IDENTIFYING DIFFERENCES IN THE GUT MICROBIOME OF EASY AND HARD KEEPER HORSES

by

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ABSTRACT

The equine gut harbors a diverse community of microbes that are crucial for proper digestion of plant materials and an essential component to the animal's overall health. As recent studies have found links between the human gut microbiome and obesity, the gut microbiota appears to play in weight and health is further being discovered. The goal of this experiment is to identify differentially abundant bacterial species between horses with differing metabolic tendencies, also known as easy keepers and hard keepers. As a secondary goal, the differences in the microbiome regarding age were also observed to determine the effect of age in addition to keeper status, as metabolic tendency is often seen in horses to change over time.

The primary methods of identifying microbial communities were 16S rRNA gene sequencing of DNA extracted from equine fecal matter. A series of analyses were used to compare microbiome data, including differential abundance, alpha diversity, beta diversity, Spearman's Rank correlation, and LDA Effect Size. All of these were performed to compare the microbiomes of horses of different keeper status, with differential abundance, beta diversity, and a Spearman's Rank correlation used to analyze and compare age groups.

Between horses who were easy and hard keepers, 32 species were found to be differentially abundant, with a definitive correlation between species and keeper status but no significance in diversity. For age, 69 taxa were found to be differentially abundant also with no significance in diversity. The taxa that were different between easy and hard keepers were from the *Lachnospiraceae* family, along with several others, suggesting a possible difference in nutrient availability for the horse due to the presence or absence of this family.

Chapter 1

INTRODUCTION

1.1 The Equine Industry

Globally, the horse is used for a wide variety of functions, including transportation, work, food, companionship, as well as the many types of sport and recreation. This puts horses in a "gray area" agriculturally, as they are not solely used for meat, dairy, or egg production as is the case with the bovine or poultry industry. Yet, the equine industry is nevertheless a substantial one. Encompassing over 7.2 million horses and at least 2 million owners in the United States, the industry provides a national economic impact of roughly $122 billion (American Equine Council, 2018). The equine community associated with the industry takes great concern for the health of these animals, as many people are affected financially and personally by their existence. Since it is a major contributor to overall equine health and well-being, the equine gut is a major area of interest and will thus be the focal point of this study.

1.2 The Equine Gut

The horse (*Equus caballus*) is a non-ruminant, monogastric, hindgut fermenter that contains an organ known as the cecum, which harbors a diverse community of microorganisms, crucial for proper digestion. The esophagus, stomach, and small intestine compose the foregut while the cecum and large intestine form the hindgut, wherein most of the microbial fermentation and fiber digestion takes place. In the cecum, microorganisms hydrolyze cellulose and dietary plant fiber, producing sugars that are fermented into short-chain fatty acids (Argenzio, 1975, Shirazi-Beechey,

2008). The equine hindgut has more anaerobic bacteria than the foregut by a factor of 100, signifying the hindgut as the main region for microbial digestion (Mackie & Wilkins, 1988).

While their stomach is relatively small, holding on average 2-4 gallons (Williams, 2004), in comparison to ruminant animals such as cows, the hindgut fermenting digestive system allows a horse to forage in small amounts all throughout the day (Al Jassim, Rafat A.M., Andrews, 2009). As their digestive system evolved to support continuous grazing throughout the day, horses require plenty of roughage in the form of grass or hay to meet their energy needs (Al Jassim, Rafat A.M., Andrews, 2009). Since the overconsumption of carbohydrates from concentrates can lead to higher lactic acid production and digestive upset, the soluble and insoluble fiber obtained from roughage can be noted as a major source of energy. (Argenzio, 1975). Microbial digestion is a major energy provider, for nearly 50% of the fiber obtained from roughage ingestion is passed to the large intestine where most microbial fermentation takes place (Argenzio, 1975).

Regardless of its ability to support continuous consumption, the equine digestive system is quite sensitive and susceptible to change. A pony's diet was abruptly shifted from all-forage to all-concentrate which resulted in a significant increase in total anaerobic hindgut bacteria as well as a significant decrease in cecal fluid pH, likely due to increased fermentation (Goodson, Tyznik, Cline, & Dehority, 1988). In this case, the sudden dietary change affected the pony's gut microbiome within hours of the experiment (Goodson, Tyznik, Cline, & Dehority, 1988).

Another review study suggests that, when overloaded rapidly with large amounts of grains, the digestive system will rapidly metabolize starch and upset the

microbiome, causing gut dysfunctionality such as colic (Shirazi-Beechey, 2008). Similarly, a rapid increase in starch ingestion can also trigger laminitis, an intense inflammation of the sensitive tissue within the hoof. It has been found that community changes are associated with starch-induced laminitis, wherein certain communities could not convert excess lactate into short fatty acids and laminitis occurred (Biddle et al., 2013). In these instances, a sudden adjustment to a newly introduced feed type or amount can greatly affect a horse's overall health, demonstrating the sensitivity of the gut.

Colic is defined as the manifestation of abdominal pain and, while there are many types and possible reasons for the disorder, is one of the most common causes of emergency, surgery, and death in horses (Shirazi-Beechey, 2008). The nature of equine colic is complex as there is a plethora of potential causes such as carbohydrate consumption, genetics, quality of care, and others, none of which are known to be exact cause (Reeves, Salman, & Smith, 1996). As seen earlier, an abrupt and recent change in diet can cause colic in horses (Shirazi-Beechey, 2008), possibly because the commensal bacteria do not have enough time to adapt to newly incoming feedstuffs (Al Jassim, Rafat A.M., Andrews, 2009). Considering this, it can be said the equine gut has a substantial effect on the animal's overall health, as a dietary disruption can cause conditions that most commonly kill horses today.

The equine digestive system is clearly a major contributor to horses' overall health, and much is currently being done to determine exactly how and to what degree. As the field progresses, methods used to define the equine gut microbiome have shifted away from a culture-based approach, which can be misleading if a species is unculturable, and towards a genomic approach to survey an entire community (Costa

& Weese, 2012, Turnbaugh et al., 2009). Some of the major phyla found inhabiting the equine hindgut using this approach are *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* (Costa & Weese, 2012, Dougal et al., 2013, Ericsson et al., 2016).

Moreover, in an attempt to define a core microbiome in the equine large intestine, Dougal et al. found similar results, finding an abundance of *Proteobacteria* and *Firmicutes* with significant dominant families found under the *Firmicutes* phylum such as *Lachnospiracea*, *Erysipelotrichaceae,* and *Ruminococcaceae* (2013). Additionally, the *Bacteriodales* and *Clostridiales* groups were found to be most abundant in the hindgut of several healthy horses, specifically the families *Lachnospiraceae* and *Ruminococcaceae* (*Clostridiales* group) (Ericsson, Johnson, Lopes, Perry, & Lanter, 2016). It was concluded that the equine gut microbiome is extremely diverse and that further consideration of bacterial function should be taken, raising question as to how influential the gut microbiome really is on equine health (Dougal et al., 2013). These maps of healthy equine microbiota can therefore help to provide reference for this study, in that any major deviations in microbiome composition found can be highlighted and questioned further.

1.3 Easy, Medium, and Hard Keeper Horses

For this study, a focus will be taken on the differences in the microbiomes of horses classified as either “easy keepers” or “hard keepers” while also considering the microbial composition of “medium keepers” as well. The phrase “easy, medium, or hard keeper” is a colloquial term well known among the equine community, referencing how much an owner needs to feed that particular animal. An “easy keeper” refers to a horse that requires very little feed to meet their nutritional needs and maintain a healthy body weight (Geor and Harris, 2013). That said, an easy keeper

often has the tendency to be overweight and at risk for metabolic issues such as laminitis, Equine Metabolic Syndrome, and obesity (Frank, Geor, Bailey, Durham, & Johnson, 2010).

Conversely, a "hard keeper" is described as an animal that requires a large amount of feed to maintain a healthy body weight. Hard keepers often tend to be underweight, a predisposition that causes their nutritional needs to be increased and thus demanding a larger feed intake for sustainment. Lastly, a "medium keeper" is considered to be a horse that requires a moderate, average amount of feed to survive and does not have the tendency to be either over or under weight. For the purposes of this study, keeper status is determined by the volunteer sending in the sample, so the measurement is slightly vague. Thus, since medium keepers can have variable definitions, they are not included in the analyses. Certain breeds often fall into the keeper categories; Thoroughbreds are often hard keepers whereas Quarter Horses or Ponies are often easy keepers. It is thought that there could be a genetic basis which determines a horse's keeper status, however this has yet to be proven (Geor and Harris, 2013).

A horse's nutritional needs may change over time, having a possible effect on their keeper status. For example, an animal that is considered an easy keeper for most of its adult life can become a hard keeper once it reaches an elderly age (>25 years). However, whether nutrient digestibility changes with age is under debate. In a study conducted by Ralston, Squires, and Nockels, horses aged 20 years and older were found to have decreased protein and fiber digestibility than horses aged 10 and under when fed the same died (1989). Yet, a more recent study found that there was no difference in nutrient digestibility between adult (ages 5-12) and aged (ages 19-28)

horses (Elzinga et al., 2014). While there are conflicting studies on the effect of age on nutrient digestibility, there is a possibility that age could still have an effect on the gut microbiome. For example, when compared to younger horses on the same diet, aged horses were found to have significantly reduced bacterial diversity, a trend that is also seen in humans (Dougal et al., 2014). Hence, age may provide insights as to why differences occur between easy and hard keepers and will therefore be kept under consideration.

1.4 The Microbiome and Obesity

Easy keepers have lower nutritional demands and often tend to be overweight. As a result, it is beneficial to observe any differences in the microbiome that occur in cases of extreme weight, i.e. obesity, for information regarding differences in easy and hard keepers. In recent studies, there have been links found between the human microbiome and obesity. According to Chakraborti's review, there were different proportions of the *Firmicutes* and *Bacteroidetes* phyla between lean and obese subjects (in both humans and a mouse model), wherein *Bacteroidetes* show reduced abundance and *Firmicutes* show a proportional increased abundance in obese subjects (2015). Yet, other studies did not find a decrease in *Bacteroidetes* in humans but still experienced an increase in *Firmicutes* in obese people (Chakraborti, 2015). Nevertheless, the *Firmicutes* to *Bacteroidetes* ratio is important when observing the microbiome in relation to obesity (Chakraborti, 2015).

In general, it is acknowledged that the human microbiome plays a large role in obesity, such as how obesity is associated with both reduced bacterial diversity and phyla-level changes (Turnbaugh et al., 2009). Additionally, an increase in both *Firmicutes* and *Bacteroidetes* was found in obese children and adults versus their lean

counterparts, thus showing a phyla-level change in other studies performed (Abdallah Ismail et al., 2010). Lastly, the *Firmicutes/Bacteroidetes* ratio was once again observed, and it was found that obese individuals had higher abundances of *Firmicutes* and lower abundances of *Bacteroidetes* than lean individuals (Koliada et al., 2017). It is evident that differing opinions on the *Firmicutes/Bacteroidetes* ratio and the relative abundance of *Bacteroidetes* in obese humans exist. Nevertheless, there is agreement on the grounds that those two dominant phyla are associated with obesity and should be noted.

Different hypotheses pertaining to the cause of differences in lean and obese microbiomes have been developed. First, it is thought that obesity can occur due to an increase in short chain fatty acid availability through the fermentation of polysaccharides by bacteria, especially *Firmicutes* (Diamant, Blaak, and de Vos, 2010). The absorption of these excess fatty acids into the body then increases body weight, sometimes to the point of obesity. *Firmicutes* in particular are the phyla associated with increased efficiency in energy harvesting, making nutrients such as shirt chain fatty acids more readily available for digestion and leading to increased weight (Turnbaugh et al., 2006). This potential change in nutrient availability due to increased *Firmicutes* may be a potential cause for differing metabolic tendencies and is thus very relevant to this study.

1.5 The Equine Gut Microbiome and Keeper Status

As previously discussed, phyla level changes have been associated with obesity in human subjects, hopefully shedding light on the possible differences in gut microbiota of horses that tend to be lean or overweight. A healthy equine hindgut is typically high in both *Firmicutes* and *Bacteroidetes*, as is the case with most mammals

(Ericsson, Johnson, Lopes, Perry, & Lanter, 2016). There is agreement on the dominance of *Firmicutes* over *Bacteroidetes* and other phyla such as *Proteobacteria* in horses, as well as most mammals (Dougal et al., 2013), noting that a change in this relationship could be relevant for relating microbiome change to metabolic tendency.

The gastro-intestinal system of the horse has proven to be very sensitive and very influential on the overall health of the animal. Further, the gut microbiome has shown to be diverse and rapid to adapt to change. Yet, the exact level of influence that the gut microbiome as well as its importance to overall equine health is yet to be determined. This raises questions that can be answered through observation of specific microbial taxa in the gut, observing changes or differences and hypothesizing why they occur. After having observed associations with the microbiome and obesity in humans as well as the sensitivity of the equine gut, the influence of the microbiome on keeper status is questioned in this project. The objective is to observe any differences in the equine gut microbiomes between easy and hard keeper horses, as it may be possible for the gut microbiome to affect nutrient digestibility and thus the level of nutrients required for horses to maintain general health and well-being. To do so, the microbiome diversity and abundance of easy and hard keepers will be compared to find differences, focusing on the abundance of Firmicutes and Bacteroidetes as well as other taxonomy.

Chapter 2

METHODS

2.1 Equine Fecal Samples Collected

In this study, equine fecal samples were sent to the University from volunteer horse owners who were notified of the Equine Microbiome Project and offered to participate. After contacting the lab and agreeing to contribute, volunteers purchase a fecal sampling kit which they use to collect their horse's fecal sample and send it back to the lab. These kits include latex gloves, an autoclaved collecting spoon, a 5 mL collection tube filled with a fecal protecting solution, tissue to provide an absorptive barrier (in case of spill), a 50 mL conical centrifuge tube to provide a second barrier, a plastic bag to put the centrifuge tube in, weight tape, and a set of detailed instructions indicating how to properly collect the fresh fecal matter in a sterile manner. A properly collected sample ready for shipment can be seen in Figure 1.

Figure 1: A fecal sample suspended in fecal protective solution, enclosed in a 5 mL collection tube, packed with tissue, enclosed in a 50 mL conical centrifuge tube, and enclosed in a plastic bag. Gloves, a collecting spoon, and instructions were also included. This sample is ready to be placed in the box above and mailed to the laboratory for DNA extraction.

Once collected, the sample is packaged and sent back to the lab via the mail, where it is kept at a temperature of -20 ˚C until use in the extraction procedure to prevent further bacterial growth, providing a more accurate representation of the horse's microbiome at the time of sampling. In addition, volunteers fill out an online questionnaire about their horse, giving a detailed description of their horse's entire health history. Information regarding all aspects of the horse's health is requested in the questionnaire, including but not limited to the horse's age, breed, sex, weight, diet, exercise regimen, vaccinations, medications, and history of disease or injury. The information collected from volunteers is then used in the analyses of data collected from the fecal samples further on in the study.

2.2 DNA Extraction Procedure

After the fecal sample is received by the lab, it is processed through a DNA extraction procedure using a QIAGEN QIAamp Powerfecal DNA Isolation Kit. A series of steps is executed to lyse the cells in the fecal sample, precipitate any organic or inorganic non-DNA material, bind the DNA, wash out non-DNA material, and elute the DNA with an elution buffer. First, the fecal sample is prepared by adding a solution containing disruption agents, including sodium dodecyl sulfate (SDS), to aid in cell membrane degradation and cell lysis. The sample is then heated at 65 ˚C and agitated to fully break the cells open. Next, a reagent with Inhibitor Removal Technology (IRT) is added to precipitate all organic and inorganic non-DNA matter including proteins, polysaccharides, and cell debris.

Once all non-DNA matter has precipitated into a pellet and removed, the DNA remaining is bound to a silica filter membrane by adding a high salt solution. This allows for the washing away of all contaminants with an ethanol-based solution while keeping the DNA intact on the membrane. Lastly, the DNA is washed off the silica membrane with a 10 mM Tris sterile elution buffer. Once fully extracted and eluted in this buffer, the DNA is then stored at -20 ˚C. Each sample is extracted in triplicate and the sample with the highest quantity and quality is sent out for sequencing. Additionally, a blank sample with no fecal matter was extracted to test for any possible kit contamination.

After extraction, the DNA is tested for quality and quantity using both Qubit and Nanodrop procedures to ensure each sample has adequate amounts for downstream sequencing. The Invitrogen Qubit utilizes the detection of target-specific fluorescence, wherein fluorescent dyes are combined with the extracted DNA sample, allowing the genomic material to be detected and quantified (Manchester, 1996). Since

samples were extracted in triplicate, the sample with the highest quantity of DNA (typically somewhere between 20-100 ng/uL) is sent out for sequencing.

The NanoDrop procedure uses spectrophotometry to determine the quantity as well as the quality of a sample by reading the absorbance ratio of a DNA sample (Huss, Festl, & Schleifer, 1983). This indicates what amount of the sample is purely DNA versus RNA or protein, with a generally pure sample having an A260/280 ratio of 1.8 (Huss, Festl, & Schleifer, 1983). Thus, the sample out of the three extracted in triplicate with the ratio closest to 1.8 was chosen to be sent for sequencing.

2.3 16S rRNA Sequencing

In order to determine the novel species of bacteria present in a sample, the extracted microbial genome is sent out for 16S rRNA sequencing. In this method, the portion of RNA from the 30s ribosomal subunit which binds to the Shine-Dalgarno sequence is referred to as 16S rRNA. The V4-V6 region of the 16S rRNA gene, which encodes for 16S rRNA, is amplified using universal primers that are then sequenced. These amplicons are then clustered, and similar reads are defined as Operational Taxonomic Units (OTU's). These provide markers for as many species as possible in each sample, which are compared and matched to a database to identify bacteria and archaea, the quantification of which is used in downstream analyses.

Two batches of samples were extracted and sent separately to RTL Genomics in Lubbock, Texas to be sequenced. Prior to the initiation of this study, the comparison of easy and hard keeper horse microbiomes, all the methods mentioned thus far have been accomplished. This produced a data set containing samples from 60 horses which was used in a previous study on differences in the equine gut microbiome pertaining to

diet. The dataset was then used for analyses in this study in conjunction with data from the newly extracted samples.

2.4 QIIME Analyses

Upon receiving the sequenced FASTQ files from RTL Genomics, paired-end reads were merged using Fast Length Adjustment of SHort reads (FLASh). After being filtered for length and quality, barcodes removed in QIIME, and paired, these reads were generated into per-sample FASTA files and concatenated into one usable FASTA file. The reads in the FASTA file, including both newly obtained reads and reads from the data set described in Chapter 2 section 3, were then clustered against the Greengenes 13.5 Reference Database with 97% accuracy in an open-reference OTU picking step. The subsequent methods of data analysis were all performed using the QIIME (Quantitative Insights Into Microbial Ecology) pipeline. The OTU table was filtered to remove singletons and doubletons, defined as reads with sequences that appear only once (singletons) or twice (doubletons) and could produce errors in the data.

Upon observation of the subjects, it was noted that the dataset for this project is unbalanced; there are many more easy keepers than hard keepers. To address this problem, a matched comparison was applied. Hard and easy keepers were matched based upon sex, age, and diet. Hard keepers were matched with easy keepers whose age is within a 5-year range i.e. is no more than 5 years older or younger. Of the total 18 easy and hard keepers, only one matched pair did not have the same diet; the hard keeper had a diet of hay/concentrates and the easy keeper had a mixed diet of hay, concentrates, and pasture. The original OTU table was then filtered to only include these 18 subjects in order to provide a more balanced data set and better analyses.

First, the relative abundance was computed using a taxa summary of the OTU's picked in which the abundances of the phyla, classes, orders, families, and genera of the samples were presented. This measurement was used in a Spearman's Rank correlation to reveal whether the ranks of easy and hard keepers covary. Hypotheses regarding bacterial symbiosis can be made using this correlation depending upon whether taxa have positive or negative relationships.

Next, the differential abundance was found, a measure which highlights which taxonomic levels have the largest differences in OTU frequency between groups. The differential abundance between easy and hard keepers was found and determined statistically significant using a parametric student's two-tailed t-test. The *Firmicutes/Bacteroidetes* ratio was found using the differential abundance data in order to observe the equine ratio compared to human ratios, a measurement that was also compared using a student's two-tailed t-test.

In addition to the abundance of bacteria between groups, diversity indices were also calculated to observe the different species in each keeper type. For alpha diversity, a rarefied richness estimator, Choa1, was used to find within-sample diversity and compared with a Kruskal-Wallis test. To determine evenness among a community while factoring in abundance, a Shannon index was used and also compared using a Kruskal-Wallis test. To compare diversity at the community level between keepers, the beta diversity was calculated and displayed using a Principal Coordinates Analysis. A Bray-Curtis dissimilarity measurement was also taken. Both the weighted and unweighted beta diversity significance were found using either weighted or unweighted significance tests. Lastly, a Linear Discriminant Analysis

Effect Size test (LEfSe/LDA) was used to determine the effect the keeper status has on the abundance.

After performing the above steps to compare easy versus hard keeper horses, a set of preliminary analyses was executed for the comparison of age with the samples of the 18 horses used in the keeper microbiome comparisons. For the purposes of simplification, the ages of the 18 horses were lumped into three categories and the categories were compared only when necessary. The categories were organized as follows; “young” horses were of ages 1-10, “adult” horses were 11-20 years old, and “aged” horses were age 21 and above. From the matched data set, there were 3 young horses, 6 aged horses, and 9 adult horses. The differential abundance of these age groups was found and found significant using a Kruskal-Wallis test, the beta diversity displayed using a PCoA plot, and the covariance of the relative abundance found using a Spearman’s Rank correlation.

Chapter 3

RESULTS

3.1 Equine Subjects

Volunteer reports were received and the information regarding the equine subjects was recorded. In this investigation, fecal samples from 104 horses total were used. Of these 104 horses, 67 were easy keepers, 28 were medium keepers, and 9 were hard keepers. There were 35 horses between the ages of 0 and 10, 47 horses between the ages of 11 and 20, and 21 horses above the age of 21. A variety of breeds composed the dataset, of which Quarter horses were dominant (30) followed by Thoroughbreds (17). Many of the breeds were combinations, resulting in a somewhat unique breed (Appaloosa/Quarter Horse/Palomino/Arabian for example). Several subjects possessed chronic diseases, including Equine Metabolic Syndrome, laminitis, colic, and Cushings. For the matched comparison, 9 easy keepers and 9 hard keepers were randomly selected out of the 104 total horses and matched by age, sex, and diet (see Chapter 2 Section 4).

3.2 Microbiome Differences Between Keepers

3.2.1 Abundance

To determine differences among the microbiomes of the two types of keepers, a number of abundance and diversity analyses were implemented. The intention is to observe the communities from several viewpoints, obtaining as much information on them as possible to draw conclusions from. An abundance measure quantifies the communities, determining how many individual bacteria are in each sample overall. The relative abundance describes the number of organisms in a sample in comparison

to the number of organisms found in the entire set of data. Relative abundance can therefore be more useful than general abundance for quantifying bacteria and using the values for other analyses. The relative abundance for the 18 equine subjects was thus found and can be observed in Figure 2.

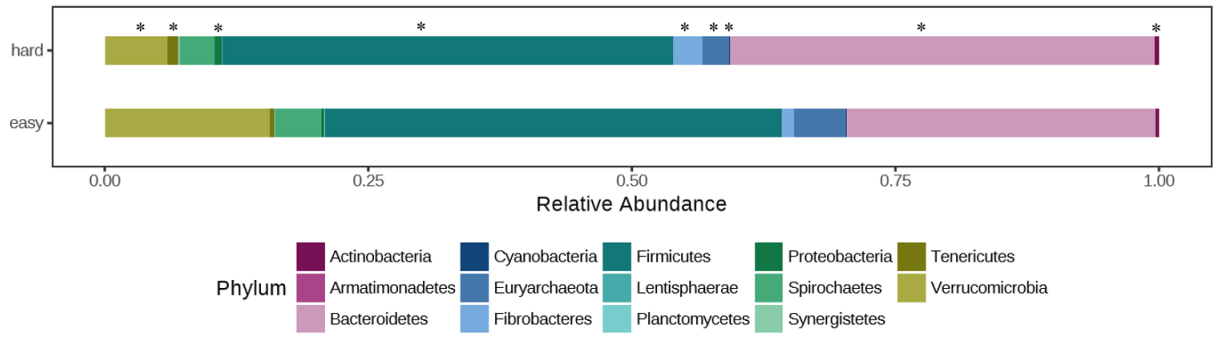

Figure 2: The relative abundance of phyla between easy and hard keeper horses. Significant differences between keepers in taxa found using a student's two-tailed t-test are starred above ($P<0.05$).

The figure above displays the percentage of relative abundance for easy and hard keepers. Both *Firmicutes* and *Bacteroidetes* compose a large majority of the gut microbiomes, as expected, with *Verrucomicrobia*, *Spirochaete*s, and *Cyanobacteria* also seen in considerable amounts. To find potential differences in microbial taxa between easy and hard keepers, the differential abundance was calculated. This measure, as the name entails, pinpoints significantly different abundance values between two sample categories such as keeper status. To determine the significance of differentially abundant taxa, a parametric student's two-tailed t-test was used since the variances of the two keeper groups were equal.

After performing the t-tests, 32 taxa were found to be significantly different in abundance between easy and hard keepers ($P<0.05$). Figure 2 shows the phyla which were found to have significantly different abundance, all seen in the relative abundance except for *Lentisphaerae*, *Planctomycetes*, and *Syngergistetes*. Table 1 displays the complete list of taxa which were differentially abundant between keepers. Of these 32 taxa, the 3 phyla to have the most frequently significant differential abundance values in descending order were *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* (Table 1). There were 16 taxa under the *Firmicutes* phylum with significant differential abundance, nearly half of all significant taxa found, 6 taxa under the *Bacteroidetes* phylum, and 2 taxa under *Actinobacteria* (Table 1).

Table 1: The 32 differentially abundant taxa that showed significance using a student's two-tailed t-test. Taxa are colorized in relation to phyla. The family level is the lowest level of taxonomy where most taxa have been identified (all $P<0.05$).

Taxa Number	Kingdom	Phyla	Class	Order	Family	Genus	Species
1	Archea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanocorpusculaceae	Methanocorpusculum	
2	Bacteria	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae		
3	Bacteria	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Aldercreutzia	
4	Bacteria	Armatimonadetes	SJA-176	RB046			
5	Bacteria	Bacteroidetes	Bacteroidia	Bacteriodales			
6	Bacteria	Bacteroidetes	Bacteroidia	Bacteriodales	[Paraprevotellaceae]	[Prevotella]	
7	Bacteria	Bacteroidetes	Bacteroidia	Bacteriodales	[Paraprevotellaceae]	CF231	
8	Bacteria	Bacteroidetes	Bacteroidia	Bacteriodales	[Paraprevotellaceae]	YRC22	
9	Bacteria	Bacteroidetes	Bacteroidia	Bacteriodales	BS11		
10	Bacteria	Bacteroidetes	Bacteroidia	Bacteriodales	Porphyromonadaceae	Paludibacter	
11	Bacteria	Cyanobacteria	4C0d-2	YS2			
12	Bacteria	Fibrobacteres	Fibrobacteria	Fibrobacteriales	Fibrobacteraceae	Fibrobacter	succinogens
13	Bacteria	Firmicutes	Clostridia	Clostridiales			
14	Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]		
15	Bacteria	Firmicutes	Clostridia	Clostridiales	Christenellaceae		
16	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae		
17	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	
18	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	butyricum
18	Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Pseudoramibacter_Eubacterium	
20	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae		
21	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia	
22	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Moryella	
23	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Roseburia	
24	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae		
25	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira	
26	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	
27	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascloractobacterium	
28	Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae		
29	Bacteria	Proteobacteria	Alphaproteobacteria	RF32			
30	Bacteria	Tenericutes	Mollicutes	RF39			
31	Bacteria	Verrucomicrobia	Verruco-5	WCHB1-41	RFP12		
32	Unassigned						

From the 32 differentially abundant taxa found to be significant, the abundance values for the taxa at the phyla level was observed (Figure 3). *Firmicutes* are the most abundant phylum for both easy and hard keepers with abundance values of 0.46 for hard keepers and 0.47 for easy keepers (Figure 3). *Bacteroidetes* are the next most abundant at 0.3 for easy keepers and 0.37 for hard keepers (Figure 3) There was a notably higher abundance in the Unassigned phylum for easy keepers than hard, 0.084 versus 0.049 (Figure 3). The 32 significant taxa were also observed at the family level, shown in Figure 4. Within the taxa, the family *Ruminococcaceae* was most abundant at 0.2 for hard keepers whereas easy keepers had an abundance of 0.000018 (Figure 4). Additionally, *Lachnospiraceae* had higher abundance in easy keepers, 0.10, than hard keepers, 0.092 (Figure 4). Despite differing abundance values, easy keepers often had higher abundance for each taxon than did hard keepers (Figures 3 & 4).

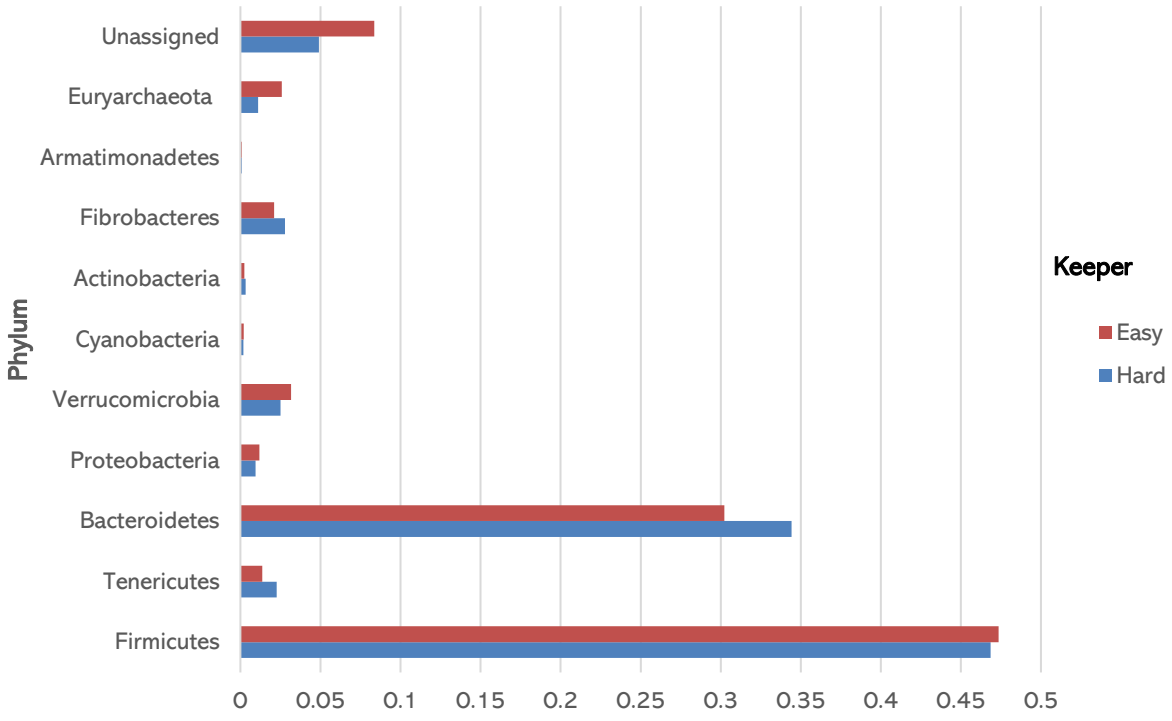

Figure 3: The distribution of significant differentially abundant taxa at the phylum level. The above phyla contain taxa which were significantly different in abundance between the two keeper types. A student's two-tailed t-test was used (all $P<0.05$)

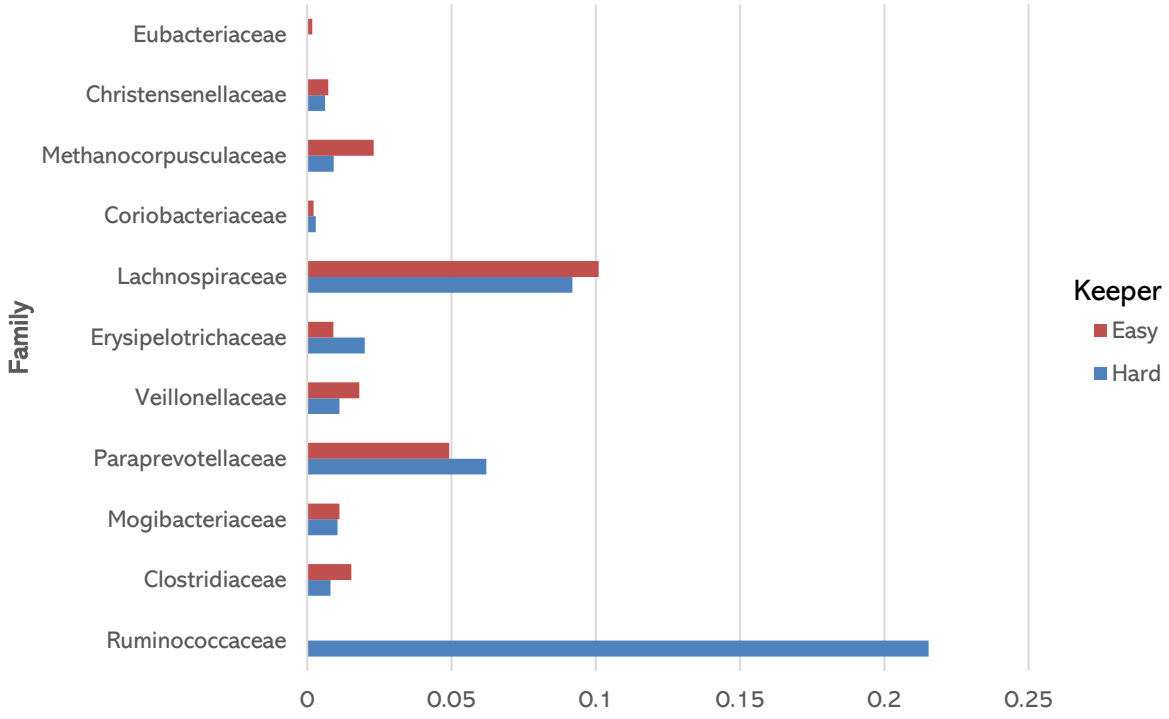

Figure 4: The distribution of significant differentially abundant taxa at the family level. The above families contain taxa which were significantly different in abundance between the two keeper types. A student's two-tailed t-test was used (all $P<0.05$)

A Spearman's Rank correlation test was used to determine whether the ranks of the two keepers covary or not, revealing if there was a correlation between keeper status and abundance of taxa. The relationship between keeper status and abundance can either be positive or negative, indicated by the coefficient's sign once produced from the test. The closer to either -1 or 1 the coefficient is, the stronger the relationship between variables and the greater the significance, however any value above 0.3 and below -0.3 was accepted as significant for this study. When comparing the keeper variable, 39 taxa were found to have a significant correlation (Figure 5).

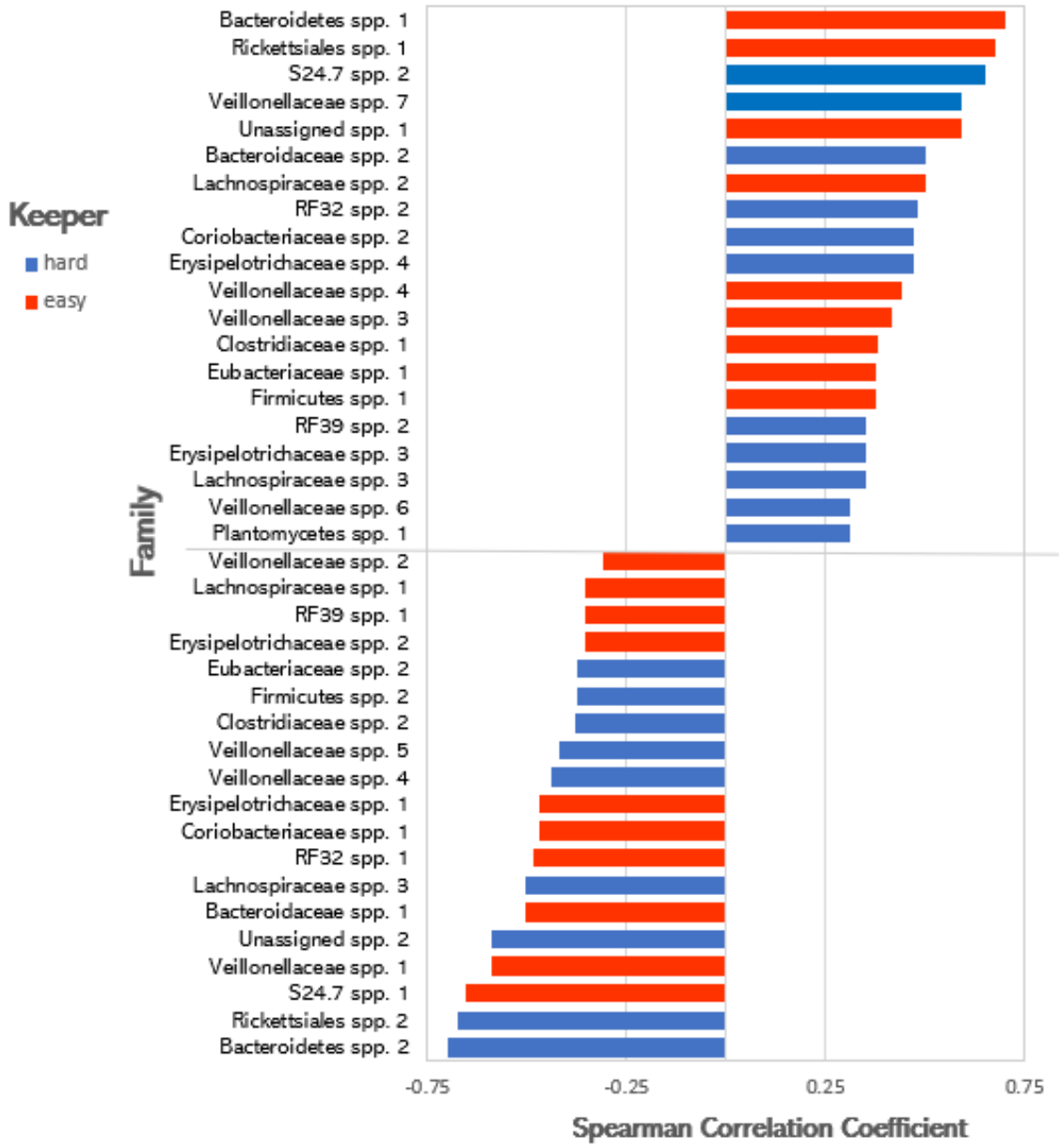

Figure 5: The results from a Spearman's Rank Correlation, with the significantly keeper-correlated taxa organized by family. For families with multiple differing lower taxa, the format "spp. #" is used to differentiate them. Families defined as "other" were labeled in the "phyla sp.. #" format. Taxa with no classified families were not included in this figure. Correlations were deemed significant if the coefficient was <-0.03 or >0.03.

The taxa were displayed at the family level for this and other figures because this taxonomic level was simultaneously the most specific and had the lowest amount of missing taxonomy (unlike genus or species level). There were also many taxa with the same family whose lower taxonomy was described as “other”. Of the 39 families, 19 were either positively or negatively correlated with easy keeper status and 20 were correlated with hard keeper status (Figure 5). Additionally, the number of taxa positively correlated to easy keeper status was relatively the same as those positively correlated to hard keeper status, with the same trend for negative correlations (Figure 5). Interestingly, the figure shows that a family that was positively correlated with easy keeper status would also be negatively correlated with hard keeper status by the same coefficient and vice versa, such as *Rickettsiales* spp. 1 and 2 (Figure 5). With all the values being within significant range, it can be said that these correlations are valid.

The last abundance analyses performed on the matched easy/hard keeper data set is a Linear Discriminant Effect Size Analysis. This test uses an algorithm to identify genomic features and determine the differences between species within a sample. The measure first finds genomic components that are significantly different through a Kruskal-Wallis test, validating that they are correctly describing the species they belong to. A Wilcoxon-rank test then compares the pairwise classes and lastly the Linear Discriminant Analysis finds the effect size of a genomic feature, or in this case keeper status. The score indicates the effect that a feature has on a particular taxon; the negative axis simply is a means of comparing the features as the actual score is found by taking the absolute value.

The LEfSe values in Figure 15 are presented at the. For easy keepers, the phyla most affected by hard keeper status are *Bacteroidetes, Tenericutes,* and *Fibrobacteres* in descending order (Figure 6). Similarly, in descending LDA value, *Verrucomicrobia, Euryarchaeota, Spirochaetes, Firmicutes,* and *Cyanobacteria* were all significantly affected by easy keeper status. There were 8 phyla affected by hard keeper status versus only 5 for easy keeper status (Figure 6).

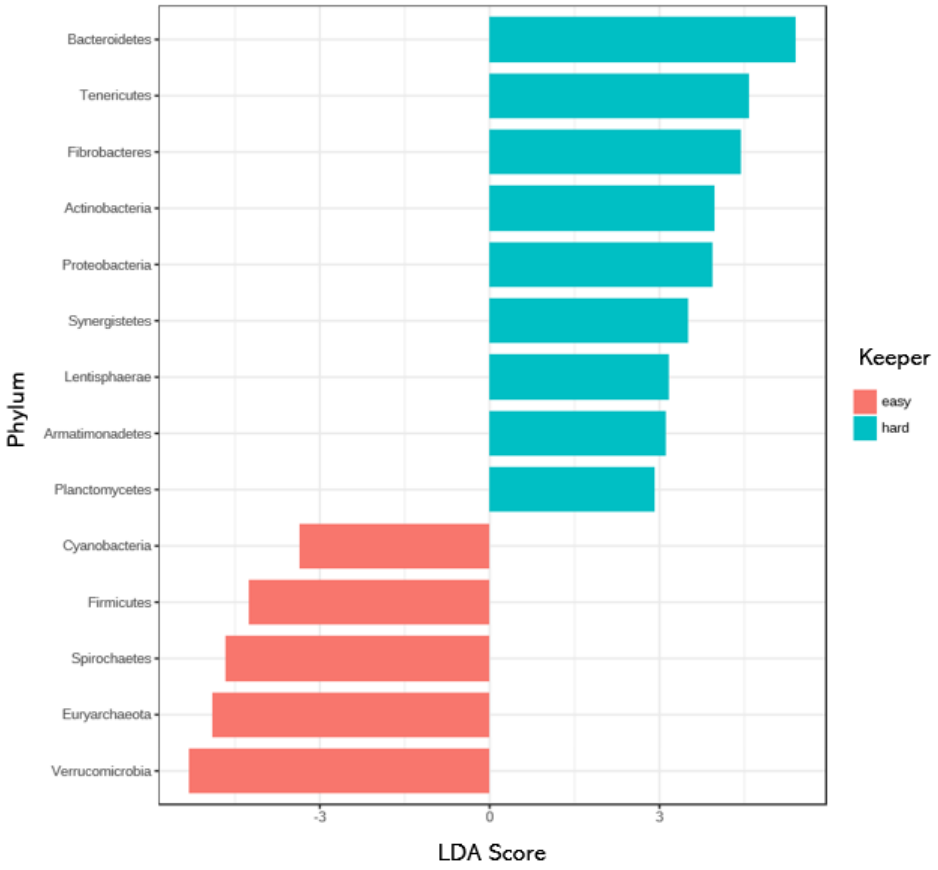

Figure 6: The results from a Linear Discriminant Analysis Effect Size (LEfSe/LDA) measurement at the phylum level. Each bar is a log10 transformed LDA score represents the effect size for that phylum and its keeper status.

The family level LDA effect sizes were found in addition to the phyla level. Those with the largest two LDA scores were species RFP12 and species BS11, affected by the easy keeper and hard keeper groups (Figure 6). Additionally, many of the families having been significantly affected by keeper status in this measure are of the *Firmicutes* phylum, with *Paraprevotellaceae, Coriobacteriaceae, Prevotellaceae, Synergistaceae, Moraxellaceae, Mycoplassmataceae, Succinivibrionaceae, Porphyromondaceae, Spirochaetaceae,* and *Methanobacteriaceae* families not having *Firmicutes* as their higher taxonomy (Figure 6).

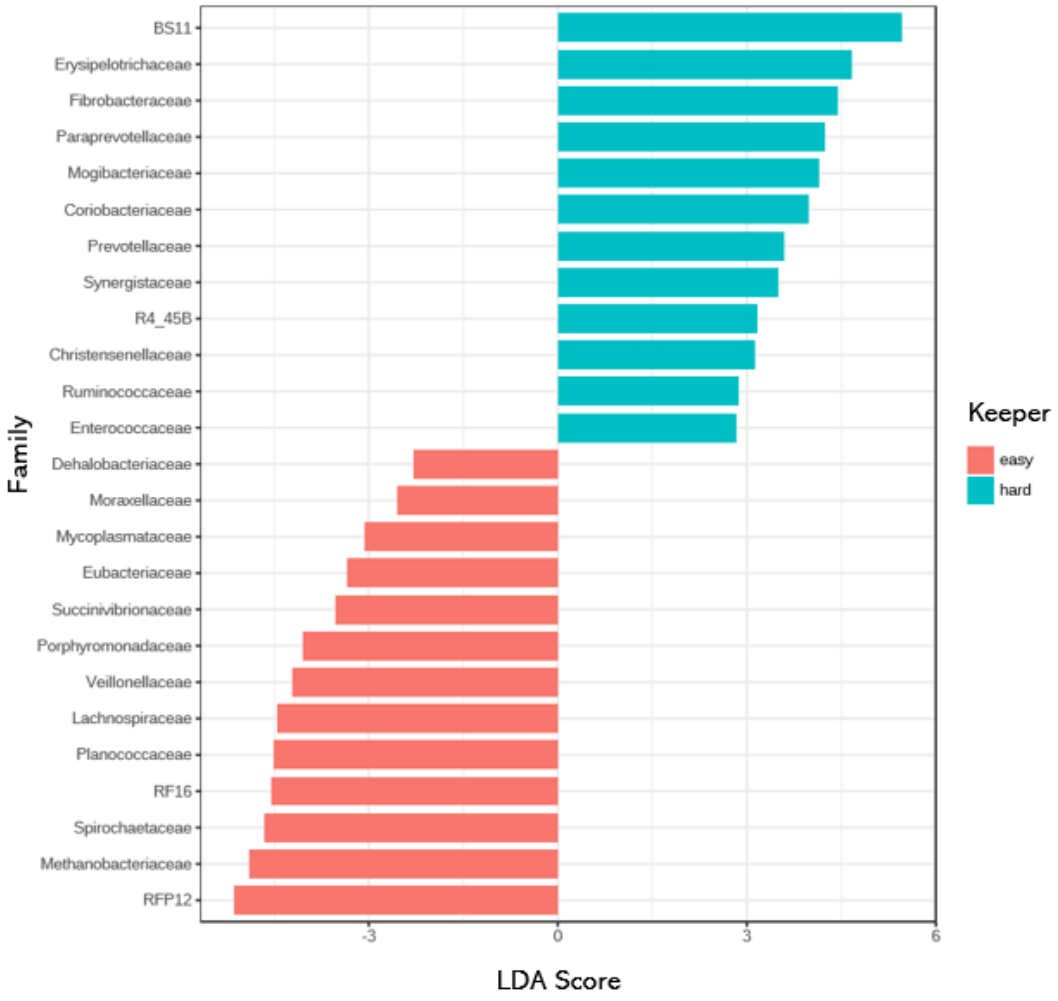

Figure 7: The results from a Linear Discriminant Analysis Effect Size (LEfSe/LDA) measurement at the family level. Each bar is a log10 transformed LDA score and represents the effect size for that family and its keeper status.

Several of the 32 taxa are within the following families affected by keeper status above; BS11, *Erysipelotrichaceae, Fibrobacteraceae, Paraprevotellaceae, Mogibacteriaceae, Coriobacteriaceae, Christensenellaceae, Ruminococcaceae, Eubacteriaceae, Porphyromonadaceae, Veillonellaceae, Lachnospiraceae,*

Methanobacteriaceae, and RFP12 (Figure 7). This indicates that the differentially abundant taxa found are in fact different due to keeper status as their LDA effect size is significant. The only taxon which was found differentially abundant between keepers but did not appear significant in the LEfSe analysis was *Clostridiaceae* (Table 1). However, there were also several families which were affected by keeper status but were not differentially abundant, including *Prevotellaceae, Synergistaceae*, R4_458, *Enterococcaceae, Dehalobacteriaceae, Moraxellaceae, Succinivibrionaceae, Planococcaceae*, RF16, and *Spirochaetaceae* (Figure 7).

3.2.2 Diversity

Determining microbiome differences requires observation of both the quantity of individuals per samples as well as sample diversity and evenness. The alpha diversity measure compares the different species within each sample, information that could be useful when comparing potential commensalism between species. Choa1 is a richness estimator used in this study to compare within-species richness. Figure 8 displays the computed alpha diversity measure Choa1 for each sample ID, where the confidence interval is shown on the y-axis and the deviation is shown for each individual sample on the plot points. Unfortunately, there is no clear correlation seen with this richness estimator. The easy keepers tend to be slightly more scattered than do the hard keepers and generally have larger deviation, yet the deviation varies for both keepers (Figure 8). A non-parametric Kruskal-Wallis was run to determine the significance of the richness and was found to be insignificant ($P>0.05$).

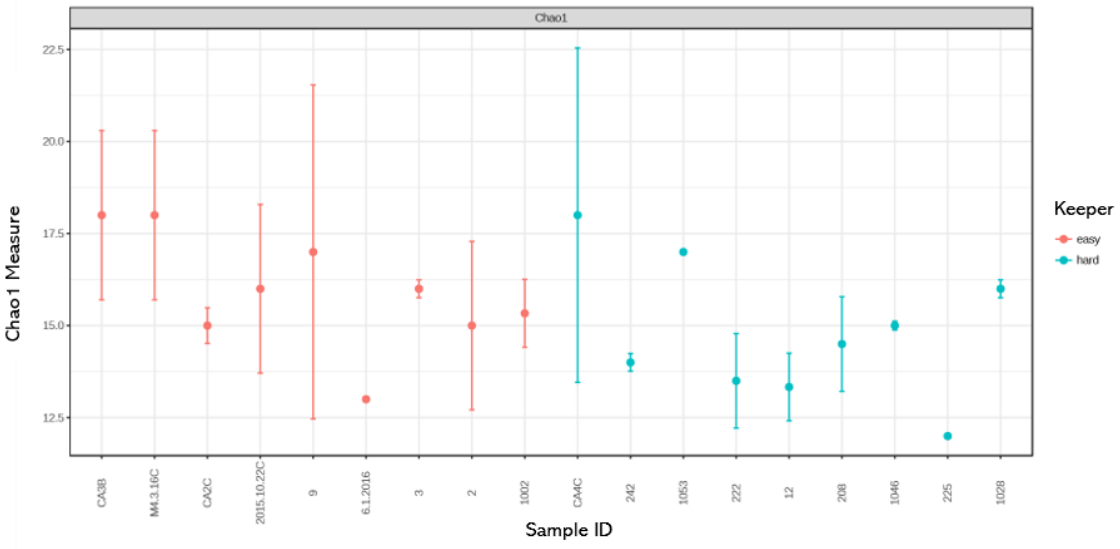

Figure 8: The richness estimator Choa1, indicating inter-sample diversity, comparing the diversity within easy and hard keeper samples using a Kruskal-Wallis nonparametric test (all P>0.05).

In addition to comparing the richness of samples between the two keepers, the evenness was also compared. A Shannon Index compares evenness while taking relative abundance into account by finding the ratio of the species present to the total number of the species. The values derived from this index are displayed for all individual samples between easy and hard keepers in Figure 9. Like Choa1, there is not a strong trend in this index. Most samples have a somewhat high Shannon index value, indicating that the abundance in each sample is relatively even. However, these are all insignificant trends as the nonparametric Kruskal-Wallis test performed did not show significance (P>0.05). The last unit of alpha diversity measurement used in this study is the Phylogenetic Diversity (or "PD") Whole Tree analysis, in which a phylogeny is used to compare the types of species within a sample. There is a moderate trend wherein most of the samples have a PD Whole Tree value above 150,

however this is also insignificant (P>0.05) (Figure 10). Conclusively, there are some interesting patterns in all alpha diversity measurements, however no significance was found for any of them.

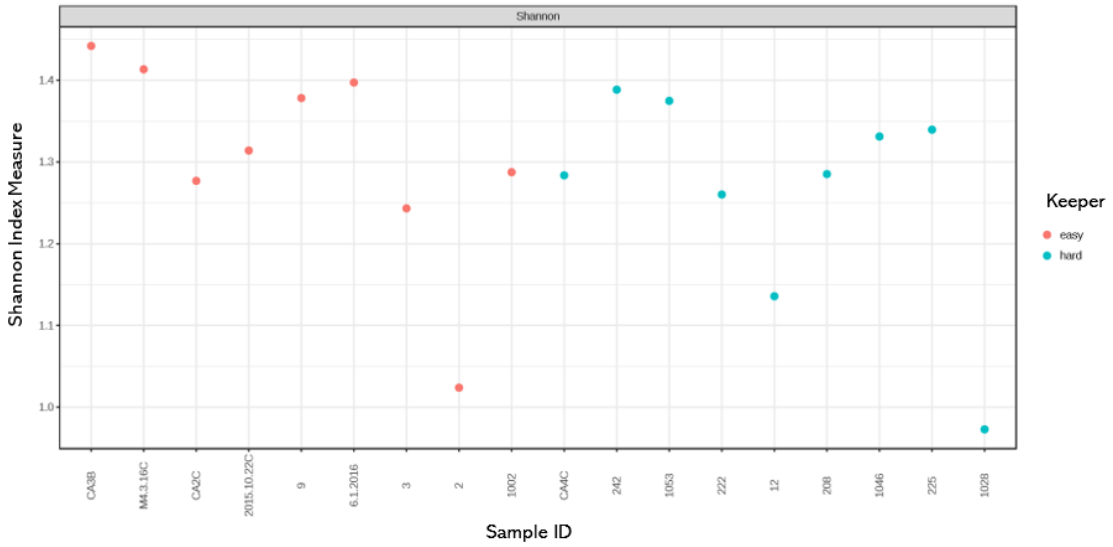

Figure 9: The Shannon Index displaying the evenness of each sample between easy and hard keepers, found using a nonparametric Kruskal-Wallis test (all P>0.05).

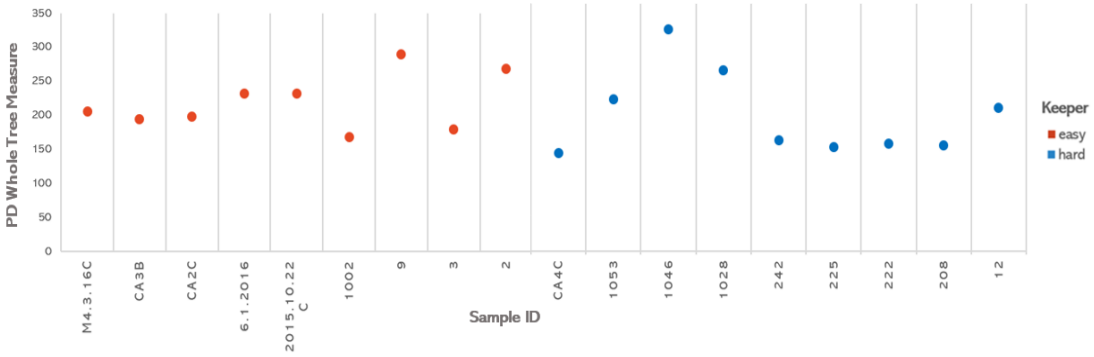

Figure 10: The results from a PD whole tree phylogenetic alpha diversity analysis comparing easy and hard keepers. A student's two-tailed t-test was used to compare the two groups, which were found insignificant (all P>0.05).

As a means of determining the diversity of bacterial species in the equine gut microbiome between easy and hard keeper groups, the beta diversity was calculated through Principal Co-ordinates Analyses. This measurement utilizes a beta diversity metric input, Unifrac, to generate and display a distance matrix for dissimilarity comparisons of samples by groups. This uses the coordinates of what is usually a 3-Dimensional graph; PC1, PC2, and PC3. Both weighted and unweighted Unifrac metrics were implemented; weighted Unifrac accounts for overall abundance and is a quantitative measure whereas unweighted does not and is a qualitative measure. Additionally, a Bray-Curtis dissimilarity measure was used to further compare group dissimilarity through 2D PCoA plots. To measure the beta diversity significance, a non-parametric PERMANOVA was used to determine the strength of the distance matrix, wherein no significance was found for all beta diversity measures (P>0.05).

By using the distance matrix, three figures were made which each compare the 3D coordinates using percent variation. The red circles in each graph represent the sample of an easy keeper and the blue squares represent the samples of hard keepers.

The unweighted, 2-Dimensional PCoA plots are shown in Figure 11; little to no clustering is occurring between the two groups. The coordinate with the largest variation explained is PC1 with 12.22% (Figure 11). This trend is also true for the weighted PCoA plots in Figure 12, where there is not clear clustering and the PC1 coordinate has the highest variation at 22.08%.

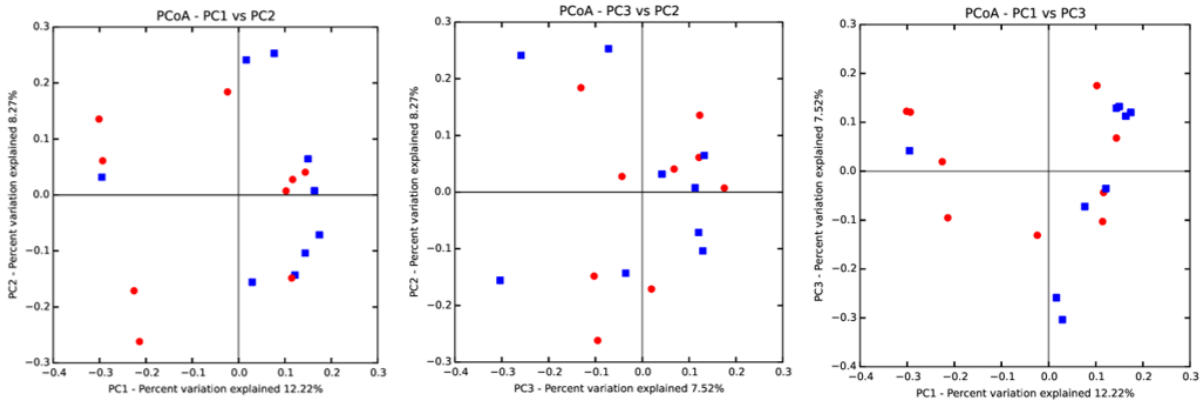

Figure 11: The beta diversity of the keeper-matched data displayed as 2-dimensional unweighted PCoA plots. Each image displays a different angle between points; PC1 vs. PC2, PC2 vs. PC3, PC1 vs. PC3. The red circles represent easy keepers whereas the blue squares represent hard keepers (all P>0.05).

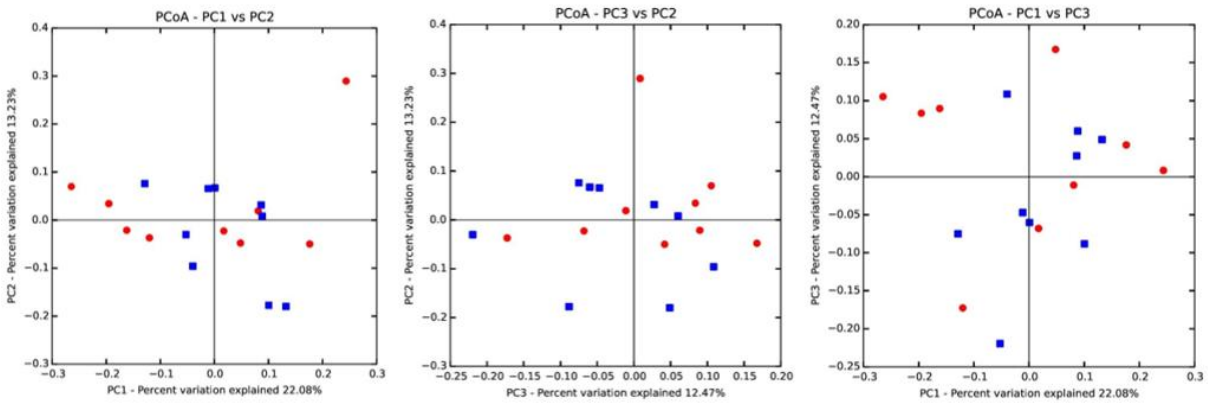

Figure 12: The beta diversity of the keeper-matched data displayed as 2-dimensional weighted PCoA plots. Each image displays a different angle between points; PC1 vs. PC2, PC2 vs. PC3, PC1 vs. PC3. The red circles represent easy keepers whereas the blue squares represent hard keepers (all P>0.05).

The 2D PCoA plots did not show any clustering or clear trends for either weighted or unweighted metrics (Figure 11 & 12). Moreover, these same measures are displayed using the 3D plots in Figures 13 and 14, as well as the Bray-Curtis dissimilarity measure in Figure 15. All these figures shared the same lack of trend and lack of significance. Variation values for each coordinate were not the same between measures, however the PC1 coordinate always had the highest variation (Figures 11-15).

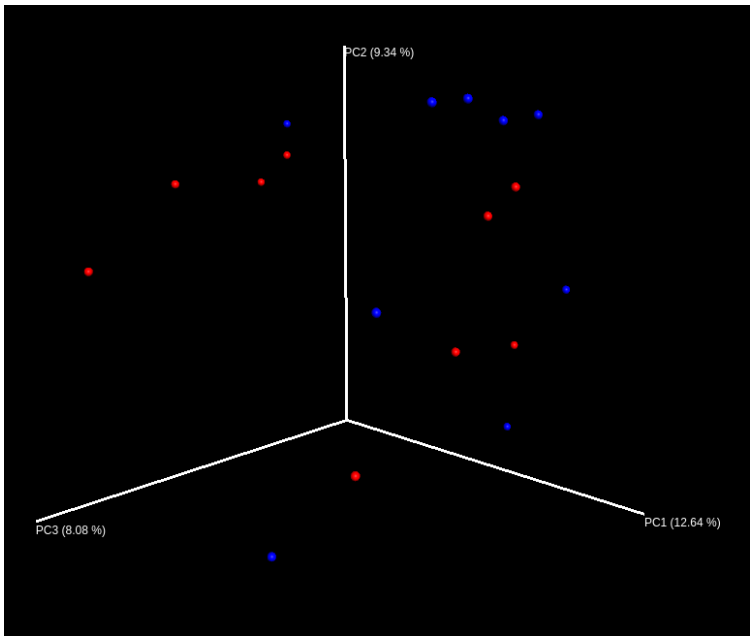

Figure 13: The beta diversity of the keeper-matched data displayed as a 3-dimensional unweighted PCoA plot. All three angles are displayed; PC1, PC2, and PC3. The red circles represent easy keepers whereas the blue circles represent hard keepers (all $P>0.05$).

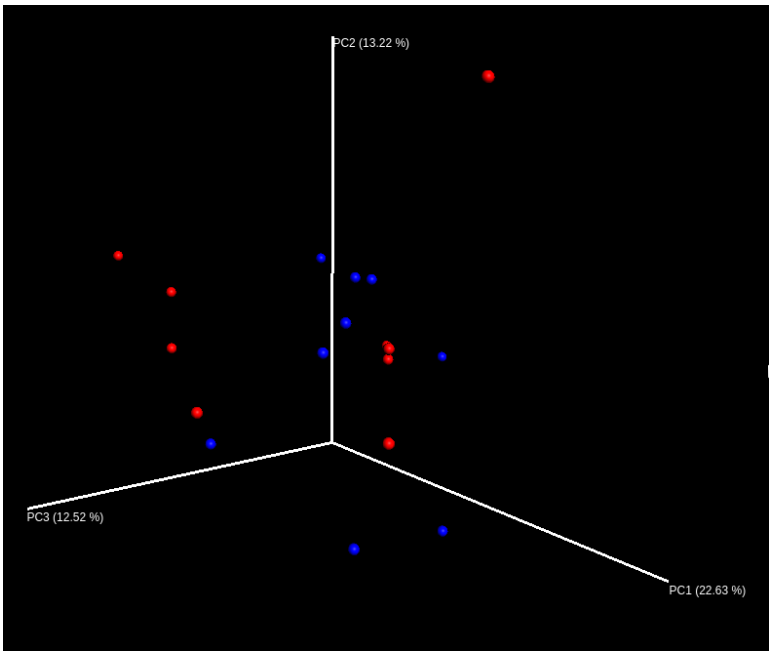

Figure 14: The beta diversity of the keeper-matched data displayed as a 3-dimensional weighted PCoA plot. All three angles are displayed; PC1, PC2, and PC3. The red circles represent easy keepers whereas the blue circles represent hard keepers (all P>0.05).

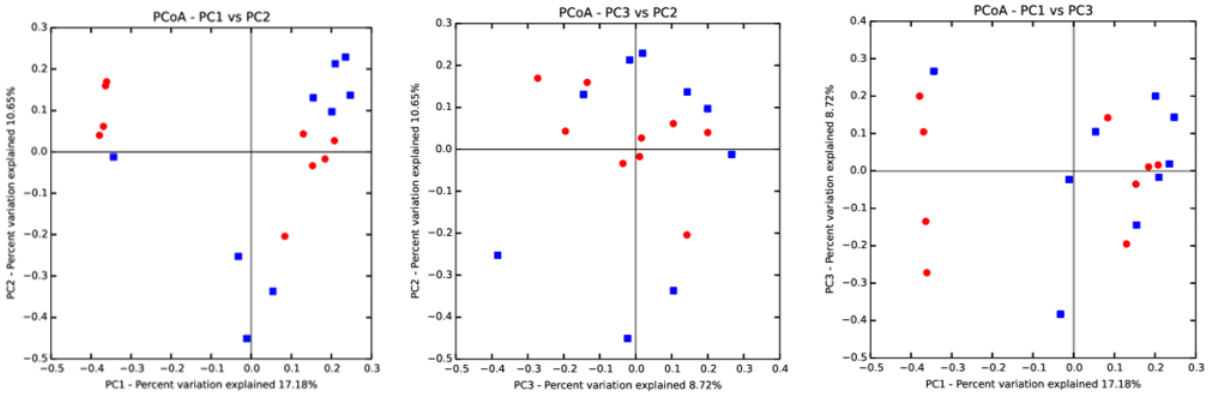

Figure 15: The result from a Bray-Curtis dissimilarity analyses comparing the dissimilarity of easy and hard keepers. All three angles are displayed; PC1, PC2, and PC3. The red circles represent easy keepers whereas the blue circles represent hard keepers (all P>0.05).

3.3 Microbiome Differences Between Age Groups

3.3.1 Abundance

After placing each horse from the matched data set into one of three age categories, a similar set of preliminary analyses was implemented. First, to observe general relationships between age groups, the relative abundance of the data was observed. This can be seen in Figure 16, where aged horses appear to have higher levels of *Firmicutes* and *Fibrobacteres* than do the young and adult age groups. Next, the differential abundance between the age groups was observed using a non-parametric Kruskal-Wallis test. The results showed that at least 69 taxa were found to be differentially abundant between the age categories. The phyla which these significant taxa were found in are starred in Figure 16: all phyla in the relative abundance except for *Armatimonadetes* and *Plantomycetes* were significantly different ($P<0.05$). Additionally, the *Firmicutes/Bacteroidetes* ratio was taken and compared using student's two-tailed t-tests for each category but proved insignificant ($P>0.05$).

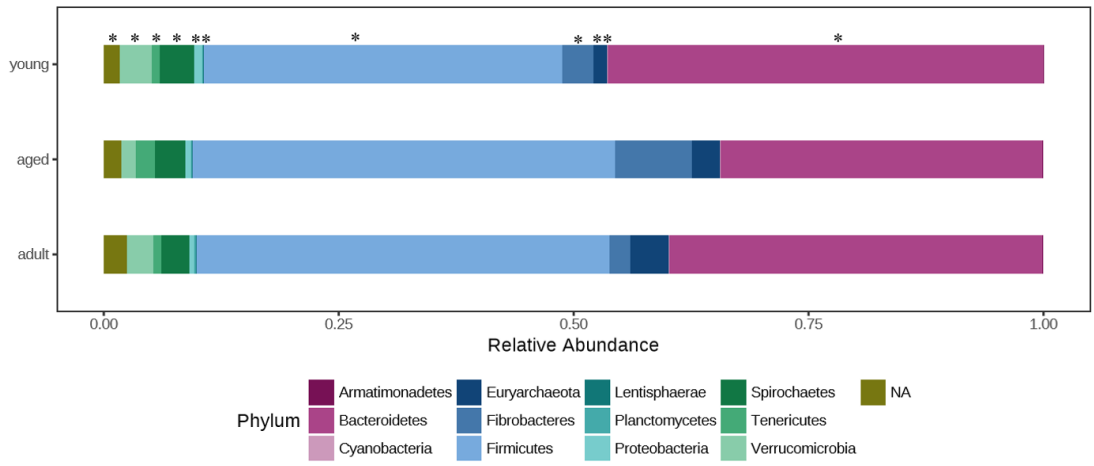

Figure 16: The relative abundance of phylum between horses of the following age groups; young, adult, and aged. Significant differences between keepers in taxa found using a non-parametric Kruskal-Wallis test are starred above ($P<0.05$).

A Spearman's Rank correlation test was used to determine relationships between bacterial species and age, similarly to how it was used with keeper status. As seen below in Figure 17, 14 taxa were found to significantly correlate with age differences. In this case, a positive coefficient indicates that a species increases with age and vice versa. A very high positive coefficient of 0.8 was found for *Christensenellaceae*, indicating that this family has a positive correlation with age (Figure 17). Additionally, *Prevotellaceae* show the highest negative coefficient and thus decreases with age (Figure 17). The most frequently correlated family, with both positive and negative correlations with age, are the *Lachnospiraceae,* having 4 significant species (Figure 17). Lastly, most of the families correlated (negatively or positively) with age were not correlated with keeper status. The species that correlate

with both age and keeper status are *Lachnospiraceae, Bacteriodaceae, Erysipelotrichaceae, and Ruminococcaceae* (Figures 5 & 17).

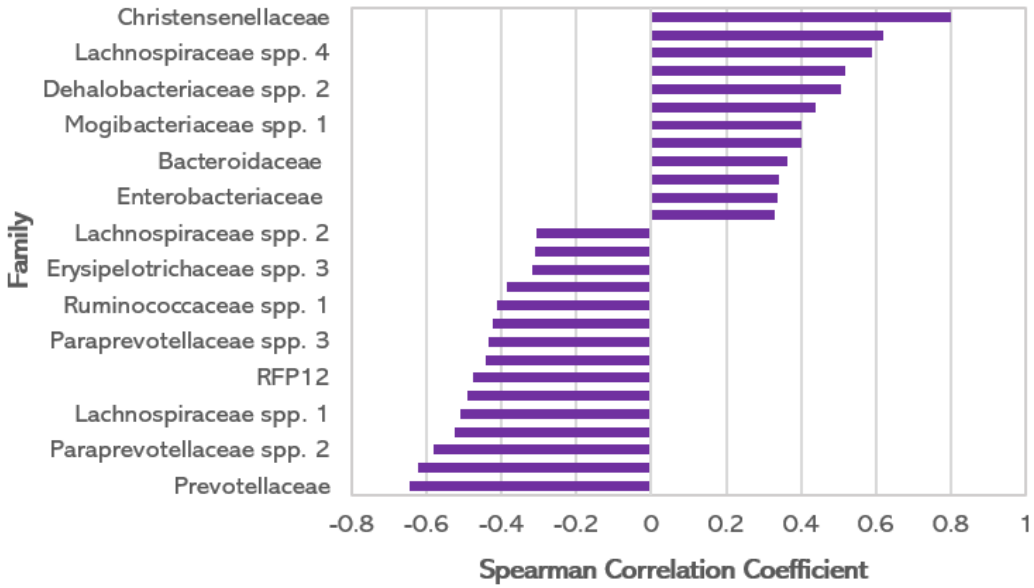

Figure 17: The results from a Spearman's Rank Correlation relating to age, organized by family. For families with multiple differing lower taxa, the format "spp. #" was used to differentiate them. Families defined as "other" were labeled in the "phyla .spp" format. Taxa with no classified families were not included in this figure. Correlations were deemed significant if the coefficient was <-0.03 or >0.03

3.3.2 Diversity

In a similar manner to the computations performed for easy and hard keepers, the beta diversity was computed to observe any differences in diversity between age groups. The beta diversity significance was also taken using a PERMANOVA and, unfortunately, proved to be insignificant for both the 2D weighted and unweighted Unifrac measures. There was, however, more clustering within age groups when

compared to keeper group; aged horses (blue squares) and adults (red triangles) were moderately clumped especially in the PC1 vs. PC3 coordinates and the PC2 vs. PC3 coordinates (Figure 18 & 19). Once again, the PC1 coordinate showed the highest variation at only 7.97% for the unweighted and 20.31% for the weighted Unifrac measures (Figure 18 and 19).

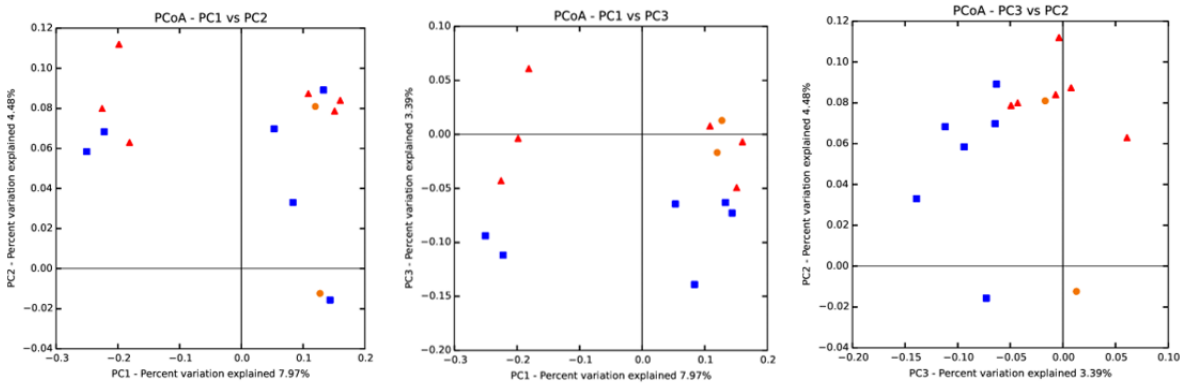

Figure 18: The beta diversity of the age-matched data displayed as 2-dimensional unweighted PCoA plots. Each image displays a different angle between points; PC1 vs. PC2, PC2 vs. PC3, PC1 vs. PC3. The orange triangles represent young horses, the red triangles represent adult horses, and the blue squares represent aged horses (all P>0.05).

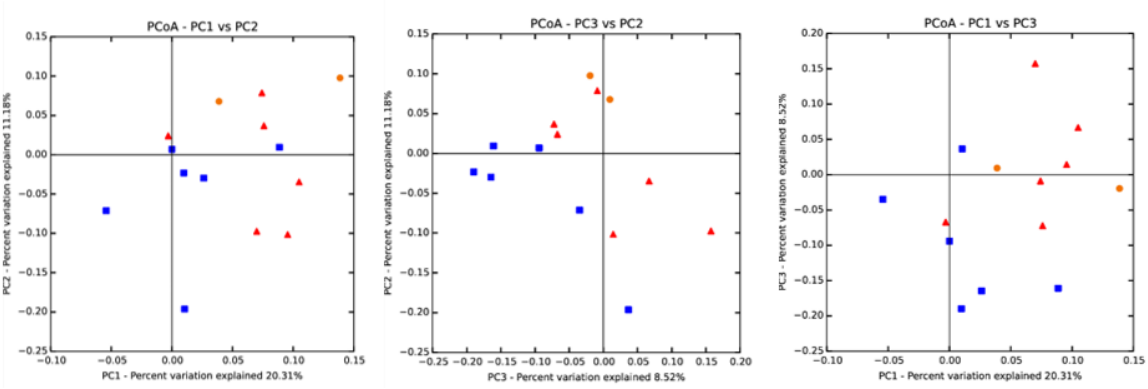

Figure 19: The beta diversity of the age-matched data displayed as 2-dimensional weighted PCoA plots. Each image displays a different angle between points; PC1 vs. PC2, PC2 vs. PC3, PC1 vs. PC3. The orange circles represent young horses, the red triangles represent adult horses, and the blue squares represent aged horses (all $P>0.05$).

Chapter 4

DISCUSSION

4.1 Interpretation of Results

In the comparison of keepers, clear differences in abundance between keepers were found in 32 taxa, supported by the fact that definitive relationships were shown at both the phyla and family levels. First, the 32 differentially abundant taxa were under many different phyla, with most being under the *Firmicutes* and *Bacteroidetes* phyla. This finding agrees with current research in that these two phyla were expected to be the most abundant and accounted for most of the differences seen.

Moreover, the phyla that are most commonly seen in the gut microbiome (*Firmicutes, Bacteroidetes, Fibrobacteres, Tenericutes*) were significantly affected by keeper status according to the LEfSe analysis. Additionally, the Spearman Rank correlations found that more species were correlated with keeper status than with age and most of the species correlated with age were not shared with those correlated with keeper status. This may suggest that those with significant correlations are mutually exclusive in whichever variable the correlation was found and could be taken into further consideration. Unfortunately, the ratio we postulated would have a correlation with easy and hard keepers, the *Firmicutes/Bacteroidetes* ratio was insignificant.

While differential abundance between keepers proved to be significant for several taxa, the diversity analyses did not. Shannon's index, Choa1, and PD Whole Tree measures were all taken to observe the alpha diversity of samples in addition to weighted Unifrac, unweighted Unifrac, and Bray-Curtis dissimilarity measures for beta diversity. Yet no significance was found for any of these tests.

Similar results were seen in the comparison of young, adult, and aged horses, where the beta diversity was insignificant as well, however there were slight trends in clustering between age groups that could be further observed. However, 69 taxa were found to be significantly different in abundance, mostly within the *Firmicutes* and *Bacteroidetes* phyla. It is interesting that twice as many taxa were found to be differentially abundant when comparing age versus comparing keeper status, as this could indicate more of a relationship between age and the microbiome rather than metabolic tendency and the microbiome.

The differential abundance between keepers may suggest a difference in microbiome functionality. For example, many of the significantly differing species were of the *Clostridiales* order, which contains the family *Lachnospiraceae*, a family known for degrading complex polysaccharides and producing SCFA like acetic and butyric acid (Meehan & Beiko, 2014). Since there was a higher abundance of taxa from this family in easy keepers than hard, perhaps these horses have a greater ability to access nutrients from complex plant substrates, providing more SCFA for energy. Additionally, this family was found to be both positively but also negatively correlated with age, suggesting that some species of this family decrease with age and may reduce a horse's access to nutrients, negatively affecting weight gain. Many other functional changes due to differential abundance may also be possible.

4.2 Conclusions and Moving Forward

One consideration with this data to be suggested is the possible bias of the sampling size. Out of the 104 horses in the entire data set, only 18 were used for the entire study. This could have caused some of the trends seen, such as the lack of alpha diversity between keepers or the clustering seen in the beta diversity between age

groups. Additionally, though they were found to be significant and have clear correlations, only 32 taxa (for keepers) and 69 taxa (for age) were found to be differentially abundant out of thousands if not millions of possible OTU's. Subsequently, these species may likely not make much of a difference in the grand scheme of things. As a result, the findings of this study will be respected but also taken with a grain of salt.

Overall, the results of this study show that, while there is not much difference in diversity, abundance between keepers as well as between age groups is different, especially since there was a clear effect of keeper status on certain taxa. Taking this into account, it may be said that the functionality of individual species is a crucial component to the microbiome versus the diversity of the community. While diversity in any ecosystem is important, this study did not show a large change occurring in communities due to diversity. Thus, the differential abundance is a large point of interest when observing the effects of certain variables on the microbiome because its increase or decrease in certain species can affect the functionality of the microbiome as a whole.

To observe the effect of certain variables on the change in abundance, it may be beneficial to observe the differential abundance and relate it to community bacterial function, for this could explain some of the effects seen. For example, the family *Ruminococcaceae* was highly more abundant in hard keepers than easy keepers, possibly because its metabolic pathways are less efficient than other families which reduces its ability to provide nutrients for its host. A study could be implemented during which the change of nutrient digestibility is observed after a species' abundance is directly modified. There are many community functions that could be

compared to observe the effect on host metabolism, such as rate of carbohydrate degradation, type of acid production, or ability to adapt to a new food source. In general, the community within two or more groups could be focused on and studied to draw conclusions as to why there are differences in metabolisms between animals who rely on bacteria for digestion.

Due to its overreaching importance in equine health, the equine gut and its diverse microbiome were of interest for this study, with a main focus on potential differences found in easy and hard keeper horses and a minor focus on differences in age groups. It was determined that diversity had little relation to keeper type and that differential abundance, which was found to be significant for several taxa, did show a relationship. The abundance should thus be focused on for future study, as the abundance can affect which metabolic functions take place depending upon which bacterial species is present in the community.

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