ABSTRACT

BURNHAM, CHRISTINA MADISON. "Drivers of Variation in the Gut Microbiome of Southern White Rhinoceros (*Ceratotherium simum simum*) Under Human Care." (Under the direction of Dr. Shweta Trivedi and Dr. Kimberly Ange-van Heugten).

As poaching incidence increases, wild populations of southern white rhinoceros (*Ceratotherium simum simum*) face an uncertain future. It is critical that reproductively healthy assurance populations be maintained under human care; however, many managed populations of southern white rhinoceros experience poor fertility and post-copulatory reproductive failure in contrast to the reproductive success of wild conspecifics. Gut microbiome and host health are inextricably linked, and reproductive outcomes in managed southern white rhinoceros may be mediated in part by their diet and gut microbial diversity. Given these considerations, understanding gut microbial dynamics within and between managed and wild populations may help improve conservation efforts. Freezing feces at -80°C is the gold standard for microbiome research; however, this practice is logistically impractical for microbiome fieldwork applications (e.g. sampling wild rhinoceros populations *in situ*). The objectives of this research were to characterize the taxonomic composition of the gut microbiome in the reproductively successful managed population of southern white rhinoceros (n = 10) at the NC Zoo, and to determine the effects of seasonality and age classes on microbial richness and community structure. We also evaluated the efficacy of three different fecal preservation methods over two timepoints (14 and 230 days) for preserving microbial communities. Fecal samples were collected from each individual once per month from July through September in 2020 and January through March in 2021. Samples were preserved via immediate freezing at -80°C, PERFORMAbiome•GUT (PB) tubes, and 95% ethanol. Samples preserved in PB tubes and in 95% ethanol were stored at ambient temperature for a minimum of two weeks before processing to simulate field conditions.

Microbial DNA was extracted from each sample and sequenced using the V3-V4 regions of the 16S rRNA gene. Total operational taxonomic units (OTUs) as well as differential abundance of taxa and the overall alpha and beta diversity were examined. Initial results show that southern white rhinoceros managed at the NC Zoo are colonized by the following phyla: Firmicutes (55% relative abundance), Bacteroidetes (21%), Spirochetes (10%), Fibrobacteres (8%), Kiritimatiellaeota (2%), and Lentisphaerae (1%). There were significant differences ($P<0.05$) in alpha and beta diversity indices among individuals, age groups, and sampling months. Samples collected during cold weather months contained statistically distinct communities and higher species richness compared to warm weather months (PERMANOVA, $P<0.05$). The alpha diversity in 95% ethanol samples also differed from both the frozen and PB samples at both Day 14 ($P \leq 0.001$) and Day 230 ($P<0.05$). Ethanol Day 230 samples differed ($P<0.05$) from all other preservation methods and time points. Ethanol preserved a wider range of OTUs across samples, but with far greater variability and a lower median number of OTUs. Notably, observed bacterial community profiles remained consistent (i.e., statistically similar) between frozen and PB preservation methods across both time points. This indicates that samples stored at ambient temperatures in PB tubes perform similarly to those frozen immediately at -80◦C. Thus, PB tubes may be advantageous in microbiome fieldwork applications. Together, our results have important implications for experimental design and increased understanding of age and seasonal related microbial variation in southern white rhinoceros at the NC Zoo.

Drivers of Variation in the Gut Microbiome of Southern White Rhinoceros (*Ceratotherium simum simum*) Under Human Care

by
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APPROVED BY:

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DEDICATION

To Pops,

You sparked in me a love of science that will never be extinguished. LLAP.

BIOGRAPHY

Christina Madison Burnham was born in Fort Bragg, North Carolina to Stephen and Laura Burnham on October 31st, 1996. She is the younger sister of Jessica Brouillet and Christopher Madrigal. Christina surrounded herself with a menagerie of creatures while growing up in Fayetteville, North Carolina, and always dreamed of a career working with animals. She possessed an inherent love of the natural world reinforced by books and frequent trips to the NC Museum of Natural Sciences. Christina graduated from Terry Sanford High School and the School of Global Studies program in 2014, where she was active in Science Olympiad and became president of the school's chapter of the National Art Honors Society.

Christina attended North Carolina State University in Raleigh, North Carolina and received Bachelor of Science degrees in Zoology and Poultry Science. She was an officer for the Zoology Club and graduated from the College of Science Honors Program. Christina also held several jobs during her undergraduate career: she spent two months with the California Academy of Science in San Francisco working as a botanical illustrator and learned the value of hard labor as a zookeeper at Sylvan Heights Bird Park in Scotland Neck, NC. She also spent four years working under the late Dr. John Brake in a poultry physiology lab where she became interested in research and gained the skills and discipline necessary to pursue it as a graduate student. Freshly instilled with a passion for research, Christina spent two and a half months studying the efficacy of drones as a poaching deterrent in Namibia. That experience, coupled with time spent shadowing wildlife veterinarians and familiarity with local culture, earned her a spot as a master's student in Dr. Shweta Trivedi's lab studying southern white rhinoceros blood biochemistry with wild populations in South Africa. Research plans were altered during the 2020

COVID-19 Pandemic, and Christina instead shifted her focus to studying southern white rhinoceros microbiome with the population of rhinos at the NC Zoo.

In her free time, Christina enjoys wandering the country, creating art, and photographing insects and other wildlife. She plans to pursue a doctorate in the future and hopes to continue working with wildlife in some capacity throughout her life.

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Many hours were spent in the laboratory during the completion of this project. Appreciation is extended to Dr. Sid Thakur for allowing me to use his lab to run my DNA extractions and to Jeff Sommer and Lyndy Harden for assistance with lab work troubleshooting. Thank you to the Genomic Science Laboratory at NC State for sequencing the extracted DNA and thank you to Dr. Jack Odle for allowing me to utilize his lab's CLC Genomics Workbench license to process my data.

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All of my appreciation is directed toward my family for their continuous support not only throughout this project but throughout the entirety of my life. They have invested their endless love and patience and I hope to continue making them proud. I extend great love and admiration to my mother, Laura Burnham, my siblings Christopher Madrigal and Jessica Brouillet, my aunt Cindy Burnham, my uncle Rick Allen, and my grandmother Lilly Burnham. My late father, Steve Burnham, was unable to see the completion of this project but nevertheless was a prominent figure in my decision to pursue a career in science.

Lastly, I extend a special thanks to the animal care and veterinary staff at the NC Zoo for the collection of samples for this project and for maintaining the highest standard of animal care.

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1.1 Microbiome Overview

The microbiome can be defined as a microbial community occupying a given host or habitat, forming a dynamic micro-ecosystem with commensal, symbiotic, and pathogenic interactions (Lederberg & Mccray, 2001; Berg et al., 2020). The taxa present within a microbiome include members of the Prokaryote, Eubacteria and Archaea domains, as well as a suite of Eukaryotes including protozoa, fungi, and algae. Microbial interactions contribute to broader ecosystem functions, including host life processes; together, the host and its microbiome form a "holobiont" (Carthey et al., 2020). Studies of animal-associated microbiomes generally involve high biomass areas such as the gut and the oral cavity; the skin, genitals, nostrils, and even bodily fluids also contain microbial communities, but can be more difficult to examine due to contamination and other biases (Selway et al., 2020). While the microbiome of the oral cavity is the most well-studied microbiome in humans to-date due to ease of sampling, it is dwarfed in both size and diversity by the gut microbiome (Deo & Deshmukh, 2019).

The gut microbiome mediates a variety of physiological functions in the host, including immune function, metabolism, and digestion (Flint et al., 2012; LeBlanc et al., 2013). Bidirectional interactions along the gut-lung, gut-brain, and gut-liver axes have all been recently described (Martin et al., 2018; Tripathi et al., 2018; Dang & Marsland, 2019). Gut microbes have been proposed to affect fertility levels and reproductive success in a variety of animal species including southern white rhinoceros (*Ceratotherium simum simum*) and zebrafish (*Danio rerio*) (Williams et al., 2019; Garrett & Uno, 2021). Abnormal changes to the gut microbiome leading

to a persistent imbalance in diversity and composition of gut communities can have downstream consequences; this imbalance, termed dysbiosis, is usually associated with decreased microbial taxonomic richness and potential increased occurrence of pathogenic species, which can result in a diseased state in the host (Lupp et al., 2007; Schippa et al., 2012; Winter et al., 2013).

Phylogeny, the evolutionary history of the host, is one of most dominant drivers affecting animal gut microbiomes: indeed, many gut microbiomes are species-specific (Ochman et al., 2010; Nashida & Ochman, 2018; Youngblut et al., 2019). However, microbiomes vary widely among individuals, even within host species; this individual variance makes up the host's microbial "cloud" (Montassier et al., 2018). Individual variation may be attributed to diet (Muegge et al., 2011; David et al., 2014; McKenney et al., 2015), gut morphology (Gillman et al., 2020), feeding strategy (McKenney et al., 2018; Greene et al., 2020), age (Adriansjach et al., 2020; Janiak et al., 2021), sex (Markle et al., 2013; Mshelia et al., 2018), geographic location (Yatsunenko et al., 2012), health status (Lewis et al., 2015), and a variety of other factors.

In mammals, family- and genus-level bacterial taxa are more associated with host lineage than diet, and closely related species tend to exhibit similar bacterial communities (Nishida & Ochman, 2018). Ley at al. (2008) found that the community compositions of individuals from many species, including Hamadryas baboons and humans, clustered together on distance matrices despite the individuals all living in different locations. Host phylogeny has been shown to dictate community membership but not the abundance of operational taxonomic units (OTU) (Youngblut et al., 2019).

Establishment of an individual's microbiome starts at birth: in mammals, infants receive microbes via vertical transmission from the vaginal canal of the mother (Dominguez-Bello et al., 2010). Those born via cesarean section instead receive microbes from the skin during direct

contact (Dominguez-Bello et al., 2010). Infants also receive microbes from breast skin during suckling (Biagi et al., 2017). As an individual ages, their microbiome continues to develop toward a mature climax community via both diet and horizontal transfer from the environment (Dominguez-Bello et al., 2010; Hehemann et al., 2010; Hills et al., 2019). The mature microbiome remains relatively stable unless disturbed by lifestyle changes including major alterations to diet, usage of antibiotics and other drugs, excessive stress, and/or disease (Faith et al., 2013; Hills et al., 2019).

1.2 Diet and Gut Morphology

Diet is the single biggest driver of gut microbial variation among conspecifics and has a strong influence on the microbial species present (Scott et al., 2013; Voreades et al., 2014). The gut microbiome plays an important role in digestion (Scott et al., 2013), vitamin synthesis (Das et al., 2019), and the breakdown of toxins across the host's lifespan (Kohl et al., 2014). Different species have evolved different gut morphologies to selectively favor microbial compositions and digestive processes that best compliment their feeding strategy. Mammalian herbivores in particular provide a compelling system for studying the dynamic relationship between hosts and gut microbes, as the mammalian genome naturally lacks genes that code for digestive enzymes needed to break down the complex polysaccharides that make up plant cell walls (e.g., cellulose) (Hehemann et al., 2010). These services are instead provided by the symbiotic gut microbiota that do possess those genes (Hehemann et al., 2010; El Kaoutari et al., 2013). Genes that gut microbes do not already possess can be obtained by horizontal transfer, which increases the diversity of dietary sources that can be utilized by the host (Hehemann et al., 2010). Symbiotic gut microbiota are also crucial for the degradation of secondary plant compounds including

toxins present in the diets of herbivorous species (Garcia-Amado et al., 2007; Amato et al., 2013; Amato et al., 2016; Borbón-García et al., 2017). Kohl et al. (2014) showed that the consumption of creosote toxins (e.g., oxalate) by desert woodrats (*Neotoma lepida*) altered community structure of the gut microbiome, leading to an increase in genes that code for enzymes that metabolize those toxins. Antibiotic treatment impeded woodrats' ability to process oxalate, suggesting a direct link between the action of the gut microbiome and the digestion of toxic plants (Kohl et al., 2014; Kohl & Dearing, 2016).

Ruminants, a suborder of herbivorous ungulate mammals including cows, sheep, and deer, have evolved enlarged stomachs comprising four specialized compartments to facilitate fiber digestion and provide a practical study system in which to study the interaction between microbes and host digestion. The rumen is the first and largest stomach compartment and contains a multitude of microbes which ferment plant fiber mats rich in polysaccharides that are otherwise indigestible (Cholewińska et al., 2020). Shabat et al. (2016) showed that the microbiota present in cow rumens directly impact cows' ability to extract energy from feed, and that the enrichment of certain microbial species (i.e., genus *Megasphaera*) increases digestive efficiency.

Reese and Dunn (2018) found that animals with simple guts host lower microbial richness than foregut and hindgut fermenting herbivores. This effect was amplified with the inclusion of ruminant herbivores into their statistical analysis, after which herbivores possessed higher gut microbial diversity than either carnivores or omnivores. Gut retention time likely also has some bearing on this trend, as short retention times in simple tracts (like those found in carnivores and some omnivores) preferentially select for fast-growing microbial taxa, inherently decreasing microbial diversity (Reese & Dunn, 2018; McKenney et al., 2018). In contrast,

herbivores tend to have complex intestinal tracts with longer retention times, which support more diverse microbiomes to facilitate digestion of high fiber diets (McKenney et al., 2018). Whereas ruminants and other foregut fermenters achieve increased gut complexity and retention time with a compartmentalized stomach, hindgut fermenters (including the southern white rhinoceros) have instead evolved an enlarged cecum (i.e., pouch between the small and large intestines) and sacculated colon (Stevens & Hume, 1998). The presence of a cecum increases tract complexity, leading to an increase in retention time and microbial diversity in turn even in species with short intestinal tracts (McKenney et al., 2018).

The host diet mediates the abundance of bacterial taxa present in the gut (Wu et al., 2011). Several studies have documented the effects of "Western" diets on the human (De Filippo et al., 2010), rodent (McNamara et al., 2021), and non-human primate gut microbiomes (Amato et al., 2015; Clayton et al., 2016). Western diets are characterized by high levels of animal protein, saturated fat, refined grains, salt, and sugar; they are also associated with reduced fiber intake corresponding to reduced intake of fruits and vegetables (Statovci et al., 2017). Individuals who convert to a Western diet experience decreased gut microbial diversity and function (Wu et al., 2011). The Western diet generally favors members of the genus *Bacteroides*, which displace members of the plant fiber-digesting genus *Prevotella* (Arumugam et al., 2011).

Diet also correlates to other microbiome drivers such as host age and seasonality. While mammalian infants receive their first inoculation of microbiota from their mother during parturition, nursing facilitates succession toward the climax community in adults. The mammalian infant microbiome is dominated by *Bifidobacterium* and, to a lesser extent, *Bacteroides*; the preferential colonization by these taxa is promoted by the oligosaccharides abundant in milk (Harmsen et al., 2000; Bezirtzoglou et al., 2011; McKenney et al., 2015; Lugli

et al., 2019). The introduction of solid foods quickly shifts the infant microbial profile towards that of the characteristic adult microbial profile, which stabilizes after weaning (Palmer et al., 2007; Koenig et al., 2011; McKenney et al., 2015). McKenney et al. (2015) found that daily consumption of solid foods and subsequent weaning drove gut microbiome convergence within species.

Changes in microbial richness and species abundance due to seasonality often corresponds to seasonal shifts in the availability of certain food items, among other factors. For example, Smits et al. (2017) found drastic seasonal microbial shifts Hadza hunter-gatherers in western Tanzania: 70% of the Bacteroidetes OTUs dropped below detectable limits during wet months, while 62% of Firmicutes OTUs disappeared at the start of the dry months. The majority of these species were restored during subsequent wet/dry seasons, a trend likely linked to diet as the Hadza people mainly consume meat during the dry season and berries and honey during the wet season (Smits et al., 2017). These seasonal distinctions in diet correspond to differential expression of carbohydrate active enzymes within the microbiome (Smits et al., 2017). Studies of wild red squirrels (*Tamiasciurus hudsonicus*), wood mice (*Apodemus sylvaticus*), ground squirrels (*Ictidomys tridecemlineatus*), giant pandas (*Ailuropoda melanoleuca*), and horses (*Equus ferus caballus*) all revealed similar seasonal shifts in microbial diversity driven by differences in diet availability (Carey et al., 2013; Maurice et al., 2015; Xue et al., 2015; Ren et al., 2017; Salem et al., 2018). In particular, Salem et al. (2018) found that horses, the domestic animal model for rhinoceros, experienced seasonal shifts in fecal microbial communities corresponding to alterations in grass availability and nutrition, sources of supplementary forage, and ambient weather conditions.

1.3 Health Status

The gut microbiome and health status of the host are inextricably linked. Previous studies have shown significant associations between gut microbial composition and phenotypic expression of body mass index (BMI), glycemic status, waist-to-hip ratio, and fasting glucose levels in humans (Rothschild et al., 2018). Bidirectional interactions along the gut-lung, gut-brain, and gut-liver axes have also been noted (Martin et al., 2018; Tripathi et al., 2018; Dang & Marsland, 2019). Gut microbiota have been shown to modulate the risk for several chronic diseases in humans including inflammatory bowel disease, celiac disease, type 2 diabetes, cardiovascular disease, colorectal cancer, and obesity (Schippa & Conte, 2014; Conlon & Bird, 2015; Brennan & Garrett, 2016; Singh et al., 2017).

The "optimal" state of the microbiome varies per individual but is generally characterized by a species- and age-specific composition of mostly non-pathogenic microbiota. These microbiota form a web of mutualistic relationships among themselves and with the host immune system, mediated by metabolic products, that culminates in a central beneficial microbiome ecosystem. Deviation from this state (i.e., dysbiosis) is usually associated with a diseased state. In dysbiotic systems, gut bacteria such as E*scherichia coli* and *Shigella* spp., which are often present in beneficial intestinal communities, can become pathogenic (i.e., pathobionts) and overwhelm and outcompete beneficial microbes; this leads to a net reduction in microbial diversity (Lupp et al., 2007; Schippa et al., 2012; Winter et al., 2013). Host responses to these pathobionts can further facilitate colonization by additional opportunistic pathogens. For example, immune responses such as inflammation negatively alter the intestinal nutritional environment for beneficial gut microbes and produce nitrate by-products that can be used as fuel by pathogenic species such as the Enterobacteriaceae family (Winter et al., 2013). In addition,

dysbiosis can affect drug metabolism and efficacy with the potential to prolong the diseased state (Clayton et al., 2009; Zaneveld et al., 2017).

Several interventions for dysbiosis have been proposed, including the usage of probiotics, prebiotics, and fecal transplants. Probiotics are live microorganisms, usually *Lactobacillus* and *Bifidobacterium* species, which are administered to reinstitute beneficial intestinal microbes; these beneficial intestinal microbes decrease colonization of pathogenic microbes in turn by lowering gut pH (Williams, 2010). By comparison, prebiotic foods supply compounds (mainly oligosaccharides) that fuel and promote the growth of beneficial gut bacteria, thus serving as a dietary modulator of gut microbial composition (Manning & Gibson, 2004). Regardless of their benefits, studies have shown that the gut microbiome is resilient to quick changes; thus, long-term usage of probiotics and prebiotics coupled with a change to a fiber-rich diet is recommended for those with dysbiotic systems (Gagliardi et al., 2018). Fecal microbiota transplantation (FMT) dramatically shifts the gut microbiome by administering fecal microbiota from a healthy donor, sparking the restoration of a stable and beneficial microbial composition. In humans, FMT has been used to treat severe diarrhea, *Clostridium difficile* infection, and Crohn's disease (Bakken et al., 2011; Zhang et al., 2013). Infant red kangaroos (*Osphranter rufus*) have also been treated for diarrheal illnesses using FMT with consistent success (Milliken, 2019). FMT has been proposed not only as a disease treatment but also as a conservation tool that could potentially be used to restore species specific microbiomes in managed animals by transplanting beneficial, non-pathogenic microbiota from wild conspecifics (Guo et al., 2020). However, further research is necessary to understand the effects of FMT on a wide variety of species and disease states, as well as the effective “dosage” per species and body mass.

1.4 The Effects of Captivity on the Microbiome

There are over 15,800 species of animals considered to be at high risk for extinction in the wild on the International Union for Conservation of Nature (IUCN) Red List as of 2021, and that number is ever-increasing due to climate change, habitat loss, and other anthropogenic effects (IUCN, 2021). As such, it is crucial that reproductively successful assurance populations of threatened wild animals be maintained in human care. One of the leading challenges in maintaining healthy managed populations of animals is producing a palatable, nutritionally balanced *ex situ* diet. It is both logistically and financially infeasible to replicate the wild diet of non-endemic animals, thus managed diets often lack the same variety and composition as wild diets (West et al., 2019). Previous comparisons of managed and wild gut microbiomes revealed that managed wildlife often have reduced microbial diversity in comparison to their wild conspecifics (Kong et al., 2014; Amato et al., 2016; Clayton et al., 2016; McKenzie et al., 2017; Metcalf et al., 2017; Gibson et al., 2019). There are many proposed explanations for this trend, including the lack of "natural" dietary diversity, relative sterility of the captive environment, lack of interaction with allospecifics, and the necessary usage of antibiotics to treat sick animals. There is, however, ample room for skepticism regarding the validity of many of these trends. Population sizes across many of these wild vs. managed comparative studies were low, as is the case with most studies involving rare and endangered animals. In addition, microbial diversity and community composition can vary with age, season of sampling, and managed facility. Due to these factors, the results of many microbiome studies based on populations in a single managed facility or wild population should not be extrapolated to the species as a whole. Regardless, it remains apparent that at least some species of animals face a decline of microbial

diversity under human management, and that decreased diversity can have implications for animal welfare.

The microbiome has been shown to influence disease susceptibility and metabolic dysregulation in managed wildlife, as well as mediating the prevalence of gastrointestinal disorders (Amato et al., 2013; Wan et al., 2016; McKenzie et al., 2017). In addition, some gut microbes can become pathogenic in managed animals experiencing compromised immune systems due to stress or infection (Wan et al., 2016; Zhao et al., 2017; Xu et al., 2018). Dysbiosis of the microbiome has also been correlated to disease states in several managed species, including: cardiac disease in gorillas (*Gorilla gorilla gorilla*), respiratory infection in red-crowned cranes (*Grus japnoensis*) and Yangtze finless porpoises (*Neophocaena phocaenoides*), and gastrointestinal illness in doucs (*P. nemaeus*) and cynomolgus macaques (*Macaca fascicularis*) (Xie et al., 2016; Wan et al., 2016; Amato et al., 2016; Krynak et al., 2017; Koo et al., 2020).

Animal-associated microbiomes are often species-specific to their host, disseminating vertically between parents and progeny (Ferretti et al., 2018). While there is some horizontal uptake of microbial species from the environment, much of the host microbiome cannot exist independently from that host (Mushegian & Ebert, 2015). If these host-specific microbial species are lost due to some disturbance such as antibiotic usage, they cannot be regained via the environment and will not be passed to offspring. This effect is compounded in captivity, where limited population sizes and reproductive partners can lead to a generational loss of microbial diversity that is nearly impossible to reestablish (Sonnenburg & Sonnenburg, 2019).

Loss of wild-type microbial diversity can lead to the loss of specific microbial species crucial in degrading certain plant toxins and fibers; while this may be of minimal consequence to

animals under human management fed easily digestible diets, it jeopardizes wild translocation or reintroduction attempts, especially for herbivores (McKenzie et al., 2017; West et al., 2019). In addition, released managed animals with dysbiotic microbiomes may be more susceptible to pathogens and disease in the wild, further harming reintroduction efforts and potentially having deleterious effects on wild conspecifics exposed to such individuals (Moeller et al., 2013; McKenzie et al., 2017). Despite this, managed animals translocated *in situ* will mostly re-acquire a wild-type microbiome and experience increased microbial diversity, if given enough time (Schmidt et al., 2019, Yao et al., 2019).

Though most managed species experience a decline in microbial diversity in captivity, there are some exceptions. McKenzie et al. (2017) showed that some families of managed mammals (bovids, giraffes, aardvarks, and anteaters) tend to experience no change in bacterial diversity when compared to their wild counterparts. The same study revealed Rhinocerotidae to be unique as one of the few taxonomic families to actually experience an increase in microbial diversity in captivity. Nevertheless, the low sample size of 13 individuals (n=6 white rhinoceros; n=7 black rhinoceros (*Diceros bicornis*)) casts doubt on the validity of this finding (McKenzie et al., 2017). Other species with more diverse managed microbiomes include the Panama golden frog (*Atelopus zeteki*), red-crowned crane, and Japanese giant salamander (*Andrias japonicus*), though managed Panama golden frogs and red-crowned cranes hosted significantly different community compositions compared to their wild counterparts (Becker et al., 2014; Xie et al., 2016; Bletz et al., 2017). We also cannot yet determine whether increased microbial diversity in managed populations might be beneficial or perhaps indicative of dysbiosis. Further research is necessary to both quantify whether these species truly have higher microbial diversity under

human management and to make more robust inferences as to why the trend is present and/or whether it has any clinical significance.

1.5 Previous Research into Rhinoceros Microbiome

Numerous studies have characterized the gut microbiomes for managed and wild members of the Rhinocerotidae family, including the southern white rhinoceros, black rhinoceros, Sumatran rhinoceros (*Dicerorhinus sumatrensis*) and greater one-horned rhinoceros (*Rhinoceros unicornis*) (Bian et al., 2013; Williams et al., 2019; Roth et al., 2019; Cersosimo et al., 2021). Roth et al. (2019) compared major taxa from all four rhinoceros species and found that Firmicutes (range; 66.3–51.0%) and Bacteroidetes (39.8–23.4%) were most abundant, followed by Verrucomicrobia (7.6–1.9%), Spirochetes (3.1–1.1%), Actinobacteria (1.04–0.03%), and Fibrobacteres (2.14–0.19%) (Roth et al., 2019). While the appearance of certain major taxa in managed southern white rhinoceros microbiomes was consistent across studies, the abundances and rankings of those taxa varied substantially despite all studies utilizing 16S rRNA sequencing of microbial DNA extracted from frozen samples. Cerososimo et al. (2021) and Williams et al. (2019) identified Bacteroidetes as the most common phyla (55%-41.6%) followed by Firmicutes (33%-29%), while Bian et al. (2013) and Roth et al. (2019) identified Firmicutes as the most common phyla (50%-44%), followed by Bacteroidetes (35%-27%). Lentisphaerae was also identified as a major phylum (>1% relative abundance) by both Bian et al. (2013) and Cersosimo et al. (2021), and Cersosimo et al. (2021) alone found a major abundance of Tenericutes (1.43%) among their population of three female southern white rhinoceros.

Though all rhinoceros are herbivores, different rhinoceros species have different feeding strategies. While southern white rhinoceros are obligate grazers, the black, Sumatran, and Javan

rhinoceros (*Rhinoceros sondaicus*) are all browsers (Hutchins & Kreger, 2006). The greater one-horned rhinoceros partakes in both feeding strategies depending on food availability and is thus difficult to categorize (Laurie, 1982). Due to the significant influence of diet on gut microbiome, one would expect the different species to cluster together in microbial composition by feeding strategy. There is some validity to this, as Sumatran and black rhinoceros had higher proportions of Bacteroidetes present than southern white and greater one-horned rhinoceros (Roth et al., 2019). The black and Sumatran rhinoceros also had less microbial diversity than the other two species (Roth et al., 2019). These results supersede expected outcomes based on phylogeny, managed diet, and historic geographical range. Incidentally, there is correlation between those species prone to iron overload disorder and those that are not, though further research is necessary to assess if there is a mechanism behind this trend (Roth et al., 2019). There has also been no published investigation into the microbiomes of Javan rhinoceros, the most endangered and illusive of the five surviving rhinoceros species.

Interestingly, the gut microbiome of an extinct woolly rhinoceros (*Coelodonta antiquitatis*) has been examined using intestinal samples preserved in the permafrost of Eastern Siberia (Mardanov et al., 2012). This female animal had a microbiome dominated by Firmicutes (68%), Proteobacteria (19.2%), Actinobacteria (5.7%), Saccharibacteria (4.3%), and Bacteroidetes (0.2%). Of the Firmicutes present, the most represented line was cellulolytic clostridia (Mardanov et al., 2012). No *Ruminococcus* species were found, though the occurrence of *Ruminococcus* is quite high in modern-day Rhinocerotidae (Mardanov et al., 2012; Bian et al., 2013; Borah et al., 2019). Though Sumatran rhinoceros are the closest living relatives to woolly rhinoceros, there were not many microbial similarities present, especially with the domination of Proteobacteria and lack of Bacteroidetes in the woolly rhinoceros microbiome. Of the rhinoceros

species, only black rhinoceros microbiomes have been characterized with some abundance of Proteobacteria (Antwis et al., 2019, Gibson et al., 2019). Though intriguing, this study had numerous limitations including the sampling of only one animal and the indeterminate amount of time that passed between the death of the animal and its preservation in the ice. Consequently, no robust characterization of the woolly rhinoceros microbiome can be made without a larger sample size.

Dedicated comparisons of managed and free-ranging rhinoceros microbiomes do not yet exist in the literature for any of the rhinoceros species except the black rhinoceros. Gibson et al. (2019) showed that the beta diversity of the microbiomes of managed black rhinoceros are significantly different than those of their wild counterparts, signifying distinct differences in microbial communities across the two populations (n=25; 17 wild vs 8 managed). Gibson et al. (2019) also revealed that managed black rhinoceros have more glycolysis and amino acid synthesis pathways compared to free-ranging rhinoceros, which is indicative of sub-optimal, glucose-rich nutrition.

Historically, the human-managed breeding population of southern white rhinoceros had been robust thanks to the reproductive success of translocated wild females in human management, which accounted for the steep rise in population numbers during the last two decades. However, that robust breeding success has seen a precipitous decline in the past few decades as facilities have struggled with infertile animals and post-copulatory reproductive failure in generations subsequent to the founder females (Swaisgood et al., 2006). Many theories abound as to why this decline has happened, with some evidence pointing towards human-formulated diets and hormonal imbalances including dietary estrogen levels being partially responsible (Tubbs et al., 2016; Williams et al., 2019). White rhinoceros fertility levels have

been shown to vary with both their fecal phytoestrogen profiles and the abundance of certain gut bacteria that metabolize those dietary phytoestrogens (Williams et al., 2019). Phytoestrogens are derived from plants and are especially prevalent in the nutrient-rich legume hay and soy/alfalfa-based concentrates typical in formulated diets (Tubbs et al., 2016). Williams et al. (2019) suggested that reproductive outcomes in white rhinoceros may be linked to gut microbiota important for converting dietary phytoestrogens into their usable metabolites. Similar work has been performed with black rhinoceros. Antwis et al. (2019) showed that certain bacteria in black rhinoceros microbiomes are associated with the production of hormones necessary to achieve breeding success, including rare microbiota that may be difficult to assimilate into the human-formulated diet. The Antwis et al. (2019) study also found significant differences in black rhinoceros gut microbial composition according to individual, facility, reproductive success, and phase of ovarian cycle.

CHAPTER 2: MICROBIOME METHODOLOGY

Chapter 2.1 Challenges of Microbiome Research

Analysis of the microbiome is still a relatively novel field and unique challenges arise depending on area of focus and study methodology. Methodological factors such as sample preservation and storage (Song et al., 2016), DNA extraction (Ferrand et al., 2014; Kennedy et al., 2014; Fiedorová et al., 2019), and bioinformatic analysis (McMurdie & Holmes, 2014) can all contribute to variation in microbial composition results. In addition, microbiomes vary widely across individuals due to age (Adriansjach et al., 2020; Janiak et al., 2021), sex (Markle et al., 2013; Mshelia et al., 2018), diet (Muegge et al., 2011; David et al., 2014; McKenney et al., 2015), geographic location (Yatsunenko et al., 2012), health status (Lewis et al., 2015), and ethnic background (Human Microbiome Project Consortium, 2012) among other factors. This individual variation makes it difficult to identify what constitutes a "normal" or core microbiome for each host. Likewise, positive and negative trends away from a proposed species core microbiome have questionable clinical relevance when the individual does not exhibit a diseased state. Studies performed within a single population in a single geographic region can likely not be extrapolated to the species as a whole. In addition, the composition of the microbiome and abundances of certain taxa does not correlate to the functional impact of those taxa in the host (DeLong & Pace, 2001). Microbial species present at <1% abundance are widely considered non-major taxa and yet may still contribute substantially to metabolite production and other physiological factors (Williams et al., 2019).

It is estimated that the majority of gut microbiota have not been cultured, leaving gaps in understanding of species-level taxonomy (Hayashi et al., 2002; Steen et al., 2019; Lewis et al.,

2021). As the majority of bacterial species are uncharacterized, the development of operational taxonomic units (OTUs) based off of reads became necessary to study microbial abundance (Bhat & Prabhu, 2017); a read is a sequence of base pairs (bp) corresponding to a DNA fragment. Microbial sequences are clustered into OTUs at an arbitrary read similarity level, canonically 95% similarity for genus and 97% for species (Bhat & Prabhu, 2017). OTUs can be assigned based on reference data available through Greengenes©, SILVA©, and other bioinformatic databases, or can be clustered *de novo* by pairwise sequence similarities. Wescott and Schloss (2015) argued that *de novo* assignment of sequences into OTUs was more optimal than using closed-references, however *de novo* OTU assignment is specific to that particular data set and dependent upon relative abundances of the sampled microbial community. As such, *de novo* OTUs sourced from two different data sets are not comparable (Callahan et al., 2017).

A novel replacement for OTU-based microbial clustering came in the form of amplicon sequence variants (ASVs). ASVs are single DNA sequences that are identified prior to amplification, removing possible sequence errors introduced during amplification (Callahan et al., 2017). ASVs can be distinguished by one nucleotide and do not need similarity thresholds, making them more accurate to true taxonomy than OTUs (Callahan et al., 2017). ASVs and OTUs cannot be compared directly due to these differences, but the field is moving towards implementing ASVs as the new standard, leading to questions of validity in relating or combining results from previous studies utilizing OTU-based microbiome analysis.

The study of the gut microbiome faces particular challenges. The fecal microbiome is commonly used as a proxy for host gut microbiome, as collection of fecal material is relatively non-invasive. However, recent research has proposed that feces is inadequate at representing all microbial taxa present in the contents and mucosa across the entirety of the gastrointestinal tract

(Donaldson et al., 2016; Tang et al., 2020). Intestinal biopsy is necessary to access those microbes but that level of invasive sampling is infeasible for all but the most extreme studies utilizing living hosts (i.e. human or wildlife-based studies). Thus, studies must continue to use fecal proxies until newer or more sensitive methods are devised.

Chapter 2.2 Common Bioinformatic and Statistical Analyses

Bioinformatic analysis of sequenced microbial DNA is commonly performed using R, RStudio, QIIME, and MOTHUR software. Other bioinformatic programs, such as the CLC Genomics Workbench, remove a degree of user error by analyzing sequence data using predetermined workflows, generating OTU abundance results, comparative plots, and heat maps. CLC is fully self-contained and is more user-intuitive than RStudio, though set workflows limit analytical freedom (e.g. rarefication levels are determined by the program and cannot be manually changed).

Rarefaction is a normalization tool used in microbiome analysis to mitigate unequal sequencing effects and differences in read sizes by standardizing all reads to one fixed size. Sample libraries are subsampled randomly to a set threshold number of sequences, while samples containing fewer than the minimum coverage threshold are discarded from the dataset. While that size threshold is selected by the user (and may therefore be considered arbitrary), for microbiome work it commonly corresponds to the smallest sequencing depth >10,000 reads (imposed to encompass most sequences while minimizing decreases in data quality) (Navas-Molina et al., 2013). In theory, rarefaction allows samples to be compared statistically without bias from differing sample sizes. Rarefaction has become controversial, however, as the omission of subsampled sequences introduces artificial variation and can reduce the amount of

OTUs recovered (McMurdie & Holmes, 2014). In addition, rarefaction inherently reduces the number of samples present, which can decrease the power of statistics depending on the number of samples retained.

Alpha diversity indices include measures of richness (number of species present), Shannon diversity (the relative abundance of taxa, incorporating both richness and evenness), and Simpson's index (which incorporates richness, evenness, and phylogenetic relatedness of taxa). The Shannon index takes into account rare species, making it very sensitive to small changes in diversity. By contrast, Simpson's index is considered a "dominance" index as it gives greater weight to common species and is not affected by less abundant species. Chao1 is a common richness estimator, based on the concept that rare species can be used to infer an accurate lower bound of species richness (Chao & Chiu, 2016). Chao1 operates by assessing the number of "singletons" (the number of species with only one occurrence) and doubletons (the number of species with two occurrences) within a sample. While singletons (i.e. rare species) are still discovered in the sample, it can be assumed that more are likely present. When all species have been identified twice, there are likely no more undocumented species present within the sample, thus generating a lower bound of species richness.

Tests of significance are needed to understand whether associations between groups and covariates are due to chance or due to legitimate differences. Kruskal-Wallis *H* tests and Wilcoxon rank-sum tests are often utilized for determining significant differences in alpha diversity indices. The Kruskal-Wallis *H* test is a nonparametric method useful for determining the presence or absence of significance (and the degree of that significance) between independent groups but does not identify exactly which groups differ nor by how much. For that

determination, the non-parametric Wilcoxon rank-sum (also known as Mann-Whitney *U* test) test is often employed (Xia et al., 2020).

In contrast, beta diversity indices measure the dissimilarity and distances between microbial communities/groups. Without a reconstructed phylogenetic tree, the Bray-Curtis dissimilarity or Jaccard index are used to compare dissimilarity, while the Euclidean method is used to calculate distance. Bray-Curtis dissimilarity values quantify compositional dissimilarity between two groups based on the presence/absence and relative abundance of community membership. Calculated Bray-Curtis values range between 0 and 1, with 0 signifying no species dissimilarity between two groups (i.e. the sites are identical in species composition) and 1 signifying complete species dissimilarity between two sites (i.e. the sites share no species). The Jaccard index operates similarly to Bray-Curtis but only uses presence/absence data, not abundance data. The Euclidean method calculates distance between data points and is used when variables are expected to have equal variance. However, Euclidean distance is inappropriate for community composition comparisons, as it does not take species identity into account. When a phylogenetic tree is present, UniFrac measures may be utilized. UniFrac (unweighted or weighted) distances are calculated by the fraction of phylogenetic tree branch length shared between communities. Unweighted UniFrac uses only the presence or absence of lineages, while weighted UniFrac measures give weight to branches based on the relative abundance of the taxa within the communities.

Permutational multivariate analysis of variance (PERMANOVA) analyses are utilized to assess significance between communities using beta diversity measures (i.e. Jaccard, Bray-Curtis, or Euclidean values). These measures can also be used to calculate distance matrices, which are then used to create ordination plots (e.g. NMDS or PCoA plots) for two-dimensional

visual comparison of community clustering, visualize differentially abundant taxa, and organize hierarchical community cladograms (Aagaard et al., 2014; Van Rensburg et al., 2015; Fourie et al., 2017).

Chapter 2.3 Effects of Different Storage Techniques

Proper sample preservation is paramount to ensuring accurate and reproducible results in microbiome research. Preservation is important for reducing changes to the original microbial community in the sample, which can confound downstream results and lead to less robust conclusions. Immediate freezing at -80°C is considered the gold standard for microbiome sample preservation, followed by freezing at -20°C (Flores et al., 2015; Loftfield et al., 2016; Song et al., 2016). However, ultra-low freezing can be logistically difficult for home-based or field-based microbiome studies, thus the exploration of preservation methods with comparable performance at ambient temperature becomes necessary.

As internal sampling is extremely invasive, fecal samples are used as a proxy to study gut microbiome (Moossavi et al., 2019). Studies have shown that fecal preservation method can affect gut microbial composition and diversity indices (Song et al., 2016; Loftfield et al., 2016; Wu et al., 2019). These preservation methods vary from sample immersion in various concentrations of ethanol to a multitude of commercially available preservatives and storage devices. The most prominently studied preservation methods include OMNIgene•GUT (DNA Genotek, Ottawa, Canada), Flinders Technology Associates (FTA) cards (Whatman plc, Maidstone, UK), 70-100% ethanol, and RNA*later* (Thermo Fisher Scientific, Waltham, MA, USA). In addition, simple refrigeration at 4°C and storage at ambient temperature have been widely assessed (Carroll et al., 2012; Cardona et al., 2012; Tedjo et al., 2015; Choo et al., 2015;

Song et al., 2016; Horng et al., 2018). While refrigeration of unstabilized samples can be utilized for limited amounts of time (<72 hours) with small risks to microbial community composition, both methods are inappropriate for any long-term storage (Tedjo et al., 2015; Choo et al., 2015; Wu et al., 2019).

Of the long-term preservation methods, OMNIgene•GUT and FTA cards have been reported to consistently perform most similarly to -80°C controls and technical replicates (Choo et al., 2015; Song et al., 2016; Wang et al., 2018). OMNIgene•GUT is a commercial, proprietary preservative and storage device and is meant to preserve fecal samples utilized for general gut microbial profile analysis. The main benefit to this device is that samples can be stabilized within the internal solution for 60 days at temperature fluctuations from -20°C to 50°C; this minimizes the chance for error during sample storage and transportation, especially for remote studies. While the device is designed to be user-friendly and optimizes sample collection for home-based microbiome studies, it can be cost prohibitive when compared to other preservation methods (DNA Genotek, 2019). OMNIgene•GUT is the predecessor of the novel PERFORMAbiome•GUT (DNA Genotek, Ottawa, Canada) product, which was formulated specifically for use in the preservation of feces for animal gut microbial profile analysis. PERFORMAbiome•GUT has relatively little published literature regarding its efficacy, but initial reports are promising; it has been validated for usage in dogs, cats, and horses internally by DNA Genotek, and has since been utilized for canine and pinniped microbiome studies (Lin et al., 2020; Steinmetz et al., 2021).

FTA cards are cotton-based cellulose paper cards infused with chemicals that lyse cells, denature proteins, and prevent DNA from degrading upon contact. They are therefore capable of inhibiting microbial growth, leading to their usage as a sample stabilizer for microbiome studies

(Song et al., 2016; Wang et al., 2018). While originally developed for usage in preserving blood and cell samples, recent work has proven their efficacy in preserving feces (Song et al., 2016; Wang et al., 2018). Similarly to OMNIgene•GUT, an advantage of this method is long-term stabilization at ambient temperature. FTA cards are also one of the more cost-effective preservation methods listed here. However, FTA cards routinely recover greater diversity of microbiota after sequencing than other methods; this effect is possibly due to the chemical cell lysing inherent to the preservative (Hale et al., 2015; Song et al., 2016). Fortunately, this effect is systemic such that it is possible to identify and remove the artifacts from FTA card samples using statistical software (Song et al., 2016). Another drawback is that extracted DNA yields from FTA cards are typically low, though methods for repeated extraction of DNA from a previously-processed FTA-disk have been published (Stangegaard et al., 2011).

RNAlater is a commercial preservation method that is used more generally to stabilize RNA in samples for further applications. The premise of RNAlater is that it can be used to store samples unfrozen, yet Choo et al. (2015) found that samples stored in RNAlater at ambient temperatures were subject to significant differences in Shannon diversity indices when compared to -80°C control samples. RNAlater also returned lower DNA extraction yields and decreased DNA purity (Choo et al., 2015). Manufacturer instructions stipulate that RNAlater was not designed for use at ambient temperatures for longer than 2 weeks, a timespan limitation later confirmed by Song et al. (2016). However, within this time frame RNAlater performs nearly as well as OMNIgene•GUT and FTA cards (Song et al., 2016).

Ethanol is a valid preservative for feces as it penetrates cell membranes and deactivates DNases which effectively halts DNA degradation; this effect is expedited at higher concentrations (King & Porter, 2004; Song et al., 2016). Varying concentrations of ethanol have

been assessed for efficacy in fecal preservation, with 95% being the most effective and widely used. While Hale et al. (2015) found that 100% ethanol preserved the microbial community in spider monkey (*Ateles geoffroyi*) feces comparably to fresh samples, this concentration has not been extensively validated otherwise. Conversely, 70% ethanol has been consistently shown to be inadequate, with significant changes in microbial communities occurring over time similar to that which occurs in unstabilized samples (Song et al., 2016). The differences were also comparable to intra-individual and interspecies differences, further confirming its inadequacy (Song et al., 2016). Any concentration of ethanol under 95% is not recommended for fecal preservation in microbiome studies. Benefits of using ethanol include its low cost and easy acquisition, especially in comparison to the other preservation methods; it has also been validated for usage at ambient temperature for several weeks. One disadvantage to using ethanol is that samples stored in ethanol yielded consistently low concentrations of DNA after extraction (Vlčková et al., 2012; Hale et al., 2015; Song et al., 2016). To mitigate this, ethanol should be removed from samples prior to DNA extraction through the use of silica beads or through evaporation of the ethanol at temperatures below 60°C (Nsubuga et al., 2004; Nagy 2010).

As stated above, multiple studies have reported high microbial community composition stability in samples collected with FTA cards, 95% ethanol, and OMNIgene•GUT stored at ambient temperature for several days to several weeks (Nechvatal et al., 2008; Loftfield et al., 2016; Song et al., 2016; Wang et al., 2018). Furthermore, it has been shown that intra-individual variation has more bearing on gut microbial composition than the variation found between most storage methods. This is a consequence of individual diet, genetic background, age, sex, and other factors that all contribute holistically to microbial diversity. Choice of preservation

technique can thus be tailored to match research interests, sampling methodology, financial limitations, and travel logistics.

Chapter 2.4 Effects of Different Extraction Methods

There are a multitude of commercial DNA extraction kits available on the market for genomic research, and previous studies have shown that the extraction kit used will influence the end result gut microbial composition sequenced (Ferrand et al., 2014; Kennedy et al., 2014; Fiedorová et al., 2019). A previous study comparing the PowerFecal DNA kit (QIAGEN, Germantown, MD, USA) to other commercial kits, including the QIAamp Stool kit, has shown the PowerFecal kit to be successful in isolating the most OTUs, a measure of microbial richness, from porcine fecal samples (Muiños-Bühl et al., 2018). Muiños-Bühl et al. (2018) also found that the QIAamp Stool kit resulted in the highest yield of extracted DNA; however, the authors stipulate this is potentially due to contamination during DNA isolation. When assessing extracted DNA fragmentation with agarose gel electrophoresis, both the PowerFecal and Stool Mini kits showed unsmeared DNA bands with high molecular weights (Lim et al., 2018). Lim et al. (2018) also found that human fecal samples extracted with the PowerFecal kit had the highest degree of microbial alpha diversity between the three kits they tested.

Gibson et al. (2019) compared wild black rhinoceros fecal samples processed with the QIAamp PowerFecal DNA kit to both wild and human-managed black rhinoceros fecal samples processed with ZymoBIOMICS DNA Miniprep extraction kit (Zymo Research, Irvine, CA, USA), due to constraints in regional kit availability. The study found that the Zymo kit produced more read results after mapped sequencing, although alpha diversity results remained comparable between the two kits (Gibson et al., 2019). However, the Zymo kit samples were

stored in Zymo DNA/RNA Shield preservation solution, which may have affected preservation of microbes and subsequent extraction results.

Bead-beating of samples (the disruption of bacterial cell walls with small beads or minerals) is a crucial step for the recovery of higher DNA yields and integrity correlating to increased recovery of microbial taxa during analysis (Zhang et al., 2020). It is thus recommended that whichever extraction kit is selected for a microbiome study contains a bead-beating step in the procedure.

Chapter 2.5 Common Microbial DNA Sequencing Methods

There are three main types of DNA sequencing utilized for microbiome studies. These include Sanger sequencing, next-generation sequencing (NGS), and shotgun sequencing. Sanger sequencing involves the amplification of target DNA by PCR and the binding of a short primer next to the region of interest on a DNA strand. DNA polymerase extends the primer to make a complementary strand, stopping once it hits an altered, fluorescently labeled chain-terminating dideoxynucleotide. The extension products are separated by capillary electrophoresis, and a laser is shined through the capillary gel to excite the fluorescent dye tag on the DNA fragments. Each color corresponds to a specific nucleotide base, and software can identify the color wavelengths and correspond them to DNA sequences. Sanger sequencing is considered a long-read sequencing technology, as it produces DNA sequences between 700-1000 nucleotides long. It is optimal for small scale projects, where it is relatively fast and cost-effective compared to NGS methods and has lower error rates. However, Sanger sequencing can only sequence one DNA fragment at a time, making it not as time- or cost-effective for projects dealing with large

amounts of DNA. Detection sensitivity in Sanger sequencing is also low (15% - 20%), so that any mutant alleles or organisms below that threshold are not detected (Rohlin et al., 2009).

There are many NGS platforms that differ by methodology, though NGS platforms as a whole differ from Sanger sequencing in distinct ways. NGS uses DNA templates which have been clonally amplified *in vitro*, and DNA sequences are determined by the addition of specific nucleotides as opposed to chain-termination. Lastly, DNA can be sequenced in a massive parallel method which allows for the reading of millions of sequences at once, making the technology "high-throughput". The most commonly utilized NGS technique for microbiome work is 16S rRNA amplicon sequencing. The 16S ribosomal RNA (rRNA) gene is a highly conserved gene present in almost all bacteria; the function of this gene has not changed over time and it is large enough (~1,500 bp) to allow for bioinformatics (Janda & Abbott, 2007). The 16S gene consists of both conserved and hypervariable sections. Conserved sections allow for amplification by providing primer binding sites, while variable sections (V1-V9) allow for discrimination of different microbiota by species-specific sequences. Prior studies using 16S sequencing overwhelmingly sampled specific hypervariable regions instead of the entire gene, as each hypervariable region is between 30 – 90 bp long and can be sequenced without the exorbitant cost that sequencing of the entire ~1,500 bp gene can incur. However, sequencing costs are declining with advances in technology and recent literature has begun advocating for the sequencing of the entire gene to achieve optimal taxonomic resolution (Johnson et al., 2019).

Lastly, shotgun sequencing is a metagenomic method whereby DNA from the entire microbial community is fragmented for sequencing. Each fragment (ranging from 2,000 – 20,000 bp) contains nucleotide sequences that are assembled based on overlap via computer program. It is much faster than Sanger or NGS sequencing but requires an existing reference genome and

extensive computational power to produce results; these results sometimes contain errors that must be fixed using NGS or other sequencing methods (Bartram et al., 2011; Langille et al., 2013: Clooney et al., 2016). Shotgun sequencing characterizes the entire microbial community and is better at identifying rare or less abundant taxa than 16S sequencing. It also allows for analysis of functional relationships between host and microbiota, a feature which 16S sequencing mostly lacked (Jovel et al., 2016).

Each sequencing method has advantages and disadvantages, and broad biological patterns in community characterization are consistent across differing methods (Rausch et al., 2019). It is important for researchers to consider cost, research question, sample size, and resource availability when deciding which method to select. Given the rapidity of technological advancement in the field, what is common practice today will likely be outdated within the next several years.

CHAPTER 3: CURRENT RESEARCH PLAN

3.1 Justification

Due to the pervasiveness of microbial reciprocal interactions with host health, digestion, and physiology, it is imperative that we catalogue and understand the gut microbiome across species, especially for those that are imperiled. Wild populations of southern white rhinoceros are under constant attack from poachers looking to sell rhinoceros horn on the black market. As southern white rhinoceros population numbers have begun declining, they are considered near threatened by the International Union for Conservation of Nature (IUCN) (Emslie, 2020). Human management of southern white rhinoceros assurance populations becomes imperative, however these populations have historically faced infertility and post-reproductive failure (Swaisgood et al., 2006; Metrione & Eyres, 2014). As reproductive outcomes in southern white rhinoceros have been linked to the abundance of several microbial taxa, it's important that we continue cataloguing the "core" microbiota of managed southern white rhinoceros populations (Williams et al., 2019). We also need to understand the microbial dynamics involved in variation between individuals.

Literature has shown that human managed southern white rhinoceros and black rhinoceros experience increased diversity compared to their wild conspecifics, though small sample sizes (n=6 southern white rhinoceros, 3 managed and 3 wild; n=7 black rhinoceros, 6 managed and 1 wild) and a limited sampling period cast doubt upon the findings (McKenzie et al., 2017). Even if the findings are valid, the implications of increased taxa diversity compared to previously published species baselines is unknown. In addition, many wild populations of southern white rhinoceros are reproductively successful where several managed populations are

not (Swaisgood et al., 2006; Tubbs et al., 2016). Thus, the need to research the microbiomes of both managed and wild southern white rhinoceros populations and devise how and why they differ is borne. Fieldwork to collect wild rhinoceros feces is necessitated, however proper preservation of fecal material and stabilization of the microbiota inside the sample becomes challenging under field conditions. The gold standard of feces preservation for microbiome studies is immediate freezing at -80°C, but is logistically difficult to achieve *in situ* (Wu et al., 2010; Choo et al., 2015).

We decided to compare the efficacy of 95% ethanol and commercial PERFORMAbiome•GUT (PB) tubes for preserving microbial diversity and community composition over time when compared to freezing at -80°C. The use of 95% ethanol for feces preservation has been extensively validated and is a relatively cheap alternative to freezing biological samples, but no previous studies have pushed its efficacy to our maximum of 230 days (Song et al., 2016; Wang et al., 2018). PB tubes were selected because they were novel and had traits that would lend well to microbiome fieldwork (i.e. the ability to stabilize fecal samples for 60 days at temperature fluctuations from -20°C to 50°C). PB tubes have only been used in two other studies, both of which studied species (canines and pinnipeds) possessing simpler gastrointestinal tracts than the hindgut-fermenting herbivorous rhinoceros (Lin et al., 2020; Steinmetz et al., 2021). In addition, the predecessor product OMNIgene•GUT had seen great success in microbial preservation but was optimized for human microbiome studies.

As for our methodology, we decided to use sequencing of the V3-V4 hypervariable region of the 16S rRNA gene to sequence microbial DNA in our samples as it has been extensively validated in the literature for use in microbiome metataxonomics. The V3-V4 region has been recommended for the profiling of microbial communities over other regions present

within the 16S gene (Rausch et al., 2019). Cost is an important consideration for any study, and 16S sequencing was also the most cost-effective high throughput sequencing technique available locally for the number of samples provided. During bioinformatics, we opted to cluster bacterial taxa into OTUs as opposed to ASVs so that data would be more comparable to previously published literature. Lastly, we selected the Powerfecal Pro kit over other DNA extraction kits as it has been shown to be successful in isolating high degrees of alpha diversity compared to other kits and produces extracted DNA with high molecular weight and quality (Muiños-Bühl et al., 2018; Lim et al., 2018). The Powerfecal Pro kit has also been previously validated with use in rhinoceros microbiome studies (Gibson et al., 2019). A similarly important factor was that the kit contained a bead-beating step, the usage of which has been shown to recover higher DNA yields and more microbial taxa during analysis (Zhang et al., 2020).

3.2 Objectives

The objectives of these studies were to:

1) Characterize the taxonomic composition of the gut microbiome in a managed population of southern white rhinoceros (n=10) at the NC Zoo, Asheboro, NC, USA

2) Assess the effects of seasonality and age classes on gut microbial community structure in managed southern white rhinoceros

3) Compare the efficacy of different fecal preservation techniques (immediate freezing at -80°C, PERFORMAbiome™•GUT tubes, and 95% ethanol) for preserving fecal samples from southern white rhinoceros

4) Test the limitations of those preservation methods (immediate freezing at -80°C, PERFORMAbiome™•GUT tubes, and 95% ethanol) for 14 days and 230 days

3.3 Hypotheses

Specifically, we tested the following hypotheses:

1. The NC Zoo population of rhinoceros would display individual, age and seasonal differences in microbiome alpha and beta diversity, as measured by Shannon index, Simpson's index, and Bray-Curtis dissimilarity
2. Of the four age-classes present, the geriatric individual would display the largest deviations in species diversity and community composition due to treatment of arthritis with medication
3. Feces collected during warm weather months would contain higher microbial species diversity and possess a different community composition compared to cold weather months, due to increased outdoor access and availability of grasses during the summer
4. Ethanol and PB samples would perform similarly to -80°C controls in preserving microbial richness and community composition in fecal samples over a 14-day period
5. Microbial richness and community composition for PB samples would be altered between 14 and 230 days of storage at ambient temperature due to degradation of both preservative solution and sample, and subsequent changes in the observed microbial community profile

REFERENCES

Aagaard, K., Ma, J., Antony, K. M., Ganu, R., Petrosino, J., & Versalovic, J. (2014). The placenta harbors a unique microbiome. *Science Translational Medicine*, *6*(237), 237ra65-237ra65.

Adriansjach, J., Baum, S. T., Lefkowitz, E. J., Van Der Pol, W. J., Buford, T. W., & Colman, R. J. (2020). Age-related differences in the gut microbiome of rhesus macaques. *The Journals of Gerontology: Series A*, *75*(7), 1293–1298.

Amato, K. R., Metcalf, J. L., Song, S. J., Hale, V. L., Clayton, J., Ackermann, G., Humphrey, G., Niu, K., Cui, D., Zhao, H., Schrenzel, M. D., Tan, C. L., Knight, R., & Braun, J. (2016). Using the gut microbiota as a novel tool for examining colobine primate GI health. *Global Ecology and Conservation*, *7*, 225–237.

Amato, K. R., Yeoman, C. J., Cerda, G., A. Schmitt, C., Cramer, J. D., Miller, M. E. B., Gomez, A., R. Turner, T., Wilson, B. A., Stumpf, R. M., Nelson, K. E., White, B. A., Knight, R., & Leigh, S. R. (2015). Variable responses of human and non-human primate gut microbiomes to a Western diet. *Microbiome*, *3*(1), 1-9.

Amato, K. R., Yeoman, C. J., Kent, A., Righini, N., Carbonero, F., Estrada, A., Rex Gaskins, H., Stumpf, R. M., Yildirim, S., Torralba, M., Gillis, M., Wilson, B. A., Nelson, K. E., White, B. A., & Leigh, S. R. (2013). Habitat degradation impacts black howler monkey (*Alouatta pigra*) gastrointestinal microbiomes. *The ISME Journal*, *7*(7), 1344–1353.

Antwis, R. E., Edwards, K. L., Unwin, B., Walker, S. L., & Shultz, S. (2019). Rare gut microbiota associated with breeding success, hormone metabolites and ovarian cycle phase in the critically endangered eastern black rhino. *Microbiome*, *7*(1), 1-12..

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., Fernandes, G. R., Tap, J., Bruls, T., Batto, J.-M., Bertalan, M., Borruel, N., Casellas, F., Fernandez, L., Gautier, L., Hansen, T., Hattori, M., Hayashi, T., Kleerebezem, M., … Bork, P. (2011). Enterotypes of the human gut microbiome. *Nature*, *473*(7346), 174–180.

Bakken, J. S., Borody, T., Brandt, L. J., Brill, J. V., Demarco, D. C., Franzos, M. A., Kelly, C., Khoruts, A., Louie, T., Martinelli, L. P., Moore, T. A., Russell, G., & Surawicz, C. (2011). Treating *Clostridium difficile* infection with fecal microbiota transplantation. *Clinical Gastroenterology and Hepatology*, *9*(12), 1044–1049.

Bartram, A. K., Lynch, M. D. J., Stearns, J. C., Moreno-Hagelsieb, G., & Neufeld, J. D. (2011). Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-and Illumina reads. *Applied and Environmental Microbiology*, *77*(11), 3846–3852.

Becker, M. H., Richards-Zawacki, C. L., Gratwicke, B., & Belden, L. K. (2014). The effect of captivity on the cutaneous bacterial community of the critically endangered Panamanian golden frog (*Atelopus zeteki*). *Biological Conservation*, *176*, 199–206.

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M.-C. C., Charles, T., Chen, X., Cocolin, L., Eversole, K., Corral, G. H., Kazou, M., Kinkel, L., Lange, L., Lima, N., Loy, A., Macklin, J. A., Maguin, E., Mauchline, T., McClure, R., … Schloter, M. (2020). Microbiome definition re-visited: Old concepts and new challenges. *Microbiome*, *8*(1), 1-22.

Bezirtzoglou, E., Tsiotsias, A., & Welling, G. W. (2011). Microbiota profile in feces of breast- and formula-fed newborns by using fluorescence in situ hybridization (FISH). *Anaerobe*, *17*(6), 478–482.

Bhat, A., & Prabhu, P. (2017). OTU Clustering: A window to analyze uncultured microbial world. *International Journal of Scientific Research in Computer Science and Engineering*, *5*, 62–68.

Biagi, E., Quercia, S., Aceti, A., Beghetti, I., Rampelli, S., Turroni, S., Faldella, G., Candela, M., Brigidi, P., & Corvaglia, L. (2017). The bacterial ecosystem of mother's milk and infant's mouth and gut. *Frontiers in Microbiology*, *8*, 1214.

Bian, G., Ma, L., Su, Y., & Zhu, W. (2013). The microbial community in the feces of the white rhinoceros (*Ceratotherium simum*) as determined by barcoded pyrosequencing analysis. *PLOS ONE*, *8*(7), e70103.

Bletz, M. C., Vences, M., Sabino-Pinto, J., Taguchi, Y., Shimizu, N., Nishikawa, K., & Kurabayashi, A. (2017). Cutaneous microbiota of the Japanese giant salamander (*Andrias japonicus*), a representative of an ancient amphibian clade. *Hydrobiologia*, *795*(1), 153–167.

Borah, P., Dutta, R., Barkalita, L. M., Buragohain, L., Deka, P., Ali, S., Choudhury, B., & Basumatary, P. (2019). Deciphering the fecal microbiome of Indian rhinoceros (*Rhinoceros unicornis*) by metagenomic approach. *Asian Journal of Conservation Biology*, *8*(2), 135–141.

Borbón-García, A., Reyes, A., Vives-Flórez, M., & Caballero, S. (2017). Captivity shapes the gut microbiota of Andean bears: Insights into health surveillance. *Frontiers in Microbiology*, *8*, 1316.

Brennan, C. A., & Garrett, W. S. (2016). Gut microbiota, inflammation, and colorectal cancer. *Annual Review of Microbiology*, *70*(1), 395–411.

Callahan, B. J., McMurdie, P. J., & Holmes, S. P. (2017). Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *The ISME Journal*, *11*(12), 2639–2643.

Cardona, S., Eck, A., Cassellas, M., Gallart, M., Alastrue, C., Dore, J., Azpiroz, F., Roca, J., Guarner, F., & Manichanh, C. (2012). Storage conditions of intestinal microbiota matter in metagenomic analysis. *BMC Microbiology*, *12*(1), 1-8.

Carey, H. V., Walters, W. A., & Knight, R. (2013). Seasonal restructuring of the ground squirrel gut microbiota over the annual hibernation cycle. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *304*(1), R33–R42.

Carroll, I. M., Ringel-Kulka, T., Siddle, J. P., Klaenhammer, T. R., & Ringel, Y. (2012). Characterization of the fecal microbiota using high-throughput sequencing reveals a stable microbial community during storage. *PLOS ONE*, *7*(10), e46953.

Carthey, A. J. R., Blumstein, D. T., Gallagher, R. V., Tetu, S. G., & Gillings, M. R. (2020). Conserving the holobiont. *Functional Ecology*, *34*(4), 764–776.

Cersosimo, L. M., Sullivan, K. E., & Valdes, E. V. (2021). Species and individual rhinoceros affect the bacterial communities, metabolites, and nutrient composition in faeces from Southern black rhinoceros (*Diceros bicornis minor*) and Southern white rhinoceros (*Ceratotherium simum simum*) under managed care. *Journal of Animal Physiology and Animal Nutrition*, *0(0),* 1-13.

Chao, A., & Chiu, C. H. (2016). Species richness: estimation and comparison. *Wiley Stats Ref: Statistics Reference Online*, 1-26

Cholewińska, P., Czyż, K., Nowakowski, P., & Wyrostek, A. (2020). The microbiome of the digestive system of ruminants – a review. *Animal Health Research Reviews*, *21*(1), 3–14.

Choo, J. M., Leong, L. E., & Rogers, G. B. (2015). Sample storage conditions significantly influence faecal microbiome profiles. *Scientific Reports*, *5*(1), 1-10.

Clayton, J. B., Vangay, P., Huang, H., Ward, T., Hillmann, B. M., Al-Ghalith, G. A., Travis, D. A., Long, H. T., Tuan, B. V., Minh, V. V., Cabana, F., Nadler, T., Toddes, B., Murphy, T., Glander, K. E., Johnson, T. J., & Knights, D. (2016). Captivity humanizes the primate microbiome. *Proceedings of the National Academy of Sciences*, *113*(37), 10376–10381.

Clayton, T. A., Baker, D., Lindon, J. C., Everett, J. R., & Nicholson, J. K. (2009). Pharmacometabonomic identification of a significant host-microbiome metabolic interaction affecting human drug metabolism. *Proceedings of the National Academy of Sciences*, *106*(34), 14728–14733.

Clooney, A. G., Fouhy, F., Sleator, R. D., Driscoll, A. O., Stanton, C., Cotter, P. D., & Claesson, M. J. (2016). Comparing Apples and Oranges?: Next generation sequencing and its impact on microbiome analysis. *PLOS ONE*, *11*(2), e0148028.

Conlon, M. A., & Bird, A. R. (2015). the impact of diet and lifestyle on gut microbiota and human health. *Nutrients*, *7*(1), 17–44.

Dang, A. T., & Marsland, B. J. (2019). Microbes, metabolites, and the gut–lung axis. *Mucosal Immunology*, *12*(4), 843–850.

Das, B., & Nair, G. B. (2019). Homeostasis and dysbiosis of the gut microbiome in health and disease. *Journal of Biosciences*, *44*(5), 1-8.

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., Ling, A. V., Devlin, A. S., Varma, Y., Fischbach, M. A., Biddinger, S. B., Dutton, R. J., & Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature*, *505*(7484), 559–563.

De Filippo, C., Cavalieri, D., Paola, M. D., Ramazzotti, M., Poullet, J. B., Massart, S., Collini, S., Pieraccini, G., & Lionetti, P. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proceedings of the National Academy of Sciences*, *107*(33), 14691–14696.

DeLong, E. F., & Pace, N. R. (2001). Environmental diversity of bacteria and archaea. *Systematic Biology*, *50*(4), 470–478.

Deo, P. N., & Deshmukh, R. (2019). Oral microbiome: Unveiling the fundamentals. *Journal of Oral and Maxillofacial Pathology : JOMFP*, *23*(1), 122–128.

Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., & Knight, R. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proceedings of the National Academy of Sciences*, *107*(26), 11971–11975.

Donaldson, G. P., Lee, S. M., & Mazmanian, S. K. (2016). Gut biogeography of the bacterial microbiota. *Nature Reviews Microbiology*, *14*(1), 20–32.

Faith, J. J., Guruge, J. L., Charbonneau, M., Subramanian, S., Seedorf, H., Goodman, A. L., Clemente, J. C., Knight, R., Heath, A. C., Leibel, R. L., Rosenbaum, M., & Gordon, J. I. (2013). The long-term stability of the human gut microbiota. *Science*, *341*(6141), 1237439.

Ferrand, J., Patron, K., Legrand-Frossi, C., Frippiat, J.-P., Merlin, C., Alauzet, C., & Lozniewski, A. (2014). Comparison of seven methods for extraction of bacterial DNA from fecal and cecal samples of mice. *Journal of Microbiological Methods*, *105*, 180–185.

Ferretti, P., Pasolli, E., Tett, A., Asnicar, F., Gorfer, V., Fedi, S., Armanini, F., Truong, D. T., Manara, S., Zolfo, M., Beghini, F., Bertorelli, R., De Sanctis, V., Bariletti, I., Canto, R., Clementi, R., Cologna, M., Crifò, T., Cusumano, G., … Segata, N. (2018). Mother-to-infant microbial transmission from different body sites shapes the developing infant gut microbiome. *Cell Host & Microbe*, *24*(1), 133-145.e5.

Fiedorová, K., Radvanský, M., Němcová, E., Grombiříková, H., Bosák, J., Černochová, M., Lexa, M., Šmajs, D., & Freiberger, T. (2019). The impact of DNA extraction methods on stool bacterial and fungal microbiota community recovery. *Frontiers in Microbiology*, *10*, 821.

Flint, H. J., Scott, K. P., Louis, P., & Duncan, S. H. (2012). The role of the gut microbiota in nutrition and health. *Nature Reviews Gastroenterology & Hepatology*, *9*(10), 577–589.

Flores, R., Shi, J., Yu, G., Ma, B., Ravel, J., Goedert, J. J., & Sinha, R. (2015). Collection media and delayed freezing effects on microbial composition of human stool. *Microbiome*, *3*(1), 1-11.

Fourie, N. H., Wang, D., Abey, S. K., Creekmore, A. L., Hong, S., Martin, C. G., Wiley, J. W., & Henderson, W. A. (2017). Structural and functional alterations in the colonic microbiome of the rat in a model of stress induced irritable bowel syndrome. *Gut Microbes*, *8*(1), 33–45.

Gagliardi, A., Totino, V., Cacciotti, F., Iebba, V., Neroni, B., Bonfiglio, G., Trancassini, M., Passariello, C., Pantanella, F., & Schippa, S. (2018). Rebuilding the gut microbiota ecosystem. *International Journal of Environmental Research and Public Health*, *15*(8), 1679.

García-Amado, M. A., Michelangeli, F., Gueneau, P., Perez, M. E., & Domínguez-Bello, M. G. (2007). Bacterial detoxification of saponins in the crop of the avian foregut fermenter *Opisthocomus hoazin*. *Journal of Animal and Feed Sciences*, *16*(Suppl. 2), 82–85.

Garrett, G., & Uno, J. (2021). The gut microbiome's influence on hormones and fertility in zebrafish. *The FASEB Journal*, *35*(S1).

Gibson, K. M., Nguyen, B. N., Neumann, L. M., Miller, M., Buss, P., Daniels, S., Ahn, M. J., Crandall, K. A., & Pukazhenthi, B. (2019). Gut microbiome differences between wild and captive black rhinoceros – implications for rhino health. *Scientific Reports*, *9*(1), 1-11

Gillman, S. J., McKenney, E. A., & Lafferty, D. J. R. (2020). Wild black bears harbor simple gut microbial communities with little difference between the jejunum and colon. *Scientific Reports*, *10*(1), 1-11.

Greene, L. K., Williams, C. V., Junge, R. E., Mahefarisoa, K. L., Rajaonarivelo, T., Rakotondrainibe, H., O'Connell, T. M., & Drea, C. M. (2020). A role for gut microbiota in host niche differentiation. *The ISME Journal*, *14*(7), 1675–1687.

Guo, W., Ren, K., Ning, R., Li, C., Zhang, H., Li, D., Xu, L., Sun, F., & Dai, M. (2020). Fecal microbiota transplantation provides new insight into wildlife conservation. *Global Ecology and Conservation*, *24*, e01234.

Hale, V. L., Tan, C. L., Knight, R., & Amato, K. R. (2015). Effect of preservation method on spider monkey (*Ateles geoffroyi*) fecal microbiota over 8 weeks. *Journal of Microbiological Methods*, *113*, 16–26.

Harmsen, H. J. M., Wildeboer–Veloo, A. C. M., Raangs, G. C., Wagendorp, A. A., Klijn, N., Bindels, J. G., & Welling, G. W. (2000). Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *Journal of Pediatric Gastroenterology and Nutrition*, *30*(1), 61–67.

Hayashi, H., Sakamoto, M., & Benno, Y. (2002). Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods. *Microbiology and Immunology*, *46*(8), 535–548.

Hehemann, J.-H., Correc, G., Barbeyron, T., Helbert, W., Czjzek, M., & Michel, G. (2010). Transfer of carbohydrate-active enzymes from marine bacteria to Japanese gut microbiota. *Nature*, *464*(7290), 908–912.

Hills, R. D., Pontefract, B. A., Mishcon, H. R., Black, C. A., Sutton, S. C., & Theberge, C. R. (2019). Gut microbiome: Profound implications for diet and disease. *Nutrients*, *11*(7), 1613.

Horng, K. R., Ganz, H. H., Eisen, J. A., & Marks, S. L. (2018). Effects of preservation method on canine (*Canis lupus familiaris*) fecal microbiota. *PeerJ*, *6*, e4827.

Hutchins, M., & Kreger, M. D. (2006). Rhinoceros behaviour: Implications for captive management and conservation. *International Zoo Yearbook*, *40*(1), 150–173.

IUCN. (2021). *The IUCN Red List of Threatened Species*. *Version 2021-2*. https://www.iucnredlist.org

Janda, J. M., & Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Journal of Clinical Microbiology*, *45*(9), 2761–2764.

Janiak, M. C., Montague, M. J., Villamil, C. I., Stock, M. K., Trujillo, A. E., DePasquale, A. N., Orkin, J. D., Bauman Surratt, S. E., Gonzalez, O., Platt, M. L., Martínez, M. I., Antón, S. C., Dominguez-Bello, M. G., Melin, A. D., & Higham, J. P. (2021). Age and sex-associated variation in the multi-site microbiome of an entire social group of free-ranging rhesus macaques. *Microbiome*, *9*(1), 1-17.

Johnson, J. S., Spakowicz, D. J., Hong, B.-Y., Petersen, L. M., Demkowicz, P., Chen, L., Leopold, S. R., Hanson, B. M., Agresta, H. O., Gerstein, M., Sodergren, E., & Weinstock, G. M. (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nature Communications*, *10*(1), 1-11.

Jovel, J., Patterson, J., Wang, W., Hotte, N., O'Keefe, S., Mitchel, T., Perry, T., Kao, D., Mason, A. L., Madsen, K. L., & Wong, G. K.-S. (2016). Characterization of the gut microbiome using 16S or shotgun metagenomics. *Frontiers in Microbiology*, *7*, 459.

Kaoutari, A. E., Armougom, F., Gordon, J. I., Raoult, D., & Henrissat, B. (2013). The abundance and variety of carbohydrate-active enzymes in the human gut microbiota. *Nature Reviews Microbiology*, *11*(7), 497–504.

Kennedy, N. A., Walker, A. W., Berry, S. H., Duncan, S. H., Farquarson, F. M., Louis, P., & Thomson, J. M. (2014). The impact of different DNA extraction kits and laboratories upon the assessment of human gut microbiota composition by 16S rRNA gene sequencing. *PLOS ONE*, *9*(2), e88982.

King, J. R., & Porter, S. D. (2004). Recommendations on the use of alcohols for preservation of ant specimens (Hymenoptera, Formicidae). *Insectes Sociaux*, *51*(2), 197–202.

Koenig, J. E., Spor, A., Scalfone, N., Fricker, A. D., Stombaugh, J., Knight, R., Angenent, L. T., & Ley, R. E. (2011). Succession of microbial consortia in the developing infant gut microbiome. *Proceedings of the National Academy of Sciences*, *108*(Supplement 1), 4578–4585.

Kohl, K. D., & Dearing, M. D. (2016). The woodrat gut microbiota as an experimental system for understanding microbial metabolism of dietary toxins. *Frontiers in Microbiology*, *7*, 1165.

Kohl, K. D., Weiss, R. B., Cox, J., Dale, C., & Denise Dearing, M. (2014a). Gut microbes of mammalian herbivores facilitate intake of plant toxins. *Ecology Letters*, *17*(10), 1238–1246.

Kong, F., Zhao, J., Han, S., Zeng, B., Yang, J., Si, X., Yang, B., Yang, M., Xu, H., & Li, Y. (2014). Characterization of the gut microbiota in the red panda (*Ailurus fulgens*). *PLOS ONE*, *9*(2), e87885.

Koo, B.-S., Baek, S. H., Kim, G., Hwang, E., Oh, H., Son, Y., Lim, K. S., Kang, P., Lee, H.-Y., Jeong, K.-J., Kim, Y.-H., Villinger, F., & Hong, J.-J. (2020). Idiopathic chronic diarrhea associated with dysbiosis in a captive cynomolgus macaque (*Macaca fascicularis*). *Journal of Medical Primatology*, *49*(1), 56–59.

Krynak, K. L., Burke, D. J., Martin, R. A., & Dennis, P. M. (2017). Gut microbiome composition is associated with cardiac disease in zoo-housed western lowland gorillas (*Gorilla gorilla gorilla*). *FEMS Microbiology Letters*, *364*(15).

Langille, M. G. I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., Clemente, J. C., Burkepile, D. E., Vega Thurber, R. L., Knight, R., Beiko, R. G., & Huttenhower, C. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology*, *31*(9), 814–821.

Laurie, A. (1982). Behavioural ecology of the Greater one-horned rhinoceros (*Rhinoceros unicornis*). *Journal of Zoology*, *196*(3), 307–341.

LeBlanc, J. G., Milani, C., de Giori, G. S., Sesma, F., van Sinderen, D., & Ventura, M. (2013). Bacteria as vitamin suppliers to their host: A gut microbiota perspective. *Current Opinion in Biotechnology*, *24*(2), 160–168.

Lederberg, J., & Mccray, A. T. (2001). `Ome Sweet `Omics—A genealogical treasury of words. *The Scientist*, *15*(7), 8.

Lewis, J. D., Chen, E. Z., Baldassano, R. N., Otley, A. R., Griffiths, A. M., Lee, D., Bittinger, K., Bailey, A., Friedman, E. S., Hoffmann, C., Albenberg, L., Sinha, R., Compher, C., Gilroy, E., Nessel, L., Grant, A., Chehoud, C., Li, H., Wu, G. D., & Bushman, F. D. (2015). Inflammation, Antibiotics, and Diet as Environmental Stressors of the Gut Microbiome in Pediatric Crohn's Disease. *Cell Host & Microbe*, *18*(4), 489–500.

Lewis, W. H., Tahon, G., Geesink, P., Sousa, D. Z., & Ettema, T. J. G. (2021). Innovations to culturing the uncultured microbial majority. *Nature Reviews Microbiology*, *19*(4), 225–240.

Ley, R. E., Hamady, M., Lozupone, C., Turnbaugh, P. J., Ramey, R. R., Bircher, J. S., Schlegel, M. L., Tucker, T. A., Schrenzel, M. D., Knight, R., & Gordon, J. I. (2008). Evolution of mammals and their gut microbes. *Science*, *320*(5883), 1647–1651.

Lim, M. Y., Song, E.-J., Kim, S. H., Lee, J., & Nam, Y.-D. (2018). Comparison of DNA extraction methods for human gut microbial community profiling. *Systematic and Applied Microbiology*, *41*(2), 151–157.

Lin, C.-Y., Cross, T.-W. L., Doukhanine, E., & Swanson, K. S. (2020). An ambient temperature collection and stabilization strategy for canine microbiota studies. *Scientific Reports*, *10*(1), 1-11.

Loftfield, E., Vogtmann, E., Sampson, J. N., Moore, S. C., Nelson, H., Knight, R., Chia, N., & Sinha, R. (2016). Comparison of collection methods for fecal samples for discovery metabolomics in epidemiologic studies. *Cancer Epidemiology and Prevention Biomarkers*, *25*(11), 1483–1490.

Lugli, G. A., Milani, C., Mancabelli, L., Turroni, F., van Sinderen, D., & Ventura, M. (2019). A microbiome reality check: Limitations of in silico-based metagenomic approaches to study complex bacterial communities. *Environmental Microbiology Reports*, *11*(6), 840–847.

Lupp, C., Robertson, M. L., Wickham, M. E., Sekirov, I., Champion, O. L., Gaynor, E. C., & Finlay, B. B. (2007). Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of enterobacteriaceae. *Cell Host & Microbe*, *2*(2), 119–129.

Manning, T. S., & Gibson, G. R. (2004). Prebiotics. *Best Practice & Research Clinical Gastroenterology*, *18*(2), 287–298.

Mardanov, A., Boulygina, E., Nedoluzhko, A., Kadnikov, V., Beletskii, A., Tsygankova, S., Tikhonov, A., Ravin, N., Prokhortchouk, E., & Skryabin, K. (2012). Molecular analysis of the intestinal microbiome composition of mammoth and woolly rhinoceros. *Doklady. Biochemistry and Biophysics*, *445*, 203–206.

Markle, J. G. M., Frank, D. N., Mortin-Toth, S., Robertson, C. E., Feazel, L. M., Rolle-Kampczyk, U., von Bergen, M., McCoy, K. D., Macpherson, A. J., & Danska, J. S. (2013). Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science*, *339*(6123), 1084–1088.

Martin, C. R., Osadchiy, V., Kalani, A., & Mayer, E. A. (2018). The brain-gut-microbiome axis. *Cellular and Molecular Gastroenterology and Hepatology*, *6*(2), 133–148.

Maurice, C. F., CL Knowles, S., Ladau, J., Pollard, K. S., Fenton, A., Pedersen, A. B., & Turnbaugh, P. J. (2015). Marked seasonal variation in the wild mouse gut microbiota. *The ISME Journal*, *9*(11), 2423–2434.

McKenney, E. A., Maslanka, M., Rodrigo, A., & Yoder, A. D. (2018). Bamboo specialists from two mammalian orders (Primates, Carnivora) share a high number of low-abundance gut microbes. *Microbial Ecology*, *76*(1), 272–284.

McKenney, E. A., O'Connell, T. M., Rodrigo, A., & Yoder, A. D. (2018). Feeding strategy shapes gut metagenomic enrichment and functional specialization in captive lemurs. *Gut Microbes*, *9*(3), 202–217.

McKenney, E. A., Rodrigo, A., & Yoder, A. D. (2015). Patterns of gut bacterial colonization in three primate species. *PLOS ONE*, *10*(5), e0124618.

McKenzie, V. J., Song, S. J., Delsuc, F., Prest, T. L., Oliverio, A. M., Korpita, T. M., Alexiev, A., Amato, K. R., Metcalf, J. L., Kowalewski, M., Avenant, N. L., Link, A., Di Fiore, A., Seguin-Orlando, A., Feh, C., Orlando, L., Mendelson, J. R., Sanders, J., & Knight, R. (2017). The effects of captivity on the mammalian gut microbiome. *Integrative and Comparative Biology*, *57*(4), 690–704.

McMurdie, P. J., & Holmes, S. (2014). Waste not, want not: Why rarefying microbiome data is inadmissible. *PLOS Computational Biology*, *10*(4), e1003531.

McNamara, M. P., Singleton, J. M., Cadney, M. D., Ruegger, P. M., Borneman, J., & Garland, T. (2021). Early-life effects of juvenile Western diet and exercise on adult gut microbiome composition in mice. *Journal of Experimental Biology*, *224*(4).

Metcalf, J. L., Song, S. J., Morton, J. T., Weiss, S., Seguin-Orlando, A., Joly, F., Feh, C., Taberlet, P., Coissac, E., Amir, A., Willerslev, E., Knight, R., McKenzie, V., & Orlando, L. (2017). Evaluating the impact of domestication and captivity on the horse gut microbiome. *Scientific Reports*, *7*(1), 1-9.

Metrione, L. C., & Eyres, A. (2014). Rhino Husbandry Manual. *International Rhino Foundation*.

Milliken, E. J. T. (2019). Fifty years of faecal microbiota transplant in Central Australia. *Gut*, *68*(8), 1536–1536.

Moeller, A. H., Shilts, M., Li, Y., Rudicell, R. S., Lonsdorf, E. V., Pusey, A. E., Wilson, M. L., Hahn, B. H., & Ochman, H. (2013). SIV-induced instability of the chimpanzee gut microbiome. *Cell Host & Microbe*, *14*(3), 340–345.

Montassier, E., Al-Ghalith, G. A., Hillmann, B., Viskocil, K., Kabage, A. J., McKinlay, C. E., Sadowsky, M. J., Khoruts, A., & Knights, D. (2018). CLOUD: A non-parametric detection test for microbiome outliers. *Microbiome*, *6*(1), 1-10.

Moossavi, S., Engen, P. A., Ghanbari, R., Green, S. J., Naqib, A., Bishehsari, F., Merat, S., Poustchi, H., Keshavarzian, A., & Malekzadeh, R. (2019). Assessment of the impact of different fecal storage protocols on the microbiota diversity and composition: A pilot study. *BMC Microbiology*, *19*(1), 1-8.

Mshelia, E. S., Adamu, L., Wakil, Y., Turaki, U. A., Gulani, I. A., & Musa, J. (2018). The association between gut microbiome, sex, age and body condition scores of horses in Maiduguri and its environs. *Microbial Pathogenesis*, *118*, 81–86.

Muegge, B. D., Kuczynski, J., Knights, D., Clemente, J. C., González, A., Fontana, L., Henrissat, B., Knight, R., & Gordon, J. I. (2011). Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science*, *332*(6032), 970–974.

Muiños-Bühl, A., González-Recio, O., Muñoz, M., Óvilo, C., García-Casco, J., & Fernández, A. I. (2018). Evaluating protocols for porcine faecal microbiome recollection, storage and DNA extraction: from the farm to the lab. *Current Microbiology*, *75*(6), 651–657.

Mushegian, A. A., & Ebert, D. (2016). Rethinking "mutualism" in diverse host-symbiont communities. *BioEssays*, *38*(1), 100–108.

Nagy, Z. T. (2010). A hands-on overview of tissue preservation methods for molecular genetic analyses. *Organisms Diversity & Evolution*, *10*(1), 91–105.

Navas-Molina, J. A., Peralta-Sánchez, J. M., González, A., McMurdie, P. J., Vázquez-Baeza, Y., Xu, Z., Ursell, L. K., Lauber, C., Zhou, H., Song, S. J., Huntley, J., Ackermann, G. L., Berg-Lyons, D., Holmes, S., Caporaso, J. G., & Knight, R. (2013). Chapter Nineteen—Advancing our understanding of the human microbiome using QIIME. *Methods in Enzymology,* 531, 371-444.

Nechvatal, J. M., Ram, J. L., Basson, M. D., Namprachan, P., Niec, S. R., Badsha, K. Z., Matherly, L. H., Majumdar, A. P. N., & Kato, I. (2008). Fecal collection, ambient preservation, and DNA extraction for PCR amplification of bacterial and human markers from human feces. *Journal of Microbiological Methods*, *72*(2), 124–132.

Nishida, A. H., & Ochman, H. (2018). Rates of gut microbiome divergence in mammals. *Molecular Ecology*, *27*(8), 1884–1897.

Nsubuga, A. M., Robbins, M. M., Roeder, A. D., Morin, P. A., Boesch, C., & Vigilant, L. (2004). Factors affecting the amount of genomic DNA extracted from ape faeces and the identification of an improved sample storage method. *Molecular Ecology*, *13*(7), 2089–2094.

Ochman, H., Worobey, M., Kuo, C.-H., Ndjango, J.-B. N., Peeters, M., Hahn, B. H., & Hugenholtz, P. (2010). Evolutionary relationships of wild hominids recapitulated by gut microbial communities. *PLOS Biology*, *8*(11), e1000546.

Palmer, C., Bik, E. M., DiGiulio, D. B., Relman, D. A., & Brown, P. O. (2007). Development of the human infant intestinal microbiota. *PLOS Biology*, *5*(7), e177.

Rausch, P., Rühlemann, M., Hermes, B. M., Doms, S., Dagan, T., Dierking, K., Domin, H., Fraune, S., von Frieling, J., Hentschel, U., Heinsen, F.-A., Höppner, M., Jahn, M. T., Jaspers, C., Kissoyan, K. A. B., Langfeldt, D., Rehman, A., Reusch, T. B. H., Roeder, T., … Baines, J. F. (2019). Comparative analysis of amplicon and metagenomic sequencing methods reveals key features in the evolution of animal metaorganisms. *Microbiome*, *7*(1), 1-19.

Reese, A. T., & Dunn, R. R. (2018). Drivers of Microbiome Biodiversity: A Review of General Rules, Feces, and Ignorance. *MBio*, *9*(4), e01294-18.

Ren, T., Boutin, S., Humphries, M. M., Dantzer, B., Gorrell, J. C., Coltman, D. W., McAdam, A. G., & Wu, M. (2017). Seasonal, spatial, and maternal effects on gut microbiome in wild red squirrels. *Microbiome*, *5*(1), 1-14.

Rohlin, A., Wernersson, J., Engwall, Y., Wiklund, L., Björk, J., & Nordling, M. (2009). Parallel sequencing used in detection of mosaic mutations: Comparison with four diagnostic DNA screening techniques. *Human Mutation*, *30*(6), 1012–1020.

Roth, T. L., Switzer, A., Watanabe-Chailland, M., Bik, E. M., Relman, D. A., Romick-Rosendale, L. E., & Ollberding, N. J. (2019). Reduced gut microbiome diversity and metabolome differences in rhinoceros species at risk for iron overload disorder. *Frontiers in Microbiology*, *10*, 2291.

Rothschild, D., Weissbrod, O., Barkan, E., Kurilshikov, A., Korem, T., Zeevi, D., Costea, P. I., Godneva, A., Kalka, I. N., Bar, N., Shilo, S., Lador, D., Vila, A. V., Zmora, N., Pevsner-Fischer, M., Israeli, D., Kosower, N., Malka, G., Wolf, B. C., … Segal, E. (2018). Environment dominates over host genetics in shaping human gut microbiota. *Nature*, *555*(7695), 210-215.

Salem, S. E., Maddox, T. W., Berg, A., Antczak, P., Ketley, J. M., Williams, N. J., & Archer, D. C. (2018). Variation in faecal microbiota in a group of horses managed at pasture over a 12-month period. *Scientific Reports*, *8*(1), 1-10.

Schippa, S., & Conte, M. P. (2014). Dysbiotic events in gut microbiota: Impact on human health. *Nutrients*, *6*(12), 5786–5805.

Schippa, S., Iebba, V., Totino, V., Santangelo, F., Lepanto, M., Alessandri, C., Nuti, F., Viola, F., Di Nardo, G., Cucchiara, S., Longhi, C., & Conte, M. P. (2012). A potential role of *Escherichia coli* pathobionts in the pathogenesis of pediatric inflammatory bowel disease. *Canadian Journal of Microbiology*, *58*(4), 426–432.

Schmidt, E., Mykytczuk, N., & Schulte-Hostedde, A. I. (2019). Effects of the captive and wild environment on diversity of the gut microbiome of deer mice (*Peromyscus maniculatus*). *The ISME Journal*, *13*(5), 1293–1305.

Scott, K. P., Gratz, S. W., Sheridan, P. O., Flint, H. J., & Duncan, S. H. (2013). The influence of diet on the gut microbiota. *Pharmacological Research*, *69*(1), 52–60.

Selway, C. A., Eisenhofer, R., & Weyrich, L. S. (2020). Microbiome applications for pathology: Challenges of low microbial biomass samples during diagnostic testing. *The Journal of Pathology: Clinical Research*, *6*(2), 97–106.

Shabat, S. K. B., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M. E., White, B. A., Shterzer, N., & Mizrahi, I. (2016). Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *The ISME Journal*, *10*(12), 2958–2972.

Singh, R. K., Chang, H.-W., Yan, D., Lee, K. M., Ucmak, D., Wong, K., Abrouk, M., Farahnik, B., Nakamura, M., Zhu, T. H., Bhutani, T., & Liao, W. (2017). Influence of diet on the gut microbiome and implications for human health. *Journal of Translational Medicine*, *15*(1), 1-17.

Smits, S. A., Leach, J., Sonnenburg, E. D., Gonzalez, C. G., Lichtman, J. S., Reid, G., Knight, R., Manjurano, A., Changalucha, J., Elias, J. E., Dominguez-Bello, M. G., & Sonnenburg, J. L. (2017). Seasonal cycling in the gut microbiome of the Hadza hunter-gatherers of Tanzania. *Science*, *357*(6353), 802–806.

Song, S. J., Amir, A., Metcalf, J. L., Amato, K. R., Xu, Z. Z., Humphrey, G., & Knight, R. (2016). Preservation methods differ in fecal microbiome stability, affecting suitability for field studies. *MSystems*, *1*(3), e00021-16.

Sonnenburg, E. D., & Sonnenburg, J. L. (2019). The ancestral and industrialized gut microbiota and implications for human health. *Nature Reviews Microbiology*, *17*(6), 383–390.

Stangegaard, M., Ferrero-Miliani, L., Børsting, C., Frank-Hansen, R., Hansen, A. J., & Morling, N. (2011). Repeated extraction of DNA from FTA cards. *Forensic Science International: Genetics Supplement Series*, *3*(1), e345–e346.

Statovci, D., Aguilera, M., MacSharry, J., & Melgar, S. (2017). The impact of Western diet and nutrients on the microbiota and immune response at mucosal interfaces. *Frontiers in Immunology*, *8*, 838.

Steen, A. D., Crits-Christoph, A., Carini, P., DeAngelis, K. M., Fierer, N., Lloyd, K. G., & Cameron Thrash, J. (2019). High proportions of bacteria and archaea across most biomes remain uncultured. *The ISME Journal*, *13*(12), 3126–3130.

Steinmetz, K., Murphy, S., Ó Cadhla, O., Barnett, J., & Mirimin, L. (2021). Enabling pinniped conservation by means of non-invasive genetic population analysis. *Conservation Genetics Resources*, *13*(2), 131–142.

Stevens, C. E., & Hume, I. D. (1998). Contributions of Microbes in Vertebrate Gastrointestinal Tract to Production and Conservation of Nutrients. *Physiological Reviews*, *78*(2), 393–427.

Swaisgood, R. R., Dickman, D. M., & White, A. M. (2006). A captive population in crisis: Testing hypotheses for reproductive failure in captive-born southern white rhinoceros females. *Biological Conservation*, *129*(4), 468–476.

Tang, Q., Jin, G., Wang, G., Liu, T., Liu, X., Wang, B., & Cao, H. (2020). Current sampling methods for gut microbiota: A call for more precise devices. *Frontiers in Cellular and Infection Microbiology*, *10*, 151.

Tedjo, D. I., Jonkers, D. M. A. E., Savelkoul, P. H., Masclee, A. A., Best, N. van, Pierik, M. J., & Penders, J. (2015). The effect of sampling and storage on the fecal microbiota composition in healthy and diseased subjects. *PLOS ONE*, *10*(5), e0126685.

The Human Microbiome Project Consortium. (2012). Structure, function and diversity of the healthy human microbiome. *Nature*, *486*(7402), 207–214.

Tripathi, A., Debelius, J., Brenner, D. A., Karin, M., Loomba, R., Schnabl, B., & Knight, R. (2018). The gut–liver axis and the intersection with the microbiome. *Nature Reviews Gastroenterology & Hepatology*, *15*(7), 397–411.

Tubbs, C. W., Moley, L. A., Ivy, J. A., Metrione, L. C., LaClaire, S., Felton, R. G., Durrant, B. S., & Milnes, M. R. (2016). Estrogenicity of captive southern white rhinoceros diets and their association with fertility. *General and Comparative Endocrinology*, *238*, 32–38.

Van Rensburg, J. J., Lin, H., Gao, X., Toh, E., Fortney, K. R., Ellinger, S., Zwickl, B., Janowicz, D. M., Katz, B. P., Nelson, D. E., Dong, Q., & Spinola, S. M. (2015). The human skin microbiome associates with the outcome of and is influenced by bacterial infection. *MBio*, *6*(5), e01315-15.

Vlčková, K., Mrázek, J., Kopečný, J., & Petrželková, K. J. (2012). Evaluation of different storage methods to characterize the fecal bacterial communities of captive western lowland gorillas (Gorilla gorilla gorilla). *Journal of Microbiological Methods*, *91*(1), 45–51.

Voreades, N., Kozil, A., & Weir, T. L. (2014). Diet and the development of the human intestinal microbiome. *Frontiers in Microbiology*, *5*, 494.

Wan, X., Ruan, R., McLaughlin, R. W., Hao, Y., Zheng, J., & Wang, D. (2016). Fecal bacterial composition of the endangered Yangtze finless porpoises living under captive and semi-natural conditions. *Current Microbiology*, *72*(3), 306–314.

Wang, Z., Zolnik, C. P., Qiu, Y., Usyk, M., Wang, T., Strickler, H. D., Isasi, C. R., Kaplan, R. C., Kurland, I. J., Qi, Q., & Burk, R. D. (2018). Comparison of fecal collection methods for microbiome and metabolomics studies. *Frontiers in Cellular and Infection Microbiology*, *8*, 301.

West, A. G., Waite, D. W., Deines, P., Bourne, D. G., Digby, A., McKenzie, V. J., & Taylor, M. W. (2019). The microbiome in threatened species conservation. *Biological Conservation*, *229*, 85–98.

Williams, C. L., Ybarra, A. R., Meredith, A. N., Durrant, B. S., & Tubbs, C. W. (2019). Gut microbiota and phytoestrogen-associated infertility in southern white rhinoceros. *MBio*, *10*(2), e00311-19.

Williams, N. T. (2010). Probiotics. *American Journal of Health-System Pharmacy*, *67*(6), 449–458.

Winter, S. E., Winter, M. G., Xavier, M. N., Thiennimitr, P., Poon, V., Keestra, A. M., Laughlin, R. C., Gomez, G., Wu, J., Lawhon, S. D., Popova, I. E., Parikh, S. J., Adams, L. G., Tsolis, R. M., Stewart, V. J., & Bäumler, A. J. (2013). Host-derived nitrate boosts growth of e. coli in the inflamed gut. *Science*, *339*(6120), 708–711.

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.-Y., Keilbaugh, S. A., Bewtra, M., Knights, D., Walters, W. A., Knight, R., Sinha, R., Gilroy, E., Gupta, K., Baldassano, R., Nessel, L., Li, H., Bushman, F. D., & Lewis, J. D. (2011). Linking long-term dietary patterns with gut microbial enterotypes. *Science*, *334*(6052), 105–108.

Wu, W.-K., Chen, C.-C., Panyod, S., Chen, R.-A., Wu, M.-S., Sheen, L.-Y., & Chang, S.-C. (2019). Optimization of fecal sample processing for microbiome study—The journey from bathroom to bench. *Journal of the Formosan Medical Association*, *118*(2), 545–555.

Xia, Y. (2020). Chapter Eleven—Correlation and association analyses in microbiome study integrating multiomics in health and disease. *Progress in Molecular Biology and Translational Science* 171, 309–491.

Xie, Y., Xia, P., Wang, H., Yu, H., Giesy, J. P., Zhang, Y., Mora, M. A., & Zhang, X. (2016). Effects of captivity and artificial breeding on microbiota in feces of the red-crowned crane (*Grus japonensis*). *Scientific Reports*, *6*(1), 1-11.

Xu, M., Pokrovskii, M., Ding, Y., Yi, R., Au, C., Harrison, O. J., Galan, C., Belkaid, Y., Bonneau, R., & Littman, D. R. (2018). C-MAF-dependent regulatory T cells mediate immunological tolerance to a gut pathobiont. *Nature*, *554*(7692), 373–377.

Xue, Z., Zhang, W., Wang, L., Hou, R., Zhang, M., Fei, L., Zhang, X., Huang, H., Bridgewater, L. C., Jiang, Y., Jiang, C., Zhao, L., Pang, X., & Zhang, Z. (2015). The bamboo-eating giant panda harbors a carnivore-like gut microbiota, with excessive seasonal variations. *MBio*, *6*(3), e00022-15.

Yao, R., Xu, L., Hu, T., Chen, H., Qi, D., Gu, X., Yang, X., Yang, Z., & Zhu, L. (2019). The "wildness" of the giant panda gut microbiome and its relevance to effective translocation. *Global Ecology and Conservation*, *18*, e00644.

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R. N., Anokhin, A. P., Heath, A. C., Warner, B., Reeder, J., Kuczynski, J., Caporaso, J. G., Lozupone, C. A., Lauber, C., Clemente, J. C., Knights, D., … Gordon, J. I. (2012). Human gut microbiome viewed across age and geography. *Nature*, *486*(7402), 222–227.

Youngblut, N. D., Reischer, G. H., Walters, W., Schuster, N., Walzer, C., Stalder, G., Ley, R. E., & Farnleitner, A. H. (2019). Host diet and evolutionary history explain different aspects of gut microbiome diversity among vertebrate clades. *Nature Communications*, *10*(1), 1-15.

Zaneveld, J. R., McMinds, R., & Vega Thurber, R. (2017). Stress and stability: Applying the Anna Karenina principle to animal microbiomes. *Nature Microbiology*, *2*(9), 1–8.

Zhang, B., Brock, M., Arana, C., Dende, C., Hooper, L., & Raj, P. (2020). Impact of bead-beating intensity on microbiome recovery in mouse and human stool: Optimization of DNA extraction. *bioRxiv*, 1-23

Zhang, F.-M., Wang, H.-G., Wang, M., Cui, B.-T., Fan, Z.-N., & Ji, G.-Z. (2013). Fecal microbiota transplantation for severe enterocolonic fistulizing Crohn's disease. *World Journal of Gastroenterology : WJG*, *19*(41), 7213–7216.

Zhao, N., Li, M., Luo, J., Wang, S., Liu, S., Wang, S., Lyu, W., Chen, L., Su, W., Ding, H., & He, H. (2017). Impacts of canine distemper virus infection on the giant panda population from the perspective of gut microbiota. *Scientific Reports*, *7*(1), 39954.

MANUSCRIPT 1: EFFECTS OF SEASONALITY AND AGE ON MANAGED SOUTHERN WHITE RHINOCEROS (*CERATOTHERIUM SIMUM SIMUM*) GUT MICROBIOME

4.1 Introduction

The southern white rhinoceros (*Ceratotherium simum simum*) is a large grazing herbivore that utilizes microbial interactions in the hindgut to facilitate the fermentation of complex plant carbohydrates (Bian et al., 2013). These rhinoceros are a bastion of conservation success stories, having been restored from fewer than 100 individuals at the start of the 20th century to over 18,000 by last estimation in 2017 (Emslie, 2020). Unfortunately, the wild population of southern white rhinoceros is once again declining due to a substantial increase in poaching and is considered near threatened by the International Union for Conservation of Nature (IUCN, 2021) (Emslie, 2020). Due to the uncertain future of southern white rhinoceros in the wild, it is critical that healthy assurance populations be maintained under human management. However, managed populations of southern white rhinoceros have historically experienced poor fertility and post-copulatory reproductive failure, with females born under human management being especially subfertile (Swaisgood et al., 2006; Tubbs et al., 2016). One proposed explanation for this trend is diet, specifically the increased levels of phytoestrogens in managed compared to wild diets, which have been negatively correlated to female southern white rhinoceros fertility (Tubbs et al., 2016). Some researchers suggest that gut microbiota may affect reproductive outcomes by metabolizing these phytoestrogens (Williams et al., 2019). Beyond digestion and metabolism, the gut microbiome also mediates host immunity and so has wide implications for host health (Shreiner et al., 2008; Kinross et al., 2011; Singh et al., 2017). Given these considerations,

understanding gut microbial dynamics is crucial for the continued conservation and population management of southern white rhinoceros and other threatened species (McKenzie et al., 2017; Carthey et al., 2020).

Some managed animal species have been noted to experience a decrease in microbial richness and diversity when compared to wild conspecifics (Amato et al., 2016; Clayton et al., 2016; McKenzie et al., 2017; Gibson et al., 2019). This may be attributed to a variety of causes, including sterile housing conditions, the usage of antibiotics and other medications, lack of interaction with allospecifics, and lack of dietary diversity comparable to wild diet. Interestingly, previous studies have shown that Rhinocerotidae are one of the few families to experience increased microbial diversity under human management compared to their wild conspecifics, although the results of this study are questionable due to a small sample size (n=6 southern white rhinoceros, 3 managed and 3 wild; n=7 black rhinoceros, 6 managed and 1 wild) and limited sampling period (McKenzie et al., 2017). Other comparisons of managed vs wild animal gut microbiomes are similarly hampered by small sample sizes and few sampling periods, thus further research is necessary to confirm the legitimacy of microbial diversity declines in managed species.

Gut microbiomes differ widely across individuals and microbial community composition can be affected by the gut morphology (Gillman et al., 2020), feeding strategy (McKenney et al., 2017; Greene et al., 2020), age (Adriansjach et al., 2020; Janiak et al., 2021), sex (Markle et al., 2013; Mshelia et al., 2018), health status (Lewis et al., 2015), and geographic location (Yatsunenko et al., 2012) of the host, among other factors. The mammalian gut microbiome has also been shown to vary by season, with previous studies revealing marked seasonal changes in the community composition in the tracts of humans, red squirrels (*Tamiasciurus hudsonicus*),

wood mice (*Apodemus sylvaticus*), ground squirrels (*Ictidomys tridecemlineatus*), giant pandas (*Ailuropoda melanoleuca*), and horses (*Equus ferus caballus*) (Carey et al., 2013; Xue et al., 2015; Maurice et al., 2015; Smits et al., 2017; Ren et al., 2017; Salem et al., 2018). Horses have been used as the domestic animal model for managed rhinoceros species due to similarities in gut morphology and hindgut microbial fermentation (Miller & Buss, 2003).

Several researchers have attempted to characterize the gut microbiome of southern white rhinoceros (Bian et al., 2013; Williams et al., 2019; Roth et al., 2019; Cersosimo et al., 2021), though none have investigated differences in microbial composition due to season or age. The objectives of this research are two-fold: 1) to characterize the taxonomic composition of the gut microbiome in a managed population of southern white rhinoceros at the NC Zoo; and 2) to determine the effects of seasonality and age on gut microbial community structure in managed southern white rhinoceros. Driven by previous research into mammalian microbiomes, we hypothesized the NC Zoo population of rhinoceros would display individual, age and seasonal differences in microbiome alpha and beta diversity, as measured by Shannon index, Simpson's index, and Bray-Curtis dissimilarity. We expected that of the four age classes present, the geriatric individual would display the largest deviations in species diversity and community composition due to both age and treatment of arthritis with medication. We also expected that feces collected during warm weather months would contain higher microbial species diversity and possess a different community composition compared to cold weather months, due to increased outdoor access and availability of grasses during the summer.

4.2 Methods

Sample population

Fecal samples were collected from a population of 10 southern white rhinoceros (9 females, 1 male) managed at the North Carolina Zoo in Asheboro, NC. Age classes for southern white rhinoceros in this study were assigned based on a previously published age classification system that includes subadult (3.5 - 7 years old) and adult (8+ years) classes (Emslie et al., 1995). For the purposes of this study, a modified juvenile class (<3.5 years old) was added which combined four separate classes of young rhinoceros between 0 and 3.5 years of age. In addition, a novel geriatric (>40 years old) age class was added. Three of the adult female rhinoceros and the geriatric female were wild-caught over twenty years prior to this study; all other individuals were born in captivity. The sex, age group, and origin of each individual are summarized in Table 1. For housing purposes, the NC Zoo population of rhinoceros was split into two major subgroups and two ungrouped individuals during the course of this study. One group consisted of four nonreproductive females (F1, F3, S1, S2), while the other consisted of the two mother-calf pairs (F2, J1, F4, J2). The two ungrouped individuals were the male individual (M1) and the geriatric female (G1). All animals were free from antibiotics, though G1 received phenylbutazone to treat inflammation from arthritis during the course of this study.

Table 1. Summary of individual characteristics of southern white rhinoceros (*Ceratotherium simum simum*) at the North Carolina Zoo (n=10).

Individual	Age (Years)	Sex	Class	Origin
J1	1	Female	Juvenile	Zoo
J2	1	Female	Juvenile	Zoo
S1	3	Female	Sub-adult	Zoo
S2	3	Female	Sub-adult	Zoo
F1	15	Female	Adult	Zoo
F2	24*	Female	Adult	Wild
F3	29	Female	Adult	Wild
F4	33*	Female	Adult	Wild
G1	53*	Female	Geriatric	Wild
M1	32	Male	Adult	Zoo

*Estimated age due to animals being wild-caught individuals.

Diets

Animal diets were standardized but varied by season and age. Year-round, the subadult and adult rhinoceros were provided with 1.36 kgs of Mazuri® Wild Herbivore Diet Hi-Fiber (St. Louis, MO, USA) pellet daily, while the calves were offered 0.68 kgs of Wild Herbivore pellet diet daily. During the summer, when the rhinoceros had access to outdoor paddocks with grass for grazing, the animals consumed around one supplementary quarter of a bale of timothy hay (*Phleum pretense*) each. During the winter, sub-adult and adult rhinoceros received roughly one bale of timothy hay per animal daily. Rhinoceros also had access to the 16-hectare Watani Grasslands habitat during the winter, where fescue (*Festuca arundinacea*), annual ryegrass (*Lolium multiflorum*), and Bermuda (*Cynodon dactylon*) grasses were available. Timothy cubes, orchard grass (*Dactylis glomerate*), and alfalfa hay (*Medicago sativa*) are all offered in rotation for training and enrichment; these supplementary feeds were used in less than 20% of their daily diet. The geriatric female rhinoceros received a slightly different diet than the main population for welfare purposes, consuming 5.44 kgs of Wild Herbivore feed and one half of a timothy/orchard grass mixed bale each day. Rhinoceros G1 also had access to outdoor grazing

areas and was provided with timothy hay cubes and alfalfa hay for training and enrichment. Managed diets have been summarized in Table 2.

Table 2. Summary of managed diets for n=10 southern white rhinoceros (Ceratotherium simum simum) at the North Carolina Zoo[1].

	Summer Daily Diet (kg)			Winter Daily Diet (kg)		
Age Class	**Mazuri® WH[2] Hi-Fiber Pellet**	**Timothy Hay**	**T/O[3] Mixed Hay**	**Mazuri® WH[2] Hi-Fiber Pellet**	**Timothy Hay**	**T/O[3] Mixed Hay**
Juvenile	0.68	-	-	0.68	-	-
Subadult	1.36	4.5	-	1.36	18	-
Adult	1.36	4.5	-	1.36	18	-
Geriatric	5.44	-	11	5.44	-	11

[1]Pasture available *ad libitum*
[2]WH- Wild Herbivore (Mazuri®, St. Louis, MO, USA)
[3]T/O- Timothy/orchard grass

Housing

The two major groups of NC Zoo rhinoceros were kept in the rhinoceros barn, while M1 and G1 were kept in a rhino annex building. The rhinoceros barn consisted of a series of 37 m² stalls and a 52 m² maternity stall. The mother-calf pairs (F2, J1, F4, J2) rotated routinely between either the one maternity stall or a combination of 2 to 3 regular stalls. Both sets of stalls had adjoining outdoor paddocks of roughly 111 m²; the substrate of these paddocks consisted of asphalt with sand beds. The mother-calf pairs had access to the outdoor paddocks unless the temperature was <4.5°C. The rhinoceros barn has forced air heating set at 10°C, with no cooling ability. The nonreproductive females, (F1, F3, S1, S2) had access to 5 stalls (for a total of 17 m² of space) when housed inside. During summer collection, this group was housed inside every other day, and was rotated into a 2000 m² outdoor habitat known as a boma, which includes a sand and rock paddock with some grass access adjacent to the 16-hectare NC Zoo Watani

Grasslands habitat. No rhinoceros were allowed access to the Grasslands habitat during the summer sampling period due to a cyanobacterial algal bloom in the lake in the habitat.

During winter collection, the nonreproductive female group was allowed access to an outdoor holding area when temperatures were above 2°C. Their outdoor holding was three paddocks totaling 1450 m², including a heated lean-to in the largest paddock. When outdoor access was restricted, the group was split into two pairs: one pair was held in 111 m² of combined stalls with access to approximately 150 m² of outdoor holding, while the other pair was held in 74 m² of combined stalls with access to 335 m² of outdoor holding. Individuals were rotated into different pairs, and pairs were rotated into different sets of stalls so that each individual was equally exposed to all group members and indoor environments. Rhinoceros groups also had access to the Grasslands habitat when the weather rose above 0°C (nonreproductive female group) and 7°C (mother-calf pair group) respectively. Both groups were rotated out and combined in this habitat.

The annex building had two 30 m² stalls and M1 and G1 each had access to a separate stall and 4,046 m² grass paddocks when temperatures rose above 2°C. The annex building had propane radiant heat that maintained 13°C during cool ambient temperatures; like the rhinoceros barn, it possessed no cooling ability and so matched ambient outdoor temperatures above 13°C. Rhinoceros G1 had limited access to the Watani Grasslands habitat with the other groups, which was no more than twice a month during the study period. Rhinoceros M1 had no access to the Grasslands habitat, having been transferred to another facility before the start of winter sampling.

Sample collection, storage, and DNA extraction

Fecal samples were collected from each individual once per month during the months of July through September in 2020 and January through March in 2021. Sampling typically began on the 20th day of the month and extended until the end of that month to provide NC Zoo animal care staff enough time to collect samples. Over this time period, 151 fecal samples were collected within 30 minutes of defecation. Fifty-one of those samples were stored in Whirl-Pak® bags (Nasco, Fort Atkinson, WI, USA) for immediate freezing at -80°C. The remaining 100 samples were aliquoted into two different preservation methods for a separate study. All 151 samples were initially analyzed using the methods described in the following section before being divided into subsets for statistical analysis.

Microbial DNA was extracted from the feces using the PowerFecal Pro DNA kit (QIAGEN, Germantown, MD, USA) after two weeks of storage. The kit was utilized per manufacturer recommendations with the following modification: after samples were placed in the provided PowerBead Pro tubes (QIAGEN, Germantown, MD, USA) and briefly vortexed, they were subjected to a bead beating step at 4 m/s for 4 min (two cycles of 2 minute shaking, with a 1 minute pause after each cycle) using a FastPrep-24 bead beater (MP Biomedicals, Santa Ana, CA, USA); this bead beating speed has previously validated for use in DNA extraction for 16S rRNA V3-V4 region sequencing (Zhang et al., 2020). Extracted DNA was eluted in 100 µl of the elution buffer (10 mM Tris) and was stored in elution tubes at -20°C until the end of the sampling season.

Sequencing

Extracted DNA was provided to the Genomic Sciences Laboratory at NC State University for 16S rRNA sequencing of the hypervariable V3 and V4 regions using established methods (PCR Amplicon, PCR Clean-up, and Index PCR, 2013). Primer pair sequences for the V3 and V4 regions (listed below) were obtained to amplify a sequence approximately 460 base pairs (bp) in length (Klindworth et al., 2013).

16S Amplicon PCR Forward Primer = 5'
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
16S Amplicon PCR Reverse Primer = 5'
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

A limited cycle polymerase chain reaction was performed, and Illumina (San Diego, CA, USA) sequencing adapters and dual-index barcodes were added to the amplicon target. The V3-V4 region was sequenced on the Illumina MiSeq platform using paired 300-bp reads and MiSeq v3 reagents, with the ends of the reads overlapped to generate full-length reads.

Raw FastQ files were imported to the CLC Genomics Workbench v21.0.4 with Microbial Genomics Module plugin (QIAGEN, Germantown, MD, USA) and were joined via the CLC default Illumina platform parameters; forward and reverse reads were paired. Workflows for data quality control and operational taxonomic unit (OTU) clustering were utilized. Reads were trimmed with a 0.05 quality limit and an ambiguous limit of 2. Read length thresholds were set between 15-1,000 nucleotides. The SILVA 16S reference database (v132) was used to define

OTUs based on a 97% taxonomic similarity cutoff. An OTU abundance table was generated and reformatted for downstream analysis in RStudio (v1.3.1073) (Boston, MA, USA).

Bioinformatics and statistical analysis

All statistical analyses were performed using R (version 4.0.2) and RStudio (v1.3.1073). A total of 59,680,646 16S rRNA sequence reads were obtained from 151 samples, with an average of 395,236 ± 12,585 (mean ± SEM) reads per sample. Total abundance was 11,445,730 with 14,247 OTUs found. Chimeric reads which align to two or more reference sequences were identified and filtered out. All samples were rarefied to a level of 29,265 reads, with the loss of one sample for a remainder of 150 samples. One hundred of these samples were not frozen at -80°C and were filtered out as part of a separate study into preservation method efficacy. The remaining 50 samples constituted frozen samples from each individual rhinoceros and were used for analysis of total population results and age class results. The data were filtered again to produce a final subset of 34 samples, constituting frozen samples from the adult and subadult females in the population only; these samples were used to analyze the effect of seasonality, avoiding possible variation due to age or sex.

The OTU abundance table was used to calculate taxonomic relative abundance as well as alpha and beta diversity indices. Linear discrimination analysis effect size (LEfSe; https://huttenhower.sph.harvard.edu/galaxy/) was used to identify any OTUs that were significantly enriched per category (i.e., individual, age, or sampling month). Alpha diversity indices include measures of richness (number of species present), Shannon diversity (which incorporates both richness and evenness, i.e. relative abundance of taxa), and Simpson's index (which incorporates richness, evenness, and phylogenetic relatedness of taxa). The Shannon

index takes into account rare species, making it very sensitive to small changes in diversity. By contrast, Simpson's index is considered a "dominance" index as it gives greater weight to common species and is not affected by less abundant species. Kruskal-Wallis tests and pairwise Wilcoxon tests were used to assess significant differences in microbial alpha diversity across individuals, age classes, and sample seasons.

Beta diversity indices measure the similarity or dissimilarity between communities (Legendre & Cáceres, 2013). To analyze microbial beta diversity, the relative abundance of each OTU was standardized using the Hellinger transformation, then Bray-Curtis dissimilarity was calculated to create distance matrices. Bray-Curtis dissimilarity values quantify compositional dissimilarity between two sites or groupings based on presence/absence and relative abundance of community membership. Possible values range between 0 and 1, with 0 signifying no species dissimilarity between two sites and 1 signifying complete species dissimilarity between two sites. Eigenvectors and eigenvalues were calculated from the distance matrices to create multidimensional scaling (MDS) plots, and PERMANOVA analysis was utilized to assess differences in community composition. P-values were adjusted using a False Discovery Rate (FDR) correction.

4.3 Results

Taxonomic relative abundance

The microbiome of southern white rhinoceros at the NC Zoo was dominated by the bacterial phylum Firmicutes (average relative abundance 55%), followed by Bacteroidetes (21%), Spirochetes (10%), Fibrobacteres (8%), Kiritimatiellaeota (2%), and Lentisphaerae (1%). Taxonomic breakdown by individual is available as supplementary material (Table S1). Phylum and genus level bar charts revealed similarities in taxonomic abundance across the majority of

individuals, though the abundance of Firmicutes and Fibrobacteres varied slightly among individuals (Fig. 1). The most notable exception was G1, the geriatric female rhinoceros, which had more Verrucomicrobia compared to the other individuals.

LefSe analysis revealed differentially abundant taxa across five age and sex classes (Fig. 2). Adult female rhinoceros were enriched in members of the Spirochaetes phylum as well as *Bacteroidetes-BD2-2*. Subadult females possessed numerous differentially abundant taxa, including members of the Kiritimatiellaeota and Tenericutes phyla, Prevotellaceae and Rikenellaceae families, and the Mollicutes class. In addition, subadult females were enriched in members of the *Quinella*, *Anaerovibrio*, and *Marvinbryantia* genera. Juvenile females were enriched in the *rumen bacterium NK4A55* and *Lachnospiraceae* species, as well as members of the Mycoplasma genus. The enrichment of Verrucomicrobia in the geriatric female was confirmed; in addition, members of the Lentisphaerae phylum, Rikenellaceae and Lachnospiraceae families, and *Victivallis*, *Sediminispirochaeta*, and *Mucinivorans* genera were shown to be enriched. The adult male rhinoceros was enriched in members of the Firmicutes phylum, class Clostridia, families Lachnospiraceae and Paenibacilliaceae, and *Eubacterium hallii*. A LefSe plot breakdown of enriched taxa by individual is available as supplemental material (Fig. S1).

LefSe analysis revealed differentially abundant taxa across all six months (Fig. 3). Of the cold weather months, January samples were enriched in members of the Coriobacteriia class and Lactobacillales order, while February samples were enriched in members of the Rhodospirillales order and *Anaeroplasma* genus. March had the highest number of differentially abundant taxa (16 taxa), including enrichment of members of the Bacteroidetes and Lentisphaerae phyla, Planctomycetacia class, Pirellulales and Micrococcales orders, Defluviitaleaceae and

Nocardiaceae families, and the *Victivallis*, *Rhodococcus*, and *Mycobacterium* genera. Of the warm weather months, August samples were enriched in members of the Elusimicrobia phylum, Bacilli class, and *Oribacterium* genus while September was enriched in the phylum Actinobacteria and genus *Alloprevotella*.

Alpha diversity

There were several significant differences in species richness, Shannon diversity, and Simpson diversity indices between individuals, sex, age classes, and month of sampling.

Kruskal-Wallis *H* tests revealed significant p-values for alpha diversity across individuals, including species richness ($p=0.016$), Shannon index ($p=0.003$), and Simpson's index ($p=0.006$). However, pairwise comparisons using the Wilcoxon rank sum test revealed no significant interactions between individuals for any measure of alpha diversity (Fig. 4).

Upon investigating age classes, Kruskal-Wallis *H* tests showed significant differences in richness ($p=0.003$), Shannon index ($p<0.001$), and Simpson's index ($p<0.001$). A Wilcoxon rank sum test found that adult females and subadult females vary in species richness ($p=0.018$), Shannon index ($p<0.001$), and Simpson's index ($p<0.001$). Adult females also varied from the geriatric female in Shannon index ($p=0.005$), and Simpson's index ($p=0.007$) (Fig. 5)

There was a significant difference (Kruskal-Wallis $p<0.05$) in species richness across six months, but no differences in Shannon or Simpson indices (Fig. 6). A Wilcoxon test showed no significant interactions among months for any measure of alpha diversity.

Beta diversity

Multidimensional scaling (MDS) plots revealed similar clustering across individuals, with the exception of the geriatric animal G1 (Fig. 7). Most non-geriatric individual clusters experienced some overlap. Pairwise comparisons made using a PERMANOVA revealed several significant differences between certain individuals. Rhinoceros G1 possessed a community composition significantly different from all other individuals ($p<0.05$). In addition, the male rhinoceros M1 had a community composition different ($p<0.05$) from all adult female animals (F1-F4) as well as a juvenile (J2) and subadult female (S2). The two non-reproductive adult females, F1 and F3, had significantly different community compositions compared to varied juvenile and subadult animals (Table 3).

An MDS plot comparing five age and sex classes showed a similar trend, with G1 once again serving as an outlier cluster (Fig. 8). Adult and subadult female clusters had broad overlap together. There was some overlap between the communities of juvenile and subadult animals. The adult male M1 shared some minor overlap with the adult female class and minimal overlap with the juvenile and subadult female animals. A PERMANOVA analysis found that every age group was significantly different in community composition (Table 4). An MDS plot comparing the community compositions of mother-calf pairs showed calves cluster tightly together and only minimally with their mothers (Fig. 9). The two mothers had some overlap in clusters but were otherwise dissimilar in composition. Limited sample size and single samples precluded the use of PERMANOVA for quantification of significance.

An MDS plot comparing community composition across six months revealed a distinct trend in clustering based on seasonality (Fig. 10). The colder months of January, February, and March shared overlap in clustering and the clusters were discrete from the warmer months of

July, August, and September. These warmer months also clustered together, with the majority of the overlap present between July and August. A PERMANOVA analysis confirmed these trends, as the cooler months had no significant differences in beta diversity. Warmer months all differ ($p<0.05$) from cooler months. Of the warmer months, there were no significant differences in beta diversity between July and August, though September was different ($p<0.01$) from every other month (Table 5).

4.4 Discussion

Total population taxa abundance

The most abundant taxa in the microbiomes of n=10 southern white rhinoceros at the NC Zoo were phylum Firmicutes (average relative abundance 55%), followed by Bacteroidetes (21%), Spirochetes (10%), Fibrobacteres (8%), Kiritimatiellaeota (2%), and Lentisphaerae (1%). The dominance of Firmicutes and Bacteroidetes coincides with a previously published overview of Rhinocerotidae gut microbial relative abundances; across southern white rhinoceros, black rhinoceros (*Diceros bicornis*), Sumatran rhinoceros (*Dicerorhinus sumatrensis*) and greater one-horned rhinoceros (*Rhinoceros unicornis*), the two most dominant phyla were Firmicutes (range; 66.3–51.0%) and Bacteroidetes (39.8–23.4%) (Roth et al., 2019). There were also high abundances of Verrucomicrobia (7.6–1.9%), Spirochetes (3.1–1.1%), Actinobacteria (1.04–0.03%), and Fibrobacteres (2.14–0.19%) present across the four species, though the rankings of these abundances do not coincide with those found in this study (Roth et al., 2019). The phylum Verrucomicrobia was not present above 1% relative abundance in the majority of the NC Zoo population of rhinoceros with the exception of the geriatric female G1. Similarly, actinobacteria was only found at <1% abundance in one individual, adult female F3.

Age-related gut microbiome differences

The geriatric female rhinoceros G1 experienced significant differences in alpha diversity indices and community composition when compared to other members of the population. Beyond age, the greatest difference between G1 and the rest of the population is that G1 was treated with phenylbutazone, a nonsteroidal anti-inflammatory drug (NSAID) used to alleviate symptoms of arthritis. NSAIDs have been associated with NSAID-induced gastrointestinal injury and the induction of microbiome dysbiosis in humans and mice, though the exact mechanism remains unknown (Mäkivuokko et al., 2010; Wallace & Redinbo., 2013; Whitfield-Cargile et al., 2016). NSAID-induced dysbiosis usually results in the reduction of Firmicutes, specifically members of the class Clostridia, as well as the order Bacteroidales and family Lachnospiraceae (Whitfield-Cargile et al., 2018). Phenylbutazone has been previously implicated in changes to microbial abundances (specifically the loss of *Pseudobutyrivibrio* of the family Lachnospiraceae) and a 3-fold increase in circulating bacterial 16S rRNA in horses, the domestic animal model for rhinoceros (Whitfield-Cargile et al., 2021). Dysbiosis of the horse gut microbiome has been linked to inflammatory diseases such as colitis and laminitis (Milinovich et al., 2006; Al Jassim and Andrews, 2009; Weese et al., 2015). Rhinoceros G1 was treated with phenylbutazone for arthritis, but without further research it is impossible to establish whether the differences in microbial alpha and beta diversity indices experienced by G1 was due to the inflammatory disease, the subsequent treatment with phenylbutazone, or some other factor. While G1 was held in a separate building to the rest of the female population, G1 consumed the same type of diet and had similar access to outdoor paddocks and the Grasslands habitat as the others. However, G1 was provided four times the amount of pellet as the subadult and adult animals and had

greater amounts of grass present in the available outdoor paddock. Interestingly, G1 had the highest abundance of Firmicutes of all individuals (Fig. S1), even though NSAID-induced dysbiosis has been mostly implicated with the loss of Firmicutes abundance (Whitfield-Cargile et al., 2018). This increased abundance of Firmicutes displaced the population-consistent abundances of Fibrobacteres and Spirochetes in G1's microbiome (Fig. 1A).

Age-related changes in gut microbial diversity and composition have been previously noted in several species, including humans and horses (Woodmansey et al., 2004; Mariat et al., 2009; Dougal et al., 2014); this study suggests similar age-related differences in the alpha and beta diversity of southern white rhinoceros gut microbiome (Fig. 5; Table 4). Each age class had significant differences in beta diversity as well as differentially abundant taxa present (Fig. 2; Table 4). One might expect the gut microbial community compositions of mother-calf pairs to cluster closely together, as mammals are believed to receive their first inoculation of microbial taxa in utero and then vertically via the mother's vaginal canal during parturition (Dominguez-Bello et al., 2010; Collado et al., 2016; Jacquay et al., 2018; Quercia et al., 2019). However, the two juvenile individuals had microbiome clusters more similar to each other than to their mothers (Figs. 7 & 9). After the initial inoculation, juvenile animals continue to establish their gut microbiomes horizontally via environmental contact and mediate microbial species abundance via the consumption of milk and feed (Jacquay et al., 2018). The difference between juveniles and adults may thus be due to the still immature juvenile microbiomes, which are likely in a transitory state during weaning after having been colonized mainly by milk metabolizing microbiota; both juveniles in this study were near the average weaning age (~1.5 years old) for female southern white rhinoceros calves (White et al., 2007; De La Torre et al., 2019). An MDS plot comparing clustering of all age-class microbiomes revealed juvenile microbiomes cluster

more similarly with subadult females than adult females, indicating a natural longitudinal progression of community composition (Fig. 8).

Juvenile animals had enriched abundances of members of the Lachnospiraceae family and *Ruminococcus*, *Ruminoclostridium,* and *Mycoplasma* genera, and uncultured *Anaerovibrio* spp. All those taxa except *Mycoplasma* have been associated with fiber fermentation and/or digestion in the guts of hindgut fermenters (Costa et al., 2015). Conversely, *Mycoplasma* spp. are considered pathogenic bacteria and have been associated with several deleterious conditions in horses, including endometritis, vulvitis, and infertility (Moorthy et al., 1977). Juveniles in this population were considered healthy, however, and had no symptoms relating to *Mycoplasma* infection.

Significant differences ($p<0.01$) in subadult female and adult female animal gut microbiome alpha and beta diversity were present (Table 4) though similarities in lifestyle make inferences challenging; both age classes overlapped in housing, outdoor access, and diet. Sex hormones may have some influence on this difference, as the subadult females here were not considered sexually mature at the time of sampling and any sex-related differences in gut microbiota appear only after puberty (Yurkovetskiy et al., 2013; Markle et al., 2013; Kim et al., 2020). Studies into human and murine models have suggested that the sex hormone estrogen has bidirectional interactions with gut microbiota, and a previous study into southern white rhinoceros dietary estrogenicity revealed similar effects (Chen & Madak-Erdogan, 2016; Williams et al., 2019). These rhinoceros studies consisted only of adult individuals, however, with no investigation into age-related differences in estrogenicity and gut microbiome interaction (Chen and Madak-Erdogan, 2016; Williams et al., 2019).

Subadult animals had enriched abundances of Rikenellaceae, Prevotellaceae, Kiritimatiellaeota, *Quinella*, *Marvinbryantia*, and *Acetitomaculum* (Fig. 2). Rikenellaceae and Prevotellaceae have been proposed to contribute to metabolite production and fiber degradation in southern white rhinoceros (Williams et al., 2019). *Quinella* is also responsible for digestion as a large propionate-producing rumen bacterium, and *Marvinbryantia* and *Acetitomaculum* have been similarly implicated in microbial fermentation processes (Kittelmann et al., 2014; Levin et al., 2015; Yu et al., 2020).

There were significant differences in alpha and beta diversity between the male (n=1) and female (n=9) individuals noted, including differences in species richness ($p<0.05$) and Bray-Curtis dissimilarity ($p<0.01$) (Table 3), as well as multiple differentially abundant taxa (Fig. 2). However, inferences based off of sex cannot be made without additional male rhinoceros added to the population; individual variation may contribute more to these differences than sex. Several mechanisms for sex differences in gut microbiota have been proposed, including sex hormones, body mass index, and colonic transit time (Yurkovetskiy et al., 2013; Haro et al., 2016; Kim et al., 2020). The male rhinoceros, M1, consumed the same managed pellet and hay diet as the female animals, but was housed near G1 in a separate building from the other individuals and had no access to the large Grasslands exhibit. While it is possible that the lack of grazing access spurred these differences, further research with additional male rhinoceros is necessary to derive a cause.

Seasonal effects on gut microbiome

Significant differences in species richness and Bray-Curtis dissimilarity across sampling months were identified and seasonal trends were observed (Table 5; Fig. 10). At the start of this

study, we hypothesized that feces collected from the population during warm weather months (July-September 2020) would reflect higher levels of species richness than cold weather months (January-March 2021). This is because the rhinoceros population would normally have outdoor access to the 16-hectare Watani Grasslands habitat during the summer, and all of the gut microbiome influences that come with it. This includes contact with environmental microbiota, potential interactions with allospecifics and their fecal microbiota, and the addition of several grass species to their diet. This expectation was based off of historical rhinoceros welfare routines at the NC Zoo but ultimately did not come to fruition due to a cyanobacterial bloom in the lake on habitat; rhinoceros were subsequently kept in holding throughout the entirety of the warm weather month sampling time frame to preserve their health. While a Kruskal-Wallis H test showed the presence of significance in richness between months ($p<0.05$), we were unable to identify which months were responsible using a Wilcoxon rank sum test. Boxplots did reveal that the cold weather months of January and March had higher median richness compared to all of the warm weather months (Fig. 6). This is unsurprising in context, as the rhinoceros were rotated back out onto the Grasslands habitat during the cold weather months subject to temperature (>0°C for nonreproductive female group and >7°C for the mother-calf pair group).

Warm weather months clustered distinctly together in microbial community composition, as did cold weather months; warm and cold weather month clusters did not share any overlap and were significantly different from each other in Bray-Curtis dissimilarity ($p<0.05$) (Fig. 10; Table 5). Cold weather months did not have significant inter-month Bray-Curtis dissimilarity differences, nor did the warm weather months of July and August. Curiously, September was different ($p<0.01$) from all other months in Bray-Curtis dissimilarity even though the only biologically relevant taxa (LDA score >3) that was differentially enriched in September were

members of the phylum Actinobacteria; these are common soil bacteria which decompose organic matter but are generally not abundant (<1%) in the gut microbiomes of this population of southern white rhinoceros (Fig. 3; Table S1) (Barka et al., 2016).

Differentially abundant taxa were also present for all other months except July, with March having the most numerous differentially abundant taxa (n=16) (Fig. 3). These include members of the phyla Bacteroidetes, Lentisphaeria, and Planctomycetes, and families Defluviitaleaceae and Norcardiaceae, order Micrococcales, and the R*hodococcus* and *Mycobacterium* genera. All aforementioned taxa are commonly occurring terrestrial and aquatic bacteria likely picked up from the soil, decaying plant matter, and freshwater lake of the Watani Grasslands habitat. The daily average temperature for March 2021 was ~12°C (Table S2), making it the first of the cool weather months to facilitate the daily rotation of both the non-reproductive female group and the mother-calf pair group onto the Grasslands habitat.

Limitations

This study has several limitations. The first of which is the small total population size (n=10) sampled, which limits the robustness of statistical inferences. Second, some animals were added and/or removed from the study population between sampling months due to external factors. For example, M1 was transferred to another facility before the sampling in March 2021 could begin, G1 was not sampled in September due to health concerns, and the juvenile animals J1 and J2 were not sampled during 2020 due to intractability. Third, the population of animals in this study belonged to one facility, thus these results are most relevant to that facility and may not be extrapolated to managed southern white rhinoceros populations as a whole. Fourth, this study sampled fecal microbiota as a proxy for gut microbiota, though recent research has

proposed that feces is inadequate at representing the microbiome present in both the contents and mucosa of the gastrointestinal tract (Tang et al., 2020). In addition, different parts of the gastrointestinal tract possess different abundances of microbial families which may not be apparent in feces (Donaldson et al., 2016). True sampling of the intestinal tract would involve highly invasive or fatal biopsies that are inappropriate for studies with endangered animals. As the majority of rhinoceros in this study were not tractable enough for per-rectal sampling, the non-invasive sampling of feces after defecation was necessary. This study used Illumina sequencing of the ~460 base pair V3 to V4 region of the 16S rRNA gene to identify bacterial OTUs and analyze composition as opposed to the usage of the full ~1500 base pair 16S gene. This compromise is known to produce high throughput results at a lower cost than Sanger sequencing but reduced the taxonomic accuracy at a species level. Finally, sequencing of samples took place after the cold-weather and warm-weather sampling periods were complete, meaning that not all samples were sequenced at the same time. While this has the potential to add sequencing variability, the utilization of the same laboratory, procedure, and technicians limited this variation as much as possible.

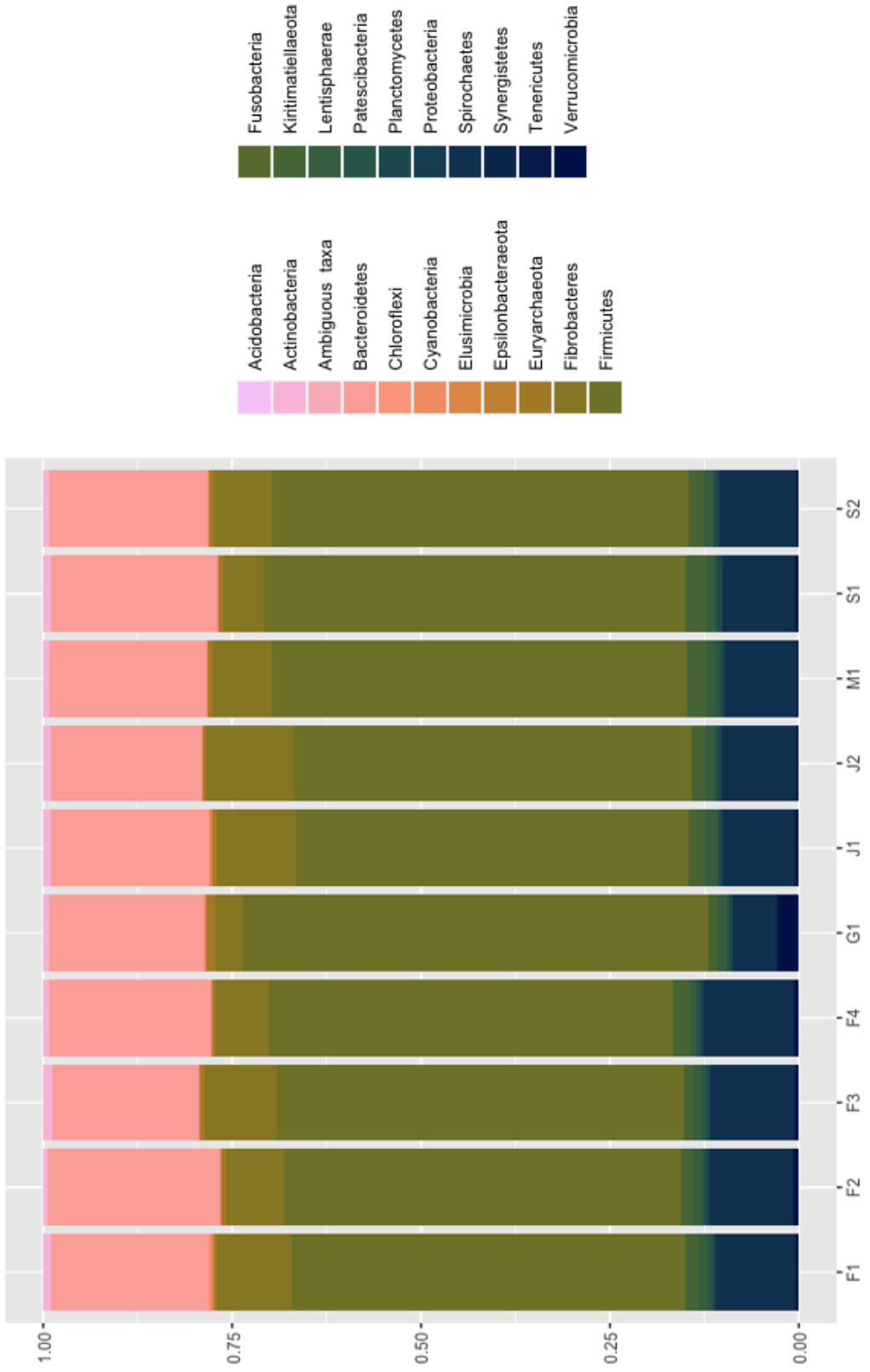
4.5 Conclusions

This study has revealed novel age and seasonal-related differences in microbial diversity and community clustering within the managed population of southern white rhinoceros at the NC Zoo. Further research with additional rhinoceros populations across similar facilities would be ideal for validating these trends for managed southern white rhinoceros as a whole, as well as discovering possible microbiome differences due to sex. As health and potential reproductive success is mediated by gut microbiota, investigating and cataloguing the microbiomes in the

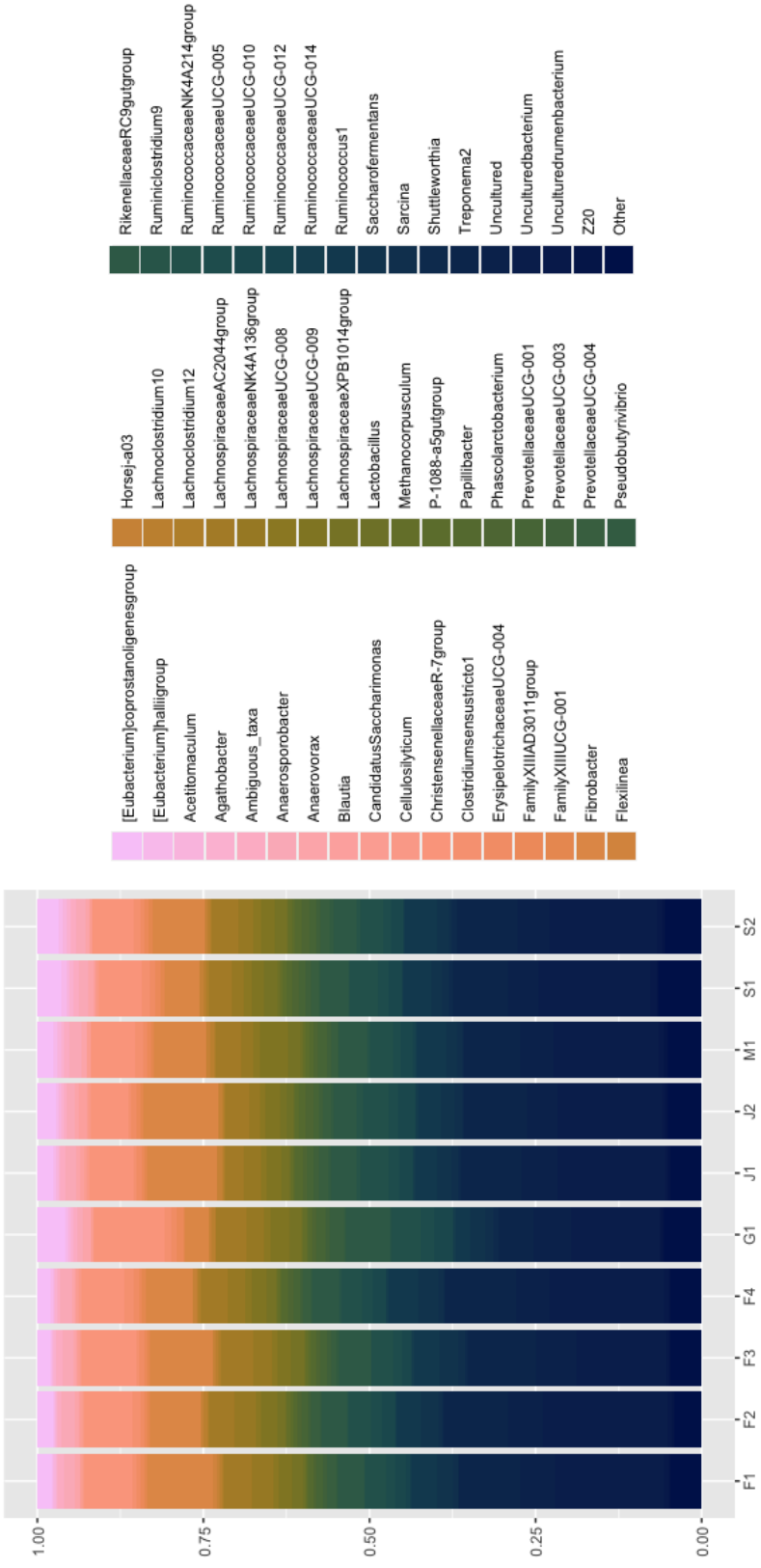
reproductively successful population of southern white rhinoceros at the NC Zoo may be of particular importance to the animal management community.

Figure 1. (A) Phylum- and (B) genus-level bar charts representing microbial community composition across n=10 southern white rhinoceros (*Ceratotherium simum simum*) at the North Carolina Zoo.

A)


Fusobacteria
Kiritimatiellaeota
Lentisphaerae
Patescibacteria
Planctomycetes
Proteobacteria
Spirochaetes
Synergistetes
Tenericutes
Verrucomicrobia
Acidobacteria
Actinobacteria
Ambiguous taxa
Bacteroidetes
Chloroflexi
Cyanobacteria
Elusimicrobia
Epsilonbacteraeota
Euryarchaeota
Fibrobacteres
Firmicutes
S2
S1
M1
J2
J1
G1
F4
F3
F2
F1
1.00
0.75
0.50
0.25
0.00

B)


RikenellaceaeRC9gutgroup
Ruminiclostridium9
RuminococcaceaeNK4A214group
RuminococcaceaeUCG-005
RuminococcaceaeUCG-010
RuminococcaceaeUCG-012
RuminococcaceaeUCG-014
Ruminococcus1
Saccharofermentans
Sarcina
Shuttleworthia
Treponema2
Uncultured
Unculturedbacterium
Unculturedrumenbacterium
Z20
Other
Horsej-a03
Lachnoclostridium10
Lachnoclostridium12
LachnospiraceaeAC2044group
LachnospiraceaeNK4A136group
LachnospiraceaeUCG-008
LachnospiraceaeUCG-009
LachnospiraceaeXPB1014group
Lactobacillus
Methanocorpusculum
P-1088-a5gutgroup
Papillibacter
Phascolarctobacterium
PrevotellaceaeUCG-001
PrevotellaceaeUCG-003
PrevotellaceaeUCG-004
Pseudobutyrivibrio
[Eubacterium]coprostanoligenesgroup
[Eubacterium]halliigroup
Acetitomaculum
Agathobacter
Ambiguous_taxa
Anaerosporobacter
Anaerovorax
Blautia
CandidatusSaccharimonas
Cellulosilyticum
ChristensenellaceaeR-7group
Clostridiumsensustricto1
ErysipelotrichaceaeUCG-004
FamilyXIIIAD3011group
FamilyXIIIUCG-001
Fibrobacter
Flexilinea
1.00
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0.00
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F2
F3
F4
G1
J1
J2
M1
S1
S2

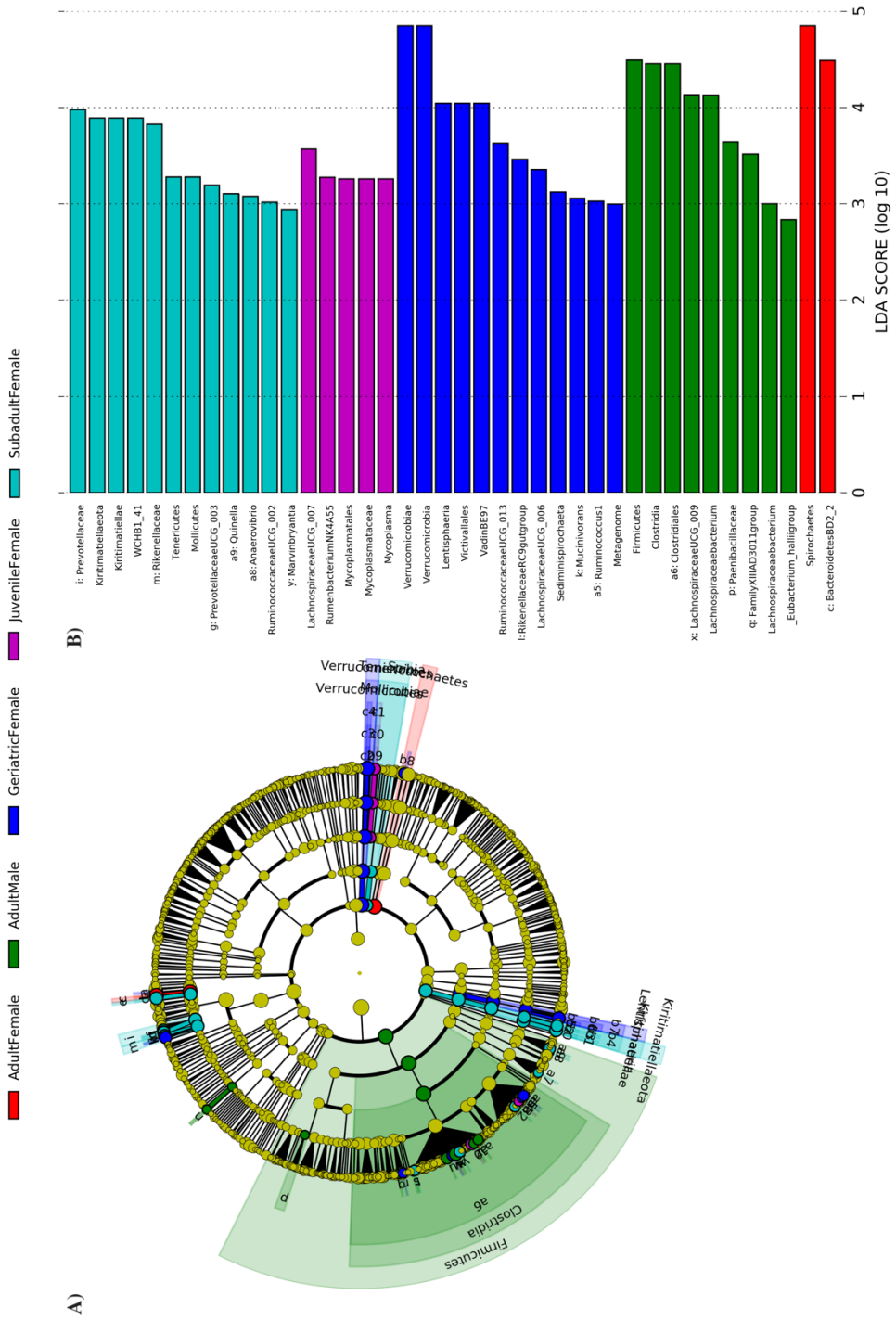


Figure 2. (A) Linear discriminate analysis Effect Size (LEfSe) cladogram and (B) plot of LDA scores comparing significantly and differentially enriched major taxa (i.e., present at >1% relative abundance) in five age/sex classes of n=10 southern white rhinoceros (*Ceratotherium simum simum*) housed at the North Carolina Zoo. $P \leq 0.05$ considered significant. An LDA score of 3 or higher is considered biologically relevant (Saito et al., 2019).

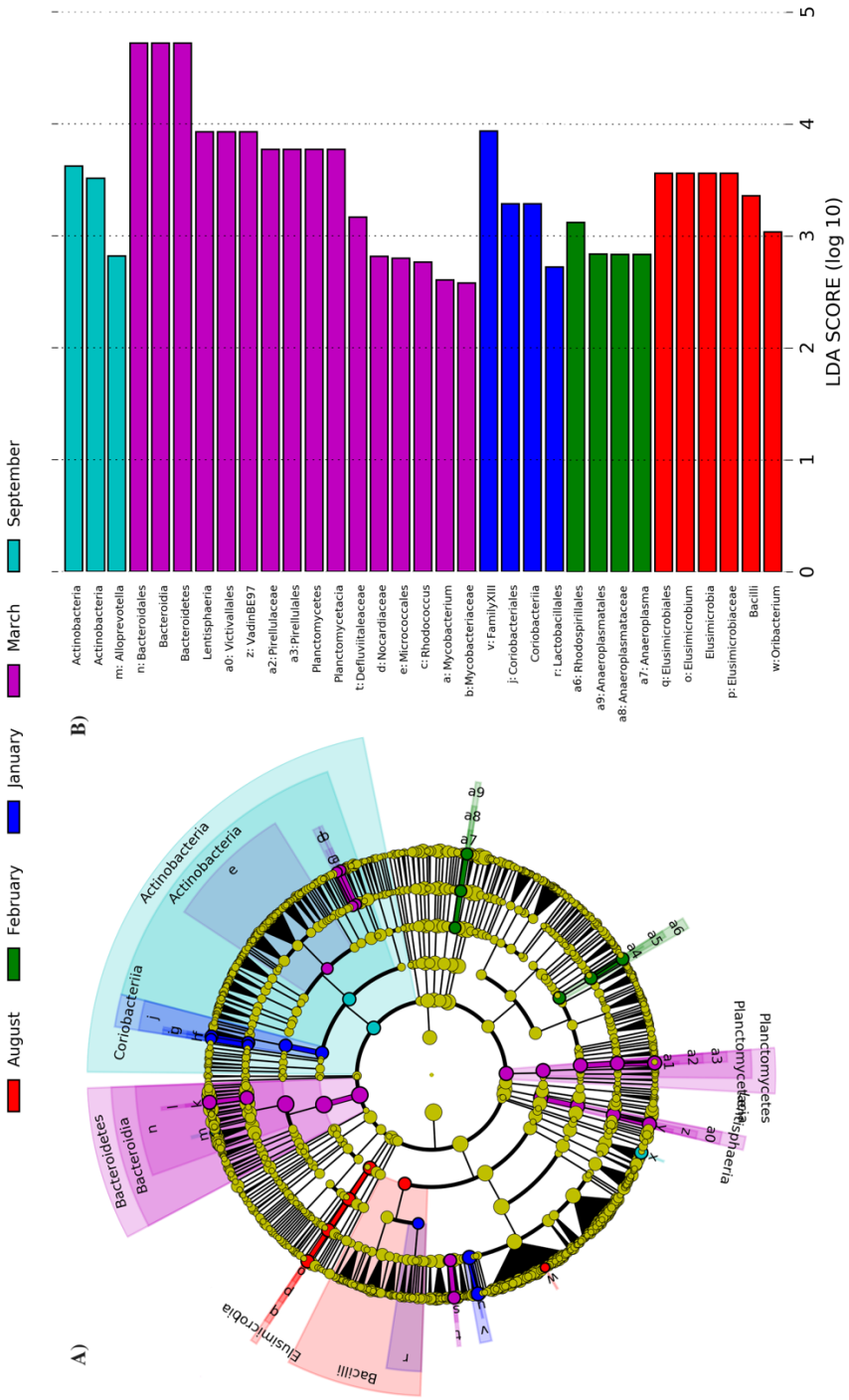


Figure 3. (A) Linear discriminant analysis Effect Size (LEfSe) cladogram and (B) plot of LDA scores comparing significantly and differentially enriched major taxa (i.e., present at >1% relative abundance) in n=6 southern white rhinoceros (*Ceratotherium simum simum*) housed at the North Carolina Zoo across six months. Data include samples from n=4 adult females and n=2 subadult females sampled between August of 2020 and March of 2021. An LDA score of 3 or higher is considered biologically relevant (Saito et al., 2019).

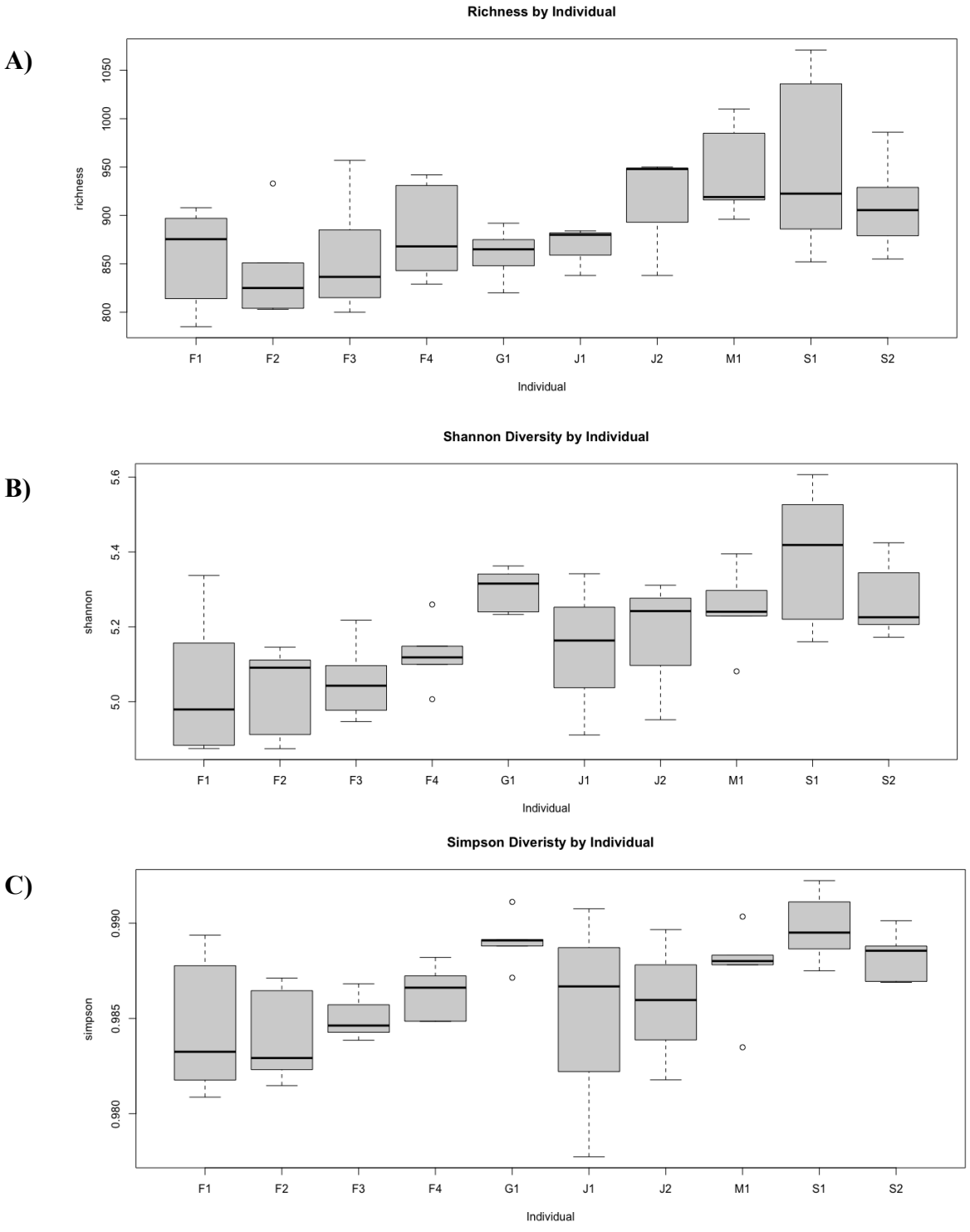
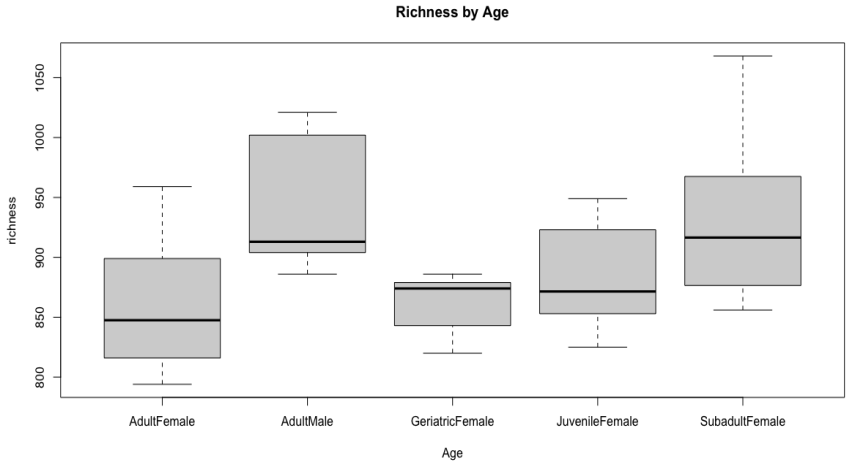
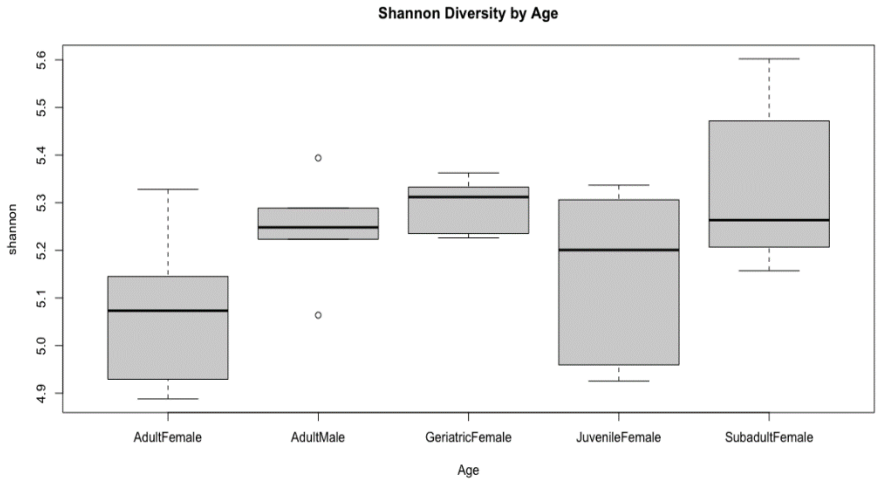


Figure 4. Boxplots comparing alpha diversity as measured by (A) species richness, (B) Shannon diversity, and (C) Simpson's diversity indices, across n=10 southern white rhinoceros (*Ceratotherium simum simum*) at the North Carolina Zoo.

A)



B)



C)

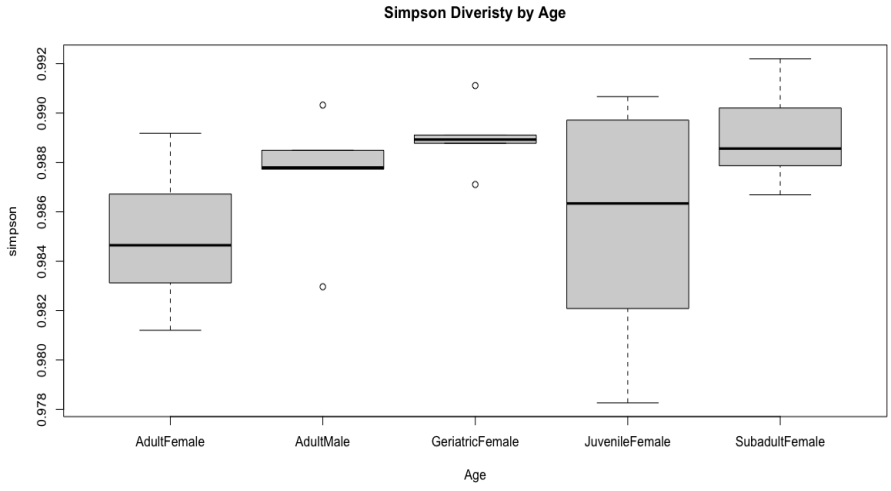
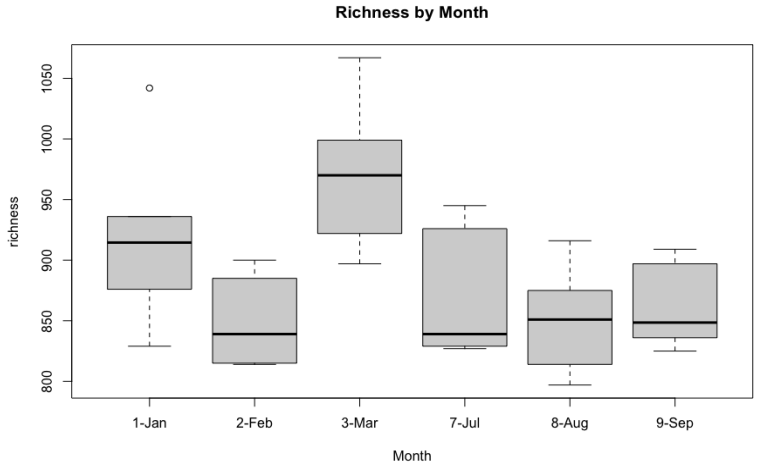
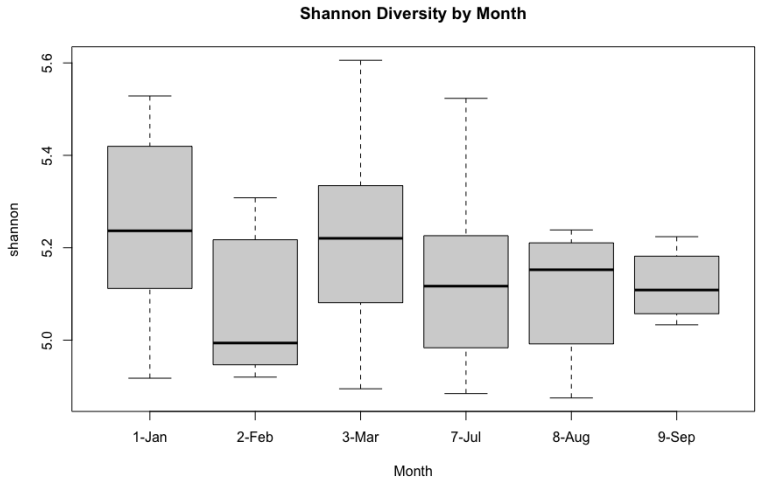


Figure 5. Boxplots comparing alpha diversity as measured by (A) species richness, (B) Shannon diversity, and (C) Simpson's diversity indices across six age classes of n=10 southern white rhinoceros (*Ceratotherium simum simum*) at the North Carolina Zoo.

A)



B)



C)

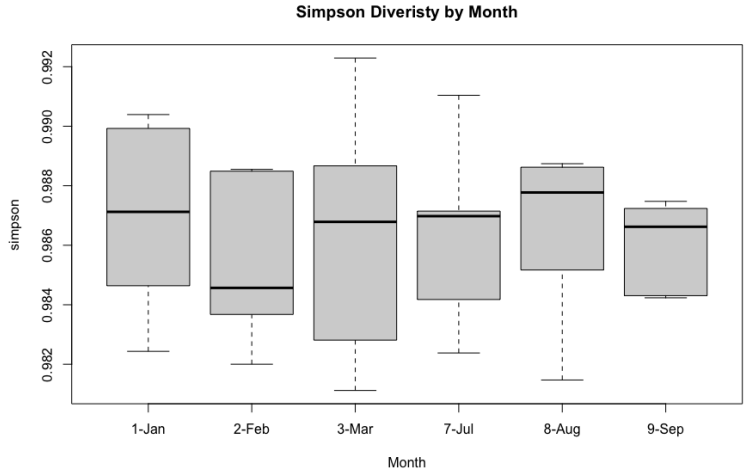


Figure 6. Boxplots comparing alpha diversity as measured by (A) species richness, (B) Shannon diversity, and (C) Simpson's diversity indices in n=6 southern white rhinoceros (*Ceratotherium simum simum*) housed at the North Carolina Zoo across six months. Data include samples from n=4 adult females and n=2 subadult females sampled between August of 2020 and March of 2021.

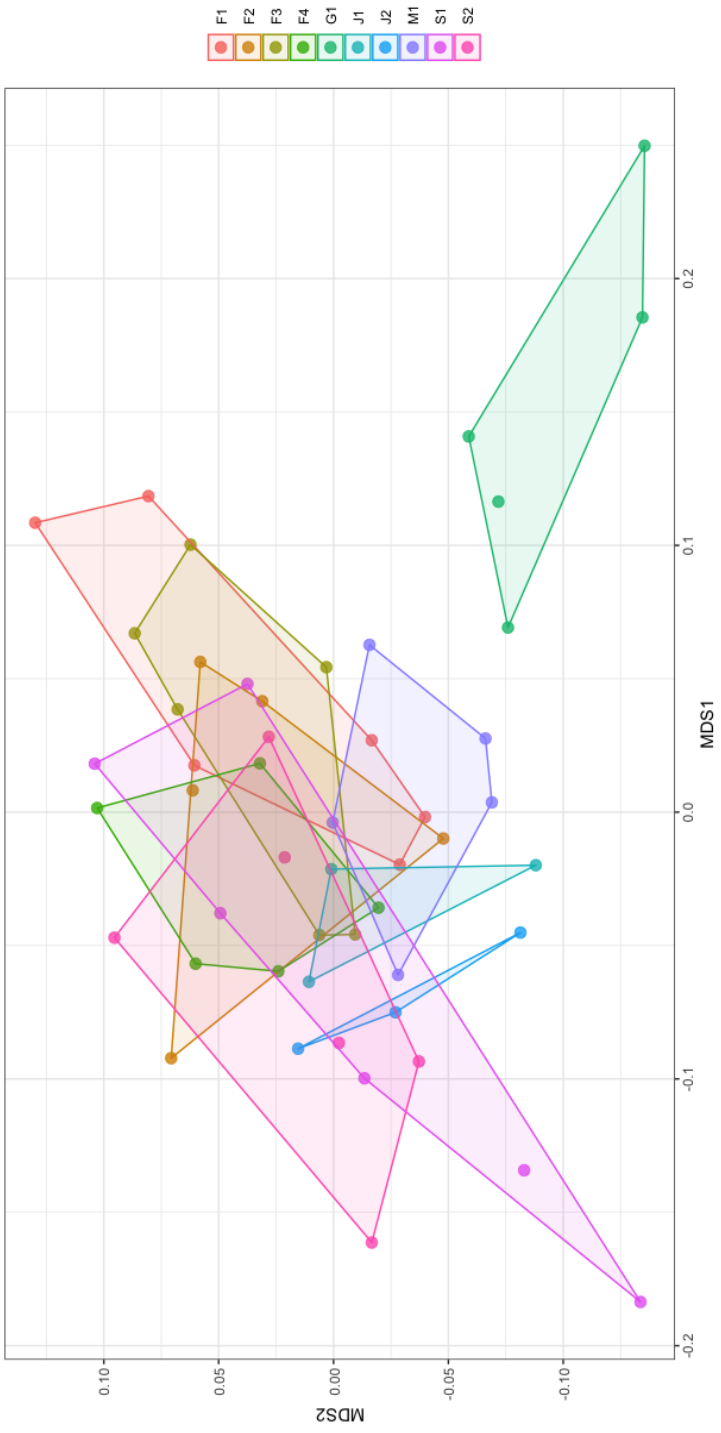


Figure 7. Multidimensional Scaling (MDS) Plots comparing individual microbiome clusters in n=10 southern white rhinoceros (*Ceratotherium simum simum*) housed at the North Carolina Zoo. Each point represents a single fecal sample; distance measured using Bray-Curtis dissimilarity.

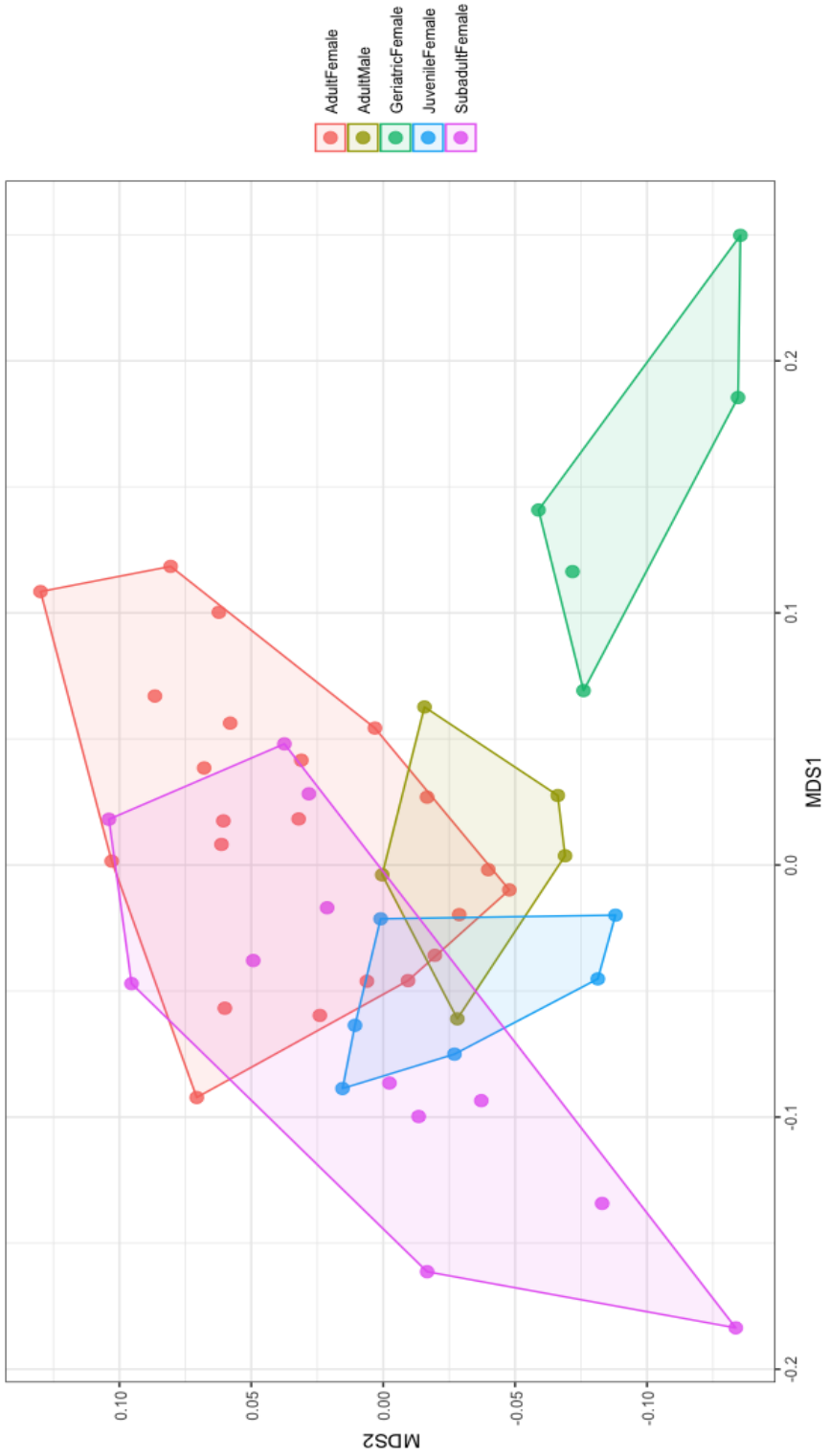


Figure 8. Multidimensional Scaling (MDS) Plots comparing microbiome clusters across five age and sex classes of n=10 southern white rhinoceros (*Ceratotherium simum simum*) housed at the North Carolina Zoo. Each point represents one fecal sample from one of n=10 southern white rhinoceros at the North Carolina Zoo.

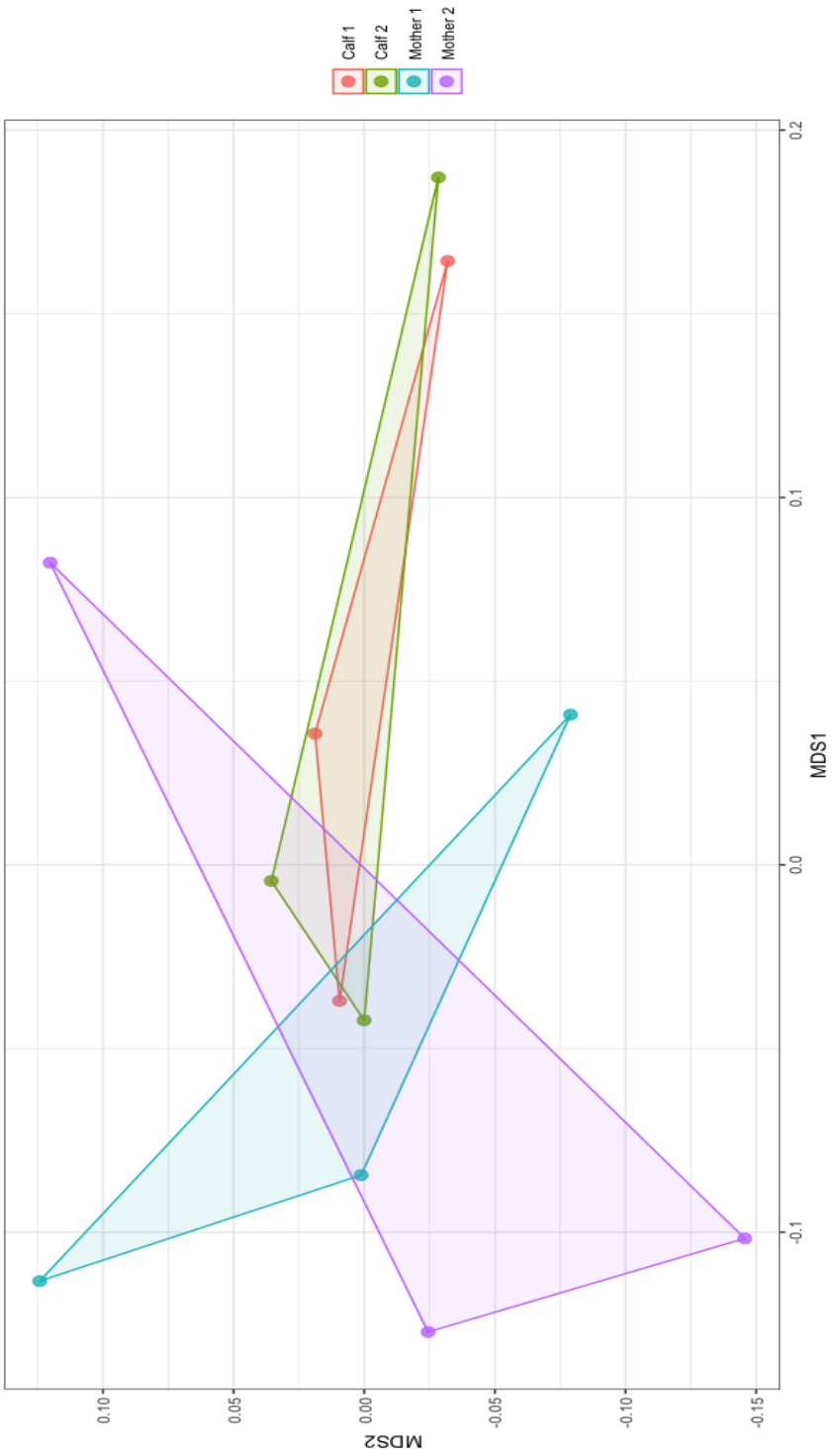


Figure 9. Multidimensional Scaling (MDS) Plots comparing clustering of microbiomes between two mother-calf pairs of southern white rhinoceros (*Ceratotherium simum simum*) housed at the North Carolina Zoo. Each point represents one fecal sample from one of n=2 adult female and n=2 juvenile female southern white rhinoceros at the North Carolina Zoo.

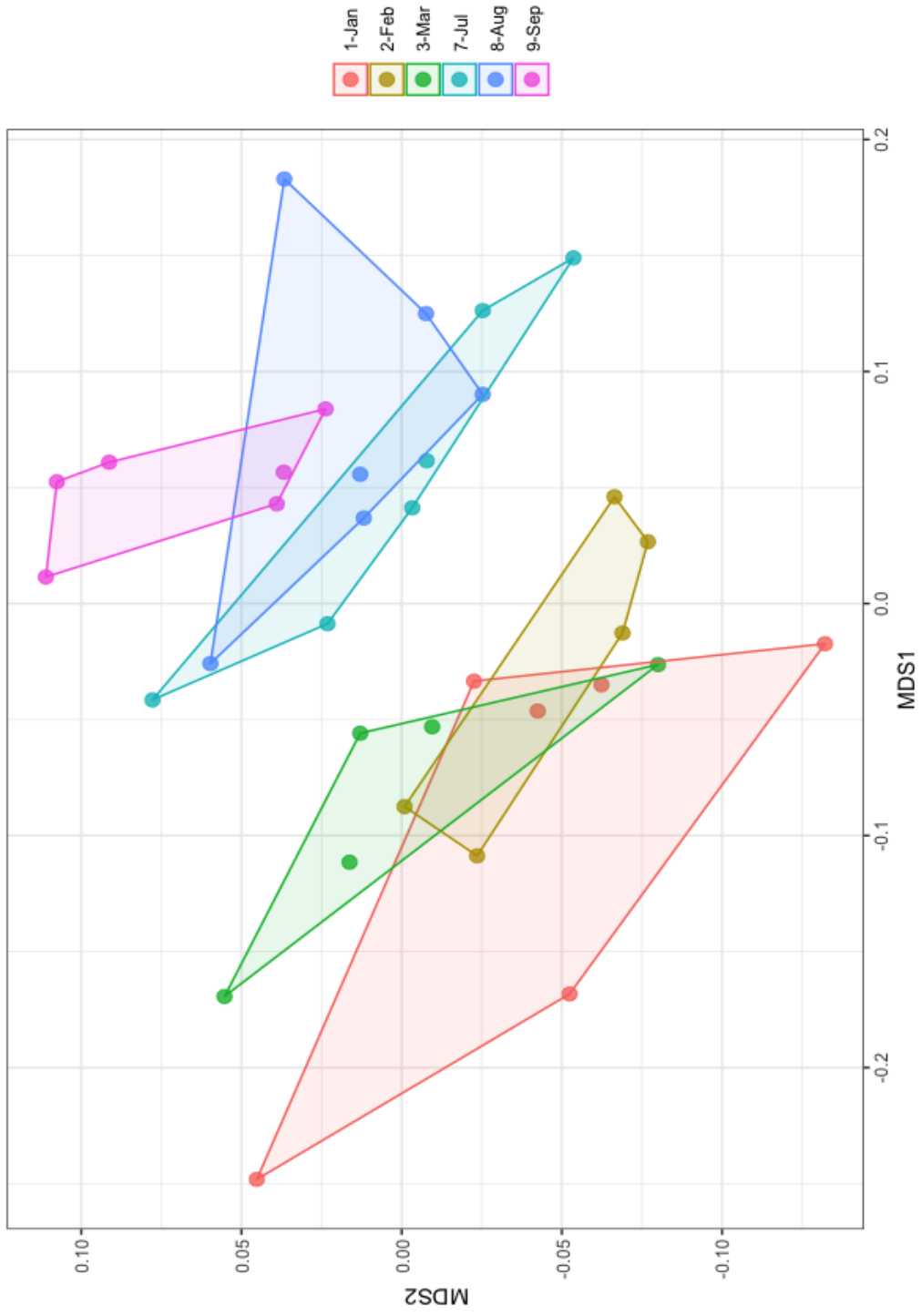


Figure 10. Multidimensional Scaling (MDS) Plots comparing microbiome clusters of n=6 southern white rhinoceros (*Ceratotherium simum simum*) housed at the North Carolina Zoo across six months. Each point represents one fecal sample from one of n=4 adult female and n=2 subadult female southern white rhinoceros at the North Carolina Zoo across six months between August of 2020 and March of 2021.

Table 3. PERMANOVA statistical comparisons of Bray-Curtis dissimilarity in microbial community composition across n=10 southern white rhinoceros (*Ceratotherium simum simum*) housed at the North Carolina Zoo.

	F1	F2	F3	F4	G1	J1	J2	M1	S1
F2	0.183	-	-	-	-		-	-	-
F3	0.183	0.264	-	-	-	-	-	-	-
F4	0.118	0.648	0.049*	-	-	-	-	-	-
G1	0.03*	0.04*	0.039*	0.04*	-	-	-	-	-
J1	0.183	0.384	0.144	0.401	0.04*	-	-	-	-
J2	0.059	0.07	0.04*	0.183	0.049*	0.628	-	-	-
M1	0.03*	0.04*	0.03*	0.03*	0.04*	0.08	0.04*	-	-
S1	0.049	0.118	0.058	0.213	0.03*	0.36	0.192	0.07	-
S2	0.04*	0.061	0.04*	0.253	0.03*	0.384	0.083	0.04*	0.648

Significance codes: '***' 0.001 '**' 0.01 '*' 0.05

Table 4. PERMANOVA statistical comparisons of Bray-Curtis dissimilarity in microbial community composition across five age/sex classes of n=10 southern white rhinoceros (*Ceratotherium simum simum*) housed at the North Carolina Zoo.

	Adult Female	Adult Male	Geriatric Female	Juvenile Female
Adult Male	0.002**	-	-	-
Geriatric Female	0.002**	0.013*	-	-
Juvenile Female	0.002**	0.005**	0.009**	-
Subadult Female	0.002**	0.01**	0.002**	0.018*

Significance codes: '***' 0.001 '**' 0.01 '*' 0.05

Table 5. PERMANOVA statistical comparisons of Bray-Curtis dissimilarity in microbial community composition across n=10 southern white rhinoceros (*Ceratotherium simum simum*) housed at the North Carolina Zoo across six months.

	January	February	March	July	August
February	0.086	-	-	-	-
March	0.056	0.077	-	-	-
July	0.007*	0.018*	0.008**	-	-
August	0.007**	0.007**	0.007**	0.164	-
September	0.007**	0.007**	0.007**	0.007**	0.007**

Significance codes: '***' 0.001 '**' 0.01 '*' 0.05

REFERENCES

Adriansjach, J., Baum, S. T., Lefkowitz, E. J., Van Der Pol, W. J., Buford, T. W., & Colman, R. J. (2020). Age-related differences in the gut microbiome of rhesus macaques. *The Journals of Gerontology: Series A*, *75*(7), 1293–1298.

Amato, K. R., Metcalf, J. L., Song, S. J., Hale, V. L., Clayton, J., Ackermann, G., Humphrey, G., Niu, K., Cui, D., Zhao, H., Schrenzel, M. D., Tan, C. L., Knight, R., & Braun, J. (2016). Using the gut microbiota as a novel tool for examining colobine primate GI health. *Global Ecology and Conservation*, *7*, 225–237.

Barka, E. A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H.-P., Clément, C., Ouhdouch, Y., & van Wezel, G. P. (2016). Taxonomy, physiology, and natural products of Actinobacteria. *Microbiology and Molecular Biology Reviews*, *80*(1), 1–43.

Bian, G., Ma, L., Su, Y., & Zhu, W. (2013). The microbial community in the feces of the white rhinoceros (*Ceratotherium simum*) as determined by barcoded pyrosequencing analysis. *PLOS ONE*, *8*(7), e70103.

Carey, H. V., Walters, W. A., & Knight, R. (2013). Seasonal restructuring of the ground squirrel gut microbiota over the annual hibernation cycle. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *304*(1), R33–R42.

Carthey, A. J. R., Blumstein, D. T., Gallagher, R. V., Tetu, S. G., & Gillings, M. R. (2020). Conserving the holobiont. *Functional Ecology*, *34*(4), 764–776.

Cersosimo, L. M., Sullivan, K. E., & Valdes, E. V. (2021). Species and individual rhinoceros affect the bacterial communities, metabolites, and nutrient composition in faeces from Southern black rhinoceros (*Diceros bicornis minor*) and Southern white rhinoceros (*Ceratotherium simum simum*) under managed care. *Journal of Animal Physiology and Animal Nutrition*, *0*(0), 1–13

Chen, K. L., & Madak-Erdogan, Z. (2016). Estrogen and microbiota crosstalk: Should we pay attention? *Trends in Endocrinology & Metabolism*, *27*(11), 752–755.

Clayton, J. B., Vangay, P., Huang, H., Ward, T., Hillmann, B. M., Al-Ghalith, G. A., Travis, D. A., Long, H. T., Tuan, B. V., Minh, V. V., Cabana, F., Nadler, T., Toddes, B., Murphy, T., Glander, K. E., Johnson, T. J., & Knights, D. (2016). Captivity humanizes the primate microbiome. *Proceedings of the National Academy of Sciences*, *113*(37), 10376–10381.

Collado, M. C., Rautava, S., Aakko, J., Isolauri, E., & Salminen, S. (2016). Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. *Scientific Reports*, *6*(1), 23129.

Costa, M. C., Silva, G., Ramos, R. V., Staempfli, H. R., Arroyo, L. G., Kim, P., & Weese, J. S. (2015). Characterization and comparison of the bacterial microbiota in different gastrointestinal tract compartments in horses. *The Veterinary Journal*, *205*(1), 74–80.

De La Torre, U. D. L., Henderson, J. D., Furtado, K. L., Pedroja, M., Elenamarie, O., Mora, A., Pechanec, M. Y., Maga, E. A., & Mienaltowski, M. J. (2019). Utilizing the fecal microbiota to understand foal gut transitions from birth to weaning. *PLOS ONE*, *14*(4), e0216211.

Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., & Knight, R. (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proceedings of the National Academy of Sciences*, *107*(26), 11971–11975.

Dougal, K., Fuente, G. De La Fuente, Harris, P. A., Girdwood, S. E., Pinloche, E., Geor, R. J., Nielsen, B. D., Ii, H. C. S., Elzinga, S., & Newbold, C. J. (2014). Characterization of the faecal bacterial community in adult and elderly horses fed a high fibre, high oil or high starch diet using 454 pyrosequencing. *PLOS ONE*, *9*(2), e87424.

Emslie, R. (2020). *Ceratotherium simum*. *The IUCN Red List of Threatened Species 2020*.

Emslie, R., Adcock, K., & Hansen, H. (1995). Fine tuning rhino management group age class system. *Rhino Management Group*, 1–17.

Gibson, K. M., Nguyen, B. N., Neumann, L. M., Miller, M., Buss, P., Daniels, S., Ahn, M. J., Crandall, K. A., & Pukazhenthi, B. (2019). Gut microbiome differences between wild and captive black rhinoceros – implications for rhino health. *Scientific Reports*, *9*(1), 7570.

Gillman, S. J., McKenney, E. A., & Lafferty, D. J. R. (2020). Wild black bears harbor simple gut microbial communities with little difference between the jejunum and colon. *Scientific Reports*, *10*(1), 20779.

Greene, L. K., Williams, C. V., Junge, R. E., Mahefarisoa, K. L., Rajaonarivelo, T., Rakotondrainibe, H., O'Connell, T. M., & Drea, C. M. (2020). A role for gut microbiota in host niche differentiation. *The ISME Journal*, *14*(7), 1675–1687.

Haro, C., Rangel-Zúñiga, O. A., Alcalá-Díaz, J. F., Gómez-Delgado, F., Pérez-Martínez, P., Delgado-Lista, J., Quintana-Navarro, G. M., Landa, B. B., Navas-Cortés, J. A., Tena-Sempere, M., Clemente, J. C., López-Miranda, J., Pérez-Jiménez, F., & Camargo, A. (2016). Intestinal microbiota is influenced by gender and body mass index. *PLOS ONE*, *11*(5), e0154090.

IUCN. (2021). *The IUCN Red List of Threatened Species*. *Version 2021-2*. https://www.iucnredlist.org

Janiak, M. C., Montague, M. J., Villamil, C. I., Stock, M. K., Trujillo, A. E., DePasquale, A. N., Orkin, J. D., Bauman Surratt, S. E., Gonzalez, O., Platt, M. L., Martínez, M. I., Antón, S. C., Dominguez-Bello, M. G., Melin, A. D., & Higham, J. P. (2021). Age and sex-associated variation in the multi-site microbiome of an entire social group of free-ranging rhesus macaques. *Microbiome*, *9*(1), 68.

Jassim, R. A. M. A., & Andrews, F. M. (2009). The bacterial community of the horse gastrointestinal tract and its relation to fermentative acidosis, laminitis, colic, and stomach ulcers. *Veterinary Clinics: Equine Practice*, *25*(2), 199–215.

Kim, Y. S., Unno, T., Kim, B.-Y., & Park, M.-S. (2020). Sex differences in gut microbiota. *The World Journal of Men's Health*, *38*(1), 48–60.

Kinross, J. M., Darzi, A. W., & Nicholson, J. K. (2011). Gut microbiome-host interactions in health and disease. *Genome Medicine*, *3*(3), 14.

Kittelmann, S., Pinares-Patiño, C. S., Seedorf, H., Kirk, M. R., Ganesh, S., McEwan, J. C., & Janssen, P. H. (2014). Two different bacterial community types are linked with the low-methane emission trait in sheep. *PLOS ONE*, *9*(7), e103171.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, *41*(1), e1.

Legendre, P., & De Cáceres, M. (2013). Beta diversity as the variance of community data: dissimilarity coefficients and partitioning. *Ecology letters*, *16*(8), 951-963.

Levin, D. B., Verbeke, T. J., Munir, R., Islam, R., Ramachandran, U., Lal, S., Schellenberg, J., & Sparling, R. (2015). Chapter 17—Omics approaches for designing biofuel producing cocultures for enhanced microbial conversion of lignocellulosic substrates. In M. E. Himmel (Ed.), *Direct Microbial Conversion of Biomass to Advanced Biofuels* (pp. 335–363). Elsevier.

Lewis, J. D., Chen, E. Z., Baldassano, R. N., Otley, A. R., Griffiths, A. M., Lee, D., Bittinger, K., Bailey, A., Friedman, E. S., Hoffmann, C., Albenberg, L., Sinha, R., Compher, C., Gilroy, E., Nessel, L., Grant, A., Chehoud, C., Li, H., Wu, G. D., & Bushman, F. D. (2015). Inflammation, antibiotics, and diet as environmental stressors of the gut microbiome in pediatric Crohn's disease. *Cell Host & Microbe*, *18*(4), 489–500.

Mäkivuokko, H., Tiihonen, K., Tynkkynen, S., Paulin, L., & Rautonen, N. (2010). The effect of age and non-steroidal anti-inflammatory drugs on human intestinal microbiota composition. *British Journal of Nutrition*, *103*(2), 227–234.

Mariat, D., Firmesse, O., Levenez, F., Guimarães, V., Sokol, H., Doré, J., Corthier, G., & Furet, J.-P. (2009). The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiology*, *9*(1), 123.

Markle, J. G. M., Frank, D. N., Mortin-Toth, S., Robertson, C. E., Feazel, L. M., Rolle-Kampczyk, U., von Bergen, M., McCoy, K. D., Macpherson, A. J., & Danska, J. S. (2013). Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science*, *339*(6123), 1084–1088.

Maurice, C. F., CL Knowles, S., Ladau, J., Pollard, K. S., Fenton, A., Pedersen, A. B., & Turnbaugh, P. J. (2015). Marked seasonal variation in the wild mouse gut microbiota. *The ISME Journal*, *9*(11), 2423–2434.

McKenzie, V. J., Song, S. J., Delsuc, F., Prest, T. L., Oliverio, A. M., Korpita, T. M., Alexiev, A., Amato, K. R., Metcalf, J. L., Kowalewski, M., Avenant, N. L., Link, A., Di Fiore, A., Seguin-Orlando, A., Feh, C., Orlando, L., Mendelson, J. R., Sanders, J., & Knight, R. (2017). The effects of captivity on the mammalian gut microbiome. *Integrative and Comparative Biology*, *57*(4), 690–704.

Milinovich, G. J., Trott, D. J., Burrell, P. C., Van Eps, A. W., Thoefner, M. B., Blackall, L. L., Al Jassim, R. a. M., Morton, J. M., & Pollitt, C. C. (2006). Changes in equine hindgut bacterial populations during oligofructose-induced laminitis. *Environmental Microbiology*, *8*(5), 885–898.

Miller, M. A., & Buss, P. E. (2015). Rhinoceridae (Rhinoceroses). In *Fowler's Zoo and Wild Animal Medicine, Volume 8* (pp. 538–547). Elsevier.

Moorthy, A. R., Spradbrow, P. B., & Eisler, M. E. (1977). Isolation of mycoplasmas from the genital tract of horses. *Australian Veterinary Journal*, *53*(4), 167–169.

Mshelia, E. S., Adamu, L., Wakil, Y., Turaki, U. A., Gulani, I. A., & Musa, J. (2018). The association between gut microbiome, sex, age and body condition scores of horses in Maiduguri and its environs. *Microbial Pathogenesis*, *118*, 81–86.

PCR Amplicon, PCR Clean-up, and Index PCR. (2013). 16S metagenomic sequencing library preparation. *Illumina*, 1-28.

Quercia, S., Freccero, F., Castagnetti, C., Soverini, M., Turroni, S., Biagi, E., Rampelli, S., Lanci, A., Mariella, J., Chinellato, E., Brigidi, P., & Candela, M. (2019). Early colonization and temporal dynamics of the gut microbial ecosystem in Standardbred foals. *Equine Veterinary Journal*, *51*(2), 231–237.

Ren, T., Boutin, S., Humphries, M. M., Dantzer, B., Gorrell, J. C., Coltman, D. W., McAdam, A. G., & Wu, M. (2017). Seasonal, spatial, and maternal effects on gut microbiome in wild red squirrels. *Microbiome*, *5*(1), 163.

Roth, T. L., Switzer, A., Watanabe-Chailland, M., Bik, E. M., Relman, D. A., Romick-Rosendale, L. E., & Ollberding, N. J. (2019). Reduced gut microbiome diversity and metabolome differences in rhinoceros species at risk for iron overload disorder. *Frontiers in Microbiology*, *10*, 2291.

Salem, S. E., Maddox, T. W., Berg, A., Antczak, P., Ketley, J. M., Williams, N. J., & Archer, D. C. (2018). Variation in faecal microbiota in a group of horses managed at pasture over a 12-month period. *Scientific Reports*, *8*(1), 8510.

Shreiner, A., Huffnagle, G. B., & Noverr, M. C. (2008). The "Microflora Hypothesis" of allergic disease. In G. B. Huffnagle & M. C. Noverr (Eds.), *GI Microbiota and Regulation of the Immune System* (pp. 113–134). Springer.

Singh, R. K., Chang, H.-W., Yan, D., Lee, K. M., Ucmak, D., Wong, K., Abrouk, M., Farahnik, B., Nakamura, M., Zhu, T. H., Bhutani, T., & Liao, W. (2017). Influence of diet on the gut microbiome and implications for human health. *Journal of Translational Medicine*, *15*(1), 73.

Smits, S. A., Leach, J., Sonnenburg, E. D., Gonzalez, C. G., Lichtman, J. S., Reid, G., Knight, R., Manjurano, A., Changalucha, J., Elias, J. E., Dominguez-Bello, M. G., & Sonnenburg, J. L. (2017). Seasonal cycling in the gut microbiome of the Hadza hunter-gatherers of Tanzania. *Science*, *357*(6353), 802–806.

Swaisgood, R. R., Dickman, D. M., & White, A. M. (2006). A captive population in crisis: Testing hypotheses for reproductive failure in captive-born southern white rhinoceros females. *Biological Conservation*, *129*(4), 468–476.

Tang, Q., Jin, G., Wang, G., Liu, T., Liu, X., Wang, B., & Cao, H. (2020). Current sampling methods for gut microbiota: A call for more precise devices. *Frontiers in Cellular and Infection Microbiology*, *10*, 151.

Tubbs, C. W., Moley, L. A., Ivy, J. A., Metrione, L. C., LaClaire, S., Felton, R. G., Durrant, B. S., & Milnes, M. R. (2016). Estrogenicity of captive southern white rhinoceros diets and their association with fertility. *General and Comparative Endocrinology*, *238*, 32–38.

Wallace, B. D., & Redinbo, M. R. (2013). The human microbiome is a source of therapeutic drug targets. *Current Opinion in Chemical Biology*, *17*(3), 379–384.

Weese, J. S., Holcombe, S. J., Embertson, R. M., Kurtz, K. A., Roessner, H. A., Jalali, M., & Wismer, S. E. (2015). Changes in the faecal microbiota of mares precede the development of *post partum* colic. *Equine Veterinary Journal*, *47*(6), 641–649.

White, A. M., Swaisgood, R. R., & Czekala, N. (2007). Differential investment in sons and daughters: Do white rhinoceros mothers favor sons? *Journal of Mammalogy*, *88*(3), 632–638.

Whitfield-Cargile, C. M., Cohen, N. D., Chapkin, R. S., Weeks, B. R., Davidson, L. A., Goldsby, J. S., Hunt, C. L., Steinmeyer, S. H., Menon, R., Suchodolski, J. S., Jayaraman, A., & Alaniz, R. C. (2016). The microbiota-derived metabolite indole decreases mucosal inflammation and injury in a murine model of NSAID enteropathy. *Gut Microbes*, *7*(3), 246–261.

Williams, C. L., Ybarra, A. R., Meredith, A. N., Durrant, B. S., & Tubbs, C. W. (2019). Gut microbiota and phytoestrogen-associated infertility in southern white rhinoceros. *MBio*, *10*(2).

Woodmansey, E. J., McMurdo, M. E. T., Macfarlane, G. T., & Macfarlane, S. (2004). Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects. *Applied and Environmental Microbiology*, *70*(10), 6113–6122.

Xue, Z., Zhang, W., Wang, L., Hou, R., Zhang, M., Fei, L., Zhang, X., Huang, H., Bridgewater, L. C., Jiang, Y., Jiang, C., Zhao, L., Pang, X., & Zhang, Z. (2015). The bamboo-eating giant panda harbors a carnivore-like gut microbiota, with excessive seasonal variations. *MBio*, *6*(3), e00022-15.

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R. N., Anokhin, A. P., Heath, A. C., Warner, B., Reeder, J., Kuczynski, J., Caporaso, J. G., Lozupone, C. A., Lauber, C., Clemente, J. C., Knights, D., … Gordon, J. I. (2012). Human gut microbiome viewed across age and geography. *Nature*, *486*(7402), 222–227.

Yu, S., Zhang, G., Liu, Z., Wu, P., Yu, Z., & Wang, J. (2020). Repeated inoculation with fresh rumen fluid before or during weaning modulates the microbiota composition and co-occurrence of the rumen and colon of lambs. *BMC Microbiology*, *20*(1), 29.

Yurkovetskiy, L., Burrows, M., Khan, A. A., Graham, L., Volchkov, P., Becker, L., Antonopoulos, D., Umesaki, Y., & Chervonsky, A. V. (2013). Gender bias in autoimmunity is influenced by microbiota. *Immunity*, *39*(2), 400–412.

Zhang, B., Brock, M., Arana, C., Dende, C., Hooper, L., & Raj, P. (2020). Impact of bead-beating intensity on microbiome recovery in mouse and human stool: Optimization of DNA extraction. *bioRxiv*, 1-23

MANUSCRIPT 2: EFFECT OF FECAL PRESERVATION METHOD ON MANAGED SOUTHERN WHITE RHINOCEROS (*CERATOTHERIUM SIMUM SIMUM*) GUT MICROBIOME

5.1 Introduction

The mammalian microbiome comprises microbial communities both on and inside of their bodies, which adapt to the unique conditions at each body site and have co-evolved to complement and meet host requirements across life processes and at each life stage. The majority of the mammalian microbiome resides within the lower gastrointestinal tract (i.e., the gut microbiome), where it facilitates the digestion of nutrients, produces vitamins and short chain fatty acids, and promotes tissue development and immune function (Geuking et al., 2011; Flint et al., 2012; LeBlanc et al., 2013). Gut microbiomes are species-specific and usually vary among individuals. Composition and abundance of the microbial taxa present are dependent on a variety of factors including diet (Muegge et al., 2011; David et al., 2014; McKenney et al., 2015), gut morphology (Gillman et al., 2020), feeding strategy (Greene et al., 2020; McKenney et al., 2018), age (Adriansjach et al., 2020; Janiak et al., 2021), sex (Markle et al., 2013; Mshelia et al., 2018), geographic location (Yatsunenko et al., 2012), and health status (Lewis et al., 2015). Dysbiosis of the gut microbiome is usually associated with diseased state in the host and the opportunistic colonization of pathogenic bacteria subsequent to the loss of beneficial gut microbes (Lupp et al., 2007; Schippa et al., 2012; Winter et al., 2013). Gut microbiome is inextricably connected to the health and functioning of the host. From a conservation standpoint, understanding the gut microbiome is thus of critical importance for the health and population management of imperiled wildlife (McKenzie et al., 2017; Carthey et al., 2020).

The stability of microbial communities in feces begins to decline after 24 hours of storage at ambient temperature (Cardona et al., 2012; Carroll et al., 2012; Tedjo et al., 2015). While refrigerating samples at 4°C does halt that degradation, this effect also only lasts up to 72 hours before the microbial community structure begins to change, favoring the growth of psychotropic and psychrophilic bacteria able to reproduce at low temperatures (Tedjo et al., 2015; Choo et al., 2015; Wu et al., 2019). Refrigeration at 4°C also encourages fungal growth, further jeopardizing samples (Song et al., 2016). As such, samples must be stored at cooler temperatures for longitudinal studies. Though immediate freezing at -80°C is the gold standard for preserving fecal samples for microbiome research, freezing at -20°C is an acceptable alternative when -80°C is unavailable (Wu et al., 2010; Choo et al., 2015). However, freezing tends to be impractical for conservation fieldwork applications because many sample sites are remote and may lack electricity and/or ultra-low freezers. In addition, any freeze-thaw cycles that occur during the transport of fecal samples to and from storage sites further jeopardize DNA quality (Song et al., 2016). To maximize the quality (e.g., molecular weight) of DNA extractions, samples must therefore be either: 1) processed (i.e., DNA extraction and possibly sequencing) where they are frozen; 2) transported at ambient temperature or on ice to a lab for possessing, at the risk of sample integrity; or 3) transported at ultra-low temperatures, which is not possible at all sample sites (Song et al., 2016; Wu et al., 2019).

In an attempt to circumvent the pitfalls associated with ambient temperatures and lack of ultra-low freezing outlined above, many field researchers store fecal samples in ethanol in an effort to halt microbial reproduction until the samples can be transported to a lab for DNA extraction (Wu et al., 2019). Fecal samples immersed in 95% ethanol can be stored safely at ambient temperature. However, these samples can be equally difficult to travel with as large

quantities of ethanol (>1L) must be shipped via private carrier, which is much more expensive and logistically challenging compared to passenger planes (Dangerous Goods Regulations, 2021). These restrictions risk loss of samples as well as increases the costs and time invested in the venture. Furthermore, several studies have indicated that samples preserved in ethanol consistently return low DNA yields after extraction, potentially affecting downstream applications (Vlčková et al., 2012; Hale et al., 2015; Song et al., 2016). While samples stored at ambient temperature in 95% ethanol for 8 weeks have shown slight compositional changes from technical replicates and were more susceptible to change under heat conditions, these effects were small compared to compositional differences due to interindividual variation (Song et al., 2016).

PERFORMAbiome™•GUT (PB), a proprietary fecal preservation method and storage device produced by DNA Genotek (Ottawa, Ontario, Canada), offers many potential benefits for microbiome fieldwork, including the ability to maintain DNA integrity at temperatures between -20°C to 50°C and to maintain a stable microbial profile at room temperature for 60 days (DNA Genotek, 2019). PB tubes have been internally validated by DNA Genotek for usage in domestic dogs, cats, and horses. However, only two published studies have used PB tubes: one for dog samples and the other for pinnipeds (harbor seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*)) (Lin et al., 2020; Steinmetz et al., 2021). These three species possess relatively short, simple digestive tracts lacking a functional cecum (Mead, 2009; Smith et al., 2009). Due to the novel nature of PB tubes, it is important that they be externally validated for usage with feces types from other species bearing diverse feeding strategies and gut morphologies, and under varying time constraints. We address this gap in information by comparing the effects of

preservation method and time on the gut microbial composition and structure of fresh fecal samples collected from managed southern white rhinoceros (*Ceratotherium simum simum*).

Southern white rhinoceros are large grazing herbivores that have evolved a complex gut morphology including a large cecum and sacculated colon to facilitate digestion of their high-fiber diet, and thus provide a novel and compelling species in which to test the efficacy of different methods for preserving the gut microbiome in herbivores. Southern white rhinoceros populations face an uncertain future due to intense poaching pressure across their home ranges, thus the management of reproductively successful assurance populations becomes imperative. Reproductive outcomes in managed southern white rhinoceros may be facilitated in part via gut microbial diversity, as female fertility levels appear to vary with both their fecal phytoestrogen profiles and the presence of specific gut bacteria that metabolize those phytoestrogens (Tubbs et al., 2016; Williams et al., 2019). However, managed populations of southern white rhinoceros in zoological facilities have historically experienced poor fertility and post-copulatory reproductive failure (Swaisgood et al., 2006; Metrione & Eyres, 2014). By contrast, wild populations of rhinoceros and those managed in reserves *in-situ* are reproductively successful to the point that the total population has risen from an estimated 50 individuals at the end of the 19th century to an estimated 18,064 wild individuals at the last estimation in December 2017, though these numbers are declining again (Emslie, 2020). It is thus of critical importance that we understand how managed populations of southern white rhinoceros differ from their wild conspecifics in ways that affect reproductive success, including their gut microbiomes. Fieldwork is needed for fecal sampling of wild populations *in-situ*; however, fecal preservation for microbiome studies can be logistically challenging in the field.

The objectives of this research are two-fold: 1) to compare the efficacy of different fecal preservation techniques (immediate freezing at -80°C, PB tubes, and 95% ethanol) for preserving fecal samples from southern white rhinoceros; and 2) to test the limitations of those preservation methods for 14 days and 230 days. We hypothesized that 95% ethanol-preserved and PB-preserved samples will perform similarly to -80°C controls in preserving microbial richness and community composition in fecal samples over a 14-day period. We also hypothesized that microbial richness and community composition in PB samples will shift between 14 and 230 days of storage at ambient temperature due to degradation of both preservative solution and sample, and subsequent changes in the observed microbial community profile. We did not expect the microbial profile of samples preserved in 95% ethanol to change significantly over 230 days given its past validation as a long-term storage preservative for fecal samples (Song et al., 2016).

5.2 Methods

Sample population and housing

Fecal samples were collected from a population of 10 southern white rhinoceros (9 females, 1 male) managed at the North Carolina Zoo in Asheboro, NC. These animals ranged in age from juvenile (n=2; J; <3.5 years old), subadult (n=2; S; 3.5 - 7 years old), adult (n=4 F and n=1 M; 8 - 40 years old), to geriatric (n=1; G; >40 years old). Age classes were assigned based on a modified version of the white rhinoceros age-class system previously published by Emslie et al. (1995). During the course of this study, NC Zoo rhinoceros were split into three subgroups for housing purposes: 1) four nonreproductive females (F1, F3, S1, S2); 2) two mother-calf pairs (F2, J1, F4, J2); 3) male individual (M1) and the geriatric female (G1). All animals were free from antibiotics, though G1 received phenylbutazone (bute) to treat arthritis symptoms.

Nonreproductive females and mother-calf pairs were housed in the rhinoceros barn, while M1 and G1 were housed separately in the rhino annex building. Groups in the rhinoceros barn were rotated between available stalls (37 m² stalls and a 52 m² maternity stall) and had access to outdoor sand and asphalt paddocks roughly 111 m² in size during temperate weather (>4.5°C). During winter sample collection, the nonreproductive female group had access to an outdoor paddock (1450 m²) when temperatures were above 2°C. When outdoor access was restricted, the group was randomly split into two pairs and rotated between combined stalls so that each individual was equally exposed to all group members and indoor environments. In the annex building, M1 and G1 were housed in separate 30 m² stalls and allowed access to 4,046 m² grass paddocks when temperatures rose above 2°C.

The NC Zoo possesses a 16-hectare mixed-species habitat called the Watani Grasslands. While rhinoceros are usually rotated onto the habitat during temperate weather, no rhinoceros were allowed access to the Grasslands during the warm weather sampling period due to a cyanobacterial algal bloom in the lake in the habitat. During the cold weather sampling period, non-reproductive females and mother-calf pairs (or a combination of both) were allowed onto the Grasslands habitat at temperatures at >0°C and >7°C, respectively. Rhinoceros G1 had bi-monthly access to the Watani Grasslands with the other groups, while M1 had no access to the Grasslands during the sampling period.

Diets

NC Zoo rhinoceros diets were standardized, but varied by season and age. Adult and subadult animals received 1.36 kgs of Mazuri® Wild Herbivore Diet Hi-Fiber (St. Louis, MO, USA) daily throughout the year, while juveniles received 0.68 kgs. During the winter, sub-adult

and adult rhinoceros received one bale of timothy hay (*Phleum pretense*) per animal. During the summer, outdoor grazing was accessible so adult and sub-adult rhinoceros were provided one quarter of a bale of timothy hay per animal. Supplementary feed in the form of timothy hay cubes, orchard grass (*Dactylis glomerate*), and alfalfa hay (*Medicago sativa*) were offered in rotation for training and enrichment, though these constituted less than 20% of the daily diet.

The diet of the single geriatric female rhinoceros (G1) was consistent throughout the year. Rhinoceros G1 received 5.44 kgs of Mazuri® Wild Herbivore Diet Hi-Fiber feed and one half of a timothy/orchard grass mixed bale each day. Rhinoceros G1 also had access to outdoor grazing areas during the summer and was similarly supplemented with timothy cubes and alfalfa hay for training and enrichment.

Sample collection and storage

Fecal samples were collected from each individual once per month from July through September in 2020 and January through March in 2021. The sampling dates remained consistent throughout, beginning on the 20th day of the month and extending until the end of that month. All sampling containers were pre-labeled with a unique identifier and collection date. A minimum of 2 grams of feces was collected from each individual within 30 minutes of defecation and aliquoted across three preservation methods: immediate freezing at -80°C in a Whirl-Pak® bag (Nasco, Fort Atkinson, WI, USA), 15 mL conical Falcon® tubes (Corning, New York, NY, USA) containing 95% ethanol, and PB tubes containing a proprietary preservative solution (Fig. 11). Samples preserved in 95% ethanol and PB tubes were stored at ambient temperature for a minimum of 14 days to simulate field conditions. A subset of frozen (n=6), PB (n=6), and 95% ethanol (n=6) samples from August 2020 were also stored for an additional 222 to 230 days

before extraction to test the longitudinal efficacy of the three preservation methods; these samples make up the “Day 230” time point data.

The PB tubes could not be used per manufacturer recommendations due to the grated secondary tube top and the physical nature of the rhinoceros fecal samples, which included long fibrous hay pieces; this feces created a mat at on the grate that could not be pushed into the preservative solution. The mat also hampered DNA extraction efforts, as there was a limited amount of preserved fecal material in solution from which to extract. As such, modifications were made whereby the grated tube top was removed during sampling and feces was manually pushed into solution using a sterile spatula, taking care that all fecal material was immersed.

DNA extraction

After 14 days, microbial DNA was extracted from the preserved fecal samples using the PowerFecal Pro DNA Kit (QIAGEN, Germantown, MD, USA) per manufacturer recommendations with the following modification: After samples were placed in the PowerBead Pro tubes and vortexed briefly to mix, they were subjected to bead beating at 4 m/s for 4 min using a FastPrep-24 bead beater (MP Biomedicals, Santa Ana, CA, USA) as opposed to being vortexed at maximum speed for 10 minutes; this bead beating speed has previously validated for use in DNA extraction for 16S rRNA V3-V4 region sequencing (Zhang et al., 2020). Extracted DNA was eluted in 15 to 100 µl of elution buffer (10 mM Tris); frozen and PB samples routinely returned high concentrations of DNA and were eluted in manufacturer recommended 100 µl of elution buffer, while samples that routinely returned minimal DNA concentrations were eluted with a minimum of 15 µl of elution buffer in order to increase concentrations for sequencing. Nucleic acid quantity and quality were measured with a NanoDrop 2000c spectrophotometer

(Thermo Fisher Scientific, Waltham, MA, USA), and DNA extractions were stored in elution tubes at -20°C until the end of the sampling season. Any samples continuously producing low DNA yields were extracted again in duplicate following manufacturer recommendations up until the final eluting step, when an additional modification was added: one replicate was eluted, then that eluate was pipetted onto the filter membrane containing DNA from the second replicate and centrifuged again, effectively doubling the final DNA concentration.

Prior to extraction, fecal samples immersed in solution were transferred to microcentrifuge tubes and centrifuged at 15,000 x g for 1 minute to concentrate the sample; the supernatant was discarded. While this was a successful technique for PB samples, 95% ethanol samples routinely returned insufficient concentrations of extracted DNA. Therefore, in March 2021 another technique was applied involving the evaporation of excess ethanol from the feces under a fume hood. DNA extractions from these samples yielded sufficient quantities of DNA for sequencing without additional processing.

Sequencing

Extracted DNA was sent to the Genomic Sciences Laboratory at NC State University for sequencing of the variable V3 and V4 regions of the 16S rRNA gene using established methods (PCR Amplicon, PCR Clean-up, and Index PCR, 2013). Primer pair sequences for the V3 and V4 region were obtained and created an amplicon approximately 460 base pairs (bp) in length; these sequences were selected from Klindworth et al. (2013) and are listed below:

16S Amplicon PCR Forward Primer = 5'

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

16S Amplicon PCR Reverse Primer = 5'

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

A limited cycle polymerase chain reaction was performed, and Illumina (San Diego, CA, USA) sequencing adapters and dual-index barcodes were added to the amplicon target. The V3-V4 region was sequenced on the Illumina MiSeq platform using paired 300-bp reads and MiSeq v3 reagents, with the ends of the reads overlapped to generate full-length reads.

Raw sequencing data were imported to the CLC Genomics Workbench v21.0.4 with Microbial Genomics Module plugin (QIAGEN, Germantown, MD, USA) for data quality control and operational taxonomic unit (OTU) clustering. FastQ files were joined via the CLC default Illumina platform parameters, and forward and reverse reads were paired. Reads were trimmed with a 0.05 quality limit and an ambiguous limit of 2. Read length thresholds were set between 15-1,000 nucleotides. SILVA 16S reference database (v132) was used to define OTUs based on a 97% taxonomic similarity cutoff. An OTU abundance table was generated for the 151 samples and reformatted for downstream analysis in RStudio (v1.3.1073) (Boston, MA, USA).

Bioinformatics and statistical analysis

All statistical analyses were performed using R (version 4.0.2) and RStudio (v1.3.1073). A total of 59,680,646 16S rRNA sequence reads were obtained from 151 samples, for an average of 395,236 ± 12,585 (mean ± SEM) reads per sample. Total abundance was 11,445,730 with 14,247 OTUs found. 211,797 chimeric reads (reads corresponding to two or more reference sequences) were identified and filtered out. All samples were rarefied to a level of 29,265 reads, with the loss of one sample for a remainder of 150 samples. The updated OTU abundance table

was used to calculate taxonomic relative abundance as well as alpha and beta diversity indices. Linear discrimination analysis Effect Size (LEfSe; https://huttenhower.sph.harvard.edu/galaxy/) was used to identify any OTUs that were significantly enriched per category (i.e., Day 14 and Day 230 preservation methods). Alpha diversity indices of richness (number of species present), Shannon diversity (the relative abundance of taxa, including both richness and evenness), and Simpson's index (constituting the richness, evenness, and phylogenetic relatedness of taxa) were measured. Kruskal-Wallis tests and pairwise Wilcoxon tests were used to assess significant differences in microbial alpha diversity across preservation methods.

To analyze microbial beta diversity, the relative abundance of each OTU was standardized using the Hellinger transformation, then Bray-Curtis dissimilarity was calculated to create distance matrices. Eigenvectors and eigenvalues were calculated from the distance matrices to create multidimensional scaling (MDS) plots, and PERMANOVA analysis was utilized to assess differences in community composition. P-values were adjusted using a False Discovery Rate (FDR) correction.

Figure 11. Overview of Fecal Storage and Preservation Methodology. Fecal samples from n=10 managed southern white rhinoceros (*Ceratotherium simum simum*) at the North Carolina Zoo were stored at -80°C and at ambient temperature in 95% ethanol and in proprietary PERFORMAbiome™•GUT (PB) tubes. Samples were stored for either 14 or 230 days.

5.3 Results

Taxonomic relative abundance

Firmicute*s* (average relative abundance 56%) was the dominant phyla across all preservation methods, followed by Bacteroidetes (20%), Spirochetes (9%), Fibrobacteres (8%), Kiritimatiellaeota (2%), Actinobacteria (1%), and Lentisphaerae (1%).

Phylum and genus level bar charts revealed apparent differences in community composition across different preservation methods and time points (Fig. 12). All Day 230 samples were enriched in Verrucomicrobia compared to Day 14 samples, and ethanol samples from both time points were enriched with Actinobacteria (Fig. 12). This increased abundance of Actinobacteria species in ethanol samples was confirmed using an LEfSe analysis (Fig. 13). Both time points were also enriched with members of the class Clostridia: Ethanol Day 14 samples had increased abundance of the Clostridia genus *Coprococcus*, while Ethanol Day 230 had increased abundance of the genera *Lachnoclostridium*, *Sarcina*, *Catenisphaer*a, *Cellulosilyticum*, and *Eubacterium.* Ethanol Day 230 samples were also specifically enriched for Bacillaceae, specifically of the genus *Bacillus*. Ethanol Day 230 samples also contained more members of the phylum Firmicutes and fewer Bacteroidetes and Spirochetes compared to other treatments and were more specifically enriched with members of the Nocardiaceae family and the *Enterorhabdus* and *Rhodococcus* genera.

Several other taxa were revealed to be differentially abundant across the different preservation methods via LEfSe analysis (Fig. 13). PB samples had the most differentially abundant taxa across both time points, with several Bacteroidetes and *Anaerovorax* species present. PB Day 230 samples were also enriched in several Clostridia taxa, including members of the families Ruminococcaceae and Lachnospiraceae as well as the genera *Papillibacter*,

Peptococcus, and *Ruminiclostridium*. Members of the Proteobacteria order Rhodospirillales were also enriched. In addition, there was more Methanomicrobia from the Archaea domain present in PB Day 230 samples than in any of the other preservation method. Frozen Day 14 samples had more Ruminococcaceae *UCG-010* present, while Frozen Day 230 samples were differentially abundant in the phyla Bacteroidetes, Firmicutes, Fibrobacteres, and Tenericutes. Mollicutes were especially abundant compared to other Tenericutes classes. Frozen Day 230 samples also had abundant levels of Peptococcaceae and *Anaeroplasma* when compared to other preservation methods and time points.

Alpha diversity

Kruskal-Wallis H tests revealed several significant differences in species richness, Shannon diversity, and Simpson diversity indices between the three preservation methods and two time points. Pairwise comparisons using the Wilcoxon rank sum test with continuity correction showed that of the species richness estimates, only Ethanol Day 14 samples were significantly different from Frozen Day 14 and PB Day 14 samples ($p=0.011$ for both). Wilcoxon comparisons detected similar patterns across Shannon and Simpson's diversity indices (Tables 6 & 7). Namely, Ethanol Day 14 samples differed significantly from Frozen Day 14 and PB Day 14 samples ($p<0.001$); and Ethanol Day 230 samples differed from all 5 other treatments and timepoints ($p<0.05$). Notably, observed bacterial community profiles remained consistent (i.e., statistically similar) between frozen and PB preservation methods across both time points (Fig. 14).

Beta diversity

Multidimensional scaling (MDS) plots revealed distinct clusters driven by the six preservation methods, though there was overlap among treatments (Fig. 15). Frozen and PB samples had the most similar bacterial community structure, though frozen samples from both time points yielded the least variable and most consistent clusters compared to all other methods. PB samples performed similarly to freezing, with the exception of three outlying values. Ethanol samples were highly variable, with large, indistinct clusters. Day 230 Ethanol samples shared overlap almost exclusively with Day 14 Ethanol samples and differed the most from the other treatments; for example, these samples shared no overlap with frozen samples at either time point or with Day 230 PB samples and showed minimal overlap with the Day 14 PB samples.

Pairwise comparisons of beta diversity using PERMANOVAs on the Bray-Curtis distance matrix verified several significant differences between preservation methods that were visible via MDS plot (Table 8). Bray-Curtis values in Day 14 Ethanol samples were different from all but Day 230 Frozen samples ($p<0.05$), and Day 230 Ethanol samples were different from all other preservation methods. Day 14 Frozen samples were different from all other non-frozen preservation methods. There were no differences in community composition between either Frozen or PB samples at either time point.

5.4 Discussion

Proper sample preservation for microbiome studies is of the utmost importance for producing accurate and reproducible results. Freezing at -80°C has long been touted as the gold standard method for sample preservation due to its ability to halt biological function without damage to genomic material. However, freezing at -80°C has logistical limitations, especially for

field research, whether it be *in situ* fieldwork abroad or sample collection at home (Song et al., 2016). One objective of this study was to determine whether 95% ethanol or the novel PB storage method is better suited for longitudinal storage of herbivore gut microbiome samples at ambient temperature, for periods of 14 days and 230 days. Our results indicate that PB provides preservation similar to freezing at -80°C, even when storage exceeds manufacturer recommendations by an order of magnitude (i.e., 230 days versus the 60-day recommended maximum).

The most abundant phyla detected across all preservation methods were Firmicutes (average relative abundance 56%), Bacteroidetes (20%), Spirochetes (9%), and Fibrobacteres (8%), which is in line with several other studies of Rhinocerotidae microbiome (Bian et al., 2013; Williams et al., 2019; Roth et al., 2019). Actinobacteria have also been found in rhinoceros before, though neither Kiritimatiellaeota (found here at 2% relative abundance) nor Lentisphaerae (1% relative abundance) have previously been noted in this host family. Both of the latter taxa have been previously identified in the *Equus* genus, however, and horses are the domestic animal model for rhinoceros digestion and nutrition (Bull et al., 2021; Gao et al., 2020). Kiritimatiellaeota has also been identified in other hind-gut fermenters including tapirs and geladas (Yanez-Montalvo et al., 2021; Baniel et al., 2021).

Samples preserved via each method had differentially enriched taxa including those subjected to immediate freezing at -80°C. Frozen samples do not escape changes in composition levels over time, and generally contain higher ratios of Bacteroidetes compared to non-frozen samples; this discrepancy is thought to arise due to alterations in cellular structure that gram-positive bacteria experience when frozen (Fouhy et al., 2015; Bahl & Licht, 2012). Our results contradict this trend: Day 230 Frozen samples did indeed have higher abundances of the gram-

negative phyla Bacteroidetes and Fibrobacteres; but they also contained an abundance of the majority gram-positive phylum Firmicutes and the unique phylum Tenericutes, which lack a cell wall entirely and whose members are thought to have developed from gram-positive bacteria through regressive evolution (Bové, 1993). All 4 phyla received LDA (log 10) scores above 3, which is hypothesized to be the lower limit for biological relevance (Saito et al., 2019).

The most promising outcome of this study was the performance of PB samples over time. PB manufacturer instructions state that samples have a shelf-life limited to 60 days at ambient temperatures between -20°C to 50°C (PB-200 Data Sheet) – a length of time that already made PB very promising for field studies. However, even after 230 days of storage, PB samples still yielded microbial profiles comparable to samples frozen at -80°C, as measured by both alpha and beta diversity indices. While there were significant differences in beta diversity between PB and frozen samples at Day 14, these differences became insignificant by day 230 of storage (Table 8).

Methanomicrobia (Archaea) were enriched in PB Day 230 samples. Archaea have been previously isolated in rhinoceros, and methanogens are thought to play a crucial role in fermentation of fibrous herbivorous diets common in rhinoceros and other hind-gut fermenters (Luo et al., 2013; Moissl-Eichinger et al., 2018). Archaea have proven difficult to cultivate in laboratories, so the development of molecular sequencing methods such as 16S rRNA sequencing has led to increased understanding of their role in microbiome ecology (Moissl-Eichinger et al., 2018). The storage solution within PB devices is proprietary and thus we cannot begin to speculate what specific preservative ingredients led to compositional differences over time, nor those differences which occur between PB and other preservation methods. However, PB is a derivative of the OMNIgene.GUT product (DNA Genotek), which was produced and

formulated to optimize home-based human fecal sample collection for gut microbial profile analysis. OMNIgene.GUT has been shown in previous studies to perform similarly to -80°C controls and technical replicates when compared against a variety of other preservation methods (Choo et al., 2015; Song et al., 2016).

The use of 95% ethanol as a preservative has been common for long term storage of many biological materials from months to years, though a previous microbiome study has only validated its usage for preserving community composition in human and dog fecal samples up to 56 days (Song et al., 2016). This longitudinal storage capability was limited based on our results, as the ethanol samples (n=6) stored for 230 days underwent significant decreases in species richness, Shannon diversity, and Simpson's diversity. Interestingly, ethanol samples stored for only 14 days were consistently significantly different from frozen at -80°C controls, as measured by both alpha and beta diversity indices. These differences were driven by differentially abundant taxa, specifically Actinobacteria, which was enriched at both time points. Actinobacteria have been previously shown to undergo ethanol-induced expansions in mice and are likely tolerant of high-alcohol environments (Bull-Otterson et al., 2013); thus, Actinobacteria may not be deleteriously affected by long term ethanol storage the way other microbial taxa would be. Actinobacteria are also resilient to another preservation method, fecal occult blood test (FOBT) cards, which utilize alpha guaiaconic acid paper (Moossavi et al., 2019). A separate issue with the use of 95% ethanol as a fecal preservation method was the consistently low DNA yields from DNA extraction, a challenge which has been documented widely in the literature (e.g., Vlčková et al., 2012; Hale et al., 2015; Song et al., 2016). This setback required additional time and funds to extract samples in duplicate, whereas frozen and PB samples consistently yielded ample, high-quality DNA.

Limitations of this study include a small sample size, especially for Day 230 samples which were a subset population (n=18). In addition, there were modifications in sample processing for ethanol samples at the start versus the end of the DNA extraction period, though early statistical comparisons revealed significant differences between 95% ethanol and other Day 14 preservation methods before these modifications were made. The volume of fecal material was not standardized across the different preservation methods due to sample collection constraints, so we cannot unequivocally state that DNA yields for one method differed significantly from another (although this trend was apparent in practice). Lastly, we realize there may be variation stemming from different sequencing run dates for samples collected in July through September of 2020 and those collected in January through March of 2021; by using the same laboratory, procedure, and technicians to process these samples, we kept that variation as limited as possible. Future studies should further evaluate the performance of PB preservation methods across different parameters including the upper limit of physical material that can be preserved using the proprietary solution without overwhelming it.

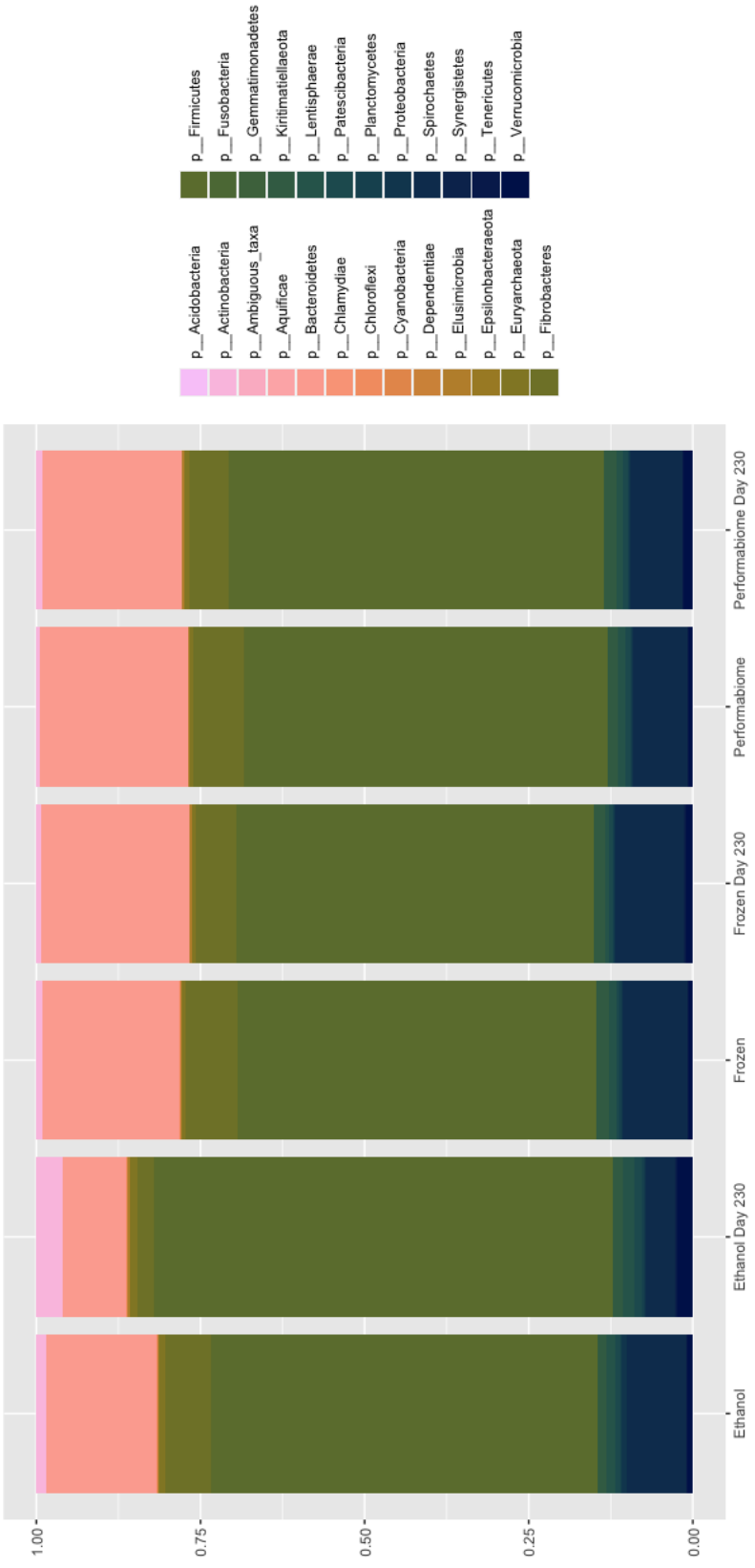
5.5 Conclusions

Of the preservation methods utilized in this study, PB tubes performed most similarly to those frozen immediately at -80°C. Observed bacterial community profiles remained consistent (i.e., statistically similar) between frozen and PB preservation methods across both time points. This study also provides the first evaluation of 95% ethanol and PB preservation performance over 230 days. PB devices showed a remarkable consistency in sample stability and preservation of microbial community composition over time at ambient temperatures, even beyond that of the manufacturer storage period recommendations. PB tubes also yielded microbial community

profiles similar to those observed for the gold standard of freezing at -80°C, with only limited differences in beta diversity. The usage of 95% ethanol, previously validated for use in microbiome studies, did not hold up during short (14 days) nor long term storage (230 days) at ambient temperature. Our results indicate that ethanol may not provide optimal storage for fecal samples from herbivorous species with complex gastrointestinal tracts, as most studies evaluating the efficacy of ethanol as a microbiome preservation method used either dog or human host subjects. By contrast, PB devices provide a viable solution to the challenges associated with microbiome fieldwork, though they may be cost prohibitive compared to previously published methods.

Figure 12. (A) Phylum- and (B) genus-level bar charts representing microbial community composition across fecal samples from n=10 North Carolina Zoo southern white rhinoceros (*Ceratotherium simum simum*) preserved via freezing at -80°C, 95% ethanol, and PERFORMAbiome™•GUT (PB) at Day 14 and Day 23

A)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p__Euryarchaeota
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p__Firmicutes
p__Fusobacteria
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p__Kiritimatiellaeota
p__Lentisphaerae
p__Patescibacteria
p__Planctomycetes
p__Proteobacteria
p__Spirochaetes
p__Synergistetes
p__Tenericutes
p__Verrucomicrobia
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0.00
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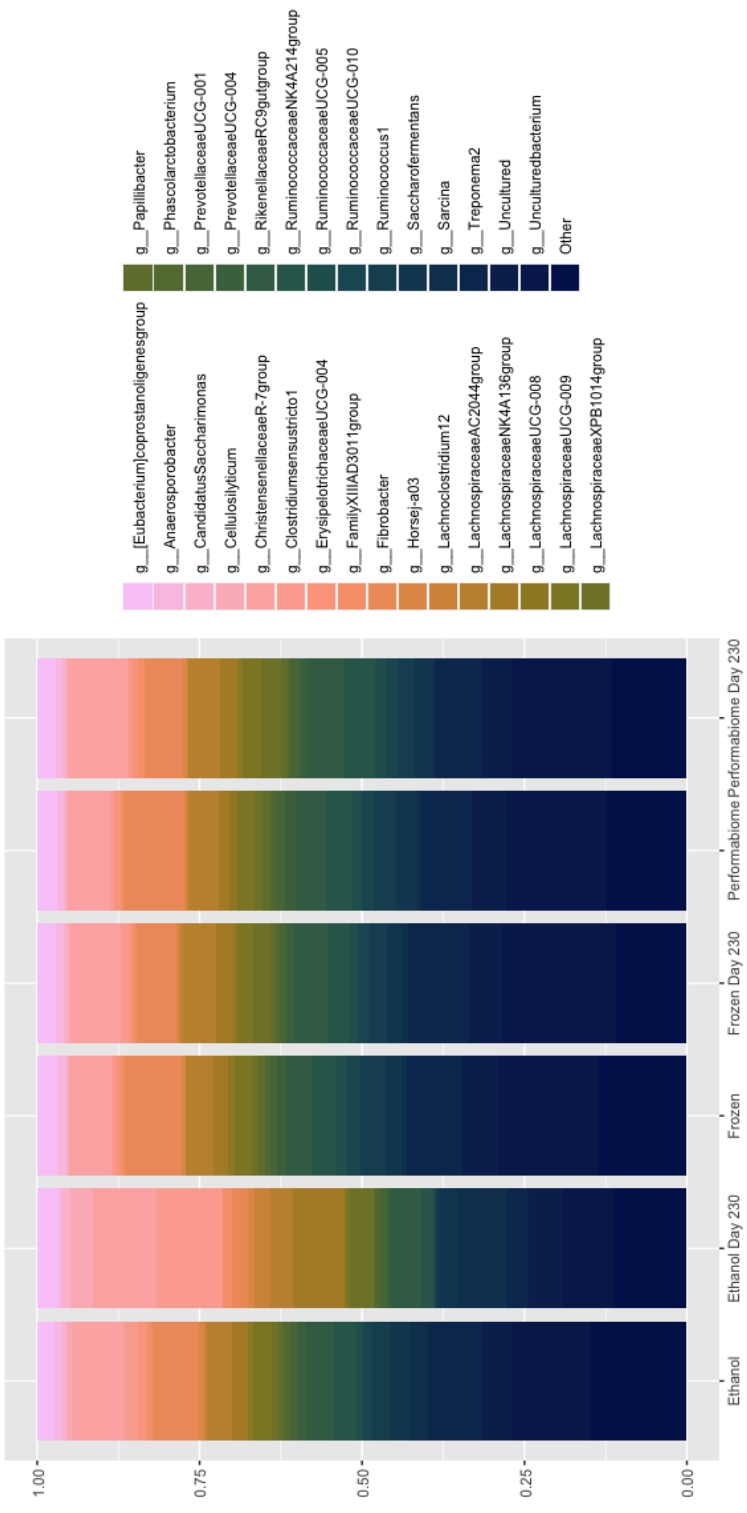
B)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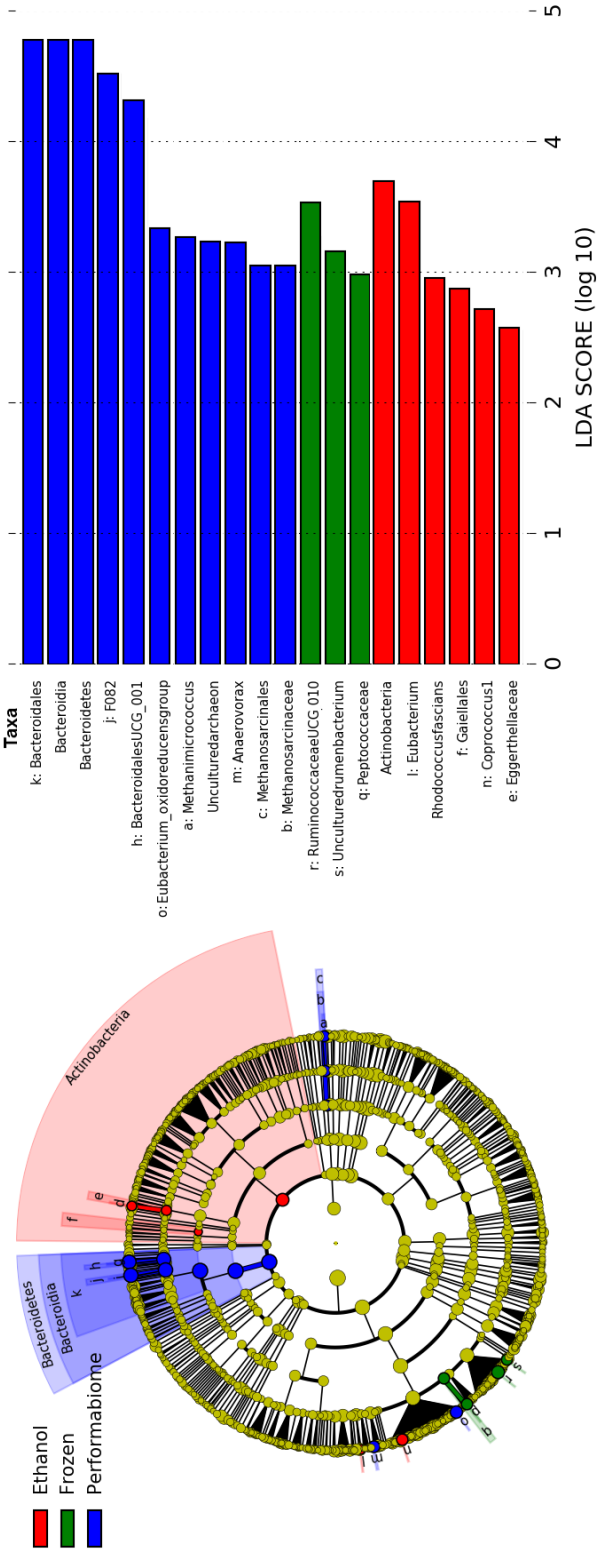
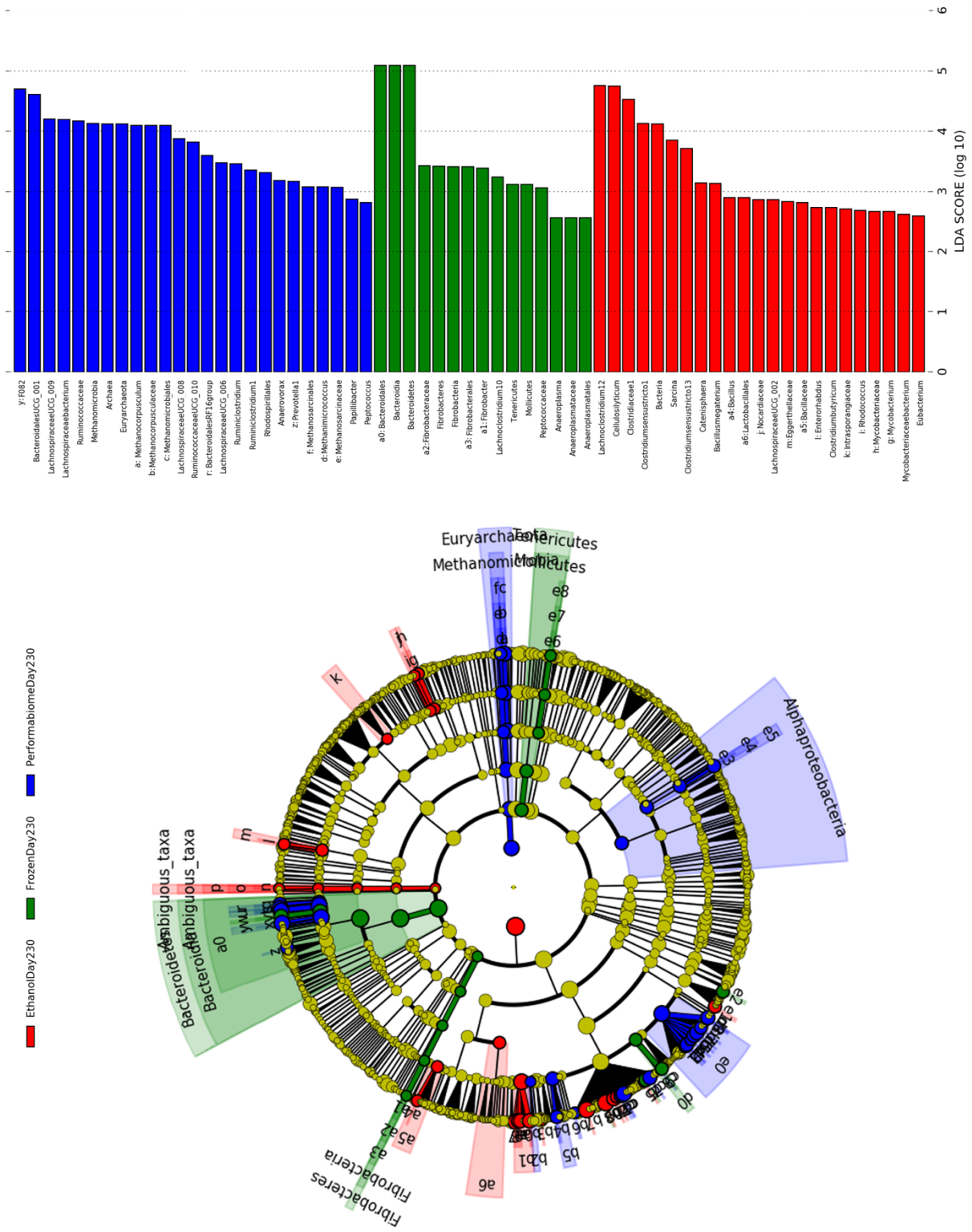
g__LachnospiraceaeUCG-008
g__LachnospiraceaeUCG-009
g__LachnospiraceaeXPB1014group
g__Papillibacter
g__Phascolarctobacterium
g__PrevotellaceaeUCG-001
g__PrevotellaceaeUCG-004
g__RikenellaceaeRC9gutgroup
g__RuminococcaceaeNK4A214group
g__RuminococcaceaeUCG-005
g__RuminococcaceaeUCG-010
g__Ruminococcus1
g__Saccharofermentans
g__Sarcina
g__Treponema2
g__Uncultured
g__Unculturedbacterium
Other
1.00
0.75
0.50
0.25
0.00
Ethanol
Ethanol Day 230
Frozen
Frozen Day 230
Performabiome
Performabiome Day 230

Figure 13. Linear discriminant analysis Effect Size (LEfSe) cladogram and plot of LDA scores comparing significantly and differentially enriched major taxa (i.e., present at >1% relative abundance) in fecal samples from n=10 North Carolina Zoo southern white rhinoceros (*Ceratotherium simum simum*) preserved via freezing at -80°C, 95% ethanol, or PERFORMAbiome™•GUT (PB) at (A) Day 14 and (B) Day 230. An LDA score of 3 or higher is considered biologically relevant

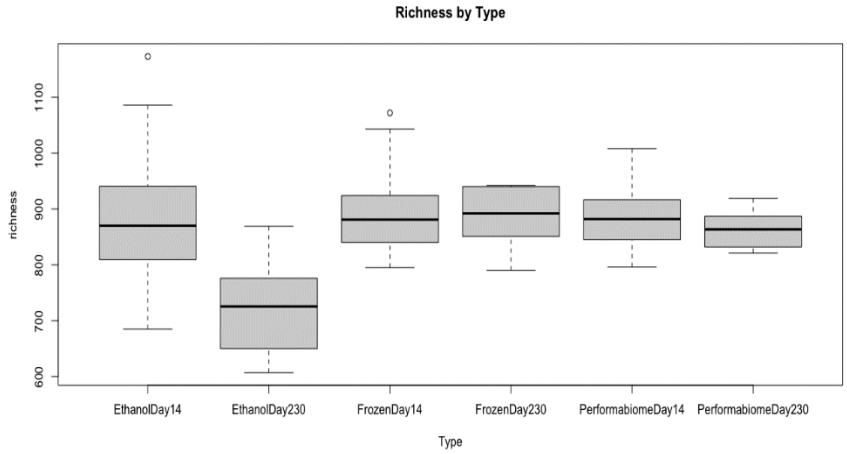
A)


Taxa
k: Bacteroidales
Bacteroidia
Bacteroidetes
j: F082
h: BacteroidalesUCG_001
o:Eubacterium_oxidoreducensgroup
a: Methanimicrococcus
Unculturedarchaeon
m: Anaerovorax
c: Methanosarcinales
b: Methanosarcinaceae
r: RuminococcaceaeUCG_010
s: Unculturedrumenbacterium
q: Peptococcaceae
Actinobacteria
l: Eubacterium
Rhodococcusfascians
f: Gaiellales
n: Coprococcus1
e: Eggerthellaceae
0
1
2
3
4
5
LDA SCORE (log 10)
Actinobacteria
Bacteroidetes
Bacteroidia
Ethanol
Frozen
Performabiome

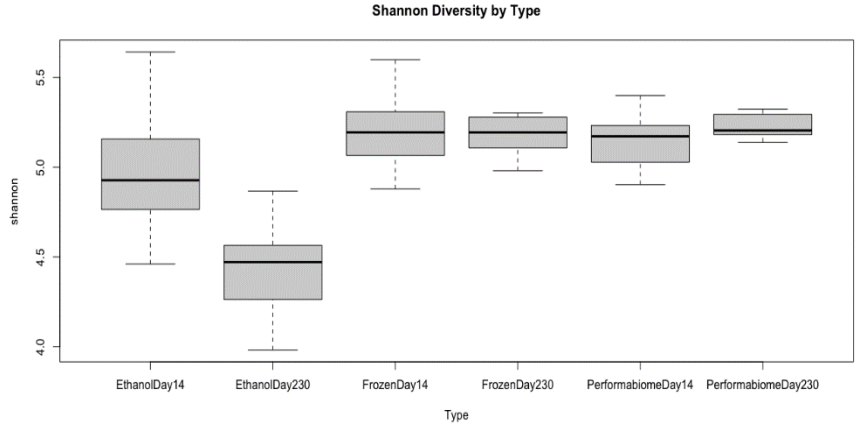
B)


LDA SCORE (log 10)
0
1
2
3
4
5
6
y:F082
BacteroidalesUCG_001
Lachnospiraceae UCG_009
Lachnospiraceaebacterium
Ruminococcaceae
Methanomicrobia
Archaea
Euryarchaeota
a: Methanocorpusculum
b:Methanocorpusculaceae
c: Methanomicrobiales
Lachnospiraceae UCG 008
Ruminococcaceae UCG_010
r: BacteroidalesRF16group
Lachnospiraceae UCG_006
Ruminiclostridium
Ruminiclostridium1
Rhodospirillales
Anaerovorax
z: Prevotella1
f: Methanosarcinales
d: Methanimicrococcus
e: Methanosarcinaceae
Papillibacter
Peptococcus
a0: Bacteroidales
Bacteroidia
Bacteroidetes
a2: Fibrobacteraceae
Fibrobacteres
Fibrobacteria
a3: Fibrobacterales
a1: Fibrobacter
Lachnoclostridium10
Tenericutes
Mollicutes
Peptococcaceae
Anaeroplasma
Anaeroplasmataceae
Anaeroplasmatales
Lachnoclostridium12
Cellulosilyticum
Clostridiaceae1
Clostridiumsensustricto1
Bacteria
Sarcina
Clostridiumsensustricto13
Catenisphaera
Bacillusmegaterium
a4: Bacillus
a6: Lactobacillales
j: Nocardiaceae
Lachnospiraceae UCG_002
m: Eggerthellaceae
a5: Bacillaceae
l: Enterorhabdus
Clostridiumbutyricum
k: Intrasporangiaceae
i: Rhodococcus
h: Mycobacteriaceae
g: Mycobacterium
Mycobacteriaceaebacterium
Eubacterium
EthanolDay230
FrozenDay230
PerformabiomeDay230
Euryarchaeota
Tenericutes
Methanomicrobia
Mollicutes
Alphaproteobacteria
Ambiguous_taxa
Ambiguous_taxa
Fibrobacteres
Fibrobacteria

A)



B)



C)

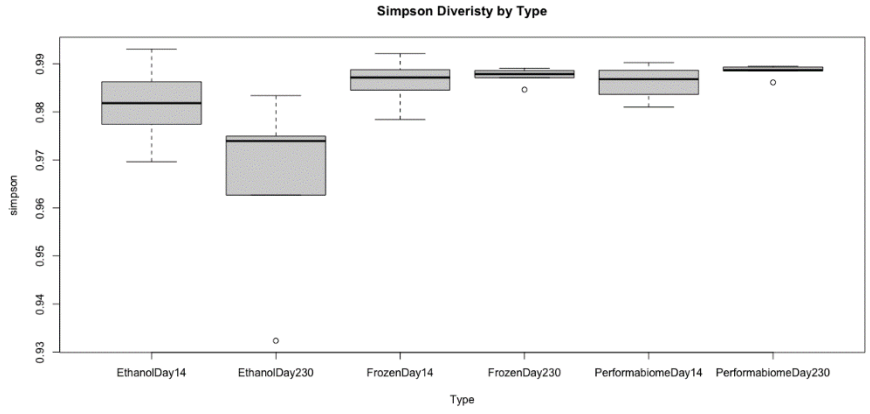
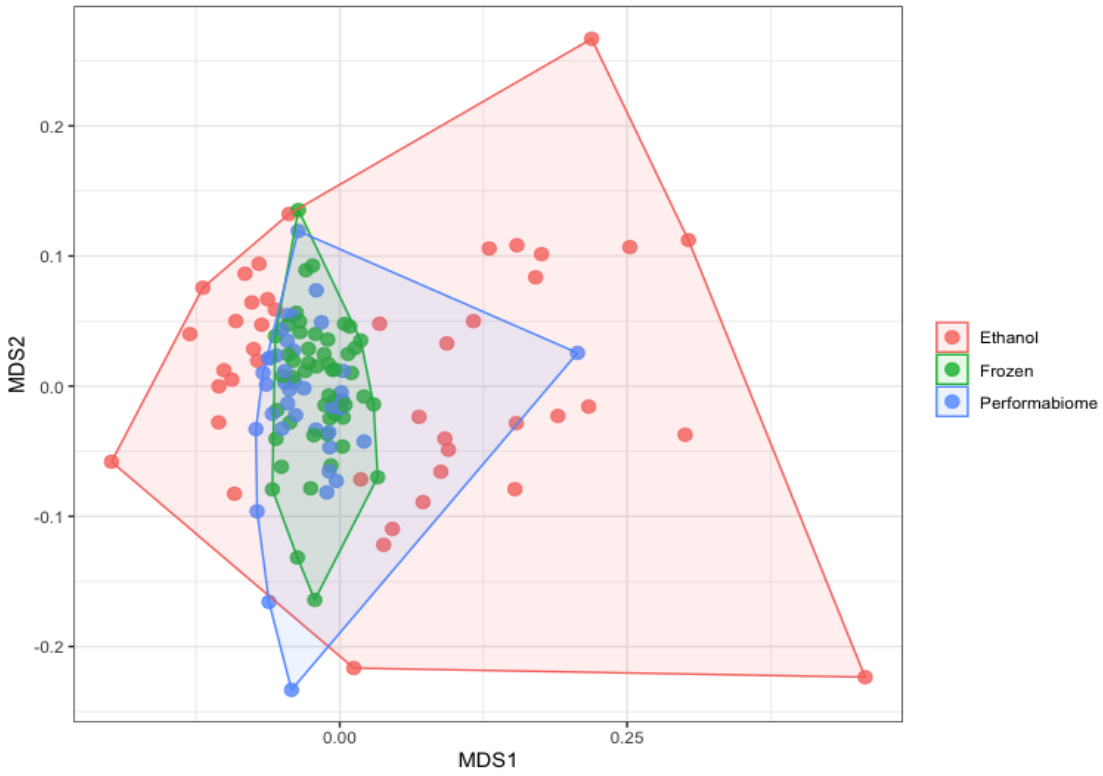


Figure 14. Boxplots comparing alpha diversity as measured by (A) species richness, (B) Shannon diversity, and (C) Simpson's diversity indices across freezing at -80°C, ethanol, and PERFORMAbiome™•GUT (PB) preservation methods at Day 14 and Day 230 for n=10 North Carolina Zoo southern white rhinoceros (*Ceratotherium simum simum*). Error bars represent SEM.

A)



B)

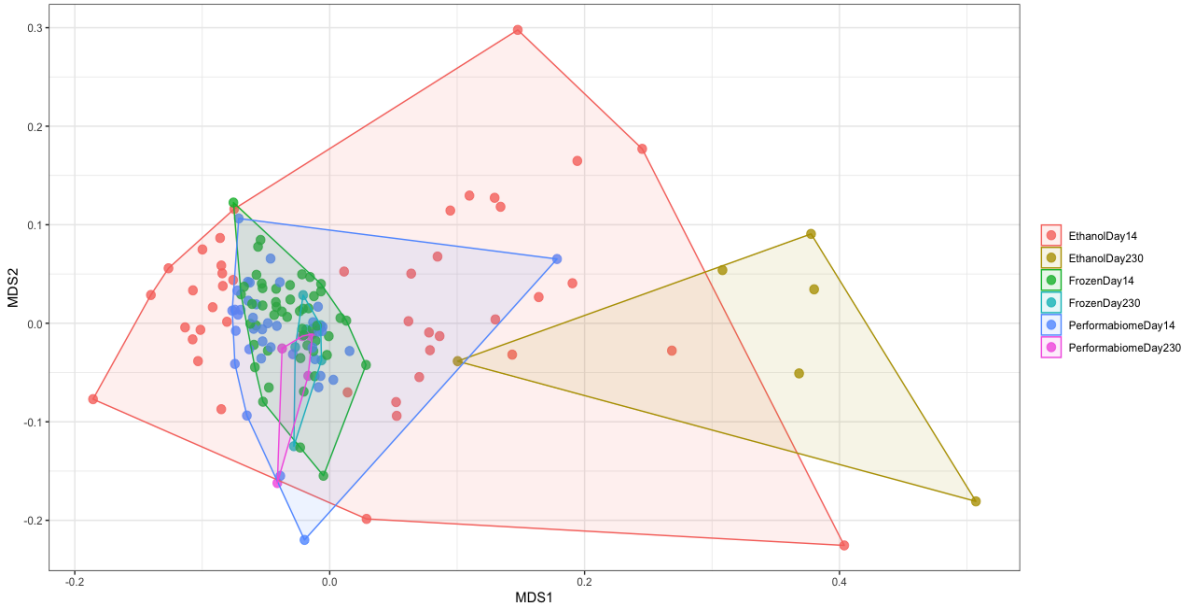


Figure 15. Multidimensional Scaling (MDS) Plots comparing (A) freezing at -80°C, ethanol, and PERFORMAbiome™•GUT (PB) preservation methods at Day 14 and (B) Day 14 versus Day 230 across all three preservation methods for n=10 North Carolina Zoo southern white rhinoceros (*Ceratotherium simum simum*). Each point represents one fecal sample from one of n=10 southern white rhinoceros, preserved using one of six preservation methods.

Table 6. Pairwise comparisons of Shannon Diversity in fecal samples from n=10 North Carolina Zoo southern white rhinoceros (*Ceratotherium simum simum*) stored via freezing at -80°C, 95% ethanol, and PERFORMAbiome™•GUT (PB) at Day 14 and Day 230 using Wilcoxon rank sum exact test.

	Frozen Day 14	Ethanol Day 14	PB Day 14	Frozen Day 230	Ethanol Day 230	PB Day 230
Ethanol Day 14	0.0003***	-	-	-	-	-
PB Day 14	1	0.0010***	-	-	-	-
Frozen Day 230	1	0.2759	1	-	-	-
Ethanol Day 230	0.0011**	0.0026**	3.7e-06***	0.0324*	-	-
PB Day 230	1	0.0986	1	1	0.0325*	-

Significance codes: '***' 0.001 '**' 0.01 '*' 0.05

Table 7. Pairwise comparisons of Simpson's Diversity in fecal samples from n=10 North Carolina Zoo southern white rhinoceros (*Ceratotherium simum simum*) stored via freezing at -80°C, 95% ethanol, and PERFORMAbiome™•GUT (PB) at Day 14 and Day 230 using Wilcoxon rank sum exact test.

	Frozen Day 14	Ethanol Day 14	PB Day 14	Frozen Day 230	Ethanol Day 230	PB Day 230
Ethanol Day 14	7.2e-05***	-	-	-	-	-
PB Day 14	1	0.0005***	-	-	-	-
Frozen Day 230	1	0.0696	1	-	-	-
Ethanol Day 230	0.0027**	0.0481*	0.0003***	0.0325*	-	-
PB Day 230	1	0.0183*	0.6182	1	0.0325*	-

Significance codes: '***' 0.001 '**' 0.01 '*' 0.05

Table 8. PERMANOVA statistical comparisons of Bray-Curtis dissimilarity in microbial community composition across fecal samples from n=10 North Carolina Zoo southern white rhinoceros (*Ceratotherium simum simum)* stored via freezing at -80°C, 95% ethanol, and PERFORMAbiome™•GUT (PB) for 14 and 230 days.

	Frozen Day 14	Ethanol Day 14	PB Day 14	Frozen Day 230	Ethanol Day 230
Ethanol Day 14	0.003**	-	-	-	-
PB Day 14	0.003**	0.003**	-	-	-
Frozen Day 230	0.1875	0.0941	0.0958	-	-
Ethanol Day 230	0.003**	0.003**	0.003**	0.0094**	-
PB Day 230	0.0167*	0.024*	0.0958	0.579	0.0064**

Significance codes: '***' 0.001 '**' 0.01 '*' 0.05

REFERENCES

Adriansjach, J., Baum, S. T., Lefkowitz, E. J., Van Der Pol, W. J., Buford, T. W., & Colman, R. J. (2020). Age-related differences in the gut microbiome of rhesus macaques. *The Journals of Gerontology: Series A*, *75*(7), 1293–1298.

Bahl, M. I., Bergström, A., & Licht, T. R. (2012). Freezing fecal samples prior to DNA extraction affects the Firmicutes to Bacteroidetes ratio determined by downstream quantitative PCR analysis. *FEMS Microbiology Letters*, *329*(2), 193–197.

Baniel, A., Amato, K. R., Beehner, J. C., Bergman, T. J., Mercer, A., Perlman, R. F., Petrullo, L., Reitsema, L., Sams, S., Lu, A., & Snyder-Mackler, N. (2021). Seasonal shifts in the gut microbiome indicate plastic responses to diet in wild geladas. *Microbiome*, *9*(1), 26.

Bové, J. M. (1993). Molecular features of mollicutes. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *17 Suppl 1*, S10-31.

Bull, K., Davies, G., Jenkins, T. P., & Peachey, L. E. (2021). The equine faecal microbiota undergoes step-wise compositional changes in accordance with increasing levels of domestication [Preprint]. In Review.

Bull-Otterson, L., Feng, W., Kirpich, I., Wang, Y., Qin, X., Liu, Y., Gobejishvili, L., Joshi-Barve, S., Ayvaz, T., Petrosino, J., Kong, M., Barker, D., McClain, C., & Barve, S. (2013). Metagenomic Analyses of Alcohol Induced Pathogenic Alterations in the Intestinal Microbiome and the Effect of Lactobacillus rhamnosus GG Treatment. *PLOS ONE*, *8*(1), e53028.

Cardona, S., Eck, A., Cassellas, M., Gallart, M., Alastrue, C., Dore, J., Azpiroz, F., Roca, J., Guarner, F., & Manichanh, C. (2012). Storage conditions of intestinal microbiota matter in metagenomic analysis. *BMC Microbiology*, *12*(1), 158.

Carroll, I. M., Ringel-Kulka, T., Siddle, J. P., Klaenhammer, T. R., & Ringel, Y. (2012). Characterization of the fecal microbiota using high-throughput sequencing reveals a stable microbial community during storage. *PLOS ONE*, *7*(10), e46953.

Carthey, A. J. R., Blumstein, D. T., Gallagher, R. V., Tetu, S. G., & Gillings, M. R. (2020). Conserving the holobiont. *Functional Ecology*, *34*(4), 764–776.

Choo, J. M., Leong, L. E., & Rogers, G. B. (2015). Sample storage conditions significantly influence faecal microbiome profiles. *Scientific Reports*, *5*(1), 16350.

Dangerous Goods Regulations (IATA--Resolution 618 Attachment “A”): Effective 1 January-31 December 2022. (2021). *International Air Transport Association*.

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., Ling, A. V., Devlin, A. S., Varma, Y., Fischbach, M. A., Biddinger, S. B., Dutton, R. J., & Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature*, *505*(7484), 559–563.

Emslie, R. (2020). *Ceratotherium simum. The IUCN Red List of Threatened Species 2020*.

Emslie, R., Adcock, K., & Hansen, H. (1995). Fine tuning rhino management group age class system. *Rhino Management Group*, 1–17.

Flint, H. J., Scott, K. P., Louis, P., & Duncan, S. H. (2012). The role of the gut microbiota in nutrition and health. *Nature Reviews Gastroenterology & Hepatology*, *9*(10), 577–589.

Fouhy, F., Deane, J., Rea, M. C., O'Sullivan, Ó., Ross, R. P., O'Callaghan, G., Plant, B. J., & Stanton, C. (2015). The effects of freezing on faecal microbiota as determined using MiSeq sequencing and culture-based investigations. *PLOS ONE*, *10*(3), e0119355.

Gao, C. (2020). Metabolic characterization of induced pluripotent stem cells from the critically endangered northern white rhinoceros. http://dspace.calstate.edu/handle/10211.3/217172

Geuking, M. B., Cahenzli, J., Lawson, M. A. E., Ng, D. C. K., Slack, E., Hapfelmeier, S., McCoy, K. D., & Macpherson, A. J. (2011). Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity*, *34*(5), 794–806.

Gillman, S. J., McKenney, E. A., & Lafferty, D. J. R. (2020). Wild black bears harbor simple gut microbial communities with little difference between the jejunum and colon. *Scientific Reports*, *10*(1), 20779.

Greene, L. K., Williams, C. V., Junge, R. E., Mahefarisoa, K. L., Rajaonarivelo, T., Rakotondrainibe, H., O'Connell, T. M., & Drea, C. M. (2020). A role for gut microbiota in host niche differentiation. *The ISME Journal*, *14*(7), 1675–1687.

Hale, V. L., Tan, C. L., Knight, R., & Amato, K. R. (2015). Effect of preservation method on spider monkey (*Ateles geoffroyi*) fecal microbiota over 8weeks. *Journal of Microbiological Methods*, *113*, 16–26.

Janiak, M. C., Montague, M. J., Villamil, C. I., Stock, M. K., Trujillo, A. E., DePasquale, A. N., Orkin, J. D., Bauman Surratt, S. E., Gonzalez, O., Platt, M. L., Martínez, M. I., Antón, S. C., Dominguez-Bello, M. G., Melin, A. D., & Higham, J. P. (2021). Age and sex-associated variation in the multi-site microbiome of an entire social group of free-ranging rhesus macaques. *Microbiome*, *9*(1), 68.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, *41*(1), e1.

LeBlanc, J. G., Milani, C., de Giori, G. S., Sesma, F., van Sinderen, D., & Ventura, M. (2013). Bacteria as vitamin suppliers to their host: A gut microbiota perspective. *Current Opinion in Biotechnology*, *24*(2), 160–168.

Lewis, J. D., Chen, E. Z., Baldassano, R. N., Otley, A. R., Griffiths, A. M., Lee, D., Bittinger, K., Bailey, A., Friedman, E. S., Hoffmann, C., Albenberg, L., Sinha, R., Compher, C., Gilroy, E., Nessel, L., Grant, A., Chehoud, C., Li, H., Wu, G. D., & Bushman, F. D. (2015). Inflammation, Antibiotics, and Diet as Environmental Stressors of the Gut Microbiome in Pediatric Crohn's Disease. *Cell Host & Microbe*, *18*(4), 489–500.

Lin, C.-Y., Cross, T.-W. L., Doukhanine, E., & Swanson, K. S. (2020). An ambient temperature collection and stabilization strategy for canine microbiota studies. *Scientific Reports*, *10*(1), 13383.

Luo, G. J., Kiese, R., Wolf, B., & Butterbach-Bahl, K. (2013). Effects of soil temperature and moisture on methane uptake and nitrous oxide emissions across three different ecosystem types. *Biogeosciences*, *10*(5), 3205–3219.

Lupp, C., Robertson, M. L., Wickham, M. E., Sekirov, I., Champion, O. L., Gaynor, E. C., & Finlay, B. B. (2007). Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell Host & Microbe*, *2*(2), 119–129.

Markle, J. G. M., Frank, D. N., Mortin-Toth, S., Robertson, C. E., Feazel, L. M., Rolle-Kampczyk, U., von Bergen, M., McCoy, K. D., Macpherson, A. J., & Danska, J. S. (2013). Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science*, *339*(6123), 1084–1088.

McKenney, E. A., Maslanka, M., Rodrigo, A., & Yoder, A. D. (2018). Bamboo specialists from two mammalian orders (Primates, Carnivora) share a high number of low-abundance gut microbes. *Microbial Ecology*, *76*(1), 272–284.

McKenney, E. A., Rodrigo, A., & Yoder, A. D. (2015). Patterns of gut bacterial colonization in three primate species. *PLOS ONE*, *10*(5), e0124618.

McKenzie, V. J., Song, S. J., Delsuc, F., Prest, T. L., Oliverio, A. M., Korpita, T. M., Alexiev, A., Amato, K. R., Metcalf, J. L., Kowalewski, M., Avenant, N. L., Link, A., Di Fiore, A., Seguin-Orlando, A., Feh, C., Orlando, L., Mendelson, J. R., Sanders, J., & Knight, R. (2017). The effects of captivity on the mammalian gut microbiome. *Integrative and Comparative Biology*, *57*(4), 690–704.

Mead, J. G. (2009). Gastrointestinal tract. In W. F. Perrin, B. Würsig, & J. G. M. Thewissen (Eds.), *Encyclopedia of Marine Mammals (Second Edition)* (pp. 472–477). Academic Press.

Moissl-Eichinger, C., Pausan, M., Taffner, J., Berg, G., Bang, C., & Schmitz, R. A. (2018). Archaea are interactive components of complex microbiomes. *Trends in Microbiology*, *26*(1), 70–85.

Moossavi, S., Engen, P. A., Ghanbari, R., Green, S. J., Naqib, A., Bishehsari, F., Merat, S., Poustchi, H., Keshavarzian, A., & Malekzadeh, R. (2019). Assessment of the impact of different fecal storage protocols on the microbiota diversity and composition: A pilot study. *BMC Microbiology*, *19*(1), 145.

Mshelia, E. S., Adamu, L., Wakil, Y., Turaki, U. A., Gulani, I. A., & Musa, J. (2018). The association between gut microbiome, sex, age and body condition scores of horses in Maiduguri and its environs. *Microbial Pathogenesis*, *118*, 81–86.

Muegge, B. D., Kuczynski, J., Knights, D., Clemente, J. C., González, A., Fontana, L., Henrissat, B., Knight, R., & Gordon, J. I. (2011). Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science*, *332*(6032), 970–974.

PB-200 Data Sheet. (2018). PD-BR-00267. *DNA Genotek*.

PCR Amplicon, PCR Clean-up, and Index PCR. (2013). 16S metagenomic sequencing library preparation. *Illumina*.

Saito, K., Koido, S., Odamaki, T., Kajihara, M., Kato, K., Horiuchi, S., Adachi, S., Arakawa, H., Yoshida, S., Akasu, T., Ito, Z., Uchiyama, K., Saruta, M., Xiao, J., Sato, N., & Ohkusa, T. (2019). Metagenomic analyses of the gut microbiota associated with colorectal adenoma. *PLOS ONE*, *14*(2), e0212406.

Schippa, S., Iebba, V., Totino, V., Santangelo, F., Lepanto, M., Alessandri, C., Nuti, F., Viola, F., Di Nardo, G., Cucchiara, S., Longhi, C., & Conte, M. P. (2012). A potential role of *Escherichia coli* pathobionts in the pathogenesis of pediatric inflammatory bowel disease. *Canadian Journal of Microbiology*, *58*(4), 426–432.

Smith, H. F., Fisher, R. E., Everett, M. L., Thomas, A. D., Randal Bollinger, R., & Parker, W. (2009). Comparative anatomy and phylogenetic distribution of the mammalian cecal appendix. *Journal of Evolutionary Biology*, *22*(10), 1984–1999.

Song, S. J., Amir, A., Metcalf, J. L., Amato, K. R., Xu, Z. Z., Humphrey, G., & Knight, R. (2016). Preservation methods differ in fecal microbiome stability, affecting suitability for field studies. *MSystems*, *1*(3), e00021-16.

Steinmetz, K., Murphy, S., Ó Cadhla, O., Barnett, J., & Mirimin, L. (2021). Enabling pinniped conservation by means of non-invasive genetic population analysis. *Conservation Genetics Resources*, *13*(2), 131–142.

Swaisgood, R. R., Dickman, D. M., & White, A. M. (2006). A captive population in crisis: Testing hypotheses for reproductive failure in captive-born southern white rhinoceros females. *Biological Conservation*, *129*(4), 468–476.

Tedjo, D. I., Jonkers, D. M. A. E., Savelkoul, P. H., Masclee, A. A., Best, N. van, Pierik, M. J., & Penders, J. (2015). The effect of sampling and storage on the fecal microbiota composition in healthy and diseased subjects. *PLOS ONE*, *10*(5), e0126685.

Tubbs, C. W., Moley, L. A., Ivy, J. A., Metrione, L. C., LaClaire, S., Felton, R. G., Durrant, B. S., & Milnes, M. R. (2016). Estrogenicity of captive southern white rhinoceros diets and their association with fertility. *General and Comparative Endocrinology*, *238*, 32–38.

Vlčková, K., Mrázek, J., Kopečný, J., & Petrželková, K. J. (2012). Evaluation of different storage methods to characterize the fecal bacterial communities of captive western lowland gorillas (*Gorilla gorilla gorilla*). *Journal of Microbiological Methods*, *91*(1), 45–51.

Williams, C. L., Ybarra, A. R., Meredith, A. N., Durrant, B. S., & Tubbs, C. W. (2019). Gut microbiota and phytoestrogen-associated infertility in southern white rhinoceros. *MBio*, *10*(2).

Winter, S. E., Winter, M. G., Xavier, M. N., Thiennimitr, P., Poon, V., Keestra, A. M., Laughlin, R. C., Gomez, G., Wu, J., Lawhon, S. D., Popova, I. E., Parikh, S. J., Adams, L. G., Tsolis, R. M., Stewart, V. J., & Bäumler, A. J. (2013). Host-derived nitrate boosts growth of *E. coli* in the inflamed gut. *Science*, *339*(6120), 708–711.

Wu, W.-K., Chen, C.-C., Panyod, S., Chen, R.-A., Wu, M.-S., Sheen, L.-Y., & Chang, S.-C. (2019). Optimization of fecal sample processing for microbiome study—The journey from bathroom to bench. *Journal of the Formosan Medical Association*, *118*(2), 545–555.

Yanez-Montalvo, A., Gaona, O., Águila, B., Arias-Domínguez, N., Falcón, L. I., & Pérez-Flores, J. (2021). *Tapirus bairdii*-Associated fecal microbiome from a critical conservation area: Calakmul, México. *Current Microbiology*, *78*(7), 2648–2659.

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R. N., Anokhin, A. P., Heath, A. C., Warner, B., Reeder, J., Kuczynski, J., Caporaso, J. G., Lozupone, C. A., Lauber, C., Clemente, J. C., Knights, D., … Gordon, J. I. (2012). Human gut microbiome viewed across age and geography. *Nature*, *486*(7402), 222–227.

Zhang, B., Brock, M., Arana, C., Dende, C., Hooper, L., & Raj, P. (2020). Impact of bead-beating intensity on microbiome recovery in mouse and human stool: Optimization of DNA extraction. *bioRxiv*, 1-23

APPENDIX

Table S1. Taxonomic abundance of phyla across individuals

Phylum	F1	F2	F3	F4	G1	J1	J2	M1	S1	S2	Average Abundance
Acidobacteria	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Actinobacteria	0.009	0.007	0.013	0.008	0.007	0.009	0.009	0.008	0.010	0.009	0.009
Ambiguous taxa	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Bacteroidetes	0.206	0.227	0.191	0.213	0.205	0.212	0.203	0.208	0.222	0.210	**0.210***
Chloroflexi	0.005	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.001
Cyanobacteria	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000
Elusimicrobia	0.003	0.002	0.002	0.003	0.004	0.002	0.001	0.001	0.002	0.002	0.002
Epsilonbacteraeota	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Euryarchaeota	0.004	0.006	0.006	0.004	0.011	0.006	0.005	0.005	0.006	0.006	0.006
Fibrobacteres	0.100	0.077	0.096	0.069	0.035	0.104	0.112	0.079	0.053	0.078	**0.080***
Firmicutes	0.523	0.525	0.541	0.538	0.617	0.519	0.529	0.551	0.558	0.549	**0.545***
Fusobacteria	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Kiritimatiellaeota	0.018	0.017	0.011	0.022	0.013	0.023	0.020	0.026	0.028	0.022	**0.020***
Lentisphaerae	0.013	0.012	0.011	0.008	0.012	0.016	0.012	0.012	0.010	0.011	**0.012***
Patescibacteria	0.005	0.004	0.006	0.005	0.005	0.003	0.002	0.005	0.003	0.003	0.004

Table S1 (Continued).

Planctomycetes	0.002	0.004	0.003	0.004	0.002	0.002	0.005	0.005	0.005	0.005	0.004
Proteobacteria	0.003	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.002	0.002
Spirochaetes	0.106	0.109	0.110	0.117	0.056	0.096	0.097	0.094	0.094	0.100	**0.098***
Synergistetes	0.000	0.001	0.002	0.001	0.002	0.002	0.001	0.000	0.002	0.001	0.001
Tenericutes	0.001	0.002	0.001	0.001	0.001	0.002	0.001	0.001	0.003	0.002	0.001
Verrucomicrobia	0.003	0.006	0.004	0.005	0.027	0.003	0.002	0.002	0.003	0.002	0.006

Top taxa (1%> abundance) are denoted by *

Table S2. High, low, and average temperatures (°F) across six sampling months.

	Temperature (°F)		
Month	**High**	**Low**	**Average**
July 2020	93	64	79
August 2020	92	63	77
September 2020	91	42	68
January 2021	58	24	39
February 2021	74	22	41
March 2021	79	25	54

Data from: https://www.timeanddate.com/weather/usa/asheboro/historic

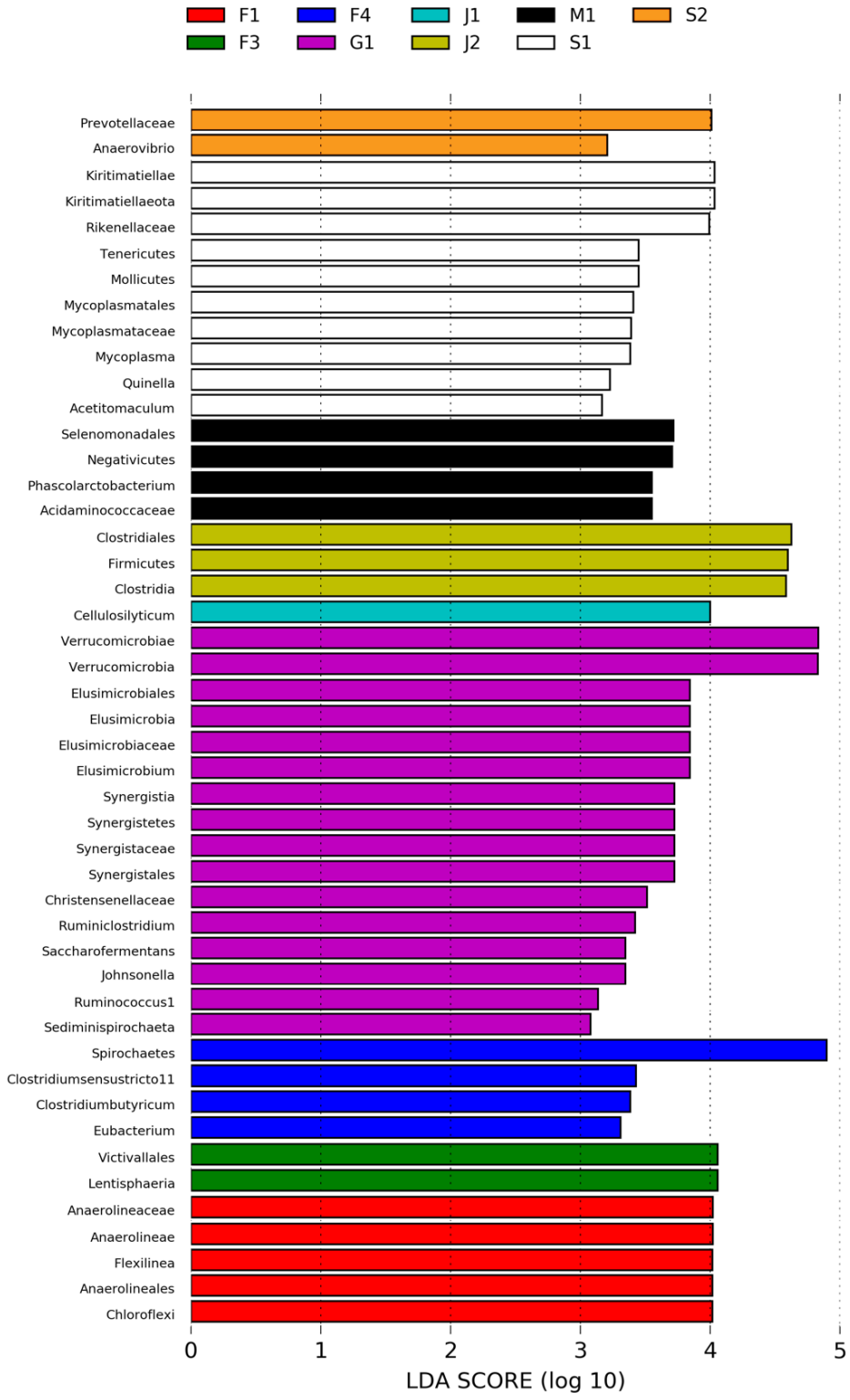


Figure S1. LEfSe LDA score plot comparing enriched major taxa present at >1% relative abundance across individuals. An LDA score of 3 or higher is considered biologically relevant.