large colon. The large colon has several sections and flexures such as the right/left ventral colon, left/right dorsal colon, and are separated by the sternal, pelvic and diaphragmatic flexures (Figure 2). Accounting for about 62%

of entire GI tract and about 75% of total GI volume (Table 1), the hindgut is a significant organ for the horse (Popesko & Getty, 1971; Sneddon & Argenzio, 1998). Being that the cecum and large colon is where the majority of the microbial populations exist, these populations contribute to the fermentation process to produce volatile fatty acids, one of the main sources of energy production for this animal (Al Jassim & Andrews, 2009; Costa & Weese, 2012). Starting at the cecum and throughout the hindgut, microbial digestion occurs by fermenting complex carbohydrates (like starch) and synthesizing essential amino acids (Dicks et al., 2014).

These microbes have been recognized as an essential ecosystem of microbial communities within the gut of mammals, and they are a host to intricate populations that consist of commensal, symbiotic and sometimes pathogenetic microorganisms. Equine researchers and the industry were quick to begin applying NGS technologies in order to enhance the understanding of the equine microbiome. Since fecal samples were identified as good indicators of the GI microbial community structures in humans and other model organisms (Turnbaugh et al., 2007), it was assumed the same for other mammals such as horses and cattle. The use of fecal samples also created a non-invasive, inexpensive 'biomarker' for researchers to begin to use for horses; however, minimal research has been conducted in order to understand how closely the microbial populations of fecal samples actually characterize the equine large intestine (Hastie et al., 2008; Sadet-Bourgeteau et al., 2010; Dougal et al., 2012), especially utilizing NGS technologies.

Some studies that have characterized the various compartments of the equine GI tract demonstrate that the microbial communities shift throughout the system in terms of abundance and similarity, but do not go into great depth of statistical comparisons that indicate similar (or dissimilar) signatures between the compartments or comparisons back to the fecal matter (Dougal et al., 2012; Costa et al., 2015; Ericsson et al., 2016; Julliand et al., 2016). Another factor that had

a large impact on all of the compartment studies is that the researchers had a variety of animals that were not consistent in terms of breed, age, housing situations, diets or where the animals originated, making it fairly challenging to remove environmental factors or individual variation that could alter the composition of the microorganisms inhabiting the GI sections and feces.

To be able to state with confidence if the fecal sample can be a predictive model of what is occurring microbially in the GI tract, we need to characterize each body site (or compartment) within the equine gut and compare the community structures back to that of the fecal microbiota structure. By implementing various multivariate statistical analyses (such as indicator species analysis), we can begin to understand which microbial families can indicate a particular compartment, and then observe if any of those specific families appear in the fecal matter (Carignan & Villard, 2002). Understanding which groups appear in the fecal samples could possibly lead equine researchers and owners to a better management strategy for the health of their animals and ultimately display the microbes residing within the GI with a simple tool such as a fecal sample.

The objectives of this study were to (1) characterize the microbial populations within three main compartments of the equine gastrointestinal tract and fecal samples and (2) to identify similar microbial communities of the hindgut compartments and compare community profiles of hindgut to profiles identified in fecal samples.

MATERIALS AND METHODS

Animal Subjects and Sample Collection

Six yearling ponies were euthanized at the University of Kentucky for reasons unrelated to gastrointestinal disease between March and July 2016. Samples were collected post mortem for reasons other than this study; an approval by the Colorado State University Institutional Animal

Care and Use Committee (IACUC) was not necessary. These animals were managed under the University of Kentucky and approved by the university's animal care committee (protocol number 2012-1046).

Prior to euthanasia, the animals had been maintained on pasture (comprised of grass hay) and managed on this pasture since birth. There was an equal distribution of males (n=3) and females (n=3) and all were similar in terms of breed (mix-breed ponies). All sires and dams of the six ponies were also managed on this pasture (this line of breed had been kept on this pasture since 1985), making the population unique in terms of possessing genetic similarity, reducing variation between subjects. This population also allowed visualization of a representative equine diet and controlled management practices. Table 2 summarizes the demographic data of the ponies included in this study.

Table 2: Demographic data from animals included in this study.

ID	Age	Breed	Sex	Date of Euthanasia
Pony I	Yearling	Mix-Breed	F	3/14/2016
Pony II	Yearling	Mix-Breed	M	3/14/2016
Pony III	Yearling	Mix-Breed	M	4/18/2016
Pony IV	Yearling	Mix-Breed	F	4/18/2016
Pony V	Yearling	Mix-Breed	F	4/18/2016
Pony VI	Yearling	Mix-Breed	M	7/11/2016

Gut digesta was collected within one hour following euthanasia for each subject. After identifying each compartment of the large intestine, digesta was consistently sampled from the following sites: cecum, right dorsal colon, right ventral colon and fecal material. Gut contents and fecal matter were placed in sterile 50 mL conical tubes and stored at -20°C until DNA extraction was performed.

DNA Extraction

Before DNA extraction began, all samples were homogenized in sterile cups and 0.25g of homogenized sample was used for extraction. DNA was isolated at Colorado State University Department of Animal Sciences using the PowerSoil® DNA Isolation Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's recommendations with minor alterations made to the protocol. After the lysis solution (C1) was added to samples, they were vortexed and incubated at 65°C for 10 minutes before placing them on the Vortex Adapter (Qiagen, Venlo, Netherlands). The only other alteration made included centrifuging samples at 13,000 x g for 1 minute at each centrifugation step of the protocol, and the remainder was followed as suggested by the manufacturer.

DNA quantification and quality were evaluated by spectrophotometry, using the NanoDrop 1000 (Roche), and PCR amplification of the V4 region of the 16S rRNA gene was performed using the Earth Microbiome Project (EMP) standard primer set. Thermocycler settings and reaction mixtures can also be found under the EMP protocols and standards (http://www.earthmicrobiome.org/protocols-and-standards/dna-extraction-protocol). All samples passed quality control if DNA concentration was above 25 ng/µl, the 260/280 ratio was 1.8 or higher and bands after gel electrophoresis were ~ 300-350 in size.

16S rRNA Amplification and Next Generation Sequencing

The V4 region of the 16S rRNA gene was amplified using the 515f/806r primer set that contained golay error-correcting barcodes on the forward primer (http://www.earthmicrobiome.org/protocols-and-standards/16s/). Samples were amplified in triplicate, pooled and visualized by gel electrophoresis (cut at 300-350 basepairs). Next generation sequencing was completed (SeqMatic LLC, Fremont, CA, USA) using a paired-end 2x150 bp

cycle run on an Illumina MiSeq platform using V3 chemistry. Demultiplexing was completed on the platform using the MiSeq Reporter Software System (Illumnia, San Diego, CA, USA) and raw DNA sequence files were then available for analysis.

Data Processing

Demultiplexed sequences were imported into Qiime2 (2017.11 distribution) and concatenated using the DADA2 pipeline, which also detects and corrects for phiX and chimeric sequences (Callahan et al., 2016). The resulting feature table was used to generate a phylogenetic tree for diversity metric analysis using the FastTree2 method (Price et al., 2010) and also for taxonomic assignments using GreenGenes (v13.8) reference database by training a BLAST+ classifier (Camacho et al., 2009). Further processing and analyses details, such as diversity metrics for community structure and taxonomic classifications, are included in the corresponding results sections.

RESULTS

From the 24 sequenced samples, a total of 2,163,951 sequencing reads were generated with a total of 4,461 (mean of 485 frequencies per feature) total features identified after quality control and construction of feature table. For downstream analysis, the feature table was sampled at a depth of 59,157 features per sample, from which 65.61% (1,419,768) of the original sequences and all 24 samples were retained for analysis.

Diversity Metrics

Alpha diversity (within sample biodiversity) was evaluated with Shannon Diversity Index, a quantitative measure of the amount of different species within a sample (Caporaso et al., 2010). This revealed that as digesta traveled through the gastrointestinal tract, alpha diversity increased

(averaged Shannon Diversity Index's based upon body site: cecum: 8.01; right ventral colon: 8.04; right dorsal colon: 8.10; feces: 8.41). Significance of intestinal site variable effect on alpha diversity was tested using a pairwise Kruskal-Wallis method between each of the body sites resulting in no significant differences between groups. However, this trend of increasing Shannon Diversity values along the tract indicates that as digesta is passing through the body, it is collecting additional microbial signatures from each area, leaving fecal sample with the highest diversity value. Other equine compartmental microbiome datasets also reflect this trend, supporting the idea that each site could encompass distinctive microbiota (Ericcson et al., 2016; Julliand & Grimm, 2016).

Beta diversity (between sample biodiversity) was analyzed using PERMANOVA, a multivariate technique that uses permutations of distances between horse and body site combinations computed with Bray-Curtis dissimilarity for each pair (Anderson, 2001). We first applied a square root transformation to the abundance (count) data due to the extreme range of values. Then, the Bray-Curtis dissimilarity was computed for each pair of horse-body site combinations. The F-test was highly significant, confirming that the microbial communities are different among body sites ($p=0.0001$; $F=5.2393$). Pairwise comparisons were also tested and all comparisons with cecum were significant ($p=0.03125$), indicating these populations differ, while dorsal to fecal, dorsal to ventral and fecal to ventral comparisons were not significant ($p=0.09375$; $p=0.09375$; $p=0.0625$, respectively), indicating similar microbial populations. Due to the fact dispersion is an assumption of PERMANOVA, we tested the multivariate homogeneity of group dispersions (variances) (Anderson, 2001). The result revealed that there is insufficient evidence to suggest the four body sites differed in respect to dispersion ($p=0.4648$). This demonstrates that the groups differ in correlation between the variables, but not in location or dispersion.

A principal component analysis was generated using the Bray-Curtis dissimilarity matrix in order to visualize the community's differences (Figure 2), and there were clustering of communities based upon body site (PCoA1). While the cecum and ventral colon communities clustered, the dorsal colon and fecal communities clustered. Interestingly, the pelvic flexure is the structure between the right ventral colon and the right dorsal colon, possibly indicating that there could be an important regulation of microbial populations occurring in this area.

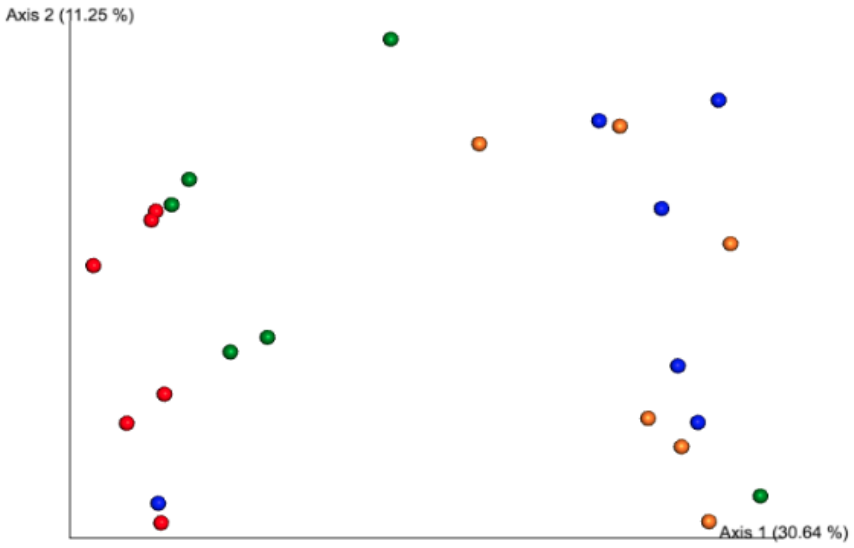

Figure 2: Principal component analysis of gut microbial populations from cecum (red), right ventral colon (green), right dorsal colon (orange) and feces (blue).

Taxonomic Classification

The main bacterial communities identified were dominated by Bacteroidetes and Firmicutes phyla, followed by Spirochaetes, Verrucomicrobia and Euryarchaeota. When broken down into the different families present, there was an apparent shift of microbial populations before and after the pelvic flexure, supporting the beta diversity clustering results we found previously. The phyla and family relative abundances can be observed in Figure 3A and 3B respectively.

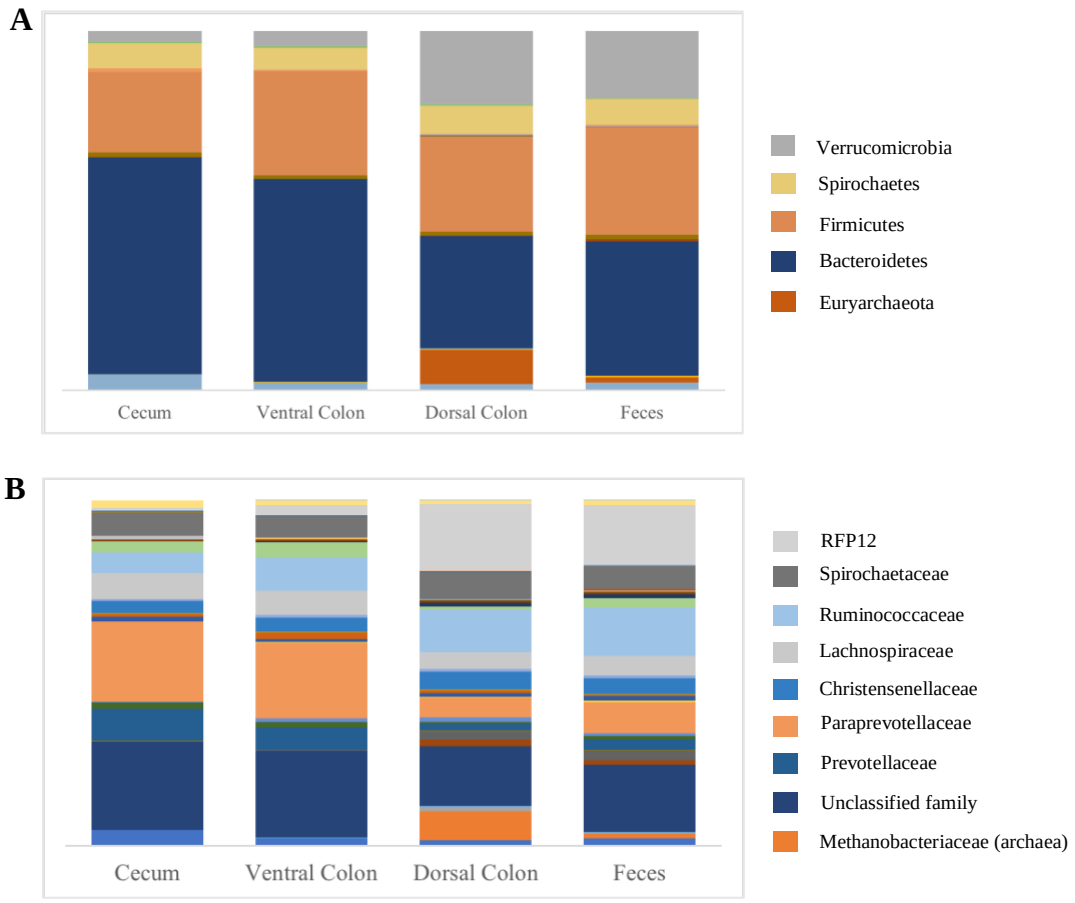

Figure 3: Relative abundance of microbial communities identified in the cecum, right ventral colon, right dorsal colon and feces, assigned to the phylum (A) and family level (B).

Indicator Species Analysis

To further understand the microbial community structures of the equine hindgut, we applied an indicator species analysis. For our purposes, we used absolute counts at the family level because there was a small amount of species identified (~27% of sequence reads were identified at the species level; ~38% down to the genus level). With that being said, we will refer to species as 'families', for this study.

This type of analysis combines information about the abundance of a family and the faithfulness of occurrence of a family in a particular group (McCune et al., 2002). Our 'groups' are the different body sites we sampled from (cecum, right ventral colon, left ventral colon, feces). An indicator value is calculated for each family in each group based off faithfulness (F_{ir}: measure of constancy of presence of a family in a particular body site) and exclusiveness (E_{ir}: measure of the family's specificity to a particular body site to the exclusion of other body sites). These can be computed with the equations below, where y_{ijr} is the abundance for the i^{th} body site (i = 1,2,3,4), the j^{th} horse (j = 1,2,3,4,5,6) and the r^{th} family (r = 1, 2, …, 87), $\bar{y}_{ir}$ is defined as the mean abundance over horses for family (r) in body site (i), and I_{ijr} is an indicator function denoting the presence or absence of family (r) in body site (i) for horse (j).

$$E_{ir} = \frac{\bar{y}_{ir}}{\sum_i \bar{y}_{ir}} \qquad F_{ir} = \frac{\sum_j I_{ijr}}{6}$$

The indicator value (IV_{ir}) for each family (r) in body site (i) is defined by the equation below. The highest IV_{ir} for a family among the body sites was taken to be the overall indicator value for the family and is denoted IV. As the indicator value for each family in the body site approaches 1, this means this family is a good indicator for a particular body site, however if the indicator value approaches 0, this means the family is a poor indicator for that body site.

$$IV_{ir} = \sqrt{E_{ir} \times F_{ir}}$$

Analysis was performed in R (v 3.5.0) using packages indicspecies (De Caceres & Legendre, 2009) and vegan (Oksanen et al., 2018). The data matrix imported consisted of 87 families across 24 body sites × horse combinations, with body site being a factor with four levels and six horses. Using a randomization test (Monte Carlo), body sites were randomly shuffled

within horse (block) 9,999 times and IV_{ir} was computed on order to obtain a p-value. To control the false discovery rate, we ran the Benjamini-Hochberg method at 0.05 to find the families with significant indicator values.

First identifying families that indicate 'single' body sites, we found that five families were high indicators of the cecum and two families were indicators of the right ventral colon, while no families were significant indicators of the right dorsal colon or feces. Refer to Table 3 for specific families, indicator values and associated p-values. This could be due to the fact that the vast majority of fermentation begins in the cecum, and the main families identified in the proximal hindgut will be aiding in that process. The families identified in the distal hindgut could be remnants of the fermentation process and other foodstuffs not fully processed yet.

Table 3: Families identified to be most significant indicators based on single body sites, which include the indicator value (IV) and p-value for each.

Body Site	Family[1]	IV	p-value[2]
Cecum	Prevotellaceae	0.6792	0.0196
	Paraprevotellaceae	0.6567	0.0435
	Planctomycetes (p)	0.8606	0.0278
	Spirochaetes (p)	0.9071	0.0195
	Synergistales (o)	0.8511	0.0195
Right Ventral Colon	S24-7	0.7379	0.0195
	Lactobacillales (o)	0.7017	0.0435

[1] Some families were identified to be 'unclassified', but the taxonomic rank is indicated (p = phylum; o = order)
[2] Corrected p-values using Benjamini-Hochberg method to control for FDR

To detect families that are indicators of body site combinations or if there were signatures in the feces that were identified in other compartments, we modified the indicator value formula and re-ran the randomization test. There was one family identified a high indicator of the cecum and right ventral colon, three families indicated for right dorsal colon and feces and two families revealed for right ventral colon, right dorsal colon, and feces (Table 4). These findings portray that the fecal sample is a good indicator for the distal hindgut, while the proximal hindgut seems to be more difficult to trace signatures from just a fecal sample. However, these results are only based on six animal subjects and the gut digesta or fecal samples were just small portions of a larger ecosystem present.

Table 4: Families identified to be most significant indicators based on combinations of body sites, which include the indicator value (IV) and p-value for each.

Body Sites	Family[1]	IV	p-value[2]
Cecum & Right Ventral Colon	Synergistales (o)	0.9285	0.0396
Right Dorsal Colon & Feces	WCHB1-41 (o)	0.9155	0.0385
	Euryarchaeota[3] (p)	0.9451	0.0385
	Methanomicrobiales[3] (o)	0.8995	0.0424
Right Ventral/Dorsal Colons & Feces	Anaerolineae (c)	0.9520	0.0424
	Pirellulaceae	0.9616	0.0385

[1]Some families were identified to be 'unclassified', but the taxonomic rank is indicated (p = phylum; o = order; c = class)
[2]Corrected p-values using Benjamini-Hochberg method to control for FDR
[3]Archaea groups

This study is the start of truly being able to identify significant indicator families of the many sections in the equine gastrointestinal system and detecting if those signatures are present in the fecal sample and different body sites. A robust statistical model of determining these specific families has been scarcely applied to new microbiome techniques, which can help to further the knowledge of a healthy equine hindgut microbiome. By utilizing this statistical technique, we were able to identify similar families across the body sites and the feces, but also were able to demonstrate that the feces might not be a full representation of the proximal hindgut, but a better view of the distal hindgut.

DISCUSSION

The present study examined the microbial communities that reside in the equine gastrointestinal tract compartments and also the fecal samples in order to understand the similarities of microbial populations between the compartments and if those populations were identified in the feces. Our results demonstrate that each compartment of the hindgut comprises a unique ecosystem, while also sharing some microbial attributes as well. While this has been described by other groups previously (Dougal et al., 2012; Dougal et al., 2013; Costa et al., 2015; Ericsson et al., 2016), there are new findings from our results that could possibly begin to bridge the gap of knowledge of the healthy equine gastrointestinal microbiota.

The differences of community structures between the compartments of the hindgut in the present data agrees with previous findings (Dougal et al., 2012; Dougal et al., 2013) in regard to the proximal and distal areas appearing dissimilar when represented on a principal coordinate analysis plot. Ericsson et al. (2016) and Costa et al. (2015) described the cecum to be similar in population structures, but the clustering on the PCoA plots says otherwise. This could be due to the fact that Ericsson et al. (2016) was comparing luminal contents to mucosa of the foregut and

hindgut, making the clustering of those areas closer together; however, there is clustering differences from dorsal and ventral colons on their plots. Dougal et al. (2012) first described the difference of the cecum and right dorsal colon microbial community structures and proceeded investigation by including other areas of the hindgut (Dougal et al., 2013) where the group recognized that the populations shifted before and after the pelvic flexure. We also recognized this phenomenon in our data which displayed the transformation of microbial populations, which paralleled the physiological structure of the pelvic flexure. Interestingly, the population of the animal subjects, from all mentioned previous studies and the present study, are vastly different in terms of geographical location, diet, breed, age, sex, management practices, etc. All of these factors have been demonstrated to have enormous impacts when exploring the gastrointestinal or fecal microbiota in other species, including horses (Debelius et al., 2016; Metcalf et al., 2017); yet, these data are still displaying similar patterns of population clustering between the various segments of the hindgut. A possible explanation of these similar results, despite the differences in animal subjects and methods or protocols used, could be suggesting the host's contribution of some control of microbiota that stays or leaves a certain area of the gastrointestinal tract. Several groups in different species have provided evidence that the host is 'shaping' the microbiota present through various physiological aspects occurring in the intestinal epithelium tissues such as secretion of immunological mediators (Okumura & Takeda, 2017), regulation of gene expression on a post-transcriptional level (Liu et al., 2016; Belcheva, 2017), aspects of cell differentiation and inflammation (Natividad & Verdu, 2013) and overall gut homeostasis. Although there are several other factors that play into why certain microbes habitat an environment, such as pH or anatomical functions (Julliand & Grimm, 2016), the present data and previous compartmental work in the horse suggest a bi-communicational pathway between host and microbe.

The taxonomic classifications for the present data seem to have inconsistencies with other equine microbiota studies. In terms of the previous compartmental studies conducted, the predominant phyla were Firmicutes, followed by Bacteroidetes (Costa et al., 2015; Dougal et al., 2013; Ericsson et al., 2016). Throughout other equine fecal microbiota studies, this also seems to be the case (Costa et al., 2012; Shepard et al., 2012, Steelman et al., 2012); however, our data had Bacteroidetes as the predominant phyla, followed by Firmicutes. This could be due to the fact that our animal subjects were all yearlings, and young age has been demonstrated to have Bacteroidetes as a predominant phylum (Mariat et al., 2009). These inconsistencies could also be explained by the different methodologies used by each group, such as the region of the 16S rRNA gene targeted, library preparation, sequencing platforms used, extraction kits utilized, geographical location, etc. While these methods did not seem to affect the diversity metrics, these could alter the taxonomic results. This ultimately demonstrates the importance of requiring a common practice amongst all equine microbiota researchers in order to be able to accurately compare these studies to each other and to be able to correctly define a healthy microbiota of the horse.

While taxonomic classifications display the groups that are similar or different across body sites in the present and previous studies, it does not indicate which groups are significantly exclusive to that area or if the fecal samples are significantly representative of sections in the gastrointestinal tract. These are also based on relative abundance, which is difficult to base exact conclusions of which groups increase or decrease, due to it being a relative measure. Previous research demonstrated that the fecal sample is a good representation of the distal hindgut, but not the proximal areas (Dougal et al., 2012), while other groups display that the fecal microbial populations are similar to the cecum (Schoster et al., 2013). The statistical methods used were not as robust or complex as they needed to be in order to fully answer this question for previous reports,

which was the main driver of utilizing an indicator species approach to begin to truly understand similarities or variances of the families residing in each body site.

There were more significant indicator families identified in the cecum compared to the other body sites, which is most likely due to the importance of fermentation process for hindgut fermenters. The Prevotellaceae family is comprised of four other genera with many functions, mainly being recognized to assist in breakdown of proteins and complex carbohydrates present in feedstuffs, especially in the rumen and hindgut of cattle, horses and sheep (Rosenburg, 2014). This aligns with the anatomical function of the cecum as this is the main area of fermentation of breakdown of complex carbohydrates that the small intestine could not perform. Other equine microbiota groups found this family to be most abundant and most culturable in the cecum of horses, further supporting our data and use of this type of analysis (Dougal et al., 2013; Julliand & Grimm, 2016; Venable et al., 2017). Another family discovered as a good indicator of the equine cecum, also found in previous studies (Julliand & Grimm, 2016), was Paraprevotellaceae. Unfortunately, little biological function is known about this group in mammals, further exemplifying the importance of other methodologies that need applied to microbiota studies besides just characterization of microbes, but also more proteomics data to understand potential function. The order Synergistales, that was also identified as a good indicator in the cecum, has been shown to inhabit anaerobic environments like animal's guts or soil and has large implications of gastrointestinal or soft tissues diseases (Vartoukian et al., 2007). This is an order classification, not a family, it is difficult to apply a significant biological function in terms of it residing in the cecum, however is still connected to tissues within the gut. This order could have other biological functions for the horse that needs to be researched further.

In terms of the family and order identified as significant in the right ventral colon, they both have interesting implications in biology of other mammals. The order Lactobacillales has been identified to be a major end product of carbohydrate fermentation with many strains and species (Sonomoto & Yokota, 2011). Being that the right ventral colon is the first structure after the cecum, this aligns with the biological function of this order. This is also naturally associated with mucosal surfaces of the gastrointestinal tract and known for the metabolism and fermentation of lactic acid (Makarova & Koonin, 2007). While the cecum is the main area for fermentation, there could possibly be residual fermentation occurring once the digesta travels into the right ventral colon. S24-7 is a family that has been associated to inhabit guts of homeothermic mammals (Ormerod et al., 2016) and many members of this family are differentiated by their degree of IgA-labeling (Palm et al., 2014; Bunker et al., 2015). This suggests that the members of this family are possibly targeted the immune system of these mammals, demonstrating a potential host-microbe interaction. Clustering of microbial populations before and after the pelvic flexure and evidence of this family was significant in the right ventral colon, could further support the host having a role in determining microbial communities in the gastrointestinal compartments.

WCHB1-41 (significant indicator in right dorsal colon and fecal sample) is an eubacterium that has been identified in biofilms (Yoon, 2011), a part of the Verrucomicrobia phylum. While this order has an insignificant described species list, this phylum has been demonstrated to be host-associated in the squirrel's gut during the hibernation cycle (Carey et al., 2012). This study demonstrated that with seasonal and diet changes, the intestinal epithelium and microbes were altered, displaying the symbiotic relationship that exists between the host and its microbiota residing in their gut. This order has been known to be fairly difficult to track, but further research

in terms of how this order interacts with intestinal epithelium and microbes could be beneficial for the poorly understood communication pathway between host and microbe.

Some archaea groups were found in this study as well, and while bacterial species are the main organisms being targeted, some archaea can be identified with 16S amplicon sequencing. Euryarchaeota, a dominant phylum discovered in several other studies, has been associated with host and gut interactions (Samuel et al., 2007; Scanlan et al., 2008; Mihajlovski et al., 2010), but with very specific genus and species in other mammals. Archaea has been gaining notice by displaying that these organisms in this kingdom are more associated with function of the gastrointestinal tract in many mammals including horses, cattle and sheep (Lwin & Matsui, 2014; Tajima et al., 2001; Yanagita et al., 2000). While bacteria are certainly the larger kingdom present, further research should include focus on other groups present like the families or orders of the archaea kingdom identified in mammal studies.

There are some limitations to this study that should be considered for further equine gastrointestinal microbiota research in the future. Some of these limitations include the small sample size of the animal subjects collected from and where the samples were taken from. Since this was a pilot study, the body sites included were due to ease of collection at the time. Ongoing research should try to sample from all areas of the gastrointestinal compartments before and after the pelvic flexure, especially incorporating all flexures and small colon. In terms of the digesta collected from each area, there was only about 300 mg collected. Although the samples were homogenized before DNA extraction occurred, this might not be a full representation of that area. Another limitation is that many families identified as being significant were unclassified. Even though our sequencing and rarefication values were one of the highest compared to other equine studies, this did not benefit the fact that our data was aligned to a model organism bacterial

database, GreenGenes, which is mainly from mouse or human research. The need for specie-specific 16S rRNA databases is crucial to being able to identify novel species in the horse and others alike, which could possibly begin to uncover some biological function beyond what is known now.

CONCLUSION

Collectively, the data presented revealed that, while the fecal sample may be a good indicator of the distal hindgut of horses, the proximal gut is not fully embodied. Microbial structures showed similarities before and after the pelvic flexure, which demonstrates that the fecal sample can be used as an estimator of the gut, but not standing as a full representation of what is occurring thought the hindgut. This study also presented some interesting findings of the host's possible contribution to the microbial populations residing in the gastrointestinal tract. By further research beyond characterizing, it can possibly begin to bridge the complex interaction of host and microbe, to add to the knowledge of gastrointestinal homeostasis.

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CHAPTER III: PROFILING MIRNA TRANSCRIPTS ALONG THE EQUINE HINDGUT

SUMMARY

Maintaining homeostasis is critical to proper gastrointestinal function. Dynamic interactions between the microbial population and its' equine host are at the center of this equilibrium. Non-coding RNAs, specifically microRNAs (miRNA), have recently been implicated as a key mechanism for regulating aspects of the interaction between host and microbiota. miRNAs are single-stranded 20-22 basepair molecules that play an active role in post-transcriptional regulation of protein-coding transcripts. Few experiments have investigated miRNA expression in equine gastrointestinal tissues or generated a profile of their expression along the tract. This pilot study is intended to address this deficiency by characterizing miRNA expression patterns in tissues of the equine hindgut. Total RNA was isolated using a TRIzol reagent protocol from intestinal epithelium collected at the cecum (base and apex), right and left ventral colon, pelvic flexure, right and left dorsal colon, and the small colon (n=3 for each tissue). RNA was reverse-transcribed using the miScript II RT kit. Relative transcript abundance was quantified using the miScript SYBR green PCR kit with primer sets designed for 346 annotated mature equine miRNAs. Biological replicates were pooled and analyzed based upon amplification thresholds. miRNA pathway analysis was also completed with the utilization of DIANA TOOLS to understand which KEGG pathways were targeted with the unique miRNA present in each tissue. Tissues from the hindgut demonstrated to have unique miRNA profiling patterns that may corresponded to the anatomical function of the gastrointestinal system. These expression profiles may get more insight and understanding of the regulatory processes involved in equine gastrointestinal homeostasis.

INTRODUCTION

While there are several different types of small non-coding RNAs that have been identified in the last few decades, one of the main classes recognized as having great impacts in many physiological and biological processes are known as microRNAs (miRNA). These are short, non-coding RNAs that are about 18-22 nucleotides in length and have been shown to be regulators of post-transactional gene expression (Lee et al., 1993; Bartel, 2004). This regulation has been demonstrated in several studies, but the main impact miRNAs have is affecting the output of protein-coding genes such as mRNA degradation, silencing, cleavage or even post-translational inhibition through different processes (Pillai, 2005; Valencia-Sanchez et al., 2006; Lee et al., 1993). Some miRNAs roles have been identified in developmental timing (Lee et al., 1993; Reinhart et al., 2000), neuronal cell fate (Johnston & Hobert, 2003), cell death and proliferation (Brennecke et al., 2003), host response to bacterial infections (Maudet et al., 2014) and an important tool for diagnostics and treatment of many diseases (Gulyaeya & Kushlinskiy, 2016). These small non-coding RNAs are important factors for many processes physiologically, which demonstrates the need to better understand them in other biological areas, such as the gastrointestinal system.

Recently, miRNAs have emerged as important regulators or mediators of gastrointestinal health in model organisms and humans through intestinal epithelial cells. Intestinal epithelium cells are known for regulating the protection of gut homeostasis by the host producing mucosal barriers, secreting different types of mediators for processes like inflammation or immunity and mainly being the barrier between the gut tissue and gut microbes (Okumura & Takeda, 2017). Being that the intestinal epithelial stem cells are the most-renewed tissues in the body, this has great effects on the gene expression within these tissues that plays a critical role in physiological

processes that occur within the gastrointestinal tract (Belcheva, 2017). Being that these short molecules are host-derived, the host has been a crucial regulator of intestinal processes, such as host miRNAs communicating with different bacterial species present in the gut, that either upregulate or downregulate certain cell lines those species are associated with (Duval et al., 2017). Duval and colleagues in 2017 demonstrated that the bacteria manipulate the expression of miRNAs to control cellular processes such as survival or proliferation. Another study revealed that different microbial community composition correlated with expression levels of specific host-derived miRNAs (Liu et al., 2016) and also showed that small RNAs secreted by bacteria in the GI influence expression of both mRNA and miRNA in epithelium (Filip et al., 2016; Choi et al., 2017). Further research has also been conducted observing single miRNA families or clusters such as the miR-30 family, which was associated with proliferation and differentiation of human intestinal epithelial cells (Peck et al., 2016). Additional research has also been done on miRNAs that are over or under-expressed with certain bacterial infections, such as miR-155, which was over-expressed during Helicobacter pylori infection in the gastrointestinal tract (Xiao et al., 2009). In terms of issues occurring of in the gastrointestinal tract, like colitis, there has been associations of complex miRNA networks in the intestinal epithelial stem cells that for important for gut homeostasis (Lee et al., 2015). These complex networks have been shown to control inflammation in terms of ulcerative colitis (Wu et al., 2008) and in innate and adaptive immunity (O'Connell et al., 2012). There are also miRNAs that aid the cell cycle processes involved in different colon cancers (Petrocca et al., 2008). While the examples of how miRNAs are involved in intestinal homeostasis, these were just a few examples of some important areas that still need further research, especially in other animal models. The interaction that is occurring between host-derived

molecules and the microbiota present adds another dimension that could possibly help better explain the regulatory processes of gastrointestinal homeostasis.

There has been a great deal of work in the horse to annotate miRNA transcripts and try to determine expression profiles in different biological systems (Kim et al., 2014; Desjardin et al., 2014; Pacholewska et al., 2016). The miRNAs that have been identified to be horse-specific thus far are now being investigated in terms of their specific roles in equine physiology such as identifying specific miRNAs associated with various mechanisms of equine reproduction (Klohonatz et al., 2015; Loux et al., 2017; Twenter et al., 2017; da Silveira et al., 2018), tissue specific or breed-related miRNAs (Pacholewska et al., 2016; Kim et al., 2014), and roles of miRNAs in equine medicine for possible therapeutic tools (van der Kolk et al., 2015). A small amount has been done in terms of the equine GI system and which miRNAs are present throughout the hindgut. While a small amount of research has showed miRNA expression in the equine colon (Kim et al., 2014; Pacholewska et al., 2016), there has not been a study conducted that begins to profile miRNA expression from tissues starting at the cecum and ending at the small colon. By providing more tissue samples from the different areas of the hindgut, it can perhaps begin to give insight on if the horse (host) has a contribution of the microbial populations present within each compartment. In order to do this, understanding which miRNAs are present in each compartment is needed. This can possibly start to give better resolution to the poor understanding of the host-microbe pathway and the normalcy of gut homeostasis of the horse, in order to prevent diseases such as colic or colitis in the future.

The main objective of this study was to profile 287 mature miRNA transcripts along the hindgut of the gastrointestinal tract to begin to improve understanding of their role in gastrointestinal function and homeostasis.

MATERIALS AND METHODS

Animal Subjects and Sample Collection

Three American Quarter horses were euthanized at Colorado State University for reasons unrelated to gastrointestinal disease in February of 2017. The samples were collected post mortem for reasons other than this study; an approval by the Colorado State University Institutional Animal Care and Use Committee (IACUC) was not necessary. Prior to the animals being euthanized, they were managed identically and were maintained on a mixture of grass hay. All three animals were four years of age, with two geldings (male) and one mare (female).

Once all structures were identified, tissues were collected from the following sections of the hindgut within 35 minutes post mortem from each subject: cecum apex (CA), cecum base (CB), right ventral colon (RVC), left ventral colon (LVC), pelvic flexure (PF), left dorsal colon (LDC), right dorsal colon (RDC) and small colon (SC). From each site, about a 1cm x 3cm section of epithelial tissue was collected and immediately rinsed with a 1X phosphate buffered saline (PBS) (Alfa Aesar, Ward Hill, MA) in order to remove mucosal layers and residual gut contents. Each section was then divided into ~0.5cm cubes (three cubes per body site) and placed in 5mL of RNALater (Invitrogen, Carlsbad, CA) at 4°C for 24 hours, per manufacturers recommendations. After 24 hours, the samples were then transferred to a new tube and stored at -80°C until further analysis.

RNA Extraction of the Hindgut Tissues

Total RNA was extracted from each tissue by placing ~ 50 mg of frozen tissue into 1 mL of TRI Reagent (Ambion, Carlsbad, CA) for tissues to be homogenized and then stored at room temperature for 5 minutes for lysing to occur. The homogenate was transferred to a new tube and chloroform was added to start the phase separation of RNA and protein layers. Each sample was

vortexed for 15 seconds and stored at room temperature for another 10 minutes. Samples were then centrifuged at 12,000 x g for 15 minutes at 4°C and after centrifugation, the aqueous phase was transferred to a new tube. Isopropanol was then added to the aqueous phase in order to begin RNA precipitation, and samples were stored at room temperature for 10 minutes. Samples then were centrifuged at 12,000 x g for 8 minutes at 4°C. After, the supernatant was removed, 1 mL of 75% ethanol was added and then the samples were centrifuged at 7,500 x g for 5 minutes at 4°C (this RNA wash was completed twice). Supernatant from the washes was removed and the pellet was then air dried for 3-5 minutes. To solubilize the pellet, nuclease-free water was added (at least 30 μl) and incubated for 15 minutes at 57°C. All samples were then processed with a DNase Treatment (Invitrogen, Carlsbad, CA) to minimize any possible DNA contamination and transferred to a new tube. RNA quantification and purity were analyzed using the NanoDrop One Spectrophotometer (Thermo Scientific, Waltham, MA) and were all normalized to about 500 ng/μl. Samples were appropriate for continuing onto qPCR if 260/280 ratio was 1.8 or higher.

Real-Time Quantitative Polymerase Chain Reaction

Reverse transcription was performed to generate cDNA using a miScript II RT kit (Qiagen, Valencia, CA). Per manufacturers suggestions, 4 μl of 5x HiSpec Buffer, 2 μl of nucleic mix, 2 μl of reverse transcriptase mix, 1 μg of total RNA and RNase-free water were added for a total reaction volume of 20 μl. There was another reaction prepared without an RNA template, which was replaced by water, for a negative control. Reactions were incubated at 37°C for 60 minutes, followed by another incubation at 95°C for 5 minutes to inactivate the reverse transcriptase and immediately placed on ice. Prior to continuing with real-time PCR, 200 μl of nuclease-free water was added to each 20 μl reverse-transcription reaction.

The cDNA template was then immediately used for real-time PCR analysis using the miScript SYBR Green PCR kit (Qiagen, Valencia, CA). A total of 286 mature equine-specific miRNA transcripts were used (Table 1) based off an in-silico detection model that identified 346 mature equine miRNAs (Zhou et al., 2009). In a 384-well plate (Applied Biosystems, Foster City, CA), each well contained a 6 μl reaction comprised of 2X QuantiTect SYBR Green Master Mix, 10X universal reverse primer, miRNA-specific forward primer (1 μl), 0.1μl of cDNA template and nuclease-free water. There was also a well with the RNA-free reaction from above and PCR reaction mixes for a negative control, totaling to 287 wells on each plate. Before analysis, each plate was briefly centrifuged for about 2 minutes to ensure all components mixing together. The 384-well plates were then analyzed using the QuantStudio 5 Real-Time PCR System (Applied Biosystems, Foster City, CA) with the following thermocycling times: 95°C for 15 minutes for the initial activation, followed by a 3-step cycling process beginning with 94°C for 15 seconds (denaturation), 55°C for 30 seconds (annealing) and 70°C for 30 seconds (extension). The 3-step cycling continued for 40 total cycles and terminated after a melt-curve analysis to support the evidence of the amplification of a single cDNA product. As a pilot study, each tissue (eight) from each subject (three) were only ran once. miRNAs were considered present if (1) the quantitative cycle (Cp) value was between 10 and 37 and (2) if miRNAs were within all three subjects at each compartment sampled from. Table 5 displays the 286 mature miRNA ID's and their respective sequences.

Table 5: Mature miRNA transcript sequences

Mature miRNA ID	Target miRNA Sequence
eca-let-7a	tgaggtagtaggttgtatagtt
eca-let-7c	tgaggtagtaggttgtatggtt
eca-let-7d	agaggtagtaggttgcatagtt
eca-let-7e	tgaggtaggaggttgtatagtt
eca-let-7f	tgaggtagtagattgtatagtt
eca-let-7g	tgaggtagtagtttgtacagtt
eca-miR-101	tacagtactgtgataactgaa
eca-miR-105	tcaaatgctcagactcctgtggt
eca-miR-106a	caaagtgcttacagtgcaggtag
eca-miR-106b	taaagtgctgacagtgcagat
eca-miR-10a	taccctgtagatccgaatttgtg
eca-miR-10b	taccctgtagaaccgaatttgtg
eca-miR-1179	aagcattctttcattggttgg
eca-miR-1180	tttccggctcgagtgggtgtgt
eca-miR-1185	agaggatacccttgtatgtt
eca-miR-1193	taggtcacccgtttgactatc
eca-miR-1197	taggacacatggtctacttct
eca-miR-1204	tcgtggcctggtccccactat
eca-miR-122	tggagtgtgacaatggtgtttg
eca-miR-1244	gagtggttggtttgtatgagatggtt
eca-miR-1248	tccttcttgtataagcactgtgctaaa
eca-miR-1255b	cggataagcaaagaaagtggtt
eca-miR-125a-3p	acaggtgaggttcttgggagcc
eca-miR-125a-5p	tccctgagacccttaacctgtga
eca-miR-125b	tccctgagaccctaacttgtga
eca-miR-126-3p	tcgtaccgtgagtaataatgcg
eca-miR-1261	gtggattaggctttggctt
eca-miR-1264	caagtcttatttgagcacctgtt
eca-miR-127	tcggatccgtctgagcttggct
eca-miR-1271	cttggcacctcgtaagcactca
eca-miR-128	tcacagtgaaccggtctcttt
eca-miR-1282	agtggttggtttgtatgagatggtt
eca-miR-1289	tggagtccaggaatctgcatttt
eca-miR-1291a	tggccctgactgaagaccagcagt
eca-miR-1291b	aggccctgaatcaagaccagcagt
eca-miR-1296	ttagggccctggctccatctcc
eca-miR-1298	ttcattcggctgtccagatgta
eca-miR-129a-3p	aagcccttaccccaaaaagtat
eca-miR-1301	ttgcagctgcctgggagtgatttc
eca-miR-13021	ttgggacatacttatactaaa
eca-miR-1302b2	ttgggacatacttatactaga
eca-miR-1302c5	ttgcgacatacttatactaaa
eca-miR-1302d4	ttgggacatacttatgctaaa

eca-miR-1302e6	ttgggatatacttatactaaa
eca-miR-1302e7	ttgggatatacttatactaaa
eca-miR-130a	cagtgcaatgttaaaagggcat
eca-miR-130b	cagtgcaatgatgaaagggcat
eca-miR-132	taacagtctacagccatggtcg
eca-miR-133a	tttggtccccttcaaccagctg
eca-miR-133b	tttggtccccttcaaccagcta
eca-miR-134	tgtgactggttgaccagagggg
eca-miR-135a	tatggctttttattcctatgtga
eca-miR-135b	tatggctttttcattcctatgtga
eca-miR-136	actccatttgttttgatgatgg
eca-miR-137	ttattgcttaagaatacgcgtag
eca-miR-138	agctggtgttgtgaatcaggccg
eca-miR-139-5p	tctacagtgcacgtgtctccag
eca-miR-140-3p	taccacagggtagaaccacgg
eca-miR-141	taacactgtctggtaaagatgg
eca-miR-142-3p	tgtagtgtttcctactttatgga
eca-miR-142-5p	cataaagtagaaagcactact
eca-miR-143	tgagatgaagcactgtagctc
eca-miR-144	tacagtatagatgatgtact
eca-miR-145	gtccagttttcccaggaatccct
eca-miR-1461	atctctacgggtaagtgtgtga
eca-miR-1468	ctccgtttgcctgttttgctg
eca-miR-146a	tgagaactgaattccatgggtt
eca-miR-146b-3p	tgccctagggactcagttctgg
eca-miR-147b	gtgtgccgaaatgcttctgcta
eca-miR-148a	tcagtgcactacagaactttgt
eca-miR-148b-3p	tcagtgcatcacagaactttgt
eca-miR-150	tctcccaacccttgtaccagtg
eca-miR-153	ttgcatagtcacaaaagtgatc
eca-miR-155	ttaatgctaatcgtgataggggt
eca-miR-1597	tgaggagctctgcgagcatgta
eca-miR-15b	tagcagcacatcatggtttaca
eca-miR-16	tagcagcacgtaaatattggcg
eca-miR-181b	aacattcattgctgtcggtgggt
eca-miR-182	tttggcaatggtagaactcacactg
eca-miR-183	tatggcactggtagaattcact
eca-miR-1839	aaggtagatagaacaggtcttg
eca-miR-184	tggacggagaactgataagggt
eca-miR-1842	tggctctgtgaggtcggctca
eca-miR-186	caaagaattctccttttgggct
eca-miR-188-3p	ctcccacatgcagggtttgca
eca-miR-1892	atttggggtgggggatgggga
eca-miR-1898	aaggtagatagaacaggtcttg
eca-miR-18a	taaggtgcatctagtgcagatag

eca-miR-18b	taaggtgcatctagtgcagttag
eca-miR-190	tgatatgtttgatatattaggt
eca-miR-1902	agaggtgcagtaggcatgactt
eca-miR-1905a	caccacgagccctaccacgcggtag
eca-miR-1905b	caccagccccactacgcggtag
eca-miR-1905c	caccaccagccccaccacgcggtag
eca-miR-190b	tgatatgtttgatattgggtt
eca-miR-191	caacggaatcccaaaagcagctg
eca-miR-1912	tacccagagcgtgcagtgtgaa
eca-miR-193a-3p	aactggcctacaaagtcccagt
eca-miR-193b	aactggcccacaaagtcccgct
eca-miR-195	tagcagcacagaaatattggc
eca-miR-196b	taggtagtttcctgttgttggg
eca-miR-197	ttcaccaccttctccacccagc
eca-miR-199a-3p	acagtagtctgcacattggtag
eca-miR-199b-3p	acagtagtctgcacattggtta
eca-miR-199b-5p	cccagtgtttagactatctgttc
eca-miR-19a	tgtgcaaatctatgcaaaactga
eca-miR-200a	taacactgtctggtaacgatgt
eca-miR-200b	taatactgcctggtaatgatga
eca-miR-200c	taatactgccgggtaatgatgga
eca-miR-205	tccttcattccaccggagtctg
eca-miR-206	tggaatgtaaggaagtgtgtgg
eca-miR-208b	ataagacgaacaaaaggtttgt
eca-miR-20a	taaagtgcttatagtgcaggtag
eca-miR-20b	caaagtgctcatagtgcaggtag
eca-miR-21	tagcttatcagactgatgttga
eca-miR-211	ttccctttgtcatcctttgcct
eca-miR-214	acagcaggcacagacaggcagt
eca-miR-216a	taatctcagctggcaactgtga
eca-miR-217	tactgcatcaggaactgattgga
eca-miR-219-5p	tgattgtccaaacgcaattct
eca-miR-221	agctacattgtctgctgggtttc
eca-miR-222	agctacatctggctactgggt
eca-miR-224	caagtcactagtggttccgtt
eca-miR-23b	atcacattgccagggattacc
eca-miR-24	tggctcagttcagcaggaacag
eca-miR-26a	ttcaagtaatccaggataggct
eca-miR-27b	ttcacagtggctaagttctgc
eca-miR-28-5p	aaggagctcacagtctattgag
eca-miR-296	gagggttgggtggaggctttcc
eca-miR-299	tatgtgggatggtaaaccgctt
eca-miR-29b	tagcaccatttgaaatcagtgtt
eca-miR-29c	tagcaccatttgaaatcggtta
eca-miR-301b-3p	cagtgcaatgatattgtcaaagc

eca-miR-302a	taagtgcttccatgttttagtga
eca-miR-302b	taagtgcttccatgttttagtag
eca-miR-302d	taagtgcttccatgttttagtgt
eca-miR-30c	tgtaaacatcctacactctcagc
eca-miR-30d	tgtaaacatccccgactggaag
eca-miR-30e	tgtaaacatccttgactggaag
eca-miR-31	aggcaagatgctggcatagct
eca-miR-323-3p	cacattacacggtcgacctct
eca-miR-323-5p	aggtggtccgtggcgcgttcgc
eca-miR-324-5p	cgcatcccctagggcattggtgt
eca-miR-326	cctctgggcccttcctccagc
eca-miR-328	ctggccctctctgcccttccgt
eca-miR-330	tctctgggcctgtgtcttaggc
eca-miR-331	gcccctgggcctatcctagaa
eca-miR-335	tcaagagcaataacgaaaaatgt
eca-miR-337-5p	gaacggcttcatacaggagct
eca-miR-338-5p	aacaatatcctggtgctgagtg
eca-miR-33a	gtgcattgtagttgcattgca
eca-miR-34	tggcagtgtcttagctggttgt
eca-miR-342-3p	tctcacacagaaatcgcacccgt
eca-miR-342-5p	aggggtgctatctgtgattgag
eca-miR-345-5p	gctgactcctagtccagtgctc
eca-miR-346	tgtctgcccgcatgcctgcctct
eca-miR-361-3p	tcccccaggcgtgattctgattt
eca-miR-362-3p	aacacacctattcaaggattca
eca-miR-363	aattgcacggtatccatctgta
eca-miR-367	aattgcactttagcaatggtga
eca-miR-369-3p	aataatacatggttgatcttt
eca-miR-369-5p	agatcgaccgtgtcatattcgc
eca-miR-371-3p	aagtgccgccatttttgagtgt
eca-miR-374a	ttataatacaacctgataagtg
eca-miR-374b	atataatacaacctgctaagtg
eca-miR-376a	atcatagaggaaaatccacgt
eca-miR-376c	aacatagaggaaattccacgt
eca-miR-377	atcacacaaaggcaacttttgt
eca-miR-378	actggacttggagtcagaagg
eca-miR-379	tggtagactatggaacgtagg
eca-miR-382	gaagttgttcgtggtggattcg
eca-miR-383	agatcagaaggtgattgtggct
eca-miR-384	attcctagaaattgttcaca
eca-miR-409-3p	gaatgttgctcggtgaacccct
eca-miR-409-5p	aggttacccgagcaactttgcat
eca-miR-411	tagtagaccgtatagcgtacg
eca-miR-412	ttcacctggtccactagccg
eca-miR-421	ggcctcattaaatgtttgttg

eca-miR-423-3p	agctcggtctgaggcccctcagt
eca-miR-423-5p	tgaggggcagagagcgagacttt
eca-miR-424	cagcagcaattcatgttttgaa
eca-miR-429	taatactgtctggtaatgccg
eca-miR-431	tgtcttgcaggccgtcatgcagg
eca-miR-432	tcttggagtaggtcattgggtgg
eca-miR-433	atcatgatgggctcctcggtgt
eca-miR-448	ttgcatatgtaggatgtcccat
eca-miR-449a	tggcagtgtattgttagctggt
eca-miR-450a	ttttgcgatgtgttcctaatat
eca-miR-450b-3p	ttgggaacattttgcatccata
eca-miR-450b-5p	ttttgcaatatgttcctgaata
eca-miR-451	aaaccgttaccattactgtgtt
eca-miR-454	tagtgcaatattgcttatagggt
eca-miR-485-3p	gtcatacacggctctcctctct
eca-miR-485-5p	tcctgtactgagctgccccgag
eca-miR-486-3p	cggggcagctcagtacaggat
eca-miR-486-5p	tcctgtactgagctgccccgag
eca-miR-487a	aatcatacagggacatccagtt
eca-miR-487b	aatcgtacagggtcatccactt
eca-miR-488	ttgaaaggctatttcttggtc
eca-miR-489	gtgacatcacatatacggcggc
eca-miR-490-3p	caacctggaggactccatgctg
eca-miR-490-5p	ccatggatctccaggtgggt
eca-miR-491-3p	cttatgcaagattcccttctac
eca-miR-491-5p	agtggggaacccttccatgagg
eca-miR-492	aggagctgcgggacaagattctt
eca-miR-493b	tgaaggtcttccgtgtgccagg
eca-miR-494	tgaaacatacacgggaaacctc
eca-miR-495	aaacaaacatggtgcacttctt
eca-miR-496	tgagtattacatggccaatctc
eca-miR-497	cagcagcacactgtggtttgt
eca-miR-499-3p	aacatcacagcaagtctgtgct
eca-miR-499-5p	ttaagacttgcagtgatgttt
eca-miR-500	taatccttgctacctgggtgaga
eca-miR-501	atccttcgtccctgggtgaga
eca-miR-502-3p	aatgcacctgggcaaggattca
eca-miR-502-5p	atccttgctatctgggtgcta
eca-miR-503	tagcagcgggaacagtactgcag
eca-miR-504	agaccctggtctgcactctatc
eca-miR-505	cgtcaacacttgctggtttcct
eca-miR-507	attggcacctcttagagtgaa
eca-miR-508-3p	tgattgtcacctttggagtaga
eca-miR-508-5p	tactccagagggtgtcattcaca
eca-miR-509-5p	tactgcagacagtggcaatca

eca-miR-514	attgacacctctgtgagtgga
eca-miR-532-3p	tgtgacagattgataactgaaa
eca-miR-532-5p	catgccttgagtgtaggaccgt
eca-miR-539	ggagaaattatccttgctgtgt
eca-miR-541	tggtgggcacagaatccagtct
eca-miR-542-3p	tgtgacagattgataactgaaa
eca-miR-542-5p	ctcggggatcatcatgtcacga
eca-miR-543	aaacattcgcggtgcacttctt
eca-miR-544b	attctgcatttttaacaagttc
eca-miR-545	tcaacaaacatttattgtgtgc
eca-miR-551a	gcgacccactcttggtttcca
eca-miR-551b	gcgacccatacttggtttcag
eca-miR-568	atgtataaatgtatacacac
eca-miR-582-3p	taaccggttgaacaactgaacc
eca-miR-582-5p	ttacagttgttcaaccagttact
eca-miR-590-3p	taattttatgtataagctagt
eca-miR-590-5p	gagcttattcataaaagtacag
eca-miR-592	ttgtgtcaatatgcgatgatgt
eca-miR-598	tacgtcatcgttgtcatcgtca
eca-miR-615-3p	tccgagcctgggtctccctctc
eca-miR-615-5p	gggggtccccggtgctcggatc
eca-miR-628a	atgctgacatatttactagagg
eca-miR-632	gtgcctgtttcctgtggga
eca-miR-652	aatggcgccactagggttgtg
eca-miR-653	gtgttgaaacaatctctgctg
eca-miR-655	ataatacatggttaacctcttt
eca-miR-656	aatattatacagtcaacctct
eca-miR-660	tacccattgcatatcggagttg
eca-miR-664	tattcatttatctcctagcctaca
eca-miR-670	gtccctgagtgtatgtggtgaa
eca-miR-671-3p	tccggttctcagggctccacc
eca-miR-671-5p	aggaagccctggaggggctggag
eca-miR-672	tgaggttggtgtactgtgtgtga
eca-miR-674-3p	aggaggccatagtggcaactgt
eca-miR-674-5p	ggtgctcacttgtcctcct
eca-miR-675	tggcgcggagagggcccacagtg
eca-miR-684	agttttcccttcaattcag
eca-miR-7	tggaagactagtgattttgttgt
eca-miR-703	aaaaccttcagaaggaaagga
eca-miR-708	aaggagcttacaatctagctggg
eca-miR-711	gggacccagggagagacgtaag
eca-miR-758	tttgtgacctggtccactaacc
eca-miR-761	gcagcagggtgaaactgacaca
eca-miR-763	ccagctgggaggaaccagtggc
eca-miR-767-3p	tctgctcatactccatggttcct

eca-miR-767-5p	tgcaccatggttgtctgagcatg
eca-miR-769-3p	ctgggatctcgggggtcttggtt
eca-miR-769-5p	ggagacctctgggttctgagct
eca-miR-769b	ggaaacctctgggttctgagct
eca-miR-770	agcaccacgtgtctgggccatg
eca-miR-802	cagtaacaaagattcatccttgt
eca-miR-872	aaggttacttgttagttcagg
eca-miR-873	gcaggaacttgtgagtctcct
eca-miR-874	ctgccctggcccgagggaccga
eca-miR-876-5p	tggatttctttgtgaatcacca
eca-miR-885-3p	aggcagcggggtgtagtggata
eca-miR-885-5p	tccattacactaccctgcctct
eca-miR-889	ttaatatcggacaaccattgt
eca-miR-92a	tattgcacttgtcccggcctgt
eca-miR-92b	tattgcactcgtcccggcctcc
eca-miR-95	ttcaacgggtctttattgagca
eca-miR-98	tgaggtagtaagttgtattgtt
eca-miR-99b	cacccgtagaaccgaccttgcg
eca-miR-9a	tctttggttatctagctgtatga

miRNA Pathway Analysis

Pathway analysis was implemented on the miRNAs that were unique (only present in that site) to each compartment using DIANA TOOLS mirPath v.3 (Vlachos et al., 2015) and the predicted targets were verified with the DIANA-microT-CDS (v5) algorithm (Paraskevopoulou et al., 2013). This pathway tool provides the KEGG pathways and the associated p-values with each pathway, which utilizes a modified Fisher's Exact Test that is coupled with false discovery rate for correction of multiple testing using the Benjamini Hochberg algorithm. All p-value thresholds were set at 0.05 for pathway analysis. This tool also provides the number of genes associated with each miRNA using the Ease score (Hosack et al., 2003) methodology.

For this analysis, the predicted pathways were aligned to a human database because this tool did not have an "equine" option due to the limited data currently in this species. However, we

ensured to identify miRNAs that were homologous to the human miRNAs when possible to ensure more accuracy of predicted pathway output

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RESULTS AND DISCUSSION

From the 286 miRNAs that were targeted in this study, 242 were present in the tissues throughout the hindgut of the gastrointestinal tract. Of these 242 miRNAs, only 36 were present in all the compartments, but there was some interesting patterning throughout the other sections of the hindgut as well. While each section of the hindgut had unique miRNAs only present in those areas, there was miRNAs specific to the proximal and distal areas. There were 6 miRNAs only present in the proximal gut (cecum to pelvic flexure) and 4 miRNAs unique to the distal area (pelvic flexure to small colon). Interestingly, one miRNA was the only one identified that was in all ventral and dorsal colon sections, but not identified anywhere else. Many other miRNAs profiles were identified in several areas, but the miRNA listed above can be found in Table 2.

The number of unique miRNAs identified at each region were as follows: cecum apex (1), cecum base (1), right ventral colon (4), left ventral colon (3), pelvic flexure (4), left dorsal colon (2), right dorsal colon (0) and small colon (5). These were the miRNAs that were used to complete pathway analyses in order to determine predicted roles of the miRNA discovered in each compartment of the hindgut (Table 3). While we sampled from two areas of the cecum, it is a one-ended fermentation vat that begins microbial digestion, largely slowing down the enzymatic digestion occurring in the small intestine before it (Dicks et al., 2014). One of the predicted targets by the miRNA present in this region (and also the right ventral colon) was the TGF beta-signaling pathway. Interestingly, this pathway has been associated with maintaining gut homeostasis through inflammatory responses (Monteleone et al., 2004). This could also affect the gastrointestinal microflora that are residing in these areas, especially if this pathway is negatively regulating gut

inflammation. Knowing this, the miRNAs that are targeting this pathway may be a route for the host to regulate gastrointestinal homeostasis by regulating immune responses, which effect the microbial populations within the cecum or other areas of the hindgut.

The miRNAs in the pelvic flexure epithelium tissues were predicted to target the Hippo signaling pathway which has been demonstrated to be associated with regulating intestinal tissue proliferative homeostasis (Piccolo et al., 2014; Ren et al., 2010) and many other physiological processes. This structure is of anatomical importance because it separates the ventral colon from the dorsal colon, by making a sharp turn in the gastrointestinal tract. In order for digesta to safely be passed through these areas from larger to smaller diameters, there are many myoelectric and mechanical activities taking place throughout the hindgut (Koenig & Cote, 2006). The Hippo signaling pathway may aid in the preservation of the epithelium tissues throughout these areas in order to help sustain intestinal homeostasis and the digestion process.

Interestingly, the right dorsal colon did not have any miRNAs identified in those tissues; however, there were several miRNAs that were present in all other tissues besides the right dorsal colon. Right dorsal displacement (which usually leads to colic) is one of the more dangerous types of colic in horses today because it only can be resolved with surgical intervention (Waguespack, 2006). Understanding why there were no miRNA present in these tissues could possibly help understand more about this type of displacement in this area of the gut; however, being that we only profiled 286 miRNA transcripts, this could be why there was not any identified in this region. More equine-specific miRNAs would be needed to be analyzed to accurately answer this question.

There are some limitations which could include the amount of animal subjects, the amount of miRNA transcripts profiled or utilizing a human database for the pathway analysis. Nonetheless, to our knowledge, this is the first study to begin to profile miRNA expression along the entire

hindgut of the horse. By advancing this type of research with utilization of other methods (such as RNA-sequencing), it may have large implications in terms of broadening the knowledge of equine gastrointestinal homeostasis and the horse's possible role in that process.

CONCLUSION

Our research suggests that each compartment of the hindgut has specific miRNAs that could have exclusive roles to those body sites. By identifying miRNAs that are specifically associated with the gastrointestinal system in the horse with utilization of other methods and improving this study, we hope to demonstrate the horse's (host's) contribution to gastrointestinal homeostasis.

Table 6: miRNAs that were identified in different tissues

All Body Sites	Proximal	Distal	Colon
eca-miR-20b	eca-miR-674-3p	eca-miR-222	eca-miR-362-3p
eca-miR-346	eca-miR-1842	eca-miR-670	
eca-miR-181b	eca-miR-543	eca-miR-541	
eca-miR-146a	eca-miR-504	eca-miR-224	
eca-miR-28-5p	eca-miR-412		
eca-miR-338-5p	eca-miR-423-5p		
eca-miR-424			
eca-miR-199b-3p			
eca-miR-16			
eca-miR-485-3p			
eca-miR-150			
eca-miR-494			
ecalet7g			
eca-miR-197			
eca-miR-21			
eca-miR-191			
ecalet7c			
eca-miR-451			
eca-miR-142-3p			
eca-miR-196b			
eca-miR-505			
eca-miR-122			
eca-miR-486-5p			
eca-miR-345-5p			
eca-miR-454			
eca-miR-145			
eca-miR-382			
eca-miR-30c			
eca-miR-7			
eca-miR-491-5p			
ecalet7f			
eca-miR-34			
eca-miR-323-5p			
eca-miR-487b			
eca-miR-664			
eca-miR-502-3p			

Table 7: miRNAs unique to the intestinal sites sampled from. If a pathway was not found for a certain miRNA, it will be denoted as '—' in the chart.

Body Sites	miRNA ID	KEGG Pathway	P-Value	# of Genes
Cecum Apex	eca-miR-33a	Adrenergic signaling in cardiomyocytes	0.00006	19
		TGF-beta signaling pathway	0.00018	12
		Adherens junction	0.00236	12
		GABAergic synapse	0.00818	8
		Biosynthesis of unsaturated fatty acids	0.01288	2
		Pantothenate and CoA biosynthesis	0.03274	5
Cecum Base	eca-miR-144	cAMP signaling pathway	0.00996	29
		Prolactin signaling pathway	0.01401	12
		Endocrine and other factor-regulated calcium reabsorption	0.04491	9
		Proteoglycans in cancer	0.04903	23
RVC	eca-miR-514	ECM-receptor interaction	0.00590	1
		Glycosphingolipid biosynthesis - lacto and neolacto series	0.00001	2
		GABAergic synapse	0.00001	5
		Ubiquitin mediated proteolysis	0.02325	8

Table 7 continued

Body Sites	miRNA ID	KEGG Pathway	P-Value	# of Genes
RVC (cont.)	eca-miR-184	Circadian entrainment	0.00217	1
		Morphine addiction	0.00217	1
		Taste transduction	0.00543	1
	eca-miR-1185	FoxO signaling pathway	0.00003	26
		Hippo signaling pathway	0.00003	25
		AMPK signaling pathway	0.00068	24
		TGF-beta signaling pathway	0.00410	16
		Colorectal cancer	0.02775	11
		Endocytosis	0.03488	26
		PI3K-Akt signaling pathway	0.03488	39
		ErbB signaling pathway	0.02784	14
	eca-miR-1291a	Thyroid hormone synthesis	0.00032	2
		Fatty acid elongation	0.02394	2
		ECM-receptor interaction	0.02394	4
		Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	0.03413	3

Table 7 continued

Body Sites	miRNA ID	KEGG Pathway	P-Value	# of Genes
LVC	eca-miR-598	Adrenergic signaling in cardiomyocytes	0.00293	1
		Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	0.00445	1
	eca-miR-551b	Lysine degradation	0.00826	1
		ErbB signaling pathway	0.00826	1
		Protein processing in endoplasmic reticulum	0.00006	25
		N-Glycan biosynthesis	0.00208	6
	eca-miR-545	--	--	--
PF	eca-miR-411	Biosynthesis of unsaturated fatty acids	0.01671	2
		Hippo signaling pathway	0.01671	13
	eca-miR-632	Biosynthesis of unsaturated fatty acids	0.00025	1
		N-Glycan biosynthesis	0.00264	3
		Fatty acid metabolism	0.04717	2
	eca-miR-499-5p	--	--	--
	eca-miR-492	Biosynthesis of amino acids	0.02698	3

Table 7 continued

Body Sites	miRNA ID	KEGG Pathway	P-Value	# of Genes
LDC	eca-miR-450b-3p	Mucin type O-Glycan biosynthesis	0.00001	2
	eca-miR-105	--	--	--
RDC	--	--	--	--
SC	eca-miR-208b	Fatty acid degradation	0.00006	4
		Fatty acid metabolism	0.04974	4
	eca-miR-653	--	--	--
	eca-miR-582-3p	Hippo signaling pathway	0.00001	7
		Sulfur relay system	0.02719	1
	eca-miR-508-5p	Drug metabolism - cytochrome P450	0.00001	1
		Hematopoietic cell lineage	0.03440	2
		Folate biosynthesis	0.04234	1
	eca-miR-876-5p	Tryptophan metabolism	0.02611	4
		Lysine degradation	0.01227	4
		Signaling pathways regulating pluripotency of stem cells	0.00291	11

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