

## ABSTRACT

ESCHWEILER, KATRINA MARIE. Longitudinal Gorilla (*Gorilla gorilla gorilla*) Microbiomes Compared Across Seven Months among Three Zoological Institutions. Under the direction of Kimberly Ange-van Heugten and Eric van Heugten.

There has been a recent wealth of microbiome research among mammalian species to characterize GI microbiome composition and diversity. A goal of this characterization was to use GI microbial analyses as a clinical tool to assess health status and potential disease states of an individual. However, baseline microbiome data for non-human primates is lacking. All gorilla species are considered critically endangered. Thus, the populations of gorillas under human care are of vital conservation importance. By combining health, nutrition, and microbiome data, caretakers of these gorilla populations can better ensure that the species thrive under human management. The current study was conducted to characterize normal GI microbiome populations and potential differences in the microbial composition and diversity for gorillas housed at three different zoological institutions (Denver Zoo, NC Zoo and Riverbanks Zoo). This study also characterized microbial communities found within the diets and environment of these individuals. Over a seven-month period from January to August, 2018, microbiome samples were collected from 19 individuals twice a month for a total of 248 fecal, 41 composited diet and 57 composited environmental samples analyzed. Individual, institution and type had a significant effect on all samples analyzed ( $p < 0.05$ ). All fecal samples contained the same top 20 taxa. Fecal samples all contained *Prevotella* as well as several genera from Prevotellaceae and Ruminococcaceae which are all capable of fiber digestion. Differences in the composition and diversity of the gorilla fecal microbiome were found to be correlated to gorilla age, diet, housing, individual gorilla, sex, social group size, and zoological institution ( $p < 0.05$ ). Antibiotic use and order in which samples were taken did not significantly affect microbial taxa richness between

samples. Individual gorilla identity was found to have the most consistent correlative effect on both diversity and richness within the GI microbiome across all three institutions ( $p < 0.001$ ). Interestingly, when each institution was analyzed separately from the others, a seasonal correlation to changes in microbial taxa abundance (based on the month of collection) was noted only at Denver Zoo ( $p = 0.005$ ). This temporal correlation may be linked to the seasonality of the environment in Colorado, seasonality of available produce, or a shift in where dietary browse was acquired for this institution. The data from this research indicate that individual animals within gorilla species likely host their own cloud of “normal” microbiome populations, supporting the notion that it is vital before characterizing the “norm” for a given host species to first understand individual variation within that host species. Thus, when comparing microbiomes among institutions, assessing differences between health states or between in human care and free-ranging locations may be more difficult than previously thought. While this study is the longest and most comprehensive study of the gorilla microbiome in populations under human management, we recommend future work with even larger populations representing more housing locations.

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Longitudinal Gorilla (*Gorilla gorilla gorilla*) Microbiome Compared Across Seven Months  
Among Three Zoological Institutions.

by  
Katrina Eschweiler

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## **BIOGRAPHY**

Katrina Eschweiler was born in Terra Haute, Indiana to Joseph and Judy Dietz on June 24<sup>th</sup> in 1989. Her family moved several times before settling down for most of her childhood in North Canton, Ohio with her two siblings Charles and Trevor. Katrina remembers spending much time with her two brothers outside exploring nature. She also fondly remembers running around the old family farm when visiting her grandparents taking care of the many farm cats. She doesn't remember a time where her family did not have at least 2 animals but often times it was many more than that. While growing up Katrina knew she wanted to work with animals but was unsure in what capacity.

Katrina attended Kent State University in Kent, Ohio for her undergraduate studies in Biological Anthropology. Through this program studying evolution and the connection between species she ultimately fell in love with Primatology declaring it her concentration. During her time at Kent State University she was able to accept a position as a field assistant under Marlyn Norconk studying golden-handed tamarins in Suriname, South American. While she loved this time in the jungle it was one fateful visit to a zoo after this position that solidified in her mind that she wanted to work with human managed populations of primates.

In the summer of 2014 Katrina accepted a seasonal position at the Denver Zoo within the Horticulture department. During this time, she was introduced and fell in love with the browse program for the zoo. This love of the program is ultimately what led her to pursue a position within the Denver Zoo's Nutrition Department and a career in exotic animal nutrition. While she had initially been driven to work with just primates in a zoological setting that drive quickly changed to one that wished to engage all species under human management. In the summer of 2015 Katrina accepted a position as a Nutrition Specialist at the Denver Zoo.

In the spring of 2017 Katrina began her MS degree distantly through NC State University seeking a co-major in Animal Science and Nutrition. In the summer of 2018 Katrina accepted the Nutrition Center Manager position at the Denver Zoo. This new position allowed her to take over the management of all animal food within the zoo as well as work in closely with the Denver Zoo's Nutritionist. Katrina hopes to continue to peruse a career in exotic animal nutrition for many years to come.

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# CHAPTER 1

## Literature Review

### 1.1. Overview of Microbiome Research

The microbiome is defined as the collection of viruses, bacteria, archaea, fungi and protists colonizing a given host (Abreu and Peek, 2014; Hollister et al., 2014; Amato, 2016; McKenney et al., 2017; Clayton et al., 2018A). A symbiotic relationship exists between the host and the microbial taxa present within gastrointestinal (GI) tract. This symbiotic relationship is built from the host providing the habitat for the microbes to thrive; while the microbial taxa provide a wealth of metabolic assistance to the host. These metabolic pathways include but are not limited to such processes as fiber digestion and the synthesis of vitamins (Flint et al., 2012; Monachese et al., 2012; LeBlanc et al 2013). In addition to metabolic functions, the microbiome also influences immune function, infant development, and disease susceptibility (Cho and Blaser, 2012; Hollister et al., 2014; Kohl et al., 2015; McKenney et al., 2015; McKenney et al., 2017; Greene et al., 2018B). The metabolome consists of complex pathways shared between the host and the taxa of the gastrointestinal (GI) microbiome and together they produce a wide range of metabolites used by the host (Nicholson et al., 2005; Gomez et al., 2015).

An individual's microbiome consists not only of those organisms inhabiting the GI tract but also those found upon the skin, oral cavity, reproductive tract and urogenital tract. However, most published research thus far has focused on the GI tract microbiome as it has the highest relative abundance of microbial taxa (Savage 1977; Sender et al., 2016). Of the species studied so far across the tree of life, from invertebrates to mammals, the GI tract of mammals has been shown to be the most densely concentrated microbial habitat, at densities of  $10^{11}$ - $10^{12}$  microbes/mL; with the colon containing more than 70% of all microbes present in the body

(Stevens and Hume, 1998; Leser and Molbak, 2009; Jandhyala et al., 2015). This multitude of microbes matches or exceeds the host's somatic cell numbers and collectively, the encoded genes surpass those of the host (Savage, 1977; Sender et al., 2016). It is with this collection of encoded genes that the microbiome can influence its host. How microbial taxa interact with the host are based off proximity to the epithelial cells found within the host GI. Those taxa that are more associated with the mucosal surface of the GI tract tend to interact with the host's immune system; while taxa found closer to the lumen correlate stronger with digestion (Nieuwdorp et al., 2014). With the plethora of genes that the organisms of the microbiome produce one can see how far reaching the effects of compositional changes to the microbiota could affect its host in both positive and negative ways.

In all species studied so far, from human and non-human primates (NPH) to fish and amphibians the main goal of the research has been to not only to classify the microbiome but to also identify the forces that shape the microbiome in different hosts (McKenney et al., 2015; Amato et al., 2016A; Clayton et al., 2016; Clayton et al., 2018B; McKenzie et al., 2017; Springer et al., 2017; Greene and McKenney, 2018; Greene et al., 2018). Within a given host species, classification is aimed at identifying optimal microbiome state and diversity to promote health and reproduction. The overarching question of the correlation between microbiome state and host health state of course is not one that is easily addressed, as the internal and external factors that affect the microbiome composition are vast and still being identified. Researchers have also begun to identify situations in which the GI microbiome composition can promote disease, and become a hindrance to the host (Abreu and Peek, 2014; Hollister et al., 2014; Devkota and Chang, 2015; Griffin et al., 2015; McKenney et al., 2017). However, direct correlation between a given microbial taxon and a disease state is difficult to prove, as the disease state may be caused

not only by the presence of a given microbe, but also its abundance as well as the relative abundance of the other microbial taxa present in the host.

The Primate order provides a unique opportunity to compare the correlation between microbiome composition, varying feeding strategies, and environments within a closely related group. Microbiome research within primates however, has generally focused on short-term sample collections, and often only a single point in time. This has left questions unanswered concerning how stable a microbiome may be over time within these host species. While in the past it was upheld that an individual's microbiome make-up is relatively stable, recent research has begun to investigate how truly stable the microbiome is over time either within an individual or within a host population (Faith et al., 2013; Hollister et al., 2014; Montassier et al., 2018). Studies assessing long term microbiome variance may give further insight into what a "healthy state" microbiome is for a given host species (Montassier et al., 2018).

## **1.2. The History of Microbiome Research**

The first discovery credited to the study of the microbiome dates to 1680's, when Anton van Leeuwenhoek described the first bacterium taken from fecal matter (Santiago et al., 2014). To date, most of the research conducted on the GI microbiome focuses on fecal sampling as fecal specimens contain one of the highest microbial densities known and have been shown to contain similar richness and diversity as the host's colon (Eckburg et al., 2005; Santiago et al., 2014; Greene et al., 2018). Additionally, fecal samples are very practical for studying non-domestic animals as they can be collected non-invasively. Little work and advancement were done after van Leeuwenhoek's discovery, due to the limitation of technology and techniques, until the 1900s when two main methodological approaches to microbiome research emerged (Bauchop,

1971; Clayton et al., 2018B). These two methods were culture-dependent methods, the most used approach, and culture independent methods (Bauchop, 1971; Clayton et al., 2018B). Culture-dependent methods relied on growing cultures *in vitro* to help identify the taxa present in the samples taken from mammalian GI tracts (Bauchop, 1971; Clayton et al., 2018B). However, this method of sampling was found to be less than ideal when it was shown to only be able to reproduce roughly 20% of the mammalian GI microbes due to the inability to create viable growing conditions (Savage, 1977; Eckburg et al., 2005; Clayton et al., 2018B). This led to a higher percentage of the GI microbes remaining uncultured and therefore unknown to research which hindered a deeper understanding of the microbiome's role to the host (Kim and Yu, 2014; Clayton et al., 2018B). Culture-independent methods became the more prominent approach in the 1970s when Woese and Fox pioneered the use of 16S rRNA in phylogenetic analysis (Woese, 1987; Woese et al., 1990; Clayton et al., 2018B). This new approach allowed researchers to survey the bacterial sequences directly from the samples and environment instead of relying on culturing methods (Woese, 1987; Woese et al., 1990; Lane et al., 1985; Clayton et al., 2018B). In the early 1900s researchers were focused on how microbes could promote longevity and treat infections (Pirisi, 2000; Metchnikoff, 2004; Keen, 2012; Hollister et al., 2014). The 20<sup>th</sup> century saw an attack on the microbes in an attempt to eradicate infectious disease, this mindset led to the limitation of the research on what benefits these microbes could convey (Hollister et al., 2014). The 21<sup>st</sup> century marked a shift in the mindset of researchers who now sought to understand the microbes that colonize a given host and to determine what defined a healthy community (Huttenhower et al., 2012; Hollister et al., 2014).

Research from early 2000 till present has focused on assessing the microbiome in terms of relative abundance and the overarching diversity of the whole microbiome of a given host by

using 16S rRNA or whole genome analysis (Hollister et al., 2014). The 16S rRNA sequence used for lineage classification is an evolutionarily conserved sequence of the small unit of the prokaryotic ribosome which contains nine hypervariable regions (Pace, 1977; Woese, 1987; Clayton et al., 2018B). Variation in these sequences allow for identification and classification of different taxonomic groups (Pace, 1977; Clayton et al., 2018B). 16S rRNA studies assume that the DNA sequences of two or more organisms sharing more than 97% 16S rRNA sequence identity belong to the same genera level taxon (Clayton et al., 2018B). These genera level taxa are now referred to as operational taxonomic units (OTU) (Clayton et al., 2018B). OTUs are not universally defined. Differences in the algorithms for calculating OTUs impacts which sequences are grouped into the same OTU (Schloss and Handelsman, 2005; Caporaso et al., 2010; Clayton et al., 2018B). A given microbial relative abundance in the microbiome is defined by the percentage of the whole microbiome each taxon makes up (Clayton et al., 2018B). The richness of the microbiome is defined by the number of taxa found within a given host's microbiome (Clayton et al., 2018B).

The increasing number of studies in microbiome research has led to the identification of microbial taxa present based on the richness and diversity found within a given hosts microbiome (Hollister et al., 2014). Several studies have demonstrated the strong impact that health, age, diet, environment, immune system, and phylogeny may have on the richness and diversity of the microbiome (Yildirim et al., 2010; McKenney et al., 2014; Abreu and Peek, 2014; McKenney et al., 2015; Amato et al., 2016A; Amato et al., 2016B; Clayton et al., 2016; Springer et al., 2017; McKenney et al., 2017; McKenzie et al., 2017, Clayton et al., 2018A; Petta et al., 2018). Work done by Yildirim et al. (2010) and McKenzie et al. (2017) have shown that host phylogeny is the one of the strongest predictors for bacterial lineage diversity in the

microbiome. Clayton et al. (2018A) and McKenzie et al. (2017) have shown, in Altelidae (wild=55, captive=4), Cercopithecidae (wild=33, captive=8), Hominidae (wild=11, captive=8) and Lerumidae (wild=22, captive=5) that captivity can decrease the bacterial lineage diversity when compared with the diversity of that same host species in the wild. However, McKenzie et al. (2017) showed an inconsistent effect of captivity on the microbiome diversity across all host species taxonomic groups: Rhinocerotidae (wild=4, captive=9) has increased diversity, while Bovidae (wild=11, captive=19), Giraffidae (wild=2, captive=4), Myrmecophagidae (wild=30, captive=11) and Orycteropodidae (wild=5, captive=18) all show no change in diversity between wild and captive states. Research in the last decade has focused around determining what level of species richness and diversity of the microbiome is considered healthy. This includes whether there are evolutionary trends to these parameters and whether certain species promote or inhibit the development of diseases such as obesity and cardiac disease (Abreu and Peek, 2014; Hollister et al., 2014; Nieuwdorp et al., 2014; Griffin et al., 2015; Clayton et al., 2018B; Soderborg et al., 2018).

The health of the GI microbiome may be assessed based on a given microbial taxon's presence or absence within a host's microbiome based on these taxa's metabolome effects within the host (Hollister et al., 2014). 60% of a human adult microbiome is considered relatively stable with more taxa retained within the host for over a 5-year period (Faith et al., 2013). While 60% of the human microbiome is considered stable there is shifting within relative abundance of these taxa that do occur over time (Faith et al 2013). This shift within microbial taxa relative abundance is poorly understood at this time. The "personal microbiome cloud" effect has since been described within the human microbiome (Montassier et al., 2018). This phenomenon describes how a given individual's microbiome may change over time but still remains within its

own specific “cloud” of overall diversity (Montassier et al., 2018). This cloud concept encompasses and explains the high diversity that can be measured across time within a given individual’s microbiome, by measuring the individual’s average across time to account for this high variability (Montassier et al., 2018). This phenomenon has only been studied in human and rodent models; no research has yet looked into the make-up of the potential “personal microbiome cloud” of NPHs.

### **1.3. Advancements in Microbiome Research**

The use of 16S rRNA methods for analyses allowed for the detection of culturable and non-culturable microbes which led to a great expansion of our understanding of what the microbiome is made up of (Pace, 1997; Kim and Yu, 2014; Clayton et al., 2018B). This method coupled with the availability of online databases such as Ribosomal Database Project©, Greengenes© and SILVA© facilitated the taxonomic classification of the 16S sequences extracted from samples (DeSantis et al., 2006; Pruesse et al., 2007; Wang et al., 2007; Cole et al., 2014; Kim and Yu, 2014). 16S sequencing paired with the ability to classify taxonomic groups aided in determining the diversity and richness of different host species microbiome (Kim and Yu, 2014). However, in the late 1990s and early 2000s this process required gene clone libraries, which were limited, making it impossible to characterize complex microbiomes (Kim and Yu, 2014). This limitation led to the development of the next generation of high throughput sequencing such as Illumina and Roche-454 which allowed for more detailed assessment of the complex microbiome (Petrosino et al., 2009; Weinstock, 2012 Kim and Yu, 2014). These next generation sequencing techniques use PCR amplicons prepared using specific primers for metagenomic DNA (Kim and Yu, 2014). These systems are able to read more bases from the

extracted 16S gene, up to 250-bases with Illumina and up to 1000-bases on Roche-454 (which is no longer in use), which results in better classification of taxon and thus a deeper and more complete understanding of the composition of the microbiome (Weinstock, 2012; Kim and Yu, 2014).

#### **1.4. Microbiome Research in Humans**

Due to the technological advancements that have enabled taxonomic identification of GI microbiome, research can now delve deeper into the relationships between these taxa and how their presence and function affects their hosts. For example, recent studies have characterized the development of the microbiome from infancy to adulthood in humans, lemur species and mice (Mackie et al., 1999; Donnet-Hughes et al., 2010; Koenig et al., 2011; Blustein et al., 2013; Nieuwdorp et al., 2014; McKenney et al., 2015; Soderborg et al., 2018). Establishment of the composition of the microbiome occurs during and after birth initially through vertical and horizontal transmission from the mother and environment respectively (Mackie et al., 1999; Donnet-Hughes et al., 2010; Koenig et al., 2011; Nieuwdorp et al., 2014). The first inoculation is directly from the mother, and is linked to the method of birth (Blustein et al., 2013; Nieuwdorp et al., 2014). Infants that are born vaginally displaying more similarities with the mother and those born via Cesarean section having more similarities with the environment (Nieuwdorp et al., 2014; Soderborg et al., 2018). Post-birth, infants continue to develop their microbial load through contact with their mother, others of their species, and the environment (Mackie et al., 1999; Yatsunenکو et al., 2012). Human infant microbiome is very simple and colonized by species such as lactic acid bacteria and enterobacteria (Koenig et al., 2011). As the infant grows and matures so does the microbiome. The next most significant change occurs at weaning with

the introduction of solid foods to the diet (Mackie et al., 1999; Koenig et al., 2011). The establishment of a mature microbiome that mimics that of an adult occurs by three years of age in humans (Koenig et al., 2011).

The microbiome does not cease to be influenced by extrinsic and intrinsic factors once it reaches the adult state. Factors such as environment, changes to diet, health, and age among others, affect the overall composition and diversity of a host's microbiome through an individual's entire life. Research has shown that diet and host species are two of the factors with the highest effect on the diversity and richness of the microbiome. Aside from the effects of diet on the microbiome the next most studied correlation in human microbiome studies is the effect on host immune response specifically through disease states, such as cardiac disease and obesity (Abreu and Peek, 2014; Hollister et al., 2014; Petta et al., 2018; Soderborg et al., 2018). Of the potential diseases that could have interplay with the GI microbiome some have received more attention than others in terms of research. These diseases include GI cancers, diabetes, obesity, and cardiac disease (Abreu and Peek, 2014; Hollister et al., 2014; Griffin et al., Petta et al., 2018; Soderborg et al., 2018). This correlation between microbiome richness and diversity and disease state is mediated through the microbiomes interplay with the host's immune system. The GI microbiome can temporarily breach the epithelial cells of the GI to stimulate immune responses (Mazmanian et al., 2005; Aidy et al., 2014). In humans and mice, this interaction, between the host's immune system and GI microbiome is established during development and have long-term implications on the host immune system and acceptance to the microbial taxa of the microbiome (Talham et al., 1999; Round and Mazmanian, 2009). Therefore, if the development, evolution, and growth of the composition of the GI microbiota is interrupted it can have long lasting implications on adulthood immune system responses.

All of these disease states have a common symptom of microbial imbalance called *dysbiosis* (Yang and Jobin, 2014; Petersen and Round 2014). Three categories of dysbiosis have been described in correlation with disease states; these categories are: loss of beneficial microbial taxa, increase of potentially harmful taxa and the decrease in microbial taxa diversity (Peterson and Round, 2014). It should be noted that while there is correlation between diseases and certain states of dysbiosis of GI microbial lineages this does not mean that all microbiomes with low diversity are pathogenic in nature. More research is needed to understand which species within the GI microbiome could be truly linked to disease states and if they have a causal relationship with diseases or if they are the result of the disease. A review of human GI malignancy discussed the links between changes to the microbiome and disease such as gastric and colonic cancers (Abreu and Peek, 2014). A gram-negative bacterial species *Helicobacter pylori* has been linked to gastric adenocarcinoma in humans (Abreu and Peek, 2014). Several researchers have shown multiple bacterial species contribute to colorectal cancer in humans and mice including *Streptococcus gallolyticus*, *Helicobacter pylori* and *Escherichia coli* (Boleij et al., 2011; Arthur et al., 2012; Abreu and Peek, 2014). It has been suggested that alterations in the richness, diversity and metabolism of the colonic microbiota can shift the homeostatic environment towards a state of dysbiosis (Abreu and Peek, 2014).

Soderborg et al. (2018) researched the effect of human obesity on the mother's microbiome and the potential long-term effect of obese mothers on their infant's microbiome. This research analyzed the infants of mothers with diet induced obesity and these offspring compared to infants from normal weight mothers. They found that infants born to obese mothers were predisposed to accelerated weight gain and the development of a fatty liver (Soderborg et

al., 2018). This research provided the first potential evidence that changes in the GI microbiome in infants born to obese mothers are predisposed disease pathways (Soderborg et al., 2018).

### **1.5. Diet and the Microbiome**

Of all of the factors that affect the microbiome research has demonstrated that diet has one of the largest influences on its composition (Ley et al., 2005; Turnbaugh et al., 2008; Abreu and Peek, 2014; Voreades et al., 2014; McKenney et al., 2015; Amato et al., 2016A; Clayton et al., 2016; Rios-Covian et al., 2016; Finlayson-Trick et al., 2017; Springer et al., 2017; Petta et al., 2018). In humans it has been demonstrated that switching from a high-fat/low-fiber diet (typical western diet) to a low-fat/high fiber diet affects the relative abundance of microbial taxa such as *Bacteroides* and *Ruminococcus* within 24 hours of the diet transition (Wu et al., 2011; David et al., 2014; Springer et al., 2017; Petta et al., 2018). Changes in the richness and diversity of the microbiome have been shown to influence metabolic processes, such as xenobiotic metabolism (metabolic pathways to modify the structures of compounds such as drugs or poisoning) and host nutritional balance (Monachese et al., 2012; Hollister et al., 2014; Desai et al., 2016). One of these processes described in humans that can be influenced by changes in abundance of certain bacterial species is the ability of the microbiome to convert non-digestible carbohydrates into short chain fatty acids (SCFA) (Rios-Covian et al., 2016; Petta et al., 2018). When this conversion is enhanced it helps reduce the risk of inflammatory disease, type 2 diabetes, obesity, and heart disease (Rois-Covian et al., 2016; Petta et al., 2018). On a high fat diet, the microbial taxa that aid in the conversion of non-digestible carbohydrates to SCFA are reduced thus reducing the production of SCFA and their benefits (Turnbaugh et al., 2008; Clayton et al., 2016; Petta et al 2018). A correlation has been found between consumption of

high fat diets and overall decrease in relative abundance in *Bacteroidetes* and an increase in *Firmicutes* (Ley et al., 2005; Turnbaugh et al., 2008; Petta et al., 2018). This reduction in *Bacteroidetes* and increase in *Firmicutes* have been associated with obesity in humans and mice (Petta et al., 2018). In mice, *Bacteroidetes* has been shown to increase IgA antibody titers and reduce illness challenges associated with rotavirus infection (Petta et al., 2018). Therefore, there is a possibility that if *Bacteroidetes* populations within a given host's GI are reduced due to the host's diet, the host's ability to deal with rotavirus infection will be reduced due to *Bacteroidetes* effects on IgA titer expression within the host.

Work has also been done in the gnotobiotic mouse model by Desai (2016) to characterize the effects of fiber deprivation on the GI microbiota as well as how this may alter disease risk within a host. The mechanism described by the authors proposes that when the GI is deprived of complex plant fibers in the diet a chain reaction occurs where microbiota taxa are triggered to feed on the colonic mucus layer (Desai et al., 2016). This mucus layer plays a major role in providing a barrier within the GI against invading pathogens (Desai et al., 2016). When the GI barrier is destroyed the host's susceptibility to enteric diseases increases and pathogen colonization within the GI is promoted (Desai et al., 2016).

Several researchers studying NPH as hosts have focused on comparing microbial lineages across hosts diet and feeding strategies (McKenney et al., 2014; Gomez et al., 2015; McKenney et al., 2015; Amato et al., 2016; Amato, 2016; McKenney et al., 2017; Greene et al., 2018). These studies have aimed to identify certain abundant bacterial lineages that may aid in the digestion of the hosts' distinct diets. Greene et al. (2018) compared 31 sifakas (*Propithecus coquereli*) under human care at Duke Lemur Institute to see how access to foliage material in the diet affected the diversity of the GI microbiome. This work showed that when foliage was

limited in the diet the microbiome content shifted towards bile tolerant species as well as those better suited to degrade starches and proteins (Kim et al, 2012; Devkota and Chang, 2015; Konikoff and Gophna, 2016; Greene et al., 2018). This shift was marked by increases in *Oscillospira*, *Bilophila* and members of the Rikenellaceae family (Kim et al., 2012; Devkota and Chang, 2015; Konikoff and Gophna, 2016; Greene et al., 2018). When sifaka's had access to foliage, *Lachnospiraceae*, a fiber degrading family, became more prevalent while *Oscillospira* and *Bilophila* decreased in abundance (Greene et al., 2018).

## **1.6. Microbiome Research in Non-Human Primates**

Due to their evolutionary relatedness to humans as well as their wide range in habitat and feeding ecology numerous studies have been conducted on the NPH microbiome, including howler monkeys (*Allouatta spp*), lemur species, chimpanzees (*Pan troglodytes*), gorillas (*Gorilla gorilla gorilla*) and many other species (Frey et al., 2006; Ochman et al., 2010; Yilidirim et al., 2010; McKenney et al., 2014; Gomez et al., 2015; Tung et al., 2015; McKenney et al., 2015; Vlckova et al., 2016; Amato et al., 2016A; Clayton et al., 2016; McKenney et al., 2017; McKenzie et al., 2017; Springer et al., 2017; Krynak et al., 2017; Moeller et al., 2017; Schulz et al., 2018; Gartland et al., 2018; Clayton et al., 2018; Greene et al., 2018; Greene and McKenney et al., 2018). These studies have been conducted on both wild and human managed populations. Research conducted on three species of lemurs under human management, *Lemur catta*, *Propithecus coquereli*, and *Varecia variegata*, demonstrated that early development of the NPH microbiome follows similar stages to those seen in human infants (McKenney et al., 2015). Each of these stages manifests within a given host species differently, with *Varecia variegata* showing a less profound shift with the introduction of solid foods compared to *Lemur catta* or

*Propithecus coquereli* (McKenney et al., 2015). This difference in succession may be due to the differences in GI morphology; *Varecia variegata* has the simplest GI morphology of the three species (McKenney et al., 2015). Therefore, an infant has less GI tissue development to go through (McKenney et al., 2015). These lemurs also showed a dominance of Firmicutes and Bacteroidetes in all libraries within this study, a finding that parallels finding in humans and other NPH (Ochman et al., 2010; McKenzie et al., 2015). All lemurs also hosted *Prevotella* and *Treponema* which contribute to the fermentation of plant-derived compounds; (Flint and Bayer, 2008; McKenzie et al., 2015). Both of these bacterial taxa are found in human populations that do not consume a western diet (McKenney et al., 2015). This finding directly links these two microbial genera to the diet consumed by the individuals hosting them showing that NPH microbiome is also strongly influenced by the diets they consume.

Clayton et al., (2016) investigated the effect human management has on wild NPH microbiomes, specifically looking at howler monkeys (n=56, both captive and wild samples) and douc langurs (n=93 both captive and wild samples). This study demonstrated that NPH lose portions of the signature microbiome under human management which is coupled by the colonization by human-associated GI bacterial genera *Bacteroides* and *Prevotella* (Clayton et al., 2016). This microbiome disruption in human managed NPH can help develop a model for how modernization may have affected and developed the current human microbiome (Clayton et al., 2016). The effects of human management may not be universal through all mammalian species or in type of human management (McKenzie et al., 2017). Small sample size and confounding factors make it hard to determine in which microbial taxa the trend of reduced richness and diversity is observed. The concern with research looking at the effects of human management is that there are the many factors that have effects on the microbiome. Such factors included diets

with less varied composition, veterinary care, exposure to human associated microorganisms, reduction in home range movement and contact with varied environments (McKenzie et al., 2017). There is also no typical form of human management, and human care ranges from semi-wild sanctuaries to fully managed populations in zoological settings throughout the world. There is no one standard to type of habitats animals may inhabit under human management. Human managed habitats can vary in type, semi-wild sanctuaries to fully managed zoo settings; but they can also vary on ecological range.

A common finding throughout the NPH research is the effect of host species vs the effect of host environment. Amato et al. (2016A) demonstrated that in howler monkeys found throughout Central America, the effect of host species on the GI microbiome is stronger than environmental factors such as forest type, habitat disturbance and seasonality of the environment (Amato et al., 2016A). Researchers have demonstrated that the mammalian host species select and shape the microbiome in terms of function and diversity through GI morphology, retention time, and phylogeny (*Colobus guereza*, *Riliocolobus tephrosceles*, *Cercopithecus Ascanius*, *Varecia variegata*, *Lemur catta*, and *Propithecus coquereli*) (Yildirim et al., 2010; McKenney et al., 2015). Other research has shown that convergence of several OTUs can occur between host species (*Colobus guereza*, *Riliocolobus tephrosceles*, and *Cercopithecus Ascanius*) when diets, digestive physiologies or environment overlap in wild populations (Yildirim et al., 2010). Moeller (2017) showed that wild chimpanzees and gorillas shared more bacterial phylotypes than allopatric populations of the two species. This was most likely due to a slight convergence in diet as well as exposure to the same environmental influences (Moeller et al., 2017).

Another interesting correlation that has come out of NPH microbiome research is the effect of social interaction on the composition of the GI microbes. This relationship between

microbiome composition and host social interactions is one of the least understood forces that can affect the microbiome (Tung et al., 2015). Tung et al. (2015) looked at wild baboons (*Papio spp.*), found in Amboseli, Kenya, and how social contact and grooming could affect and predict similarities in richness and diversity of the microbiome between individuals. This research looked at 48 individuals within two social groups of wild baboons, fecal samples were combined with behavioral data to track social interaction. The authors found that closer grooming partners harbored more similar microbial communities in the GI, and grooming networks could predict composition (Tung et al., 2015). They also found baboons are not coprophagic, therefore similarities in microbial composition between non-related individuals are facilitated through other avenues such as grooming bouts and indirect mediation through the soil, environment, similar diets and exposure to transmissible diseases (Tung et al., 2015).

### **1.7. Microbiome Research in Hominidae**

Our closest relatives the great apes, are of greater interest to human microbiome research. Free-ranging gorillas provide insight into unique morphological and diet adaptations because they tend towards high folivory for the majority of the year and frugivory during certain restricted seasons (Gomez et al., 2015). However, the seasonality of fruit forces them to fall back on other food sources such as leaves, bark, and pith during lean times (Masi et al., 2009; Gomez et al., 2015). This seasonal shift in food availability has caused most GI adaptation within gorillas to tend towards the digestion of high fiber food sources. These fallback food sources are much higher in plant structural polysaccharides, which no mammalian digestive enzyme can breakdown (Flint et al., 2008; Gomez et al., 2015). Due to this diet niche, gorillas rely heavily on the GI microbial fermentation to produce up to 57% of their daily energetic needs (Popovich et

al., 1997; Gomez et al., 2015). Gorillas are classified as a frugivorous herbivore with a long small intestine and a haustrated colon (Savage, 1847; Stevens and Hume, 1998; McKenney et al., 2014). A number of studies have also demonstrated that increased folivory by the host increases fecal eubacterial diversity (McKenney et al., 2014). Gomez (2015) demonstrated that gorillas display an increased abundance of microbes involved in fiber breakdown during the times they must rely on their fallback food sources, with Firmicutes and Bacteroidetes dominating the microbiome of wild gorillas (Gomez et al., 2015). Gorillas under human management have shown to have microbiomes dominated by *Provetella* and *Bacteroides* species when compared to their wild counterparts (Clayton et al., 2016).

Most gorilla studies, both on wild and human managed populations, are relatively short-term studies. The longest collection period found during this review was a four-month study conducted on a single wild male gorilla in Uganda (Frey et al., 2006). That study looked to define wild gorilla microbiome richness and diversity as well as what the stability of the individual's microbiome (Frey et al., 2006). While the authors were able to document richness and diversity from the samples collected, they characterized a single individual over this time frame and therefore they were unable to conclude whether the microbial variation was typical for the species, or if the individual was a population outlier (Frey et al., 2006).

Vlckova et al. (2016) conducted research to evaluate the effects of antibiotics on gorilla microbiomes, and if changes were similar to those seen in humans. Specifically, they looked at the effect of intramuscular cephalosporin administered via dart in eight wild gorillas found in Central Africa Republic. Fecal samples were collected for three months before the antibiotic treatment and then post antibiotic treatment (Vlckova et al., 2016). The authors found that the administration of antibiotics affects the stability of the microbial community in the GI and the

relative abundance of particular taxa such. For example, they detected a decrease in Bacteroidetes and an increase in Firmicutes post treatment (Vlckova et al., 2016). This decrease in microbial diversity is based on certain microbial taxa being susceptible to antibiotics. Administering an antibiotic can decrease or even whip out the presence of these microbial taxa, which in turn can promote the over-growth of resistant strains (Panda et al., 2014; Vlckova et al., 2016). While there was perturbation of the richness and diversity of the gorilla microbiome post-antibiotic treatments, the subjects eventually recovered carbohydrate processing microbial strains (Vlckova et al., 2016). Vlckova et al. (2016) suggested that gorillas may be more resistant to disturbances to the microbiota than humans and mice. This may also be reflective of antibiotics being administered to these individuals only once. Changes seen in human populations associated with antibiotic use may be correlated to long term usage of the antibiotics throughout individual's life.

Recent gorilla microbiome research has also focused on the factors that influence the richness and diversity as well as the correlations between taxa within microbiome and diseases states, following the research previously done in humans (Frey et al., 2006; Moeller et al., 2013; McKenney et al., 2014; Gomez et al., 2015; Vlckova et al., 2016; Krynak et al., 2017; Schulz et al., 2018). Krynak et al. (2017) looked at the correlation between cardiac disease and microbiome composition in eight male gorillas housed at Zoo Atlanta, as a first step in determining if certain microbiome compositions would result in higher cardiac disease risk. One sample was collected per week from each individual for a total of 32 samples were collected from June 9<sup>th</sup> to June 21<sup>st</sup> in 2013. The authors found that OTUs belonging to Proteobacteria, Bacteroidetes, Spirochaetes, and Firmicutes were significant indicators of gorillas with cardiac disease (Krynak et al., 2017). Variation within individuals over the collection period was found

to be attributed to difference in medical treatments and diet, though these parameters were not tested (Krynak et al., 2017). Effect of the sex of wild gorillas on the composition and diversity of their microbiome has also been researched. This research has found a correlation between age of individual and sex of individual (Pafco et al., 2019). An analyses on immature male and female gorillas showed a higher OTU richness in females (Pafco et al., 2019). This age group also showed differences in GI microbial compositions based on sex (Pafco et al., 2019). Differences based on sex in adult gorillas was only significantly different in the dry season (Pafco et al., 2019).

## CHAPTER 2

### 2.1. Introduction

All gorilla populations are considered critically endangered, with future population decrease expected (IUCN, 2019). Individuals under human managed care are of vital conservation importance for the survival of this genus. Additionally, human managed gorilla populations offer a unique ability to study the primate gastrointestinal (GI) microbiome in a controlled environment. In zoological settings, diets are controlled and have minimal annual fluctuations unlike most western human diets (AZA Gorilla Species Survival Plan, 2017; Statovci et al., 2017). Microbiome studies conducted in human managed gorilla populations can also offer a unique insight into the effects that diet, medical treatment, season, sex, age and various other variables may have on the composition and diversity of the microbiome.

The GI microbiome can provide a wealth of benefits to the host (Hollister et al., 2014; McKenney et al., 2015; Clayton et al., 2017; Greene et al., 2018). However, the microbial taxa found within the GI microbiome are not always beneficial (Frey et al., 2006; Moeller et al., 2013; McKenney et al., 2014; Gomez et al., 2015; Vlckova et al., 2016; Krynak et al., 2017; Schulz et al., 2018). Presence or abundance of certain microbial taxa within a host may therefore increase disease risk. However, the GI microbiome of the gorilla (*Gorilla gorilla gorilla*) has not been studied well enough distinguish microbial communities associated with health or disease states.

The study of non-human primates (NHP) has yielded interesting data to develop analytical tools for health in human managed populations (Frey et al., 2006; Ochman et al., 2010; Yilidirim et al., 2010; McKenney et al., 2014; Gomez et al., 2015; Tung et al., 2015; McKenney et al., 2015; Vlckova et al., 2016; Amato et al., 2016; Clayton et al., 2016; McKenney et al., 2017; McKenzie et al., 2017; Springer et al., 2017; Krynak et al., 2017; Moeller et al., 2017;

Clayton et al., 2018A; ; Gartland et al., 2018; Greene et al., 2018 Schulz et al., 2018). Trends across NHP microbiomes found thus far include a strong influence of diet and lifestyle (Ley et al., 2005; Turnbaugh et al., 2008; Abreu and Peek, 2014; Voreades et al., 2014; Rios-Covian et al., 2016; Finlayson-Trick et al., 2017; Springer et al., 2017; Clayton et al., 2018A; Petta et al., 2018). For example, several studies of the microbiome have shown that feeding strategies can help define the microbial taxa present in the NHP GI tract (McKenney et al., 2014; Gomez et al., 2015; McKenney et al., 2015; Amato et al., 2016; Amato, 2016; McKenney et al., 2017; Greene et al., 2018).

However, one weak point in many of the prior NHP studies has been the limited duration of microbial evaluations. Research studies that extend beyond 4 months are rare, and those that include greater than 10 human managed individuals are even more rare. The most extensive sampling from a gorilla found to date was collected from a single wild male across four months (Frey, 2006). The highest number of individuals included in a study of gorillas was 40 wild individuals; however, these individuals were only sampled once (Gomez et al., 2015). The highest number of individual gorillas' sampled under human care for a single study was eight (McKenzie et al., 2017; Krynak et al., 2017). Both of these studies were relatively short with the longest period of sample collection lasting only 4 weeks (Krynak et al., 2017). Studies of humans and mice, which have revealed long-term variation within an individual's microbiome, have raised the question of whether sampling an individual at a single time point can accurately capture an individual's true variation (Faith et al., 2013; Montassier et al., 2018). This also leads to the question of whether point sampling of a few individuals will give a clear picture of host's GI microbial taxa variance.

In this thesis we present the most extensive longitudinal study of human managed gorilla (*Gorilla gorilla gorilla*) to date. This study provides a 7-month analysis of the GI microbial composition and diversity of nineteen individual gorillas housed at three different zoological institutions in North America. Samples were collected bi-weekly to assess the stability of gorilla microbiome of individuals under human management across institutions, diets, and housing units.

Human managed NHP populations suffer from similar disease states to humans; but researchers will be unable to link states within the microbiome to the diseases carried by these individuals without long term data on microbiome diversity and richness. It is also unclear how various metadata parameters including age, sex and diet affect the composition and diversity of the microbiome in populations of human managed gorillas.

The push to understand the host species “norm” of microbial composition and diversity within the GI has fueled a recent surge in research. However, defining this “norm” has become increasingly difficult. Bolstering the long-term data pool in human managed primate populations not only aides in the care of these animal, but also provide a dataset within which to test concepts that have come out of human microbiome research such as the individual “cloud” of variance over time. This study can also inform management strategies that are under current debate, such as the inclusion of pelleted feed within human managed populations of gorilla. Therefore, the aim of the study was:

1. Determine if there are long-term fluctuations in the microbiome composition of gorillas under managed care.
2. Determine if different zoo environments have an effect on GI microbial variation within the same species of primate.

3. Determine if the inclusion of pelleted feed and differences in dietary management in human managed gorilla populations affect the microbial diversity and richness.
4. Determine if whether individuals' age, social group size, sex, and antibiotic use are significantly correlated to changes in the GI microbiome within human managed populations of gorilla.

Specifically, we tested the following hypothesis:

1. The gorilla microbiome is stable overtime and no time correlated changes in the microbiome composition will be detected.
2. Gorilla microbiome is shaped by their environment; therefore, differences between the three zoological institutions will be seen in microbial taxa richness and diversity.
3. Differences in dietary management at different institutions will affect the richness and diversity of the microbial taxa detected for each diet regime.
4. Gorilla diversity parameters of age, social group and sex will affect the composition of gorilla microbiomes
5. Administering antibiotics will restrict the diversity and richness of the microbiome of those recipient individuals.

## CHAPTER 3

### Materials and Methods

#### 3.1. Animal Population

Nineteen western lowland gorillas (*Gorilla gorilla gorilla*) were enrolled in this project. They were housed at three North American zoos: Denver Zoological Foundation in Denver, Colorado (3.3); Riverbanks Zoo in Columbia, South Carolina (2.3); NC Zoo in Asheboro, North Carolina (5.3). The overall age range for the entire study group was two years of age to forty-five years of age. Overall, ten individuals were male while nine were female.

Housing situations and social group size varied by zoo. Denver Zoo maintained two troops a 2.0 bachelor troop and a 1.3 family troop. NC Zoo housed a single troop of eight individuals (5.3). Riverbanks Zoo housed two groups a 1.0 bachelor troop and a 1.3 family group. None of the three zoos fed the same diet. Main differences included the percentage of feed type inclusion into a individual's diet as well as pelleted feed inclusion. Only Denver Zoo fed a pelleted feed every day to their individuals. Riverbanks zoo fed a pelleted feed to one female during her pregnancy.

##### 3.1.1. Animal Health Observations

In general, all animals (except two elderly females) within the research population were of good health. The two elderly females, one from Denver Zoo (age 41) and one from NC (age 45), passed away during the collection period March 2018 and June 2018, respectively. The female at Denver Zoo was being treated for ovarian cancer that predated the collection period. The female at NC Zoo suffered from age related health decline that ultimately resulted in the decision to euthanize.

Three individuals were placed on antibiotics during the collection period. This included the elderly female at NC Zoo for 10 days prior to euthanasia on June 11<sup>th</sup>, a 23-year-old female from Riverbanks Zoo for fourteen days starting January 30<sup>th</sup>, and a 2-year-old juvenile at the Denver Zoo for two weeks starting June 4<sup>th</sup> to treat a hand injury. NC Zoo treated with sulfamethoxazole/trimethoprim, Denver Zoo treated with azithromycin, and Riverbanks zoo treated with sulfamethoxazole/trimethoprim.

One female at Riverbanks Zoo was pregnant during the collection period. She gave birth to a healthy infant at the beginning of June. Infant fecal samples were not collected.

A female at Riverbanks Zoo was treated for a fungal infection with fluconazole during the collection period on June 26<sup>th</sup>. This same individual was treated with antacids on May 16<sup>st</sup> for a forty-two-day period. Another female at the Riverbanks Zoo was given Ethinylestradiol (birth control, 81mg) during the entire collection period. This same female had food coloring added to a portion of her diet to make it easier to identify her fecal samples.

### **3.1.2. Diet Information**

No zoos diet was comprised of the exact same percentages of the offered diet items. However, all three zoos fed similar food type categories. All three zoos fed leafy greens, vegetables and browse. NC Zoo and Riverbanks Zoo both fed higher quantities of leafy greens and vegetables than Denver Zoo. These two zoos also offered fruit as a part of the standard daily diet to their individuals. Denver Zoo was the only zoo to feed a pelleted feed consistently, while Riverbanks Zoo only fed a pelleted feed during pregnancy. Denver Zoo unlike the other zoos

also offers alfalfa hay to the gorillas every other day. Browse quantity offered daily was only quantified at the Denver Zoo. For full breakdown of each institution's diet see Appendix A.

## **3.2. Sample Collection**

### **3.2.1. Fecal Collection**

Fecal samples were collected from individual gorillas every two weeks starting February 1<sup>st</sup> 2018 and ending August 31<sup>st</sup> 2018. Samples were collected within 30 minutes post defecation and placed in Whirl-Pak ® bags (Nasco, Fort Atkinson, WI) using sterile tongue depressors and nitrile gloves (SensiCare®Silk Powder Free Nitrile Exam gloves, Medline Industries© Northfield, IL) to reduce contamination from the environment or collector. Each sample was labeled with the gorilla name, collector's initials, institution, time and the date the sample was collected. The samples were immediately transferred to a -80° C freezer at each zoo facility. The samples were stored in the -80°C freeze until picked up or shipped on dry ice to NC State University, Raleigh, NC where they were transferred to another -80°C freezer upon arrival until DNA extraction.

### **3.2.2. Diet sample collection**

Diet samples were collected bi-weekly via zoo keeper staff at the same time as the fecal collections. These samples were obtained by taking a representative sample of all items offered that day. These included both base diet items as well as food enrichment. For a full list of diet items offered at each institution see Appendix A. Diet samples were placed into Ziploc® bags (©S.C. Johnson & Son, Inc Racine, WI) and labeled with the institution and date collected. These samples were also stored at -80°C until shipped or transported to NC State University.

### **3.2.3. Environmental sample collection**

Zoo keeper staff used BBL™ Culture swabs (COPAN ITALIA SpA Brescia, Italy; distributed by Becton, Dickinson and Company Sparks, MD) to swab inside and outside surfaces of the gorilla housing spaces. For schematics and pictures of the three zoo's housing spaces see Appendix B. The number of swabs collected per sampling at each zoo varied; therefore, all environmental swabs for a given collection every two weeks were pooled into one sample per collection, per zoo, during the extraction phase. These swabs were stored in the tubes provided by the manufacturer and labeled with the institution, date of collection, and the area swabbed.

At Denver Zoo and NC Zoo, soil and bedding samples were also collected once a month. Riverbanks zoo soil samples were also collected, but were misplaced within their collection freezer and ultimately lost before shipment to NC State. These samples were collected from around the entirety of the outside exhibit space for the gorillas. The soil / bedding samples were collected into a Ziploc® bags (S.C. Johnson & Son, Inc Racine, WI) and labeled with date collected, exhibit location and zoo name. All environmental samples were placed into the -80°C freezer until shipped or transported to NC State University.

### **3.3. DNA Extraction**

DNA was extracted from all sample types were conducted at NC State University in two rounds. The first round took place in June 2018, and comprised of 134 fecal samples collected up to that point at all zoos. The second extraction round occurred in September 2018 and was comprised of the remaining 221 samples. DNA was extracted from a total of 355 samples, including 257 fecal samples, 41 diet samples, and 57 environmental samples which included 14

soil samples and 43 swabs. The workstation was washed with a 95% ethanol solution between each sample to prevent contamination.

The outside layer of each fecal sample was shaved away to expose the inside of the feces that had not come into contact with the environment during collection. A sample was then removed from the inner portion of the feces for DNA extraction.

Each frozen composited diet sample was crushed with a hammer to break the diet components into small particles. The crushed diet was then homogenized by shaking the collection bag. After the diets were crushed, shaken and thoroughly mixed 0.25 g of representative diet matter was removed for extraction and analyses.

All environmental swabs collected within each two-week period were pooled into a single sample by trimming the tips of all swabs for a given collection round and combining into a single extraction tube. For soil/bedding samples, 0.25 g of matter was removed from the total collected material to be used for extraction and analyses.

DNA extractions were conducted using the QIAGEN DNEasy PowerSoil kit from QIAGEN (Hilden, Germany). Extractions were performed using modifications of the manufacturer specifications in ten samples per round, as previously described (McKenney et al. 2015). See Appendix C for the detailed protocol. At the end of each extraction round, each DNA sample was labeled with its corresponding sample ID. DNA yields were quantified and placed into a -80°C freezer until all extractions were completed.

### **3.4. DNA Sequencing**

After all extractions were completed DNA aliquots were shipped on dry ice to The Nebraska Food for Health Center in Lincoln, Nebraska. All samples were prepared for pair end

sequencing 2 x 250 on the Illumina© MiSeq™ (San Diego, California). The 16S v4 region of rRNA was amplified and sequenced as previously described (Kozich et al., 2013). Completed DNA sequencing produced raw .fastq files that were then used to produce operational taxonomic unit (OTU) table QIIME 2 (Caporaso et al., 2010). Trimmomatic was used for quality control, flash was used for splicing, and search was used for chimerism to remove low-quality sequences (Edgar, 2010). OTU classifying was carried out using a Bayesian classifier against SILVA132 database using an 80% pseudobootstrap confidence score (Quast et al., 2013; Kozich et al., 2013). OTU assignment was based at a 3% dissimilarity level (Kozich et al., 2013). Nine samples were misplaced between storage after extraction and shipment to the lab for analysis and therefore were missing from the final data set, leaving a total of 346 samples sequenced.

### **3.5. Statistical Analysis**

Statistical analysis was performed using R studio version 1.1.463 statistical program using the vegan, biom, mctoolsr, stats, ggplot2 and PMCMRplus packages (Oksanen et al 2019). Operational taxonomic unit (OTU) tables were combined with a metadata mapping file of potential host diversity parameters. These ten parameters included age, antibiotic use, diet, housing (which individuals were housed together), social group size (number of individuals within a group), individual, institution (Denver Zoo, NC Zoo or Riverbanks Zoo), month the sample was collected, sex, and order (the order in which the samples were collected). Samples were rarefied to 1235 reads for the full dataset and 10000 reads for the fecal sample only dataset to compensate for differences in sequencing coverage across samples (Gotelli and Colwell 2001).

Alpha diversity (i.e. richness and Shannon Diversity) and beta diversity (i.e. Bray-Curtis dissimilarity) were calculated for all samples.

Richness is a measure of how many distinct OTUs were detected. Shannon diversity is the measure of both abundance and evenness within samples. Evenness is a measure of how similar abundance levels the represented OTUs are within a sample. Kruskal-Wallis rank sum tests were performed on both Shannon diversity and richness to detect differences across each parameter using R's *vegan* package (Oksanen et al. 2019).

Beta diversity measures differences in microbial composition across samples. ANOVA statistical tests were used to detect differences associated with host and management parameters. Bray-Curtis dissimilarity measures were used to analyze the differences between samples based on relative abundance of microbial taxa in each sample.  $R^2$  values closer to 0 mean the samples share the same abundance, while values closer to 1 mean the samples have different species abundances. Wilcoxon Rank Sum test was performed to detect if the relative abundances of the OTUs significantly differed between samples.

The effect of differing potential host diversity parameters on the microbial beta diversity was analyzed using ANOVA ANOSIM based on Euclidian distance metrics using R (Oksanen et al. 2019). Initial analysis was based on the full dataset to test whether the host parameters caused observable differences in the microbial abundance between samples. Single Permutova (999 permutations) was carried out for sample type, zoological institution collected from, individual sample ID, month and order (The order in which the samples were collected).

Diet and environmental microbiome samples were then removed from the data set to leave only fecal samples. These fecal samples were then tested against the ten metadata potential diversity parameters age, antibiotic, diet, housing, individual, institution, month, order, social

group size, and sex. Single permanova were used to analyze variance within the fecal samples. Permanova pairwise multiple comparison (999 computations) was also carried out testing each potential diversity parameter against the other nine parameters for a total of 90 computations (i.e. age+diet). Finally, Permanova interaction analysis was performed to test for pairwise interactions across potential diversity parameters, for a total of 90 computations (i.e. age\*diet). All results were considered significant at  $p < 0.05$ , although stronger significances were found at  $p < 0.01$  and  $p < 0.001$ .

## CHAPTER 4

### Results

#### 4.1. All Samples

After the loss of nine samples prior to sequencing a total of 346 microbiome samples were sequenced and used to produce the OTU summary tables for downstream analyses in R (R Team, 2016). Of these 120 were collected from Denver Zoo, 125 were collected from NC Zoo, and 101 were collected from Riverbanks Zoo. All libraries were rarefied to 1235 reads for each sample to remove any low yield sequencing that could skew the data. This low rarefaction threshold was used to accommodate the relatively low coverage of the environmental and diet samples compared to fecal samples. After rarefaction 329 samples remained for statistical analyses. A list of individual samples can be found in Appendix D.

##### 4.1.1. Alpha Diversity Across All Microbiome Samples

When the potential diversity parameters were analyzed, microbiome sample type (fecal, environmental or diet), zoological institution and gorilla individual affected microbial richness across all samples collected (Table 1, Figure 1). Riverbanks Zoo fecal samples contained significantly more microbial taxa (130-155 genera), compared to the other two institutions (Figure 1), while Denver Zoo samples had the lowest richness (120-140 genera). The environmental samples from all three institutions show high variation (Figure 1), and higher mean values for the number of microbial taxa reported (200 to 600 reported microbial taxa). The diet samples contained lower overall richness compared to the fecal and environmental samples (10 to 100 reported microbial genera) (Figure 1).

Sample type, institution and individual ( $p < 0.001$ ) were linked to differences in the microbial OTUs measured by Shannon Diversity Index (Table 1, Figure 2). For these three parameters, the microbial relative abundance was uneven between samples, suggesting that some samples were dominated by relatively few microbial OTUs (Figure 3). Environmental samples contained a lowest abundance by unclassified microbial taxa (~6%,  $p < 0.001$ ) (Figure 4). Environmental samples from Riverbanks displayed a higher abundance of *Alkanindiges* (13.4%,  $p < 0.001$ ) (Figure 4). Denver samples had a higher abundance of *Laceyella* (13.2%,  $p < 0.001$ ) than environmental samples from the other institutions. Overall, diet samples were dominated by unclassified microbial taxa (~15% of reported taxa), but also contained many low abundance taxa that make up less than 1% of the overall diversity of microbial taxa reported in these samples (Figure 5). Fecal samples contained higher abundance of unclassified microbial taxa similar to the levels seen in the diet samples (~13,  $p < 0.001$ ) (Figure 6). The diet samples have little to no abundance of the *Pediococcus*, *Phocaeicola*, Prevotellaceae and Ruminococcaceae (Figure 3, Figure 5). While environmental samples did have an abundance between 1-2% for *Pediococcus*, *Phocaeicola*, Prevotellaceae and Ruminococcaceae, they had fewer distinct OTUs within Prevotellaceae and Ruminococcaceae than fecal samples (Figure 3, Figure 4). Fecal and environmental samples showed *Cloacibacillus* at abundance levels  $> 1\%$  while diet samples showed abundances  $< 1\%$  ( $p < 0.001$ ) (Figure 4, Figure 6).

#### **4.1.2. Beta Diversity Across All Microbiome Samples**

When comparing all microbiome samples types the potential diversity parameters institution, type and individual had a significant effect on the relative abundance of microbial

taxa (Table 2). The highest indicator of difference in microbial taxa abundance is type ( $p=0.002$ ,  $R=0.9776$ ) followed by institution ( $p=0.001$ ,  $R=0.8007$ ) for Anova ANOSIM (Table 2).

Overall, environmental and diet samples have significantly lower abundance of *Prevotella*, Prevotellaceae, and Ruminococcaceae ( $p<0.001$ ) (Figure 3, Figure 4, Figure 5, Figure 6). Environmental samples (CEDZ, CENC and CERB) are spread across the ordination plot, indicating that they contain a greater variety of microbial taxa across the collection period compared to diet and fecal samples (Figure 7).

Analyses based on the individual gorilla parameter (ANOVA  $p=0.001$ ,  $R=0.7598$ ) and institution parameter ( $p=0.001$ ,  $R=0.8007$ ) collected from showed a high degree of dissimilarity therefore the microbial composition differs (Table 2, Figure 4, Figure 5, Figure 6). Based on individual one can see that the fecal samples all cluster with each other, while the diet and environmental samples cluster farther away from the fecal samples (Figure 7). This suggests that, despite variation among individuals, the gut induces similar selective pressures that favor specific microbial taxa. For example, all fecal samples had similar reported abundance of *Prevotella*, Prevotellaceae, and Ruminococcaceae ( $p<0.001$ ). The exception is RBPa which had a higher relative abundance for *Pediococcus* and *Phocaelicola* ( $p<0.001$ ). Diet and environmental samples at all three institutions had a higher percentage of genera in the “Other” reported taxa category for the top 20 taxa reported than what was reported in the fecal samples (Figure 3).

#### **4.2. Fecal Microbiome Samples Only**

For further analyses, environmental and diet microbiome samples were removed from the dataset leaving only the fecal microbiome samples. This reduced the samples from 346 to 248

(84 samples for Denver Zoo, 92 samples for NC Zoo and 72 samples for Riverbanks Zoo). The average number of reads before rarefaction was 19680, with the lowest number of reads at 4 and the highest number of reads at 55721. The samples were rarefied to 10006 reads for each sample to remove any low yield sequencing that could skew the data. After rarefaction 187 fecal samples remained for statistical analyses.

#### **4.2.1. Alpha Diversity Across Fecal Microbiome Samples**

When only fecal microbiome samples were analyzed the microbial taxa richness among samples was different for the potential diversity parameters age, diet, housing, individual, institution, and social group size ( $p$ -value $<0.05$ ) (Table 3, Figure 1, Figure 8, Figure 9, Figure 10). This means that each of these parameters had a significant effect on the number of microbial taxa reported among groups based on a given diversity parameter. Denver Zoo samples, consistently displayed lower species richness than the other two institutions (Figure 8, Figure 9, Figure 10). Housing and social group size showed the highest number of microbial taxa for the singly housed male, followed by the large seven individual troop at NC Zoo (Figure 9). Denver Zoo troops had the lowest reported number of microbial taxa (Figure 9). Diet followed the same trend as housing and social group size. The highest number of reported microbial taxa were in gorillas between the ages 22 and 28 (Figure 10). There was however no clear trend of younger individuals with lower amounts of reported taxa compared to the older individuals as the youngest individual richness overlaps the spread of several of the other age groups (Figure 10).

Age and individual displayed dissimilarities between samples based on Shannon diversity ( $p=0.0004$  and  $p=0.004$ , respectively) (Table 3). This points towards there being dominance of certain microbial taxa over others. The dominate genera included *Cloacibacillus* (18.1%,

$p < 0.001$ ) for two Denver Zoo individuals DZBa and DZJi, while Norank (unclassified genera of bacterium) in all NC samples and one Riverbanks individual RBPa ( $p < 0.001$ ) (Figure 6, Figure 11). Riverbanks Zoo individuals presented a higher percentage in the “Other” category which are taxa found at  $< 1\%$  of the overall relative abundance and therefore did not meet the analysis threshold cut off ( $p < 0.001$ ) (Figure 6, Figure 11). NC Zoo samples displayed the highest abundance for *Phocaeicola* and unclassified microbial taxa ( $p < 0.0010$ ) (Figure 6, Figure 11). However, all samples exhibited 10-20% of the overall relative abundance to be unclassified microbial taxa (Norank and uncultured), indicating that a large percentage of the gorilla microbiome is still undescribed (Figure 6, Figure 11).

#### **4.2.2. Beta Diversity Across Fecal Microbiome Samples**

When microbiome fecal samples only were analyzed, the potential diversity parameters age, diet, housing, social group size, individual, institution and sex had an effect on the relative abundance of the microbial taxa found (Table 4). The two genera with the highest difference based on individual and institution were *Cloacibacillus* and *Phocaeicola* ( $p < 0.001$ ) (Figure 11). Denver Zoo had significantly higher relative abundance of *Cloacibacillus* ( $p < 0.001$ ), but little to no abundance of *Phocaeicola* ( $p < 0.001$ ) (Figure 6, Figure 11), compared to the other institutions. However, overall clustering of a given institution overlapped the cluster of another institution showing no true distinguishable grouping based by this parameter (Figure 12).

Individual presented the strongest effect on relative abundance ( $p = 0.001$ ,  $R = 0.8094$ ) (Table 4, Figure 11, Figure 12). Individuals NCAp, NCBo and RBPa exhibited the highest relative abundance for *Phocaeicola* compared to the other individuals ( $p < 0.001$ ) (Figure 11). All individuals exhibited similar abundance for *Prevotella*, Prevotellaceae and Ruminococcaceae

(Figure 11). RBP<sub>a</sub> presented the highest relative abundance for the “Other” category ( $p < 0.001$ ) (Figure 11). Because taxa were reported based on an abundance threshold of  $>1\%$  in the relative abundance figures for fecal samples, it is difficult to predict what effect these “Other” taxa might have in this individual (Figure 11).

Dissimilarity based on diets, housing and social group size had a similar pattern to individual. Individuals consuming the two diets at Denver Zoo showed the highest relative abundance of *Prevotella*, with the youngest individual at the zoo DZWh having the highest abundance of all gorillas samples ( $p < 0.001$ ) (Figure 11, Figure 13). Regardless of which diet type was consumed, all fecal samples had higher and similar abundance of Prevotellaceae and Ruminococcaceae than compared to the environmental and diet samples ( $p < 0.05$ ) (Figure 3).

ANOVA revealed significant dissimilarities based on gorilla housing groups (ANOVA  $p = 0.001$ ,  $R = 0.6817$ ) (Figure 13). The NC troop (5.3) and Riverbanks family group (1.3) showed lowest percentages of *Cloacibacillus* compared to the other fecal samples. By comparison, the two Denver groups (i.e. 2.0 and 1.3) showed higher percentages of Prevotellaceae and Ruminococcaceae than any of the other three groups (Figure 9). The Riverbanks solitary male showed the lowest abundance for Prevotellaceae and Ruminococcaceae (Figure 13).

Social group size showed higher percentages of unclassified microbial taxa ( $\sim 4\text{-}10\%$ ) and *Cloacibacillus* ( $\sim 12\text{-}17\%$ ) in the smaller group sizes (i.e. groups of one to four individuals) ( $p < 0.001$ ) (Figure 13). The middle-sized groups, two to four individuals, showed the highest abundance for Prevotellaceae ( $\sim 7\text{-}10\%$ ) and *Prevotella* (3-9%) (Figure 13). The bachelor group of two individuals showed no abundance for *Phocaeicola* while the Denver family troop of three showed no abundance for *Gemmata* (Figure 13). The largest troop from NC Zoo showed the

lowest abundances for *Cloacibacillus*, *Prevotella*, and Prevotellaceae (<0.001%, p<0.001) (Figure 13).

There was no clear trend in differences in microbial taxa relative abundance based on groups by the diversity parameter age.

Dissimilarity based on sex is significant for fecal samples (ANOVA p=0.001, R=0.4864). Ordination shows an overlap of males and females showing that this clustering of the two sexes is not mutually exclusive (Figure 12). Males do show a higher percentage of *Phocacaeicola* (9.32%) and unclassified taxa (13.52%) than females (4.46% and 12.4%) (p<0.001) (Figure 14). While females had a higher percentage of *Cloacibacillus* (13.06%), *Prevotella* (4.43%), Prevotellaceae (8.36%) and Ruminococcaceae (6.5%) than males (12.03%, 4.26%, 6.86%, 5.06%, respectively) (p<0.001) (Figure 14).

Comparison and interaction analysis were carried out to identify potential confounding variables (Table 5, Table 6). Individual presented the highest value as the first variable for the comparison analysis (p=0.001, R<sup>2</sup>=0.53984) (Table 5), indicating individual had the highest interaction with other parameters. Individual+month was the highest of the comparison tests (individual p=0.001, R<sup>2</sup>=0.53984; month p=0.002, R<sup>2</sup>=0.02588) (Table 5), indicating that these two parameters together explained the highest percentage of the variation seen among samples microbial taxa relative abundance. The month, order and antibiotic did not show significance as the first variable when tested against age, diet, institution, sex and social group size (Table 5). The order in which samples were collected only showed significance when compared with individual, both as the first variable and as the second variable (individual+order; individual p=0.001, R<sup>2</sup>=0.53984, order p=0.04, R<sup>2</sup>= 0.04793; order+individual; order p=0.52831, R<sup>2</sup>=

0.52831) (Table 5). Age, diet, institution, social group size, and sex all show significant tests with seven other parameters in explaining the differences in microbial taxa relative abundance.

Individual\*month demonstrated the highest interaction between parameters to explain the differences in microbial taxa abundance between samples (combined  $p=0.002$ ,  $R^2= 0.25740$ ) (Table 6). When held as the first variable, age, social group size and sex each significantly interact with five other parameters (Table 6). The most significant interactions detected for each of these variables were, age\*institution (combined  $p=0.001$ ,  $R^2= 0.05060$ ), social group size\*diet (combined  $p=0.001$ ,  $R^2=0.07301$ ), and sex\*age (combined  $p=0.001$ ,  $R^2= 0.03591$ ), respectively (Table 6). However, these combined effects are still relatively lower when compared to that of individual\*month, indicating that individual explains the most differences in sample composition.

#### **4.2.3. Beta Diversity Across Denver Zoo Fecal Microbiome**

Within the Denver Zoo samples, the highest correlative effect on variance seen in the microbial taxa relative abundance was the diversity parameters social group size and housing ( $p=0.001$ ) (Table 7). The bachelor troop hosted a significantly higher abundance of unclassified microbial taxa and lower abundance of *Cloacibacillus* compared to the family group ( $p<0.001$ ). Month in which the sample was collected was also found affect variation in the abundance of microbial taxa ( $p=0.005$ ,  $R^2=0.1218$ ) (Table 7, Figure 15). Individual also exhibited significance with some distinct clustering per individual occurring within the ordination plot ( $p=0.032$ ,  $R^2=0.0924$ ) (Table 7, Figure 16). Sex and order did not show significance in this population ( $p>0.05$ ) (Table 7). The individual DZWh underwent antibiotic treatment during the collection

period; however, we detected no significant differences between samples collected before and after the antibiotic treatment ( $p>0.05$ ).

#### **4.2.4. Beta Diversity Across NC Zoo Fecal Microbiome**

The diversity parameter individual explains the largest portion of variation in reported relative abundances of microbial taxa at the NC Zoo ( $p=0.001$ ,  $R^2=0.8032$ ) (Table 8, Figure 17). Compared to Denver Zoo, gorillas at NC Zoo presented greater dissimilarity between individuals, with distinct clusters of groups of individuals occurring (Figure 17). NC Zoo month, sex and order parameters all showed no significance ( $p>0.05$ ) (Table 8). When the individual NCRo was analyzed for effect of antibiotic treatment no significant effect was detected ( $p>0.05$ ). However, no comparison to post antibiotic treatment could be made as this individual was euthanized before antibiotic treatment was completed.

#### **4.2.5. Beta Diversity Across Riverbanks Zoo Fecal Microbiome**

Riverbanks zoo samples were significantly affected by five diversity parameters (age, housing, individual, social group size, and sex) ( $p=0.001$ ) (Table 9). Individual explained the highest percentage of variance within the Riverbanks Zoo's samples ( $p=0.001$ ,  $R^2=0.8523$ ) (Table 9, Figure 18). Overlap between individuals is minimal compared to Denver Zoo (Figure 16, Figure 18). This is indicative of a stronger individual effect at Riverbanks Zoo than there is at either Denver Zoo ( $p=0.001$ ,  $R^2=0.1323$ ) or NC Zoo ( $p=0.001$ ,  $R^2=0.8032$ ) (Table 9). RBAc individual underwent antibiotic treatment at the beginning of the collection period. Like the individual at Denver Zoo antibiotic treatment in this individual did not show significance differences between samples taken before and after this treatment ( $p>0.05$ ).

## CHAPTER 5

### Discussion

#### 5.1. Individual is the strongest driver of variation among samples

Individual gorilla identity affected alpha and beta diversity of gastrointestinal (GI) microbiomes significantly across institutions. While operational taxonomic unit (OTU) abundance is similar between individuals within the same institution, no two individuals' microbial communities looked exactly the same. This finding is similar to research conducted in humans that have showed each individual hosts their own "cloud" of variance (Montassier et al., 2018). This may indicate that, like humans', gorillas display a strong individual identity with regard to gut microbial membership.

Relative abundance of microbial taxa differed between individuals. Two individuals at Denver Zoo (DZJi and DZBa) had the highest overall percentage of *Cloacibacillus*. This genera is known to be pathogenic in nature. DZBa was actively under treatment for ovarian cancer and this higher percentage of this potentially pathogenic taxon may have a correlation. DZJi while considered healthy is the alpha male of the family troop that also houses DZBa. Association between the two individuals instead of disease state may be the reason for elevated *Cloacibacillus* in DZJi. An individual at Riverbanks zoo (RBPa) had the highest relative abundance for the other genera category of the reported top 20 taxa. This other category is made up of microbial taxa found at less than 1% abundance in the microbiome. This higher percentage of low abundance taxa may potentially be due to an effect that social contact with other individuals has, as RBPa is a singly housed male. Individuals NCDe, NCHa, NCJa, NCMo, NCOI and RBCe displayed the largest percentages of the unclassified microbial taxa group. DZ individuals had a higher percentage of *Prevotella*, with the youngest individual DZWh having

the highest relative abundance of this taxa of all individuals studied. This higher abundance of *Prevotella* (a carbohydrate generalist) may indicate differences in dietary management differences between Denver Zoo and the other two zoos.

Differences in microbial community composition among gorilla individuals may be linked to several factors. Factors shown within this study to have a significant interaction with the individual parameter include age, month sample was collected and social group size. There are also several factors that could affect individual variation that were not within the scope of this study. For example, the number of times an individual has been moved to another zoological institution, and any previous medical treatments.

Surprisingly, the two individuals that were euthanized (DZBa and NCRo) showed similar microbial taxa abundances to the other individuals within their institutions, indicating that their gut microbiome were not clearly affected by their terminal conditions.

## **5.2. Environment affects microbial richness and abundance**

This research has shown that zoological institution affects gut microbial richness and Shannon diversity. Each zoo varies in habitat design, as well as the gorillas' daily schedule (i.e. length of time individuals spend outside). The environments among the zoos also vary based on regional differences. For example, Riverbanks Zoo and NC Zoo are located in similar ecological biomes, while Denver Zoo is located in a steppe climate and experiences the most drastic seasonal changes in weather and temperature of the three institutions studied (Young et al., 2017). Due to the harsh winter, the Denver gorillas experience limited access to their outside habitats from November to March. By contrast, the troops at NC Zoo and Riverbanks Zoo have nearly year-round access to their outside habitat space. Denver Zoo environmental samples also

displayed a larger spread of richness across the collection. This may be indicative of the environments more extreme change over time. Environmental samples from the three zoos differed based on the relative abundances of *Alkanindiges*, *Laceyella* and *Sphingopyxis*. Fecal samples have low reported abundance for both *Alkanindiges* and *Laceyella*, and no fecal samples reported an abundance for *Sphingopyxis*. The presence of *Alkanindiges* and *Sphingopyxis* in fecal samples is indicative of the environments influence on microbial taxa present.

Previous work has indicated wild gorillas in different regions of Africa show variations in species richness (Moeller et al., 2013). Dissimilar ecological biomes offer diverse environmental bacterial species that a given individual will come in contact with (Moeller et al., 2013). Differences in the environmental pool of microbes will, in turn affect the microbial taxa are present within an individual's microbiome.

Despite differences in relative abundance, however, gorillas' at all three zoos hosted the same top 20 taxa. Of those most prominent taxa, several have been identified in previous studies of the gorilla microbiome. For example, *Prevotella*, Prevotellaceae and Ruminococcaceae are associated with fiber degradation which is very important to gorillas and other folivores (Yildirim et al., 2010; Wu et al., 2011; Gomez et al., 2015; Clayton et al, 2018A).

Denver Zoo individuals hosted a greater abundance of *Cloacibacillus*, which can be pathogenic in humans (Domingo et al., 2015). NC Zoo individuals showed the highest relative abundance for *Phocaeicola* while Denver Zoo individuals had the lowest relative abundance for this taxon. This microbial taxon has also been documented as pathogenic in humans and link to abscesses (Masaima et al., 2009). Most pathogenic microbes do not exhibit pathogenic properties unless they occur above a specific relative abundance (Abreu and Peek, 2014). The pathogenic

potential or threshold of *Cloacibacillus* has not been studied in gorillas; but all individuals in this study (except the two previously discussed) were considered healthy.

While known membership (i.e. top 20 reported taxa/taxonomic groups) was relatively consistent, the relative abundances of unclassified microbial taxa (i.e. unclassified taxa, Unknown and other) was more variable across institutions. Riverbanks Zoo's individuals showed a higher percentage of "Other" genera meaning they had a lower abundance of the named top 19 taxa than the other two institutions. NC Zoo showed a higher relative abundance of the unclassified taxa category, which include unclassified genera. In comparison Denver Zoo shows the lowest relative abundance for the unclassified taxa category.

### **5.3. Diet affects microbial diversity**

Analysis of diet samples showed that these samples had the lowest Shannon diversity values. This means that these samples are dominated by many low abundance taxa. Influence of the microbial taxa present on the food consumed is poorly understood. The spatial relationship between environmental, diet and fecal samples suggest that the environmental pool of microbes and the microbes associated with diet may play a role in shaping the GI microbiome, by affecting different classes of membership. The unclassified microbial taxa is dominant within diet samples. *Prevotella*, *Prevotellaceae*, *Cloacibacillus*, *Phocaeicola*, and *Succiniclaticum* were all detected in both fecal samples and diet samples. However, relative abundance of these taxa within the diet samples are extremely low. Presence of these taxa are not consistent across all three institutions. For example *Cloacibacillus* is detected in both Denver Zoo and NC Zoo diet samples. However, overall individuals at NC Zoo have the lowest relative abundance of *Cloacibacillus* compared to the individuals at the other institutions. This is a similar finding to a

previous study that detected greater microbiome similarities among chimpanzees and gorillas that share environments, than in groups of chimpanzees or gorillas that live in other environments (Moeller et al., 2013). This pattern was driven by the sympatric chimpanzees' and gorillas' consumption of similar diet items (Moeller et al., 2013).

All three zoos offer similar quantities of fresh produce in their gorilla diets. However, microbial analyses suggest that diets differ enough across institutions to affect not only the number of microbial taxa found, but also the relative abundance of these taxa. Different diets collected from the same institution carry a more similar microbial load than diets from different institutions. Within a given zoo, the same base diet is feed to all gorillas; weights of offered food and animal preferences are the only major dietary variable that differs between individuals. These weight percentages are based off of each individual's body weight (Appendix A).

No single diet regime stands apart from the other for all fecal microbiome samples. Fecal samples for individuals at Denver Zoo plus the pregnant individual at Riverbanks Zoo had the highest percentage of *Cloacibacillus*. These individuals all had pelleted feed included in their diets. Individuals at the Denver Zoo also had the highest percentage of *Prevotella* and Prevotellaceae. Presence of *Prevotella* has been linked to human managed non-human primates and higher levels of polysaccharide digestion (Rosenberg, 2014; Clayton et al., 2016). Prevotellaceae has been linked to fiber digestion in several species including gorillas (Gomez et al., 2015). This higher relative abundance of *Prevotella* may be linked to the inclusion of pelleted feed at Denver Zoo which may have a higher carbohydrate load than other diet items. While the enrichment of Prevotellaceae in the Denver Zoo group may be linked to a consistent daily browse content in their diets.

The use of pelleted feed at the Denver Zoo may contribute to the observed microbial dissimilarities. Inclusion of pelleted feed within gorilla diets is a highly debated topic within the zoological community. This diet item contains more starch and less fiber than wild gorillas are thought to consume, and this carbohydrate “imbalance” is sometimes considered detrimental to the health of animals in populations under human management. Pelleted feed is never fed at North Carolina and is only minimally included at Riverbanks Zoo; the latter only offered pelleted feed to their pregnant female. Denver Zoo and Riverbanks also offer different types of pelleted feed. The Denver Zoo fed Mazuri® L/S Primate Biscuit (St. Louis, MO), with banana and cinnamon flavors mixed together, while the Riverbanks zoo fed their pregnant female the Mazuri® Leafeater Large biscuit (St. Louis, MO). Both zoos, however, fed a very low inclusion of pellet in their diets less than 2% at both institutions, and therefore further research is needed to determine if this is a concern. Additionally Denver Zoo may be the only zoo that is nutritionally balanced due to the inclusion of pelleted feed which allows for balance of micronutrients. The effect of these differences on the individuals that consume a given diet are unknown at this time.

The way each zoo rotated produce within their diets was also different. Denver Zoo rotated produce varieties on a daily basis (i.e., red pepper on one day and eggplant the next). The leafy greens and vegetables portions of this diet were rotated this way such that every day the diet is slightly different. The NC Zoo rotated diet items on a weekly basis; but not every produce item rotated every week. For example, the leafy greens portion of the diet may change one week, while the vegetable portion of the diet rotated the next week. Produce items at the Riverbanks Zoo are rotated on a monthly basis.

Each zoo acquired their produce from different growers within the United States. Regional effects on the produce may contribute to the strong dietary effect on the fecal microbial

composition. This regional factor could also explain why the richness at NC Zoo was more similar to Riverbanks (in South Carolina) than either was to Denver Zoo: the former most likely source from similar growing regions in southern North American, while Denver pulls from western growing regions. Each zoo also varied in the species and quantity of browse offered to the gorillas, based on what could be grown in the region. Denver Zoo was the only institution to give a standard quantity of browse to their individuals daily. Browse offered at NC Zoo and Riverbanks Zoo varied daily based on availability both within the exhibit space and cut browse supplied by the animal keepers. Because Denver Zoo cannot grow browse on grounds during winter months, that zoo also had an added dietary variance of browse that was shipped in from other areas.

#### **5.4. Housing and social group size affect microbial abundance and richness**

Housing and social size are not mutually exclusive variables. There were five distinct housing groups, which correlated to the social group size count. Denver Zoo maintains 2 troops, a 1.0 bachelor troop and a 1.2 family troop. At the beginning of the study the 1.2 family troop was a 1.3 family troop but one adult female was euthanized three months into collection. NC Zoo also lost a female from their troop of 5.2 (originally a group of 5.3) five months into collection. Riverbanks Zoo house one 1.0 troop and a 3.1 troop. These parameters affected the reported microbial richness for the samples that fall within a given housing and social group. The richness by size included the transitions at both Denver Zoo and NC Zoo's when an individual was euthanized. The mean microbial richness did change when group size changed at NC Zoo. However, when Denver Zoo's family troop went from 4 to 3 individuals the effects on the microbial richness was less clear. This is due to Riverbanks Zoo also housing a troop of four

individuals, therefore an institution effect is also in play when Denver Zoo's shift from four to three individuals. The observed shift in the NC Zoo troop microbiome when an individual was lost could be due to several factors. These factors include stress to the troop due to the loss of an individual. As well as loss of the contact with that individual. Social contact with individuals within a troop have been shown to significantly affect the microbiome of the individual (Tung et al., 2015).

Only Riverbanks zoo housed an individual alone, this individual (RBP<sub>a</sub>) shows the lowest relative abundance for Prevotellaceae compared to the other housing and social groups. This microbial taxa is important in fiber digestion. The lower abundance of this group in RBP<sub>a</sub> may be linked to dietary preference away from food containing fiber content. This individual also had the highest percentage of low abundance taxa (<1% of the overall microbiome). Relative distances between RBP<sub>a</sub> the remaining four individuals at Riverbanks Zoo on the NMDS plot may be an indication of effect of daily social contact. Social contact has been shown to correlate to similarities between individual baboons within a much larger troop (Tung et al., 2015). The lack of this social contact is a potential driver of the differences seen between RBP<sub>a</sub> and the rest of the individuals housed at Riverbanks Zoo since they rotate between the sample habitat spaces.

The troop at NC Zoo shows the highest relative abundance for unclassified microbial taxa and *Phocaeicola*, an anaerobic gram-negative strain, within the order Bacteroidales (Masaima et al., 2009). This order has been detected several times within the gorilla microbiome for both wild and human manage populations (Moeller et al., 2013; McKenney et al., 2014; Gomez et al., 2015; Clayton et al 2016). In humans *Phocaeicola* has been isolated from brain abscess and may

have a pathogenic nature (Masaima et al., 2009). However, the metabolic properties of this genus have not been reported in NPH.

### **5.5. Effect of host's age and sex on microbial composition and diversity**

Age showed an effect on not only the richness and Shannon diversity of the reported microbial taxa, but also the variation between individuals in the same age group. Different microbial taxa appear to dominate each age group over others, although more individuals are needed in each age group to determine whether these trends are significant. *Cloacibacillus* was dominant in gorillas aged 27-31 and 41, which included individuals housed at Denver and Riverbanks zoos. No individual at NC Zoo fell within this age range, and their eldest individual (45 years of age) did not show the higher abundance of *Cloacibacillus*. The adolescent at Denver Zoo showed a striking similarity to the adults. This may suggest that the gorilla microbiome matures by the age of two, a similar colonization timeframe to lemurs (McKenney et al., 2015). More data is needed to verify the age of microbiome maturity in gorillas but should correlate of the age of weaning (McKenney et al., 2015). *Prevotella* and *Prevotellaceae* show high relative abundance for all ages pointing towards their importance in digesting the diet gorillas consume.

Sex of individual was shown to interact with the effects of age of individual on the diversity and richness of the gorilla microbiome. Overall in this study sex was shown to be a significant driver of the relative abundance of species present within the samples. Other work however, has shown a stronger sex driven correlation within the immature individuals during all seasons (Pafco et al., 2019). While differences between sexes in adult individuals was not found to be significant (Pafco et al., 2019). This difference between previous work and this study may be the link to this research population being under human management. Individuals under human

management do not go through external diet variation through the year like wild individuals do. Wild individuals must cope with seasonal availability of preferred diet items through the year, and therefore alter their base diets based on availability (Pafco et al., 2019). Under human management these changes do not occur as diets are standardized.

## **5.6. Comparison to previous studies**

Many of the microbial taxa reported in this work have been previously detected in both other human managed populations of gorillas as well as wild populations (McKenney et al., 2014; Gomez et al., 2015; Vlckova et al., 2016; Moeller et al., 2017; Krynak et al., 2017). Most common reported taxa for human managed gorilla populations are *Prevotella*, Prevotellaceae and Ruminococcaceae (McKenney et al., 2014; Krynak et al., 2017). Prevotellaceae and Ruminococcaceae are reported in wild gorillas in high relative abundance (Gomez et al., 2015; Vlckova et al., 2016; Moeller et al., 2017). While *Prevotella* has previously been described in some wild populations, gorillas near Bai-Hokou in Central Africa, some research suggested that the presence of this microbial taxa is linked to human management (Gomez et al., 2015; Clayton et al., 2016). *Prevotella* is capable of polysaccharide degradation and is linked to the presence of high abundance of polysaccharides in the diet (Clayton et al., 2016). All three of these genera have been linked to fiber digestion within the hindgut and therefore may be evolutionarily upheld within the gorilla species (Gomez et al., 2015). The genus *Phocaeicola*, detected in 17 of the individuals of in this study, has not previously been mentioned in either human managed or wild gorilla microbiome work. However, the order Bacteroidales and family Bacteroidetes that this genus is within has been reported often (McKenney et al., 2014; Gomez et al., 2015; Vlckova et al., 2016; Krynak et al., 2017).

In all gorilla microbiome work there is a large portion of unclassified and uncultured genera. These levels of unclassified taxa suggest that gorillas under human management maintain a reservoir of uncharacterized microbial diversity. Until these genera are described it will be impossible to fully understand the diversity of the gorilla microbiome and what subtle changes in these populations mean to the host.

## **5.7. Conclusion**

The microbiome data presented in this thesis represents the most extensive longitudinal study, and the largest number of individual gorillas studied to date. This project aimed to identify drivers of gut microbial diversity in human managed populations across institutions in North America. The results suggest that host identity is the strongest driver of composition of the gorilla gut microbiome, followed by diet management and institution of the individual. While these correlations can help explain the variation between individual gorillas under human management, none of these parameters alone can explain the variation observed between samples. This is due to the confounding nature of many of the parameters studied such as diet management and institution. Future research that includes more individuals from different zoos, over a longer timeframe, could help verify whether gorillas, like humans, truly display an individual “cloud” of conserved variation over time. Further long-term studies on more individuals will also help to understand the full extent of microbial variation within the gorilla genus. By including more institutions in future research, we can hope to further understand how ecological biome dissimilarities (correlated with the geographic location of zoos) effect bacterial lineage composition within the microbiome of human managed gorillas.

Diet related differences seen between individual microbiomes could be linked to the dissimilarities in diet management at the three institutions. Microbial differences on the diets, themselves, may also be correlated to different food growing regions within the United States that these institutions use. This study is unable to characterize whether this shift in the microbiome is harmful or helpful, only that a difference was seen. Further research should be done to link microbial composition differences between institutions, to the growing regions used by those institutions. This may give insight into agricultural practices that affect food composition, and which may also affect bacterial membership within the GI tracts of human managed gorillas. Further research should also be carried out to help characterize what the inclusion of pelleted feed within gorilla diet means to the individuals consuming the pellets.

The present study did not look into any changes in the metabolome that may have resulted from the reported differences in microbial taxa richness and abundance. This study did show that a significant portion of the gorilla microbiome remains unclassified and uncultured. Until research is carried out to understand what these genera are and how they affect the host we cannot say for certain how changes in the relative abundances of these genera might affect their hosts. Thus, further research should be carried out to gain a full picture of diversity and functionality within the gorilla microbiome and metabolome.

As one of human's closest relatives, gorillas can lend insight to aspects of our own GI microbiome. Once we are better able to understand the gorilla microbiome patterns and if they can indicate disease states in a controlled environment, we may be able to better predict other primate (including human) disease states. Of even more importance than the gorilla:human microbiome relationship is the endangered status of our study species. It is of the utmost importance to understand how human management can affect the microbiome and what the

changes mean to the hosts health. This information can potentially be used to ensure gorillas thrive to their full health and reproductive potential under human management for years to come.

## TABLES

Table 1: Kruskal-Wallis Rank Sum Tests Revealed Significant Diversity Parameter Drivers of Gorilla (*Gorilla gorilla gorilla*) Fecal, Diet and Environmental Microbiome Richness and Shannon Diversity in Samples Collected from Denver, NC and Riverbanks Zoos<sup>1</sup>.

<b>Diversity Parameter</b>	<b>Richness</b>	<b>Shannon Diversity</b>
<b>Individual Sample</b>	<0.001 *** <sup>1</sup>	<0.001 ***
<b>Institution</b>	<0.001 ***	<0.001 ***
<b>Month</b>	0.4458	0.7429
<b>Order</b>	0.9022	0.9792
<b>Sample Type</b>	<0.001 ***	<0.001 ***

<sup>1</sup>Asterisks Indicate that Diversity Parameter was Associated with Significant Differences in Microbial Diversity, as Follows:

\*\*\*p<0.001

Table 2: All Diversity Parameters Except Month and Order of Collection Significantly Affected Bray-Curtis Dissimilarity Across Gorilla (*Gorilla gorilla gorilla*) Fecal, Diet, and Environmental Microbiome Samples Collected at Denver, NC and Riverbanks Zoos<sup>1,2</sup>.

<b>Parameter</b>	<b>p-value</b>		<b>R-value</b>
<b>Individual Sample</b>	0.001	*** <sup>1</sup>	0.7522
<b>Institution</b>	0.001	***	0.8007
<b>Month</b>	0.18		0.0068
<b>Order</b>	1		-0.0306
<b>Sample Type</b>	0.001	***	0.9776

<sup>1</sup>Diversity Parameter Effects Were Tested Using Anova ANOSIM. 999 Permutations Ran Per Parameter Test.

<sup>2</sup>Asterisks Indicate that Diversity Parameter was Associated with Significant Differences in Microbial Composition, as Follows:

\*\*\*p<0.001

Table 3: Kruskal-Wallis Rank Sum Tests Revealed Significant Diversity Parameter Drivers of Gorilla (*Gorilla gorilla gorilla*) Microbiome Fecal Richness and Shannon Diversity in Samples Collected from Denver, NC and Riverbanks Zoos<sup>1</sup>.

Diversity Parameter	Richness	Shannon Diversity
<b>Age</b>	0.03485 * <sup>1</sup>	0.0004 *** <sup>1</sup>
<b>Diet</b>	0.01435 *	0.1959
<b>Housing</b>	0.007733 ** <sup>1</sup>	0.2194
<b>Social Group Size</b>	0.004093 **	0.1604
<b>Gorilla Individual</b>	0.02698 *	0.0037 **
<b>Institution</b>	0.002894 **	0.0861
<b>Month</b>	0.5052	0.2657
<b>Sex</b>	0.6291	0.8202
<b>Order</b>	0.825	0.9272

<sup>1</sup>Asterisks Indicate that Diversity Parameter was Associated with Significant Differences in Microbial Diversity, as Follows:

\* p<0.05

\*\* p<0.01

\*\*\*p<0.001

Table 4: All Diversity Parameters Except Month and Order of Collection Significantly Affected Bray-Curtis Dissimilarity Across Gorilla (*Gorilla gorilla gorilla*) Fecal Microbiome Samples Collected at Denver, NC and Riverbanks Zoos<sup>1,2</sup>.

<b>Diversity Parameter</b>	<b>p-value</b>		<b>R-value</b>
<b>Age</b>	0.001	***	0.4563
<b>Diet</b>	0.001	***	0.7766
<b>Housing</b>	0.001	***	0.6487
<b>Social group size</b>	0.001	***	0.4660
<b>Gorilla Individual</b>	0.001	***	0.8094
<b>Institution</b>	0.001	***	0.5722
<b>Month</b>	0.341		0.0039
<b>Sex</b>	0.001	***	0.1585
<b>Order</b>	0.922		-0.0213

<sup>1</sup>Diversity Parameter Effects Were Tested Using Anova ANOSIM. 999 Permutations Ran Per Parameter Test.

<sup>2</sup>Asterisks Indicate that Diversity Parameter was Associated with Significant Differences in Microbial Composition, as Follows:

\*\*\*p<0.001

Table 5: Multiple Comparisons Permanova for Gorilla (*Gorilla gorilla gorilla*) Fecal Microbiome Among Several Potential Diversity Parameters for all Fecal, Diet and Environmental Samples Collected at Denver, NC and Riverbanks Zoos<sup>1,2,3,4</sup>.

Diversity Comparison computation	P value		R <sup>2</sup>	Diversity Comparison computation	P value		R <sup>2</sup>
<b>Age</b> <sup>2</sup>	0.001	**** <sup>4</sup>	0.02173	<b>Individual</b>	0.001	***	0.53984
+Diet	0.001	***	0.02887	+Diet	NA <sup>3</sup>		NA
+Institution	0.001	***	0.21656	+Institution	NA		NA
+Housing	0.001	***	0.28001	+Housing	NA		NA
+Sex	0.001	***	0.05564	+Sex	NA		NA
+Month	0.531		0.03111	+Month	0.002	**** <sup>4</sup>	0.02588
+Individual	0.001	***	0.52258	+Age	0.025	* <sup>4</sup>	0.00459
+Order	0.999		0.05947	+Order	0.040	*	0.04793
+Social Group Size	0.001	***	0.09887	+Social Group Size	0.003	**	0.00736
<b>Institution</b>	0.001	***	0.21246	<b>Diet</b>	0.001	***	0.03003
+Diet	0.001	***	0.03386	+Age	0.001	***	0.02057
+Age	0.001	***	0.02584	+Institution	0.001	***	0.21629
+Housing	0.001	***	0.07218	+Housing	0.001	***	0.27908
+Sex	0.001	***	0.04006	+Sex	0.001	***	0.05628
+Month	0.335		0.02718	+Month	0.510		0.03096
+Individual	0.001	***	0.32738	+Individual	0.001	***	0.50981
+Order	1		0.04836	+Order	0.999		0.05927
+Social Group Size	0.001	***	0.04278	+Social Group Size	0.001	***	0.09896
<b>Housing</b>	0.001	***	0.28464	<b>Sex</b>	0.001	***	0.05649
+Diet	0.001	***	0.02447	+Diet	0.001	***	0.02982
+Institution	NA		NA	+Institution	0.001	***	0.19604
+Age	0.001	***	0.01710	+Age	0.001	***	0.02088
+Sex	0.001	***	0.02606	+Housing	0.001	***	0.25421
+Month	0.165		0.02687	+Month	0.429		0.03110
+Individual	0.001	***	0.25520	+Individual	0.001	***	0.48335
+Order	0.986		0.04889	+Order	0.998		0.06017
+Social Group Size	0.012	**	0.00842	+Social Group Size	0.001	***	0.08701

Table 5: (continued)

<b>Diversity Comparison computation</b>	<b>P value</b>		<b>R<sup>2</sup></b>	<b>Diversity Comparison computation</b>	<b>P value</b>		<b>R<sup>2</sup></b>
<b>Social Group Size</b>	0.001	***	0.09560	<b>Month</b>	0.022	**	0.03132
+Diet	0.001	***	0.03340	+Housing	0.001	***	0.28020
+Institution	0.001	***	0.15964	<b>Month</b>	0.001	***	0.03132
+Age	0.001	***	0.02501	+Individual	0.001	***	0.53441
+Sex	0.001	***	0.04790	+Sex	0.001	***	0.03373
+Housing	0.001	***	0.19746	<b>Order</b>	0.001	***	0.05946
+Individual	0.001	***	0.45161	+Individual	0.001	***	0.52831
+Order	1.00		0.05216	<b>Month</b>	0.001	***	0.03132
+Month	0.374		0.02391	+Individual	0.001	***	0.53441

<sup>1</sup>Comparisons are based on Euclidian Distances with 999 Permutations Run Per Test.

<sup>2</sup>Order of Diversity Parameter is Significant for this Test. 72 Computations in total were Run. First Variable of the Computation Indicated By Bold Lettering, i.e. **Age**+Individual.

<sup>3</sup>Inability to Test One Diversity Parameter Against Another Diversity Parameter Indicated by NA.

<sup>4</sup>Asterisks Indicate that Diversity Parameter was Associated with Significant Differences in Microbial Composition, as Follows: \* p<0.05; \*\* p<0.01; \*\*\*p<0.001

Table 6: Permanova Pairwise Interaction Test for Gorilla (*Gorilla gorilla gorilla*) Fecal Microbiome Among Several Potential Diversity Parameters for all Fecal, Diet and Environmental Samples Collected at Denver, NC and Riverbanks Zoo<sup>1,2,3,4</sup>.

Diversity Parameter Interaction Computation	P value		R <sup>2</sup>	Combined P value		Combined R <sup>2</sup>
<b>Age<sup>2</sup></b>	0.001	*** <sup>4</sup>	0.02173			
*Diet	0.001	***	0.02887	0.001	***	0.04239
*Institution	0.001	***	0.21656	0.001	***	0.05060
*Housing	0.001	***	0.28001	0.001	***	0.04062
*Sex	0.001	***	0.05564	0.001	***	0.03543
*Month	0.566		0.0311	1.00		0.01817
*Individual	0.001	***	0.52258	0.145		0.00670
*Order	1.00		0.05847	1.00		0.04345
*Social Group Size	0.001	***	0.09887	0.001	***	0.03362
<b>Institution</b>	0.001	***	0.21246			
*Diet	0.001	***	0.03386	0.002	*** <sup>4</sup>	0.01070
*Age	0.001	***	0.02584	0.001	***	0.05060
*Housing	0.001	***	0.07218	NA		NA
*Sex	0.001	***	0.04006	0.001	***	0.03948
*Month	0.297		0.02718	0.297		0.05408
*Individual	0.001	***	0.32738	NA		NA
*Order	1.00		0.04836	1.00		0.09015
*Social Group Size	0.001	***	0.04278	0.001	***	0.03671
<b>Housing</b>	0.001	***	0.28464			
*Diet	0.001	***	0.02447	NA		NA
*Institution	NA		NA	NA		NA
*Age	0.001	***	0.01710	0.001	***	0.04062
*Sex	0.001	***	0.02606	0.001	***	0.03735
*Month	0.141		0.02687	0.335		0.09661
*Individual	0.001	***	0.25520	NA		NA
*Order	1.00		0.04889	1.00		0.16172
*Social Group Size	0.010	**	0.00842	0.032	* <sup>4</sup>	0.00707
<b>Month</b>	0.001	***	0.03132			
*Individual	0.001	***	0.53441	0.001	***	0.25740
<b>Diet</b>	0.001	***	0.03003			
*Month	0.551		0.03096	0.912		0.02597
*Institution	0.001	***	0.21629	0.001	***	0.01070
*Housing	0.001	***	0.27908	NA		NA
*Sex	0.001	***	0.05628	0.001	***	0.05103
*Age	0.001	***	0.02057	0.001	***	0.04239
*Individual	0.001	***	0.50981	NA		NA
*Order	1.00		0.05927	1.00		0.04687
*Social Group Size	0.001	***	0.09896	0.001	***	0.07301

Table 6: (continued)

<b>Diversity Parameter Interaction Computation</b>	<b>P value</b>		<b>R<sup>2</sup></b>	<b>Combined P value</b>		<b>Combined R<sup>2</sup></b>
<b>Individual</b>	0.001	***	0.53984			
*Diet	NA		NA	NA		NA
*Institution	NA		NA	NA		NA
*Housing	NA		NA	NA		NA
*Sex	NA		NA	NA		NA
*Month	0.001	***	0.02588	0.002	**	0.25740
*Age	0.055		0.00447	0.169		0.00670
*Order	0.263		0.04793	0.418		0.40972
*Social Group Size	0.004	**	0.00736	0.136		0.02753
<b>Sex</b>	0.001	***	0.05649			
*Diet	0.001	***	0.02982	0.001	***	0.05103
*Institution	0.001	***	0.19604	0.001	***	0.03948
*Housing	0.001	***	0.25421	0.001	***	0.03735
*Age	0.001	***	0.02088	0.001	***	0.03543
*Month	0.445		0.03110	0.994		0.02153
*Individual	0.001	***	0.48335	NA		NA
*Order	1.00		0.06017	1.00		0.05253
*Social Group Size	0.001	***	0.08701	0.003	**	0.01285
<b>Social Group Size</b>	0.001	***	0.09560			
*Diet	0.001	***	0.03340	0.001	***	0.07301
*Institution	0.001	***	0.12793	0.001	***	0.07020
*Housing	0.001	***	0.19746	0.031	*	0.00707
*Sex	0.001	***	0.04790	0.001	***	0.01285
*Month	0.491		0.02899	0.646		0.02757
*Individual	0.001	***	0.45161	0.134		0.02753
*Order	1.00		0.05216	1.00		0.04200
*Age	0.001	***	0.02501	0.001	***	0.03362

<sup>1</sup>Comparisons are based on Euclidian Distances with 999 Permutations Run Per Test.

<sup>2</sup>Order of Diversity Parameter is Significant for this Test. 72 Computations in total were Run. First Variable of the Computation Indicated by Bold Lettering, i.e. **Age**\*Individual.

<sup>3</sup>Inability to Test One Diversity Parameter Against Another Diversity Parameter Indicated by NA.

<sup>4</sup>Asterisks Indicate that Diversity Parameter was Associated with Significant Differences in Microbial Composition, as Follows: \* p<0.05; \*\* p<0.01; \*\*\*p<0.001

Table 7: All Diversity Parameters Except Month and Order of Collection Significantly Affected Bray-Curtis Dissimilarity Across Gorilla (*Gorilla gorilla gorilla*) Fecal Microbiome Samples Collected at Denver Zoo<sup>1,2</sup>.

<b>Diversity Parameter</b>	<b>p-value</b>		<b>R-value</b>
<b>Antibiotic</b>	1.000		-0.3200
<b>Age</b>	0.004	*** <sup>2</sup>	0.2144
<b>Housing</b>	0.001	***	0.1976
<b>Individual</b>	0.002	* <sup>2</sup>	0.2069
<b>Month</b>	0.023	*	0.1165
<b>Social Group Size</b>	0.001	***	0.2298
<b>Sex</b>	0.164		0.0681
<b>Order</b>	0.876		-0.0983

<sup>1</sup>Diversity Parameter Effects Were Tested Using ANOSIM. 999 Permutations Ran Per Parameter Test.

<sup>2</sup>Asterisks Indicate that Diversity Parameter was Associated with Significant Differences in Microbial Composition, as Follows:

\* p<0.05

\*\*\*p<0.001

Table 8: All Diversity Parameters Except Month and Order of Collection Significantly Affected Bray-Curtis Dissimilarity Across Gorilla (*Gorilla gorilla gorilla*) Fecal Microbiome Samples Collected at NC Zoo<sup>1,2</sup>.

<b>Diversity Parameter</b>	<b>p-value</b>		<b>R-value</b>
<b>Antibiotic</b>	0.682		-0.1412
<b>Age</b>	0.005	** <sup>2</sup>	0.1593
<b>Individual</b>	0.001	*** <sup>2</sup>	0.8032
<b>Month</b>	0.342		0.0114
<b>Sex</b>	0.417		0.0110
<b>Order</b>	1.00		-0.1286

<sup>1</sup>Diversity Parameter Effects Were Tested Using ANOSIM. 999 Permutations Ran Per Parameter Test.

<sup>2</sup>Asterisks Indicate that Diversity Parameter was Associated with Significant Differences in Microbial Composition, as Follows:

\*\* p<0.01

\*\*\*p<0.001

Table 9: All Diversity Parameters Except Month and Order of Collection Significantly Affected Bray-Curtis Dissimilarity Across Gorilla (*Gorilla gorilla gorilla*) Fecal Microbiome Samples Collected at Riverbanks Zoo<sup>1,2</sup>.

<b>Diversity Parameter</b>	<b>p-value</b>		<b>R-value</b>
<b>Antibiotic</b>	0.875		-0.2641
<b>Age</b>	0.001	** <sup>2</sup>	0.6414
<b>Housing</b>	0.001	*** <sup>2</sup>	0.5513
<b>Social group size</b>	0.001	***	0.5513
<b>Individual</b>	0.001	***	0.8523
<b>Month</b>	0.860		-0.0351
<b>Sex</b>	0.001	***	0.5600
<b>Order</b>	1.000		-0.1454

<sup>1</sup>Diversity Parameter Effects Were Tested Using ANOSIM. 999 Permutations Ran Per Parameter Test.

<sup>2</sup>Asterisks Indicate that Diversity Parameter was Associated with Significant Differences in Microbial Composition, as Follows:

\*\* p<0.01

\*\*\*p<0.001

## FIGURES

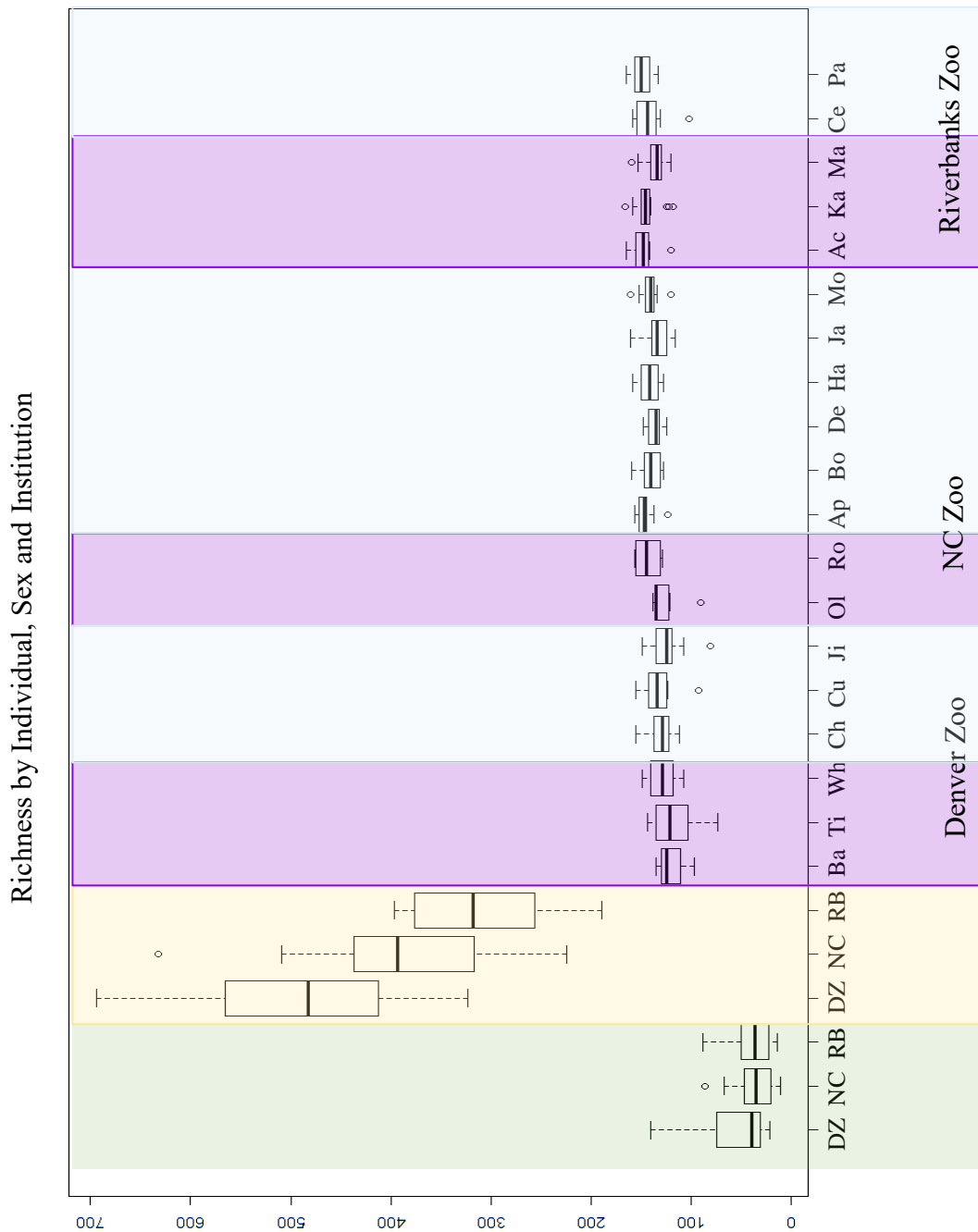


Figure 1: Plot of Species Richness of Gorilla (*Gorilla gorilla gorilla*) Fecal, Diet and Environmental Microbiome Samples Collected at Denver, NC and Riverbanks Zoos. Based on Individual, Institution, and Sample Type<sup>1,2,3</sup>.

<sup>1</sup>For fecal samples purple box indicated female, blue box indicates male

<sup>2</sup>Diet samples indicated by the green box. DZ=Denver Zoo, NC= NC Zoo, RB= Riverbanks Zoo

<sup>3</sup>Environmental samples indicated by the yellow box, DZ=Denver Zoo, NC= NC Zoo, RB= Riverbanks Zoo.

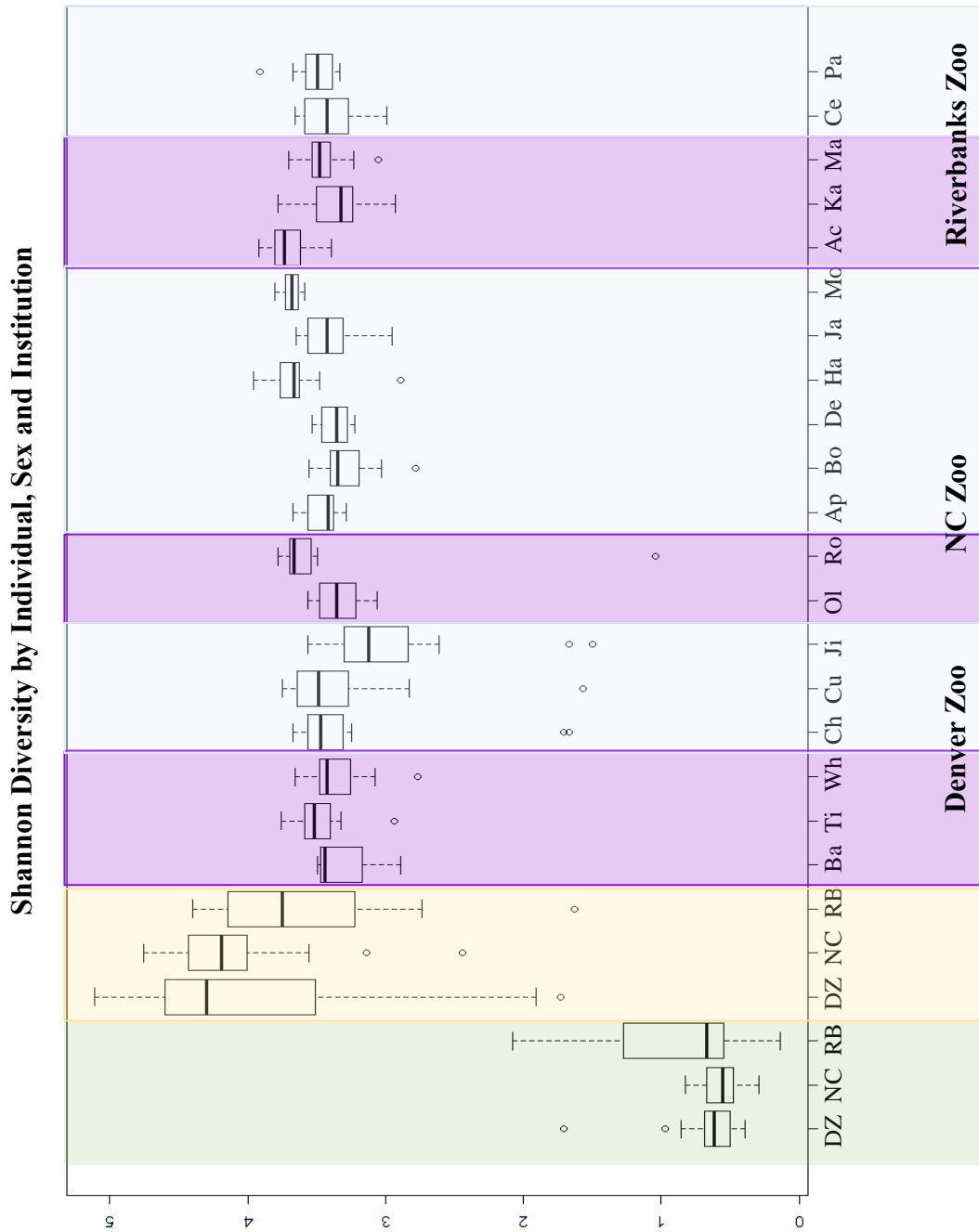


Figure 2: Plot of Shannon Diversity of Gorilla (*Gorilla gorilla gorilla*) Fecal, Diet and Environmental Microbiome Samples Collected at Denver, NC and Riverbanks Zoos. Based on Individual, Institution, and Sample Type<sup>1,2,3</sup>.

<sup>1</sup>For fecal samples purple box indicated female, blue box indicates male

<sup>2</sup>Diet samples indicated by green box, DZ=Denver Zoo, NC= NC Zoo, RB= Riverbanks Zoo

<sup>3</sup>Environmental samples indicated by the yellow box, DZ=Denver Zoo, NC= NC Zoo, RB= Riverbanks Zoo.

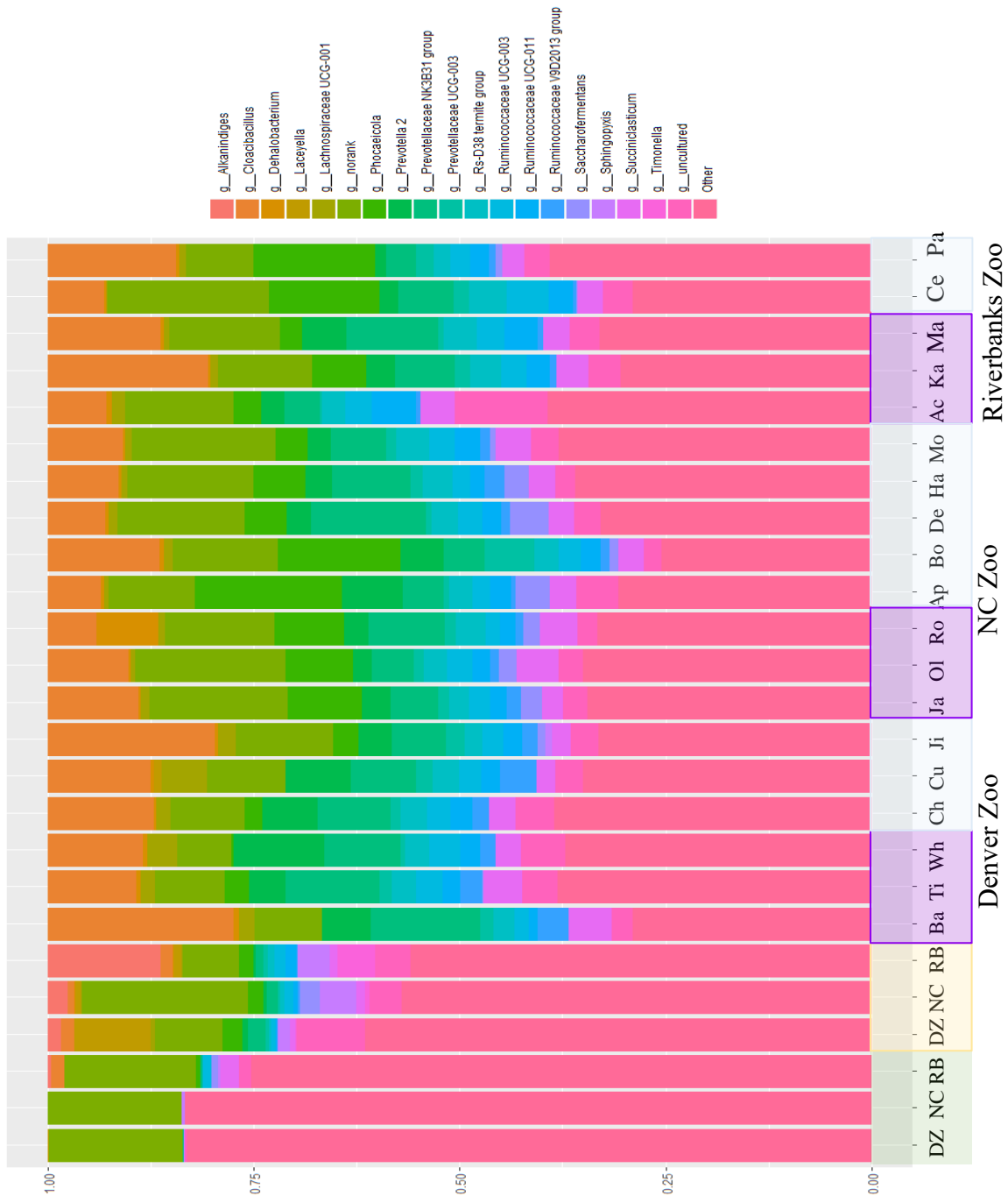


Figure 3: Relative Abundance of the Top 20 Microbial Genera Reported for Gorilla (*Gorilla gorilla gorilla*) Fecal, Diet and Environmental Microbiome Samples Collected at Denver, NC and Riverbanks Zoos. Grouped by Individual<sup>1,2,3</sup>.

<sup>1</sup>For fecal samples purple box indicated female, blue box indicates male

<sup>2</sup>Diet samples indicated by green box, DZ=Denver Zoo, NC= NC Zoo, RB= Riverbanks Zoo.

<sup>3</sup>Environmental samples indicated by the yellow box, DZ=Denver Zoo, NC= NC Zoo, RB= Riverbanks Zoo.

RBE	2.8	13.4	1.5	0.7	0.9	0.9	1.1	2.6	6.7	1.2	0.8	1.8	1.1	2	1.2	1.5	4	3.7	4.2	4.2	13.1	30.7
NCE	0.6	3	1.3	2.8	1	1.7	1.2	2.1	7.3	1.9	1.9	2.2	2	2.7	1.7	1.1	5.7	1.7	0.8	4.5	14.2	38.4
DZE	0.4	1.8	0.6	0.1	13.2	0.9	2.5	0.6	5.9	0.9	0.5	0	1	0.3	0.7	0.4	1.5	4	0.3	8.7	8.9	46.8
	g__Aerosphaera	g__Alkanindiges	g__Cloacibacillus	g__Corynebacterium 1	g__Laceyella	g__Larkinella	g__Lawsonella	g__Micrococcus	g__norank	g__Noviherbaspirillum	g__Pediococcus	g__Phocaeicola	g__Prevotellaceae NK3B31 group	g__Proteus	g__Pseudovibrio	g__Ruminococcaceae UCG-003	g__Sphingopyxis	g__Thiopseudomonas	g__Timonella	g__uncultured	Other	Other1

Figure 4: Heat Map of Detected Microbiome Taxa Based on Relative Abundance for Taxa Found at Abundance level 0.1% or Greater for Environmental Microbiome Samples at Denver, NC and Riverbanks Zoo<sup>1</sup>.

<sup>1</sup>RBE= Riverbanks Zoo, NCE= NC Zoo, DZE= Denver Zoo.

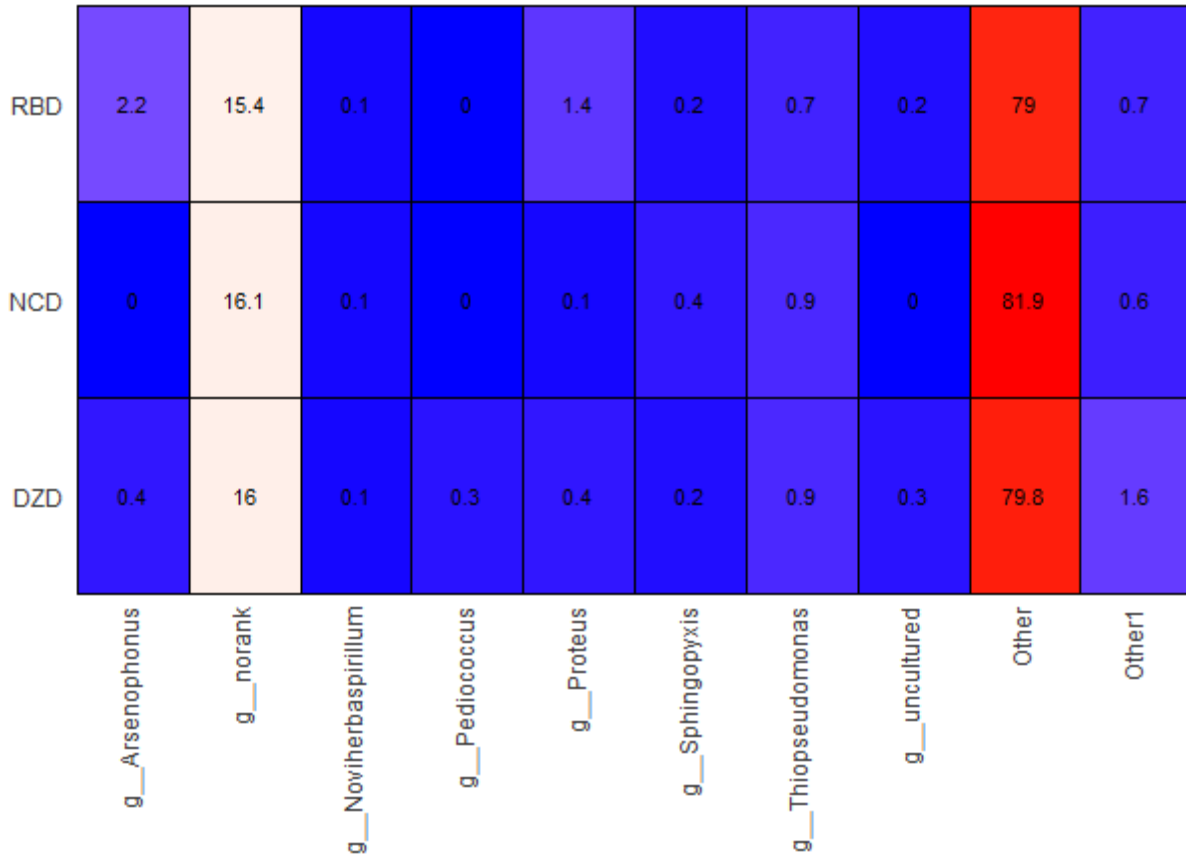


Figure 5: Heat Map of Detected Microbiome Taxa Based on Relative Abundance of Microbial Taxa Found at 0.1% or Greater Abundance Level for Diet Microbiome Samples at Denver, NC and Riverbanks Zoo<sup>1</sup>.

<sup>1</sup>RBD= Riverbanks Zoo, NCD= NC Zoo, DZD= Denver Zoo.

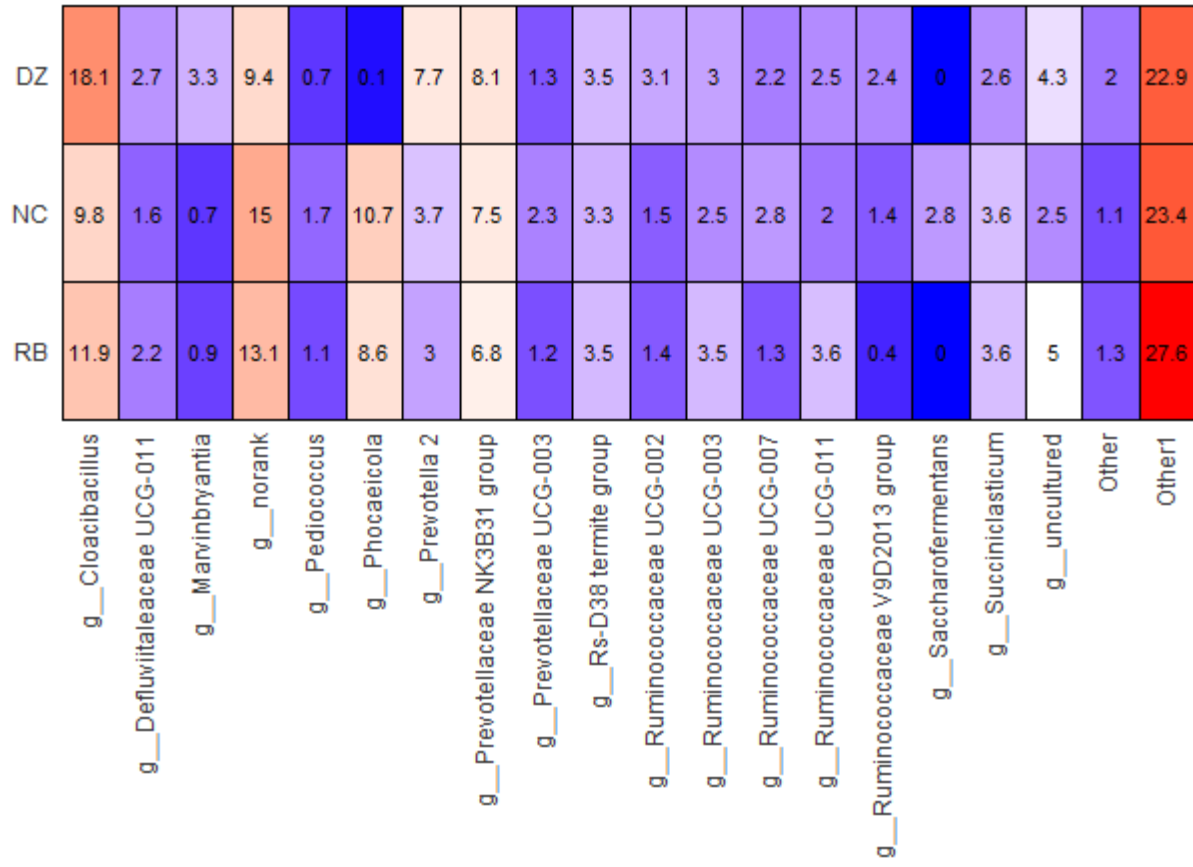


Figure 6: Heat Map of Detected Microbiome Taxa Based on Relative Abundance of Microbial Taxa Found at 1% or Greater Abundance Level for Gorilla (*Gorilla gorilla gorilla*) Fecal Microbiome Samples at Denver, NC and Riverbanks Zoo<sup>1</sup>.  
<sup>1</sup>RB= Riverbanks Zoo, NC= NC Zoo, DZ= Denver Zoo.

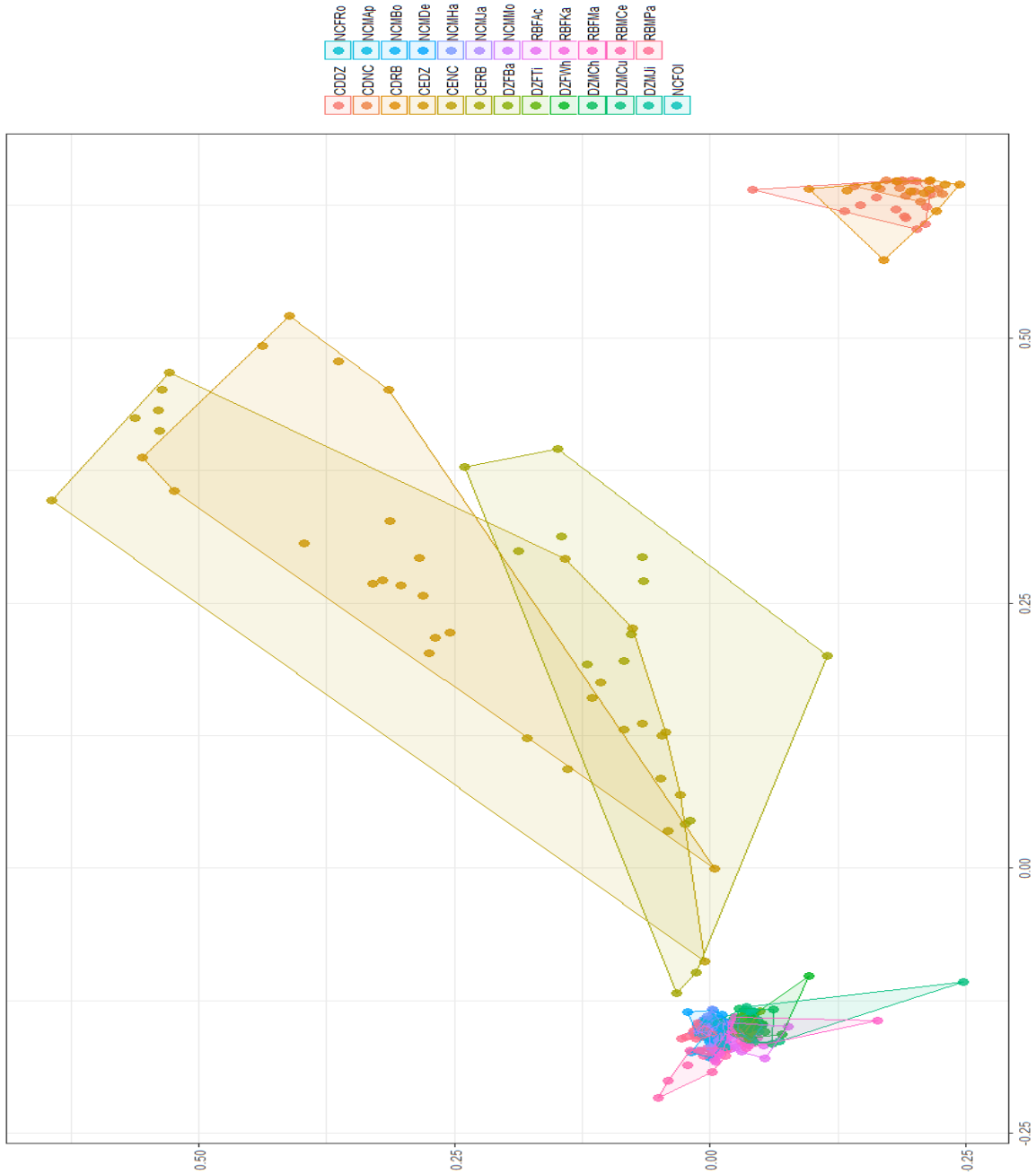


Figure 7: Diet, Environment and Fecal Microbiome Samples Form Distinct Clusters of Sample Type Based on Microbial Membership, as Measured by Non-Metric Multidimensional Scaling (NMDS). Grouping Based on Individual Sample Name<sup>1,2,3,4</sup>.

<sup>1</sup>For Fecal Samples First Letter Indicates institution sample was collected at, final two letters indicate first two letters of individual gorilla's name.

<sup>2</sup>Diet samples coded CD for the first two letters, final two letter indicate institution collected from.

<sup>3</sup>Environmental samples as coded CE for the first two. Final two letters indicate institution collected from.

<sup>4</sup>Samples closer together contain more similar microbial communities.

### Richness by Institution

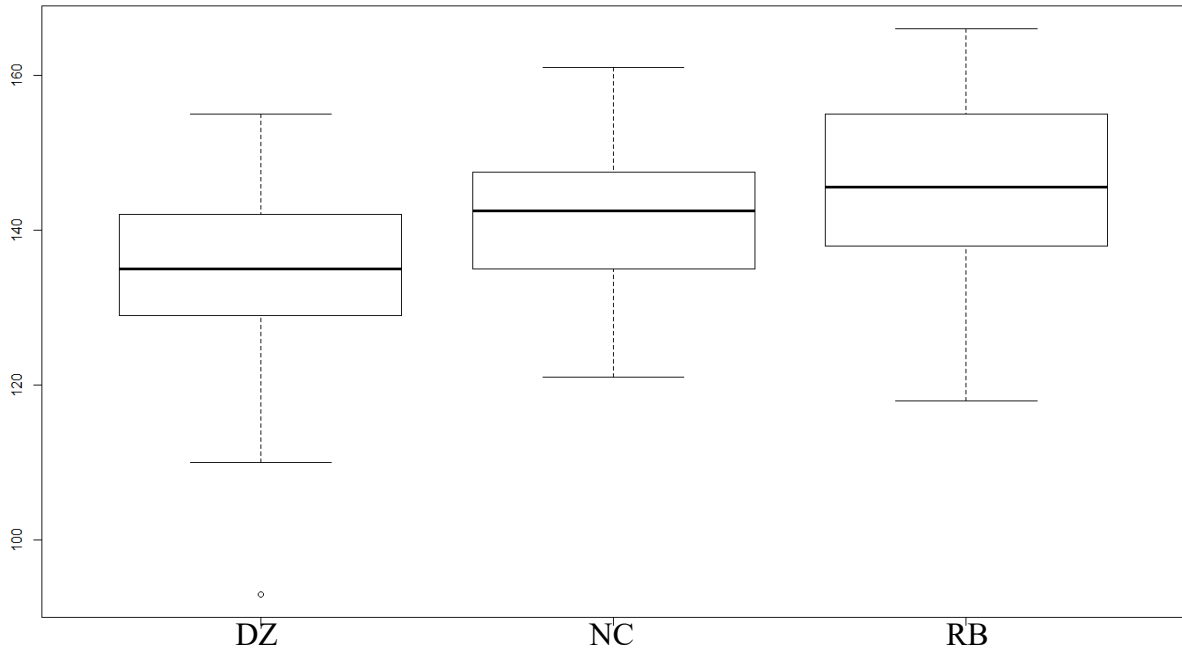


Figure 8: Plot of Species Richness of Gorilla (*Gorilla gorilla gorilla*) Fecal Microbiome Samples Collected at Denver, NC and Riverbanks Zoos. Grouped by the Institution Sample was Collected From<sup>1</sup>.

<sup>1</sup>DZ= Denver Zoo, NC=NC Zoo, and RB= Riverbanks Zoo

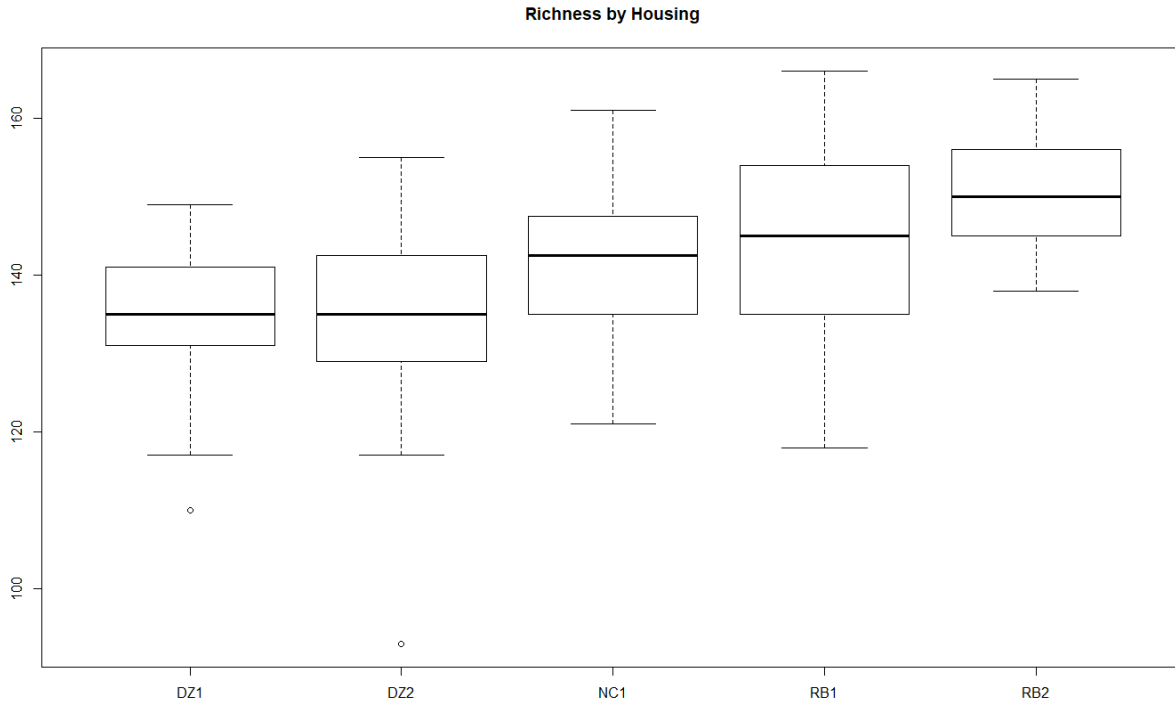


Figure 9: Plot of Species Richness of Gorilla (*Gorilla gorilla gorilla*) Fecal Microbiome Samples Collected at Denver, NC and Riverbanks Zoos. Grouped by the Housing of Individual Gorilla<sup>1,2</sup>.  
<sup>1</sup>Number of individuals per group are reported (number of males.number of female) (i.e. 1.1= one male. one female).  
<sup>2</sup>Social Group Size for each housing group are DZ1=1.2, DZ2=2.0, NC1=5.2, RB1=1.3, RB2=1.0.

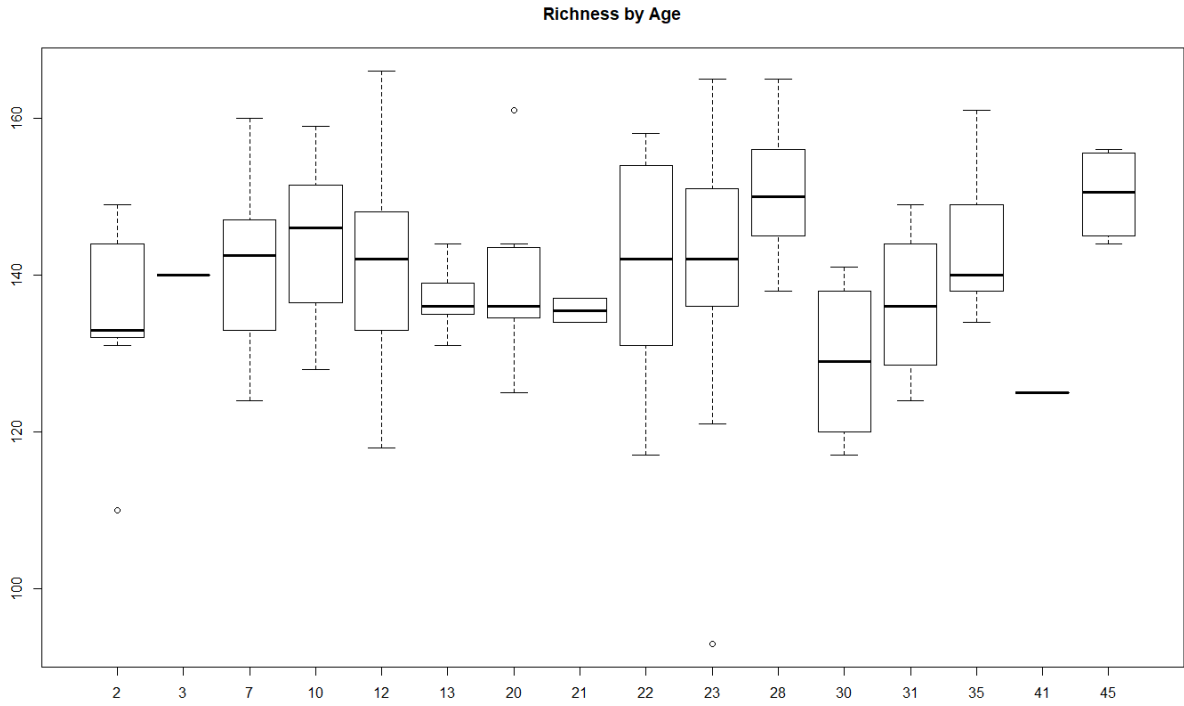


Figure 10: Plot of Species Richness of Gorilla (*Gorilla gorilla gorilla*) Fecal Microbiome Samples Collected at Denver, NC and Riverbanks Zoos. Grouped by Age of the Individual Gorilla.

RBMPa	13.1	1.4	1.9	7.7	3.7	15.8	0.9	2.7	2.2	1.8	1.4	2.3	0.6	2.5	0.3	0	3.5	3.8	1	33.4	
RBMCe	6.6	1.1	0.1	18.8	0	13.9	2.9	6.8	1.5	4.4	1.3	5.1	0.9	2.7	0.3	0	3.1	3.7	1	25.8	
RBFMa	14.3	4	0	11.1	0.3	0.6	6.1	11.6	0.3	2.6	1	3.9	1.4	4.8	0.7	0	3	3.9	1.8	28.7	
RBFKa	16.4	1.5	1.2	14.2	0.7	8.7	2.2	7.9	1.6	5.5	1.3	2.8	1.5	2.7	0.3	0	4.4	3.9	1.1	21.8	
RBFaC	7.3	3	1.5	13.9	1	3.6	2.6	4.1	0.3	2.9	1.9	3.2	2.2	5.7	0.6	0	4.3	1.1	1.7	29.2	
NCMMo	8.9	2	0.4	19.3	3.9	5.8	1.9	4.1	1.6	4.3	1.7	2.8	2.6	1.8	1.4	1.6	4.8	2.7	0.7	27.7	
NCMHa	8.3	1	0.5	15.9	2.8	5.8	3.1	7.7	1.3	4.3	1.3	1.7	2.5	1.2	2.3	3.1	3.7	1.9	1.1	30.6	
NCMDe	7.6	1.2	0.4	16.1	2	6.5	2.6	15.6	0.9	2.6	1.7	2.9	3.6	2.2	1.3	5.3	2.9	2.5	1.2	21.1	
NCMBo	13.6	1.9	0.8	13.7	0.1	15.9	4.4	4.3	6.7	3.2	1.2	2.7	1.8	2.5	1.1	0.6	3.2	1.9	0.9	19.2	
NCMAp	7	1.3	1	10.9	0.9	18.8	8.4	5.4	0.8	2.1	1.4	1.9	2.3	2.1	0.6	4.7	3	3.5	0.9	23.2	
NCFRo	6	1.5	0.8	11.1	4.9	8.5	4.2	7.2	2.4	4.8	1.6	1.9	3.1	2.2	1.2	2.4	5.9	2.6	0.9	27	
NCFoI	13.3	1.8	0.9	15.1	0.6	10.4	2.1	8.9	0.9	2.3	1.1	3.3	3.6	1.8	1.5	1.1	5.3	2.4	1.1	22.6	
NCFJa	12.3	2.3	1	16.6	0.5	12.4	2.6	5.9	1.7	3.2	1.9	2.9	4.2	2.7	2.2	2.7	1.5	3.3	2	18	
DZMJi	27.3	1.8	3.4	6.9	1	0.2	7	7.7	2.5	1.9	2.7	2.7	1.7	2.2	1.4	0	3.2	3.4	1.7	21.5	
DZMCu	15.6	3.8	2	11.2	0.9	0.2	8.5	7.7	1.1	4.4	3	3	2.4	2.8	2.7	0	2.4	4	2.1	22.2	
DZMCh	17.4	3.1	3.8	10.1	0.5	0.2	7	7.1	1	3.5	3.6	3.1	2.7	3.3	2.1	0	1.7	5	2.1	22.7	
DZFWH	12.1	2.1	4.8	8.6	0.6	0	11.3	10.1	0.4	4	4.1	3.3	1.9	2.3	1	0	2.3	5.7	2.4	22.8	
DZFTi	16	2.2	3.1	9	0.9	0.1	5.2	9.3	1.2	3.6	2.1	3	1.9	1.7	5	0	3.7	4	1.7	26.3	
DZFBa	23.2	1.2	3.1	13.2	0.2	0	3	6.4	0.7	3.3	1.8	1.6	2.3	1.3	5.1	0	4.7	2.5	0.5	25.8	
g__Cloacibacillus																					
g__Defluviitaleaceae UCG-011																					
g__Marvinbryantia																					
g__norank																					
g__Pediococcus																					
g__Phocaeicola																					
g__Prevotella 2																					
g__Prevotellaceae NK3B31 group																					
g__Prevotellaceae UCG-003																					
g__Rs-D38 termite group																					
g__Ruminococcaceae UCG-002																					
g__Ruminococcaceae UCG-003																					
g__Ruminococcaceae UCG-007																					
g__Ruminococcaceae UCG-011																					
g__Ruminococcaceae V9D2013 group																					
g__Saccharofermentans																					
g__Succiniclasticum																					
g__uncultured																					
Other																					
Other1																					

Figure 11: Heat Map of Detected Microbiome Taxa Based on Relative Abundance of Microbial Taxa Found at 1% or Greater Abundance Level for Gorilla (*Gorilla gorilla gorilla*) Fecal Microbiome Samples Collected at Denver, NC and Riverbanks Zoos. Grouped by the Individual<sup>1,2,3</sup>.

<sup>1</sup>First two letter of individual code stand for institution: DZ=Denver Zoo, NC= NC Zoo, and RB= Riverbanks Zoo.

<sup>2</sup>Third let of individual code stands for sex: F= female, M= male.

<sup>3</sup>Last two letters of the individual code are the first two letters of their name.

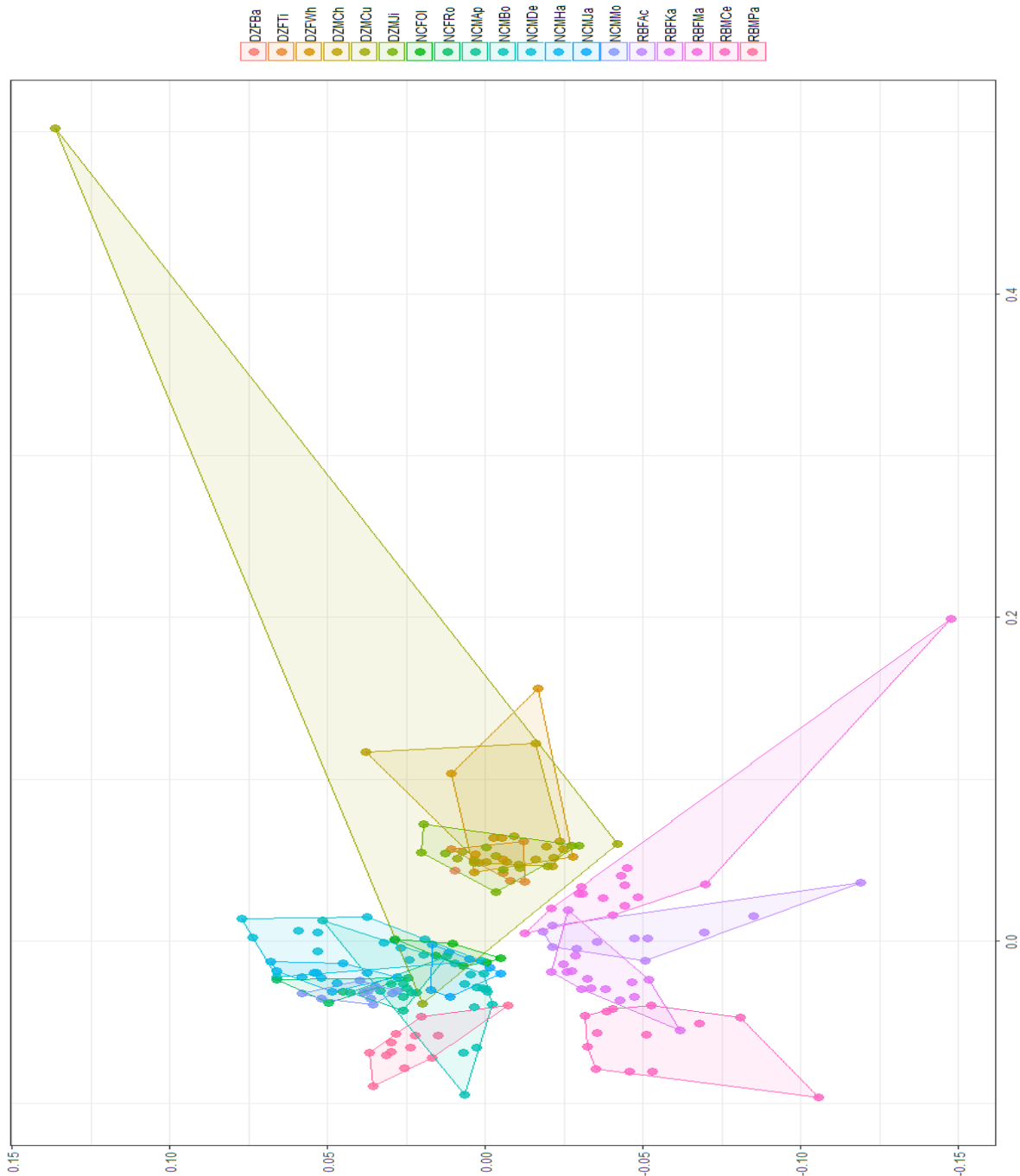


Figure 12: Plot of Ordination Analysis based on non-metric multidimensional scaling (NMDS) for Gorilla (*Gorilla gorilla gorilla*) for Fecal Samples Collected at Denver, NC and Riverbanks Zoos. Grouping Based by Individual Gorilla<sup>1,2,3,4</sup>.

<sup>1</sup>First letter indicates institution sample was collected: DZ= Denver Zoo, NC= NC Zoo, RB= Riverbanks Zoo

<sup>2</sup>Third letter in individual code stands for sex: F= female, M= male.

<sup>3</sup>Last two letters of the individual code are the first two letters of their name.

<sup>4</sup>Samples closer together contain more similar microbial communities.

RB2	13.1	1.4	1.9	7.7	3.7	15.7	0.9	2.8	2.3	1.8	1.3	2.3	0.7	2.4	0.3	0	3.4	3.8	1	33.5
RB1	11.6	2.4	0.7	14.4	0.5	6.8	3.4	7.8	1	3.9	1.3	3.8	1.5	3.8	0.5	0	3.7	5.3	1.4	26.2
NC1	9.8	1.6	0.7	15.1	1.8	10.7	3.8	7.6	2.3	3.3	1.5	2.5	2.8	2	1.4	2.7	3.5	2.5	1.1	23.3
DZ2	16.5	3.5	2.9	10.6	0.7	0.2	7.8	7.5	1.1	4	3.3	3.1	2.6	3	2.4	0	2	4.4	2.1	22.5
DZ1	19.7	1.9	3.7	8.3	0.8	0.1	7.7	8.7	1.5	2.9	2.9	2.9	1.9	2	2.4	0	3.1	4.2	1.8	23.4
	g__Cloacibacillus	g__Defluviitaleaceae UCG-011	g__Marvinbryantia	g__norank	g__Pediococcus	g__Phocaeicola	g__Prevotella 2	g__Prevotellaceae NK3B31 group	g__Prevotellaceae UCG-003	g__Rs-D38 termite group	g__Ruminococcaceae UCG-002	g__Ruminococcaceae UCG-003	g__Ruminococcaceae UCG-007	g__Ruminococcaceae UCG-011	g__Ruminococcaceae V9D2013 group	g__Saccharofermentans	g__Succiniasticum	g__uncultured	Other	Other1

Figure 13: Heat Map of Detected Microbiome Taxa Based on Microbial Taxa Relative Abundance Found at 1% or Greater Abundance Level for Gorilla (*Gorilla gorilla gorilla*) Fecal Microbiome Samples Collected at Denver, NC and Riverbanks Zoos. Grouped by Housing<sup>1,2</sup>.

<sup>1</sup>Number of individuals per group are reported number of males.number offemale (i.e. 1.1= one male, one female)

<sup>2</sup>Social group size in each housing group: DZ1=1.2, DZ2=2.0, NC1=5.2, RB1=1.3, RB2=1.0.

M	12	1.8	1	1.3	13.5	1.5	9.3	4.3	6.9	2.1	3.2	1.8	2.8	2.2	2.3	1.3	1.6	3.1	3.2	1.3	23.4
F	13.1	2.5	1	1.5	12.4	0.9	4.5	4.4	8.4	0.9	3.7	1.7	3.2	2	3.4	1.2	0.3	4	5	1.5	24.6
	g__Cloacibacillus	g__Defluviitaleaceae UCG-011	g__Lachnospiraceae UCG-001	g__Marvinbryantia	g__norank	g__Pediococcus	g__Phocaeicola	g__Prevotella 2	g__Prevotellaceae NK3B31 group	g__Prevotellaceae UCG-003	g__Rs-D38 termite group	g__Ruminococcaceae UCG-002	g__Ruminococcaceae UCG-003	g__Ruminococcaceae UCG-007	g__Ruminococcaceae UCG-011	g__Ruminococcaceae V9D2013 group	g__Saccharofermentans	g__Succinielasticum	g__uncultured	Other	Other <sup>1</sup>

Figure 14: Heat Map of Detected Microbiome Taxa Based on Microbial Taxa Relative Abundance Found at 1% or Greater Abundance Level for Gorilla (*Gorilla gorilla gorilla*) Fecal Samples Collected at Denver, NC and Riverbanks Zoos. Grouped by Sex of Gorilla<sup>1</sup>.  
<sup>1</sup>F= Female, M=Male.

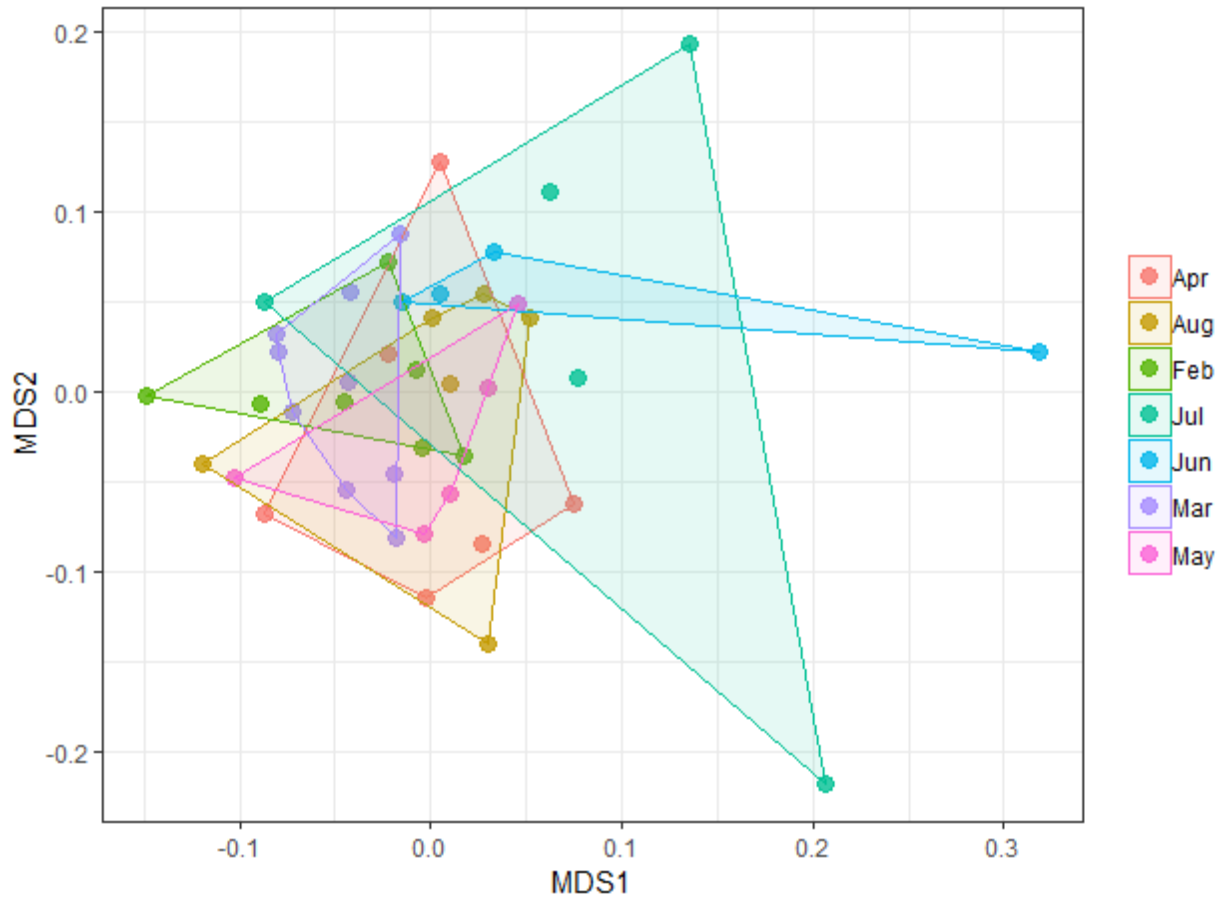


Figure 15: Plot of Ordination Analysis Based on Non-Metric Multidimensional Scaling (NMDS) for Gorilla (*Gorilla gorilla gorilla*) for Fecal Microbiome Samples Collected at Denver Zoo. Grouping Based on Month Sample Collected.

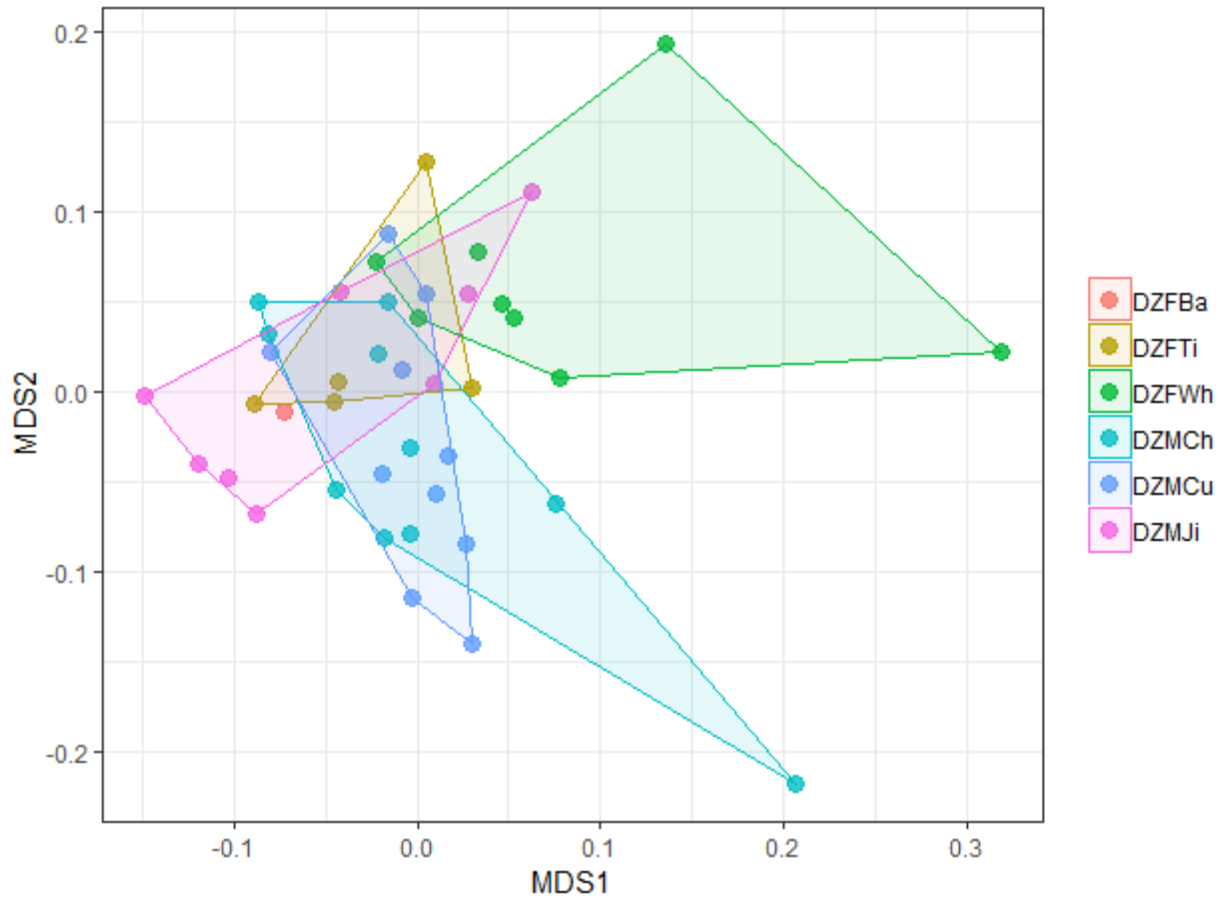


Figure 16: Plot of Ordination Analysis Based on Non-Metric Multidimensional Scaling (NMDS) for Gorilla (*Gorilla gorilla gorilla*) for Fecal Microbiome Samples Collected at Denver Zoo. Grouping Based on Individual Gorilla<sup>1,2,3,4</sup>.

<sup>1</sup>First two letters indicate institution collected from.

<sup>2</sup>Third letter indicated sex of individual: F=female, M=males.

<sup>3</sup>Final two letters stand for the first two letters of the individual's name.

<sup>4</sup>Samples closer together contain more similar microbial communities.

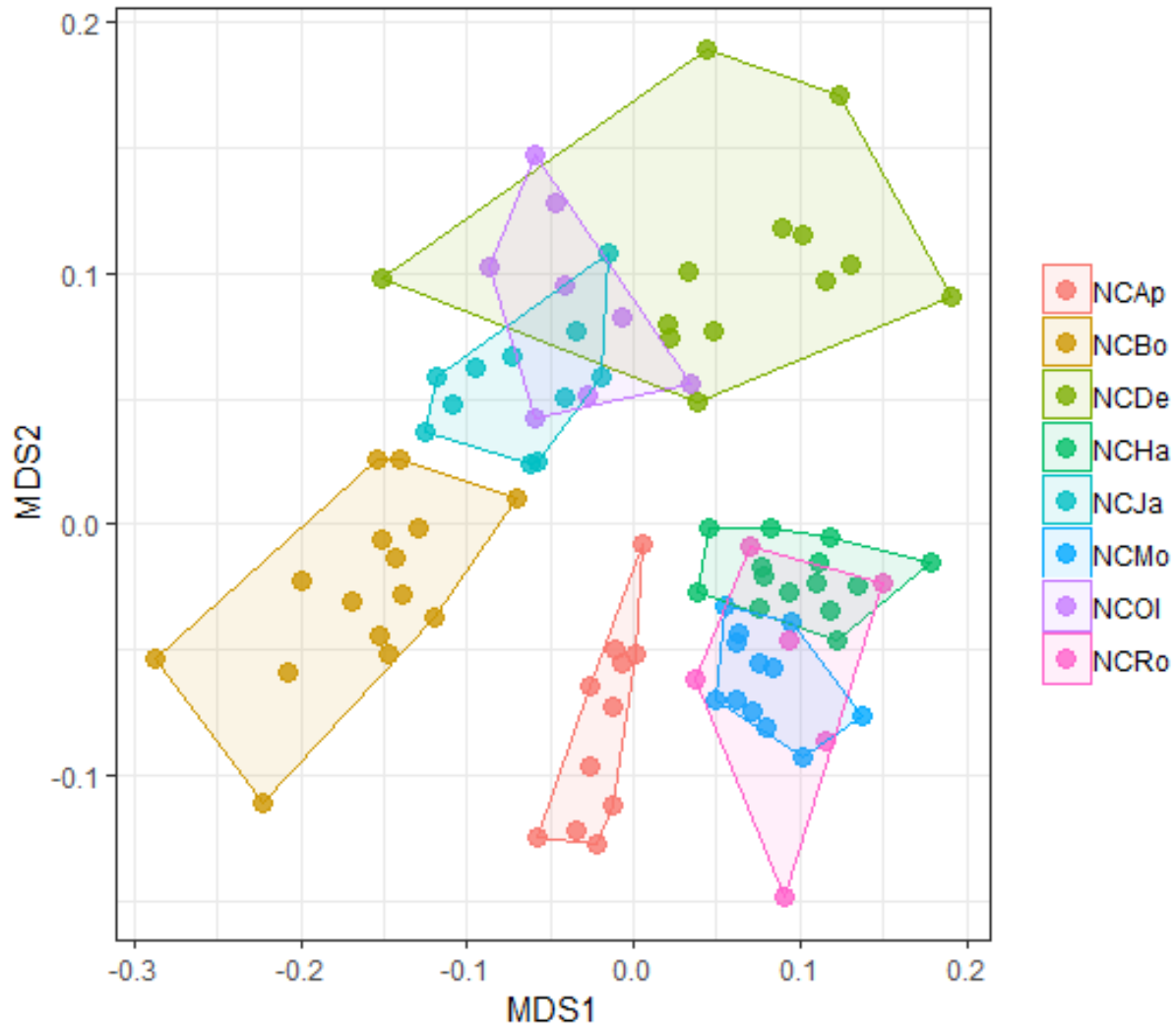


Figure 17: Plot of Ordination Analysis Based on Non-Metric Multidimensional Scaling (NMDS) for Gorilla (*Gorilla gorilla gorilla*) for Fecal Microbiome Samples Collected at NC Zoo.

Grouping Based on Individual Gorilla<sup>1,2,3,4</sup>.

<sup>1</sup>First two letters indicate institution collected from.

<sup>2</sup>Third letter indicated sex of individual: F=female, M=males.

<sup>3</sup>Final two letters stand for the first two letters of the individual's name.

<sup>4</sup>Samples closer together contain more similar microbial communities.

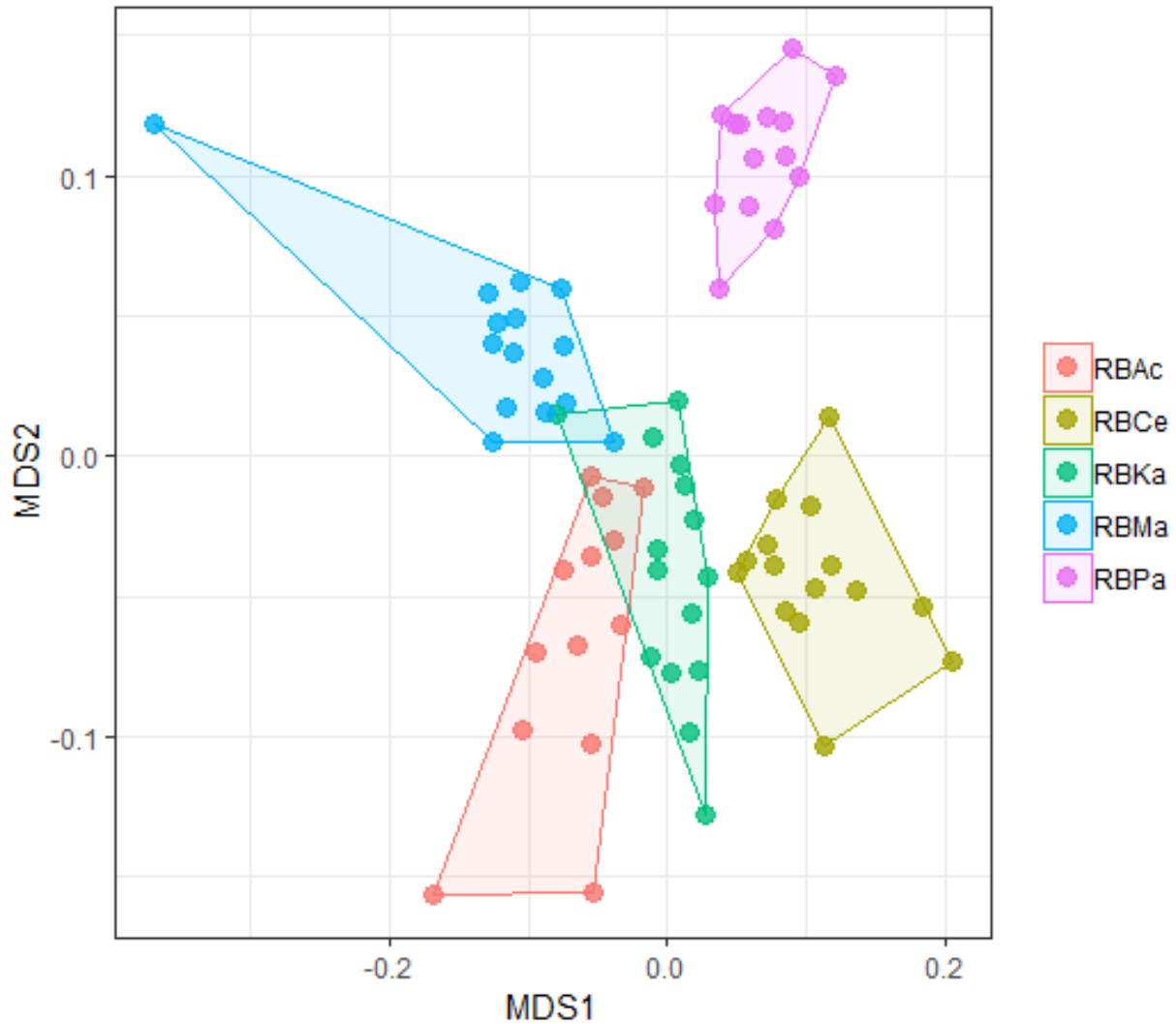


Figure 18: Plot of Ordination Analysis Based on Non-Metric Multidimensional Scaling (NMDS) for Gorilla (*Gorilla gorilla gorilla*) for Fecal Microbiome Samples Collected at Riverbanks Zoo. Grouping Based on Individual Gorilla<sup>1,2,3,4</sup>.

<sup>1</sup>First two letters indicate institution collected from.

<sup>2</sup>Third letter indicated sex of individual: F=female, M=males.

<sup>3</sup>Final two letters stand for the first two letters of the individual's name.

<sup>4</sup>Samples closer together contain more similar microbial communities.

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## APPENDICES

## Appendix A

### Denver Zoo Diet

Diet Item	Greens	Vegetables	Mazuri® L/S Primate Biscuit	Browse	Celery	Alfalfa Hay
Diet percentage	40%	20%	1.8%	15%	11.7%	11.5%

Greens include: Romaine, red leaf, green leaf, nappa cabbage, bok choy, kale and bibb lettuce. Two types of greens fed daily, rotated every day to two different greens than previous day. All types are fed within a single week.

Vegetables include: Green beans, red pepper, green pepper, eggplant, tomato, cauliflower, broccoli. Two types of vegetable fed daily, rotated every day to two different vegetables than previous day. All types fed within a single week.

Browse species vary depending on availability, consistent quantity given daily to a total of 1 kg per individual. Seasonal shift from browse grown at the zoo during the summer to browse grown in Arizona and Florida during the winter.

Alfalfa Hay, offered every other day at ½ flake per troop.

### NC Zoo Diet

Diet Item	Greens	Vegetable	Fruit	Avocado	Browse
Diet percentage	69%	26.6%	3%	1.4%	Few sticks

Greens include: Green leaf, kale, romaine, cabbage as the basic daily diet

Vegetable included: green pepper, green bean, carrot, and cucumber as the basic daily diet

Fruit included: Apple, pear, kiwi, banana, orange, grapes

Browse: offered daily at 2-3 limbs per individual of locally available species of plants.

### Riverbanks Zoo Diet

#### Standard Diet

Diet Item	Greens	Celery	Vegetable	Fruit	Browse
Diet Percent	62%	10%	26%	2%	variable

#### Pregnancy Diet

Diet Item	Butternut Squash	Sweet Potato	Mazuri® Leafeater Large Biscuit

Daily quantity (grams)	3.6%	2%	2%
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Greens include: Cabbage, kale and romaine

Vegetables include: bell pepper, carrot, cucumber and green beans

Fruits include: Kiwi, grapes and peeled orange

Browse is grown with the outside exhibit of the gorillas, therefore gorillas have voluntary access.

Occasionally browse is offered in the inside holding overnight.

## Appendix B

### Pictures of Denver Zoo Enclosure

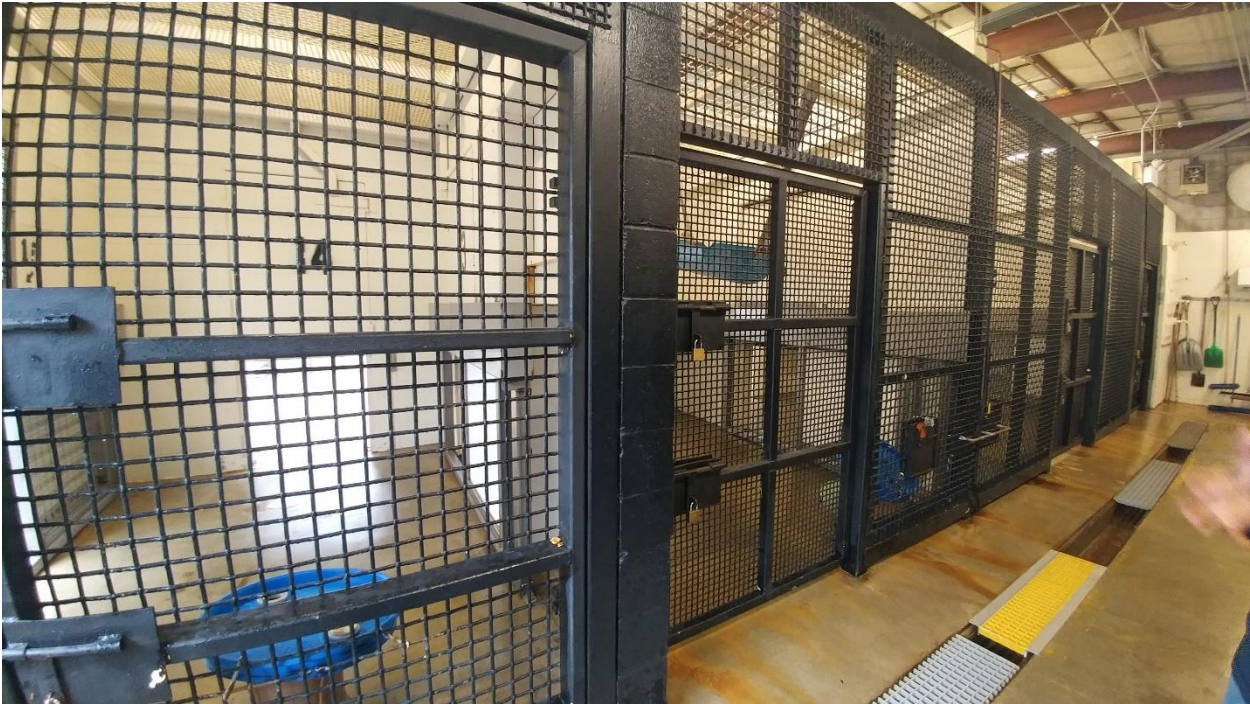








**Pictures for NC Zoo Enclosure**





**Pictures for Riverbanks Zoo Enclosure**





## Appendix C

### Modified DNA extraction from stool using QIAGEN DNEasy PowerSoil Kit

1. Add 60 uL Solution C1 to PowerBead Tube.
2. Add 0.25 g of sample to PowerBead Tube. Vortex ~3 seconds to mix.
3. Heat at 65°C for 10 minutes.
4. Vortex (horizontal) at maximum speed for 10 minutes ( 20 minutes for more than 10 samples)
5. Centrifuge at 10,000x g for 1 minute
6. Pipette supernatant into a 2-mL collection tube with C2 (250uL). (*Expect 400-500 uL supernatant.*)
7. Vortex for 1 minute.
8. Incubate at 2-8°C for 5 minutes.
9. Centrifuge at 10,000x g for 2 minutes.
10. Avoiding the pellet, pipette up to 600 uL supernatant into collection tube w/ Solution C3 (200uL)
11. Vortex briefly.
12. Incubate at 2-8°C for 5 minutes.
13. Centrifuge at 10,000x g for 2 minutes.
14. Shake Solution C4 preset collection tube
15. Avoiding the pellet, pipette up to 750 uL supernatant to Solution C4(1200 uL) collection tube
16. Vortex for 5 seconds.
17. Load 657 uL onto an MB Spin Column and centrifuge at 10,000x g for 2 minutes.
18. Discard flow-through.
19. Repeat step 17-18 until all of the sample has been processed.
20. Add 500 µL of Solution C5. Centrifuge at 10,000x g for 1 minute
21. Discard flow-through.
22. Centrifuge again at 10,000x g, for 2 minute.
23. Carefully place the MB Spin Column into a clean 2-mL Collection Tube. *Avoid splashing any Solution C5 onto the column.*
24. Add 100 µL of Solution C6 directly to the center of the white filter membrane.
25. Close cap and incubate at RT for 5 minutes.
26. Centrifuge at 10,000x g for 1 minute. →*Elute a second time* to increase yield.
27. SAVE FILTRATE AND DISCARD COLUMN.

28. Measure and record [DNA], i.e. using Nanodrop or Qubit.
29. Store DNA product at -80°C

### Appendix D

<b>Location</b>	<b>Description</b>	<b>Individual</b>
RB	Acacia, Riverbanks Zoo	RBAc
NC	Apollo, NC Zoo	NCAp
DZ	Bassa, Denver Zoo	DZBa
NC	Bomassa, NC Zoo	NCBo
RB	Cenzoo, Riverbanks Zoo	RBCe
DZ	Charlie, Denver Zoo	DZCh
DZ	Curtis, Denver Zoo	DZCu
NC	Dembe, NC Zoo	NCDe
NC	Hadari, NC Zoo	NCHa
NC	Jamani, NC Zoo	NCJa
DZ	Jim, Denver Zoo	DZJi
RB	Kazi, Riverbanks Zoo	RBKa
RB	Macy, Riverbanks Zoo	RBMa
NC	Mosuba, NC Zoo	NCMo
NC	Olympia, NC Zoo	NCOI
RB	Patrick, Riverbanks Zoo	RBPa
NC	Rosie, NC Zoo	NCRo
DZ	Tinga, Denver Zoo	DZTi
DZ	Whimsie, Denver Zoo	DZWh
DZ	Composite Diet Sample, Denver Zoo	DZCD
NC	Composite Diet Sample, North Carolina	NCCD
RB	Composite Diet Sample, Riverbanks Zoo	RBCD
DZ	Composite Environmental, Denver Zoo	DZCE
NC	Composite Environmental, NC Zoo	NCCE
RB	Composition Environmental, Riverbanks Zoo	RBCE
DZ	Exhibit Soil Sample, Denver Zoo	DZCS
NC	Exhibit Soil Sample, North Carolina	NCCS

<b>Sample ID</b>	<b>Location</b>	<b>Description</b>	<b>Individual</b>
1	RB	Acacia, Riverbanks Zoo	RBAc
2	RB	Acacia, Riverbanks Zoo	RBAc
3	RB	Acacia, Riverbanks Zoo	RBAc
4	RB	Acacia, Riverbanks Zoo	RBAc
5	RB	Acacia, Riverbanks Zoo	RBAc
6	RB	Acacia, Riverbanks Zoo	RBAc
7	NC	Apollo, North Carolina Zoo	NCAp
8	NC	Apollo, North Carolina Zoo	NCAp
9	NC	Apollo, North Carolina Zoo	NCAp
10	NC	Apollo, North Carolina Zoo	NCAp
11	DZ	Bassa, Denver Zoo	DZBa

12	DZ	Bassa, Denver Zoo	DZBa
13	DZ	Bassa, Denver Zoo	DZBa
14	NC	Bomassa, North Carolina Zoo	NCBo
15	NC	Bomassa, North Carolina Zoo	NCBo
16	NC	Bomassa, North Carolina Zoo	NCBo
17	NC	Bomassa, North Carolina Zoo	NCBo
18	NC	Bomassa, North Carolina Zoo	NCBo
19	NC	Bomassa, North Carolina Zoo	NCBo
20	NC	Bomassa, North Carolina Zoo	NCBo
21	NC	Bomassa, North Carolina Zoo	NCBo
22	RB	Cenzoo, Riverbanks Zoo	RBCe
23	RB	Cenzoo, Riverbanks Zoo	RBCe
24	RB	Cenzoo, Riverbanks Zoo	RBCe
25	RB	Cenzoo, Riverbanks Zoo	RBCe
26	RB	Cenzoo, Riverbanks Zoo	RBCe
27	RB	Cenzoo, Riverbanks Zoo	RBCe
28	RB	Cenzoo, Riverbanks Zoo	RBCe
29	RB	Cenzoo, Riverbanks Zoo	RBCe
30	DZ	Charlie, Denver Zoo	DZCh
31	DZ	Charlie, Denver Zoo	DZCh
32	DZ	Charlie, Denver Zoo	DZCh
33	DZ	Charlie, Denver Zoo	DZCh
34	DZ	Charlie, Denver Zoo	DZCh
36	DZ	Charlie, Denver Zoo	DZCh
37	DZ	Charlie, Denver Zoo	DZCh
38	DZ	Charlie, Denver Zoo	DZCh
39	DZ	Curtis, Denver Zoo	DZCu
40	DZ	Curtis, Denver Zoo	DZCu
41	DZ	Curtis, Denver Zoo	DZCu
42	DZ	Curtis, Denver Zoo	DZCu
43	DZ	Curtis, Denver Zoo	DZCu
44	DZ	Curtis, Denver Zoo	DZCu
45	DZ	Curtis, Denver Zoo	DZCu
46	DZ	Curtis, Denver Zoo	DZCu
47	DZ	Curtis, Denver Zoo	DZCu
48	NC	Dembe, North Carolina Zoo	NCDe
49	NC	Dembe, North Carolina Zoo	NCDe
50	NC	Dembe, North Carolina Zoo	NCDe
51	NC	Dembe, North Carolina Zoo	NCDe
52	NC	Dembe, North Carolina Zoo	NCDe
53	NC	Dembe, North Carolina Zoo	NCDe
54	NC	Hadari, North Carolina Zoo	NCHa
55	NC	Hadari, North Carolina Zoo	NCHa

56	NC	Hadari, North Carolina Zoo	NCHa
57	NC	Hadari, North Carolina Zoo	NCHa
58	NC	Hadari, North Carolina Zoo	NCHa
59	NC	Hadari, North Carolina Zoo	NCHa
60	NC	Hadari, North Carolina Zoo	NCHa
61	NC	Hadari, North Carolina Zoo	NCHa
62	NC	Jamani, North Carolina Zoo	NCJa
63	NC	Jamani, North Carolina Zoo	NCJa
65	NC	Jamani, North Carolina Zoo	NCJa
66	NC	Jamani, North Carolina Zoo	NCJa
67	DZ	Jim, Denver Zoo	DZJi
68	DZ	Jim, Denver Zoo	DZJi
69	DZ	Jim, Denver Zoo	DZJi
70	DZ	Jim, Denver Zoo	DZJi
71	DZ	Jim, Denver Zoo	DZJi
72	DZ	Jim, Denver Zoo	DZJi
73	DZ	Jim, Denver Zoo	DZJi
74	DZ	Jim, Denver Zoo	DZJi
76	RB	Kazi, Riverbanks Zoo	RBKa
77	RB	Kazi, Riverbanks Zoo	RBKa
78	RB	Kazi, Riverbanks Zoo	RBKa
79	RB	Kazi, Riverbanks Zoo	RBKa
80	RB	Kazi, Riverbanks Zoo	RBKa
81	RB	Kazi, Riverbanks Zoo	RBKa
82	RB	Kazi, Riverbanks Zoo	RBKa
83	RB	Kazi, Riverbanks Zoo	RBKa
84	RB	Macy, Riverbanks Zoo	RBMa
85	RB	Macy, Riverbanks Zoo	RBMa
86	RB	Macy, Riverbanks Zoo	RBMa
87	RB	Macy, Riverbanks Zoo	RBMa
88	RB	Macy, Riverbanks Zoo	RBMa
89	RB	Macy, Riverbanks Zoo	RBMa
90	RB	Macy, Riverbanks Zoo	RBMa
91	RB	Macy, Riverbanks Zoo	RBMa
93	NC	Mosuba, North Carolina Zoo	NCMo
94	NC	Mosuba, North Carolina Zoo	NCMo
95	NC	Mosuba, North Carolina Zoo	NCMo
96	NC	Mosuba, North Carolina Zoo	NCMo
97	NC	Mosuba, North Carolina Zoo	NCMo
98	NC	Mosuba, North Carolina Zoo	NCMo
100	NC	Olympia, North Carolina Zoo	NCOI
101	NC	Olympia, North Carolina Zoo	NCOI
102	NC	Olympia, North Carolina Zoo	NCOI

103	RB	Patrick, Riverbanks Zoo	RBPa
104	RB	Patrick, Riverbanks Zoo	RBPa
105	RB	Patrick, Riverbanks Zoo	RBPa
106	RB	Patrick, Riverbanks Zoo	RBPa
107	RB	Patrick, Riverbanks Zoo	RBPa
108	RB	Patrick, Riverbanks Zoo	RBPa
109	RB	Patrick, Riverbanks Zoo	RBPa
110	NC	Rosie, North Carolina Zoo	NCRo
111	NC	Rosie, North Carolina Zoo	NCRo
112	NC	Rosie, North Carolina Zoo	NCRo
113	NC	Rosie, North Carolina Zoo	NCRo
114	NC	Rosie, North Carolina Zoo	NCRo
116	NC	Rosie, North Carolina Zoo	NCRo
117	NC	Rosie, North Carolina Zoo	NCRo
118	DZ	Tinga, Denver Zoo	DZTi
119	DZ	Tinga, Denver Zoo	DZTi
120	DZ	Tinga, Denver Zoo	DZTi
121	DZ	Tinga, Denver Zoo	DZTi
122	DZ	Tinga, Denver Zoo	DZTi
123	DZ	Tinga, Denver Zoo	DZTi
124	DZ	Tinga, Denver Zoo	DZTi
125	DZ	Tinga, Denver Zoo	DZTi
127	DZ	Whimsie, Denver Zoo	DZWh
128	DZ	Whimsie, Denver Zoo	DZWh
129	DZ	Whimsie, Denver Zoo	DZWh
130	DZ	Whimsie, Denver Zoo	DZWh
131	DZ	Whimsie, Denver Zoo	DZWh
132	DZ	Whimsie, Denver Zoo	DZWh
133	DZ	Whimsie, Denver Zoo	DZWh
134	DZ	Whimsie, Denver Zoo	DZWh
136	RB	Acacia, Riverbanks Zoo	RBAc
137	RB	Acacia, Riverbanks Zoo	RBAc
138	RB	Acacia, Riverbanks Zoo	RBAc
139	RB	Acacia, Riverbanks Zoo	RBAc
140	RB	Acacia, Riverbanks Zoo	RBAc
141	RB	Acacia, Riverbanks Zoo	RBAc
142	RB	Acacia, Riverbanks Zoo	RBAc
143	NC	Apollo, North Carolina Zoo	NCAp
144	NC	Apollo, North Carolina Zoo	NCAp
145	NC	Apollo, North Carolina Zoo	NCAp
146	NC	Apollo, North Carolina Zoo	NCAp
147	NC	Apollo, North Carolina Zoo	NCAp
148	NC	Apollo, North Carolina Zoo	NCAp

149	NC	Apollo, North Carolina Zoo	NCAp
150	NC	Bomassa, North Carolina Zoo	NCBo
151	NC	Bomassa, North Carolina Zoo	NCBo
152	NC	Bomassa, North Carolina Zoo	NCBo
153	NC	Bomassa, North Carolina Zoo	NCBo
154	NC	Bomassa, North Carolina Zoo	NCBo
155	NC	Bomassa, North Carolina Zoo	NCBo
156	RB	Cenzoo, Riverbanks Zoo	RBCe
157	RB	Cenzoo, Riverbanks Zoo	RBCe
158	RB	Cenzoo, Riverbanks Zoo	RBCe
159	RB	Cenzoo, Riverbanks Zoo	RBCe
160	RB	Cenzoo, Riverbanks Zoo	RBCe
161	RB	Cenzoo, Riverbanks Zoo	RBCe
162	RB	Cenzoo, Riverbanks Zoo	RBCe
163	DZ	Charlie, Denver Zoo	DZCh
164	DZ	Charlie, Denver Zoo	DZCh
165	DZ	Charlie, Denver Zoo	DZCh
166	DZ	Charlie, Denver Zoo	DZCh
167	DZ	Charlie, Denver Zoo	DZCh
168	DZ	Charlie, Denver Zoo	DZCh
169	DZ	Charlie, Denver Zoo	DZCh
170	DZ	Charlie, Denver Zoo	DZCh
171	DZ	Curtis, Denver Zoo	DZCu
172	DZ	Curtis, Denver Zoo	DZCu
173	DZ	Curtis, Denver Zoo	DZCu
174	DZ	Curtis, Denver Zoo	DZCu
175	DZ	Curtis, Denver Zoo	DZCu
176	DZ	Curtis, Denver Zoo	DZCu
177	DZ	Curtis, Denver Zoo	DZCu
178	DZ	Curtis, Denver Zoo	DZCu
179	NC	Dembe, North Carolina Zoo	NCDe
180	NC	Dembe, North Carolina Zoo	NCDe
181	NC	Dembe, North Carolina Zoo	NCDe
182	NC	Dembe, North Carolina Zoo	NCDe
183	NC	Dembe, North Carolina Zoo	NCDe
184	NC	Dembe, North Carolina Zoo	NCDe
185	NC	Dembe, North Carolina Zoo	NCDe
186	NC	Hadari, North Carolina Zoo	NCHa
187	NC	Hadari, North Carolina Zoo	NCHa
188	NC	Hadari, North Carolina Zoo	NCHa
189	NC	Hadari, North Carolina Zoo	NCHa
190	NC	Hadari, North Carolina Zoo	NCHa
191	NC	Hadari, North Carolina Zoo	NCHa

192	NC	Jamani, North Carolina Zoo	NCJa
193	NC	Jamani, North Carolina Zoo	NCJa
194	NC	Jamani, North Carolina Zoo	NCJa
195	NC	Jamani, North Carolina Zoo	NCJa
196	NC	Jamani, North Carolina Zoo	NCJa
197	NC	Jamani, North Carolina Zoo	NCJa
198	DZ	Jim, Denver Zoo	DZJi
199	DZ	Jim, Denver Zoo	DZJi
200	DZ	Jim, Denver Zoo	DZJi
201	DZ	Jim, Denver Zoo	DZJi
202	DZ	Jim, Denver Zoo	DZJi
203	DZ	Jim, Denver Zoo	DZJi
204	DZ	Jim, Denver Zoo	DZJi
205	DZ	Jim, Denver Zoo	DZJi
206	RB	Kazi, Riverbanks Zoo	RBKa
207	RB	Kazi, Riverbanks Zoo	RBKa
208	RB	Kazi, Riverbanks Zoo	RBKa
209	RB	Kazi, Riverbanks Zoo	RBKa
210	RB	Kazi, Riverbanks Zoo	RBKa
211	RB	Kazi, Riverbanks Zoo	RBKa
212	RB	Kazi, Riverbanks Zoo	RBKa
213	RB	Macy, Riverbanks Zoo	RBMa
214	RB	Macy, Riverbanks Zoo	RBMa
215	RB	Macy, Riverbanks Zoo	RBMa
216	RB	Macy, Riverbanks Zoo	RBMa
217	RB	Macy, Riverbanks Zoo	RBMa
218	RB	Macy, Riverbanks Zoo	RBMa
219	RB	Macy, Riverbanks Zoo	RBMa
220	NC	Mosuba, North Carolina Zoo	NCMo
221	NC	Mosuba, North Carolina Zoo	NCMo
222	NC	Mosuba, North Carolina Zoo	NCMo
223	NC	Mosuba, North Carolina Zoo	NCMo
224	NC	Mosuba, North Carolina Zoo	NCMo
225	NC	Mosuba, North Carolina Zoo	NCMo
226	NC	Olympia, North Carolina Zoo	NCOl
227	NC	Olympia, North Carolina Zoo	NCOl
228	NC	Olympia, North Carolina Zoo	NCOl
229	NC	Olympia, North Carolina Zoo	NCOl
230	RB	Patrick, Riverbanks Zoo	RBPa
231	RB	Patrick, Riverbanks Zoo	RBPa
232	RB	Patrick, Riverbanks Zoo	RBPa
233	RB	Patrick, Riverbanks Zoo	RBPa
234	RB	Patrick, Riverbanks Zoo	RBPa

235	RB	Patrick, Riverbanks Zoo	RBPa
236	RB	Patrick, Riverbanks Zoo	RBPa
237	DZ	Tinga, Denver Zoo	DZTi
238	DZ	Tinga, Denver Zoo	DZTi
239	DZ	Tinga, Denver Zoo	DZTi
240	DZ	Tinga, Denver Zoo	DZTi
241	DZ	Tinga, Denver Zoo	DZTi
242	DZ	Tinga, Denver Zoo	DZTi
243	DZ	Tinga, Denver Zoo	DZTi
244	DZ	Tinga, Denver Zoo	DZTi
245	DZ	Whimsie, Denver Zoo	DZWh
246	DZ	Whimsie, Denver Zoo	DZWh
247	DZ	Whimsie, Denver Zoo	DZWh
248	DZ	Whimsie, Denver Zoo	DZWh
249	DZ	Whimsie, Denver Zoo	DZWh
250	DZ	Whimsie, Denver Zoo	DZWh
251	DZ	Whimsie, Denver Zoo	DZWh
252	DZ	Whimsie, Denver Zoo	DZWh
253	NC	Jamani, North Carolina Zoo	NCJa
254	NC	Hadari, North Carolina Zoo	NCHa
255	NC	Bomassa, North Carolina Zoo	NCBo
101.5	NC	Olympia, North Carolina Zoo	NCOl
D1	DZ	Composite Diet Sample, Denver Zoo	DZCD
D10	DZ	Composite Diet Sample, Denver Zoo	DZCD
D11	DZ	Composite Diet Sample, Denver Zoo	DZCD
D12	DZ	Composite Diet Sample, Denver Zoo	DZCD
D13	DZ	Composite Diet Sample, Denver Zoo	DZCD
D14	DZ	Composite Diet Sample, Denver Zoo	DZCD
D15	NC	Composite Diet Sample, North Carolina	NCCD
D16	NC	Composite Diet Sample, North Carolina	NCCD
D17	NC	Composite Diet Sample, North Carolina	NCCD
D18	NC	Composite Diet Sample, North Carolina	NCCD
D19	NC	Composite Diet Sample, North Carolina	NCCD
D2	DZ	Composite Diet Sample, Denver Zoo	DZCD
D20	NC	Composite Diet Sample, North Carolina	NCCD
D21	NC	Composite Diet Sample, North Carolina	NCCD
D22	NC	Composite Diet Sample, North Carolina	NCCD
D23	NC	Composite Diet Sample, North Carolina	NCCD
D24	NC	Composite Diet Sample, North Carolina	NCCD
D25	NC	Composite Diet Sample, North Carolina	NCCD
D26	NC	Composite Diet Sample, North Carolina	NCCD
D27	RB	Composite Diet Sample, Riverbanks Zoo	RBCD
D28	RB	Composite Diet Sample, Riverbanks Zoo	RBCD

D29	RB	Composite Diet Sample, Riverbanks Zoo	RBCD
D3	DZ	Composite Diet Sample, Denver Zoo	DZCD
D30	RB	Composite Diet Sample, Riverbanks Zoo	RBCD
D31	RB	Composite Diet Sample, Riverbanks Zoo	RBCD
D32	RB	Composite Diet Sample, Riverbanks Zoo	RBCD
D33	RB	Composite Diet Sample, Riverbanks Zoo	RBCD
D34	RB	Composite Diet Sample, Riverbanks Zoo	RBCD
D35	RB	Composite Diet Sample, Riverbanks Zoo	RBCD
D36	RB	Composite Diet Sample, Riverbanks Zoo	RBCD
D37	RB	Composite Diet Sample, Riverbanks Zoo	RBCD
D38	RB	Composite Diet Sample, Riverbanks Zoo	RBCD
D39	RB	Composite Diet Sample, Riverbanks Zoo	RBCD
D4	DZ	Composite Diet Sample, Denver Zoo	DZCD
D40	RB	Composite Diet Sample, Riverbanks Zoo	RBCD
D41	RB	Composite Diet Sample, Riverbanks Zoo	RBCD
D5	DZ	Composite Diet Sample, Denver Zoo	DZCD
D6	DZ	Composite Diet Sample, Denver Zoo	DZCD
D7	DZ	Composite Diet Sample, Denver Zoo	DZCD
D8	DZ	Composite Diet Sample, Denver Zoo	DZCD
D9	DZ	Composite Diet Sample, Denver Zoo	DZCD
E1	DZ	Composite Environmental, Denver Zoo	DZCE
E10	DZ	Composite Environmental, Denver Zoo	DZCE
E11	DZ	Composite Environmental, Denver Zoo	DZCE
E12	DZ	Composite Environmental, Denver Zoo	DZCE
E13	DZ	Composite Environmental, Denver Zoo	DZCE
E14	DZ	Composite Environmental, Denver Zoo	DZCE
E15	DZ	Composite Environmental, Denver Zoo	DZCE
E16	NC	Composite Environmental, North Carolina Zoo	NCCE
E17	NC	Composite Environmental, North Carolina Zoo	NCCE
E18	NC	Composite Environmental, North Carolina Zoo	NCCE
E19	NC	Composite Environmental, North Carolina Zoo	NCCE
E2	DZ	Composite Environmental, Denver Zoo	DZCE
E20	NC	Composite Environmental, North Carolina Zoo	NCCE
E21	NC	Composite Environmental, North Carolina Zoo	NCCE
E22	NC	Composite Environmental, North Carolina Zoo	NCCE
E23	NC	Composite Environmental, North Carolina Zoo	NCCE

E24	NC	Composite Environmental, North Carolina Zoo	NCCE
E25	NC	Composite Environmental, North Carolina Zoo	NCCE
E26	NC	Composite Environmental, North Carolina Zoo	NCCE
E27	NC	Composite Environmental, North Carolina Zoo	NCCE
E28	NC	Composite Environmental, North Carolina Zoo	NCCE
E29	NC	Composite Environmental, North Carolina Zoo	NCCE
E30	RB	Composition Environmental, Riverbanks Zoo	RBCE
E31	RB	Composition Environmental, Riverbanks Zoo	RBCE
E32	RB	Composition Environmental, Riverbanks Zoo	RBCE
E33	RB	Composition Environmental, Riverbanks Zoo	RBCE
E34	RB	Composition Environmental, Riverbanks Zoo	RBCE
E35	RB	Composition Environmental, Riverbanks Zoo	RBCE
E36	RB	Composition Environmental, Riverbanks Zoo	RBCE
E37	RB	Composition Environmental, Riverbanks Zoo	RBCE
E38	RB	Composition Environmental, Riverbanks Zoo	RBCE
E39	RB	Composition Environmental, Riverbanks Zoo	RBCE
E4	DZ	Composite Environmental, Denver Zoo	DZCE
E40	RB	Composition Environmental, Riverbanks Zoo	RBCE
E41	RB	Composition Environmental, Riverbanks Zoo	RBCE
E42	RB	Composition Environmental, Riverbanks Zoo	RBCE
E43	RB	Composition Environmental, Riverbanks Zoo	RBCE
E5	DZ	Composite Environmental, Denver Zoo	DZCE
E6	DZ	Composite Environmental, Denver Zoo	DZCE
E7	DZ	Composite Environmental, Denver Zoo	DZCE
E8	DZ	Composite Environmental, Denver Zoo	DZCE

E9	DZ	Composite Environmental, Denver Zoo	DZCE
ET1	DZ	Exhibit Soil Sample, Denver Zoo	DZCS
H1	DZ	Exhibit Soil Sample, Denver Zoo	DZCS
H10	NC	Exhibit Soil Sample, North Carolina	NCCS
H11	NC	Exhibit Soil Sample, North Carolina	NCCS
H12	NC	Exhibit Soil Sample, North Carolina	NCCS
H13	NC	Exhibit Soil Sample, North Carolina	NCCS
H14	NC	Exhibit Soil Sample, North Carolina	NCCS
H2	DZ	Exhibit Soil Sample, Denver Zoo	DZCS
H3	DZ	Exhibit Soil Sample, Denver Zoo	DZCS
H4	DZ	Exhibit Soil Sample, Denver Zoo	DZCS
H5	DZ	Exhibit Soil Sample, Denver Zoo	DZCS
H6	DZ	Exhibit Soil Sample, Denver Zoo	DZCS
H7	DZ	Exhibit Soil Sample, Denver Zoo	DZCS
H8	NC	Exhibit Soil Sample, North Carolina	NCCS
H9	NC	Exhibit Soil Sample, North Carolina	NCCS