ABSTRACT

MALY, MORGAN ANN. Interactions Among Diet, Gut Microbiota, and Gut Health in the Cheetah (*Acinonyx jubatus*). (Under the direction of Drs. Reade Roberts and Matthew Breen).

The cheetah (*Acinonyx jubatus*) is a member of the *Felidae* family and the only living member of the genus *Acinonyx*. Cheetahs are listed as vulnerable by the IUCN. Having been extirpated from 90% of their habitat, there are less than 7,100 cheetahs left in the wild. This decline in wild populations highlights the importance of maintaining *ex situ* cheetah populations as an insurance policy against extinction. Unfortunately, *ex situ* cheetahs face their own set of challenges, including low reproductive rates, and kidney and gastrointestinal (GI)-related diseases. GI-related diseases, such as gastritis, are one of the top reasons for death or euthanasia for *ex situ* populations. As a species with extremely low genetic variation due to two historical bottlenecks, each cheetah loss has a significant negative impact on maintaining a healthy self-sustaining population. Additionally, with increased appreciation for the role of microbiomes in the general health of humans and animals, there is great interest amongst zoos and animal management facilities to study the microbiomes of their animals and compare them to their wild conspecifics. As more wildlife are faced with shrinking habitats and extinction, management facilities are becoming increasingly important for the survival of entire species. A better understanding of animal microbiomes, including the gut, are now included as a key component in conservation efforts.

In Chapter 1, I provide a brief overview of current knowledge on wild and managed cheetahs, as well as data derived from using the domestic cat as a model for understanding relationships among diet, gut microbiota, and gut health.

In Chapter 2, I determine the appropriate sampling protocol for collecting non-invasive cheetah fecal samples from an *ex situ* facility in North America under hot and humid conditions. I also identify potential patterns within cheetah microbiota that may affect their fecal microbiota stability and hint at fecal microbial differences among cheetahs.

In Chapter 3, I determine the appropriate sampling protocol for collecting non-invasive cheetah fecal samples during the dry season from their native habitat in Namibia. I also performed a preliminary study to compare fresh samples of two separate populations to identify potential impacts of diet and location on the fecal microbiota and predicted functional pathways.

In Chapter 4, I identify interactions among diet, fecal microbiota, and gut health in managed cheetahs. I found an association between diet types and prevalence of GI symptoms in cheetahs. I determined that diet was a major driver of cheetah fecal microbiota and identified both animal fiber (carcass versus commercial fed) and species of diet (horse versus beef) as factors that contribute to microbial community composition. I also found that age is important for microbial composition but not microbial diversity. Additionally, neither sex nor presence of GI symptoms influenced the fecal microbiota. Lastly, I found that while there were no associations between GI symptoms and overall microbial diversity, I did identify bacteria that were correlated with absence or presence of GI symptoms.

In Chapter 5, I summarize how this dissertation contributes to the current knowledge on cheetah diets, fecal microbiota, and gut health. I describe how these data can influence how we think about managed animal diets and health in zoo facilities, and possibly even for our own domestic pets. Finally, I examine how these data can provide hypotheses and directions for future research, not just for cheetahs, but for other felids and carnivores.

Interactions Among Diet, Gut Microbiota, and Gut Health in the Cheetah (*Acinonyx jubatus*).

by
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APPROVED BY:

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DEDICATION

I would like to dedicate this dissertation to our friend gone too soon, Sarah Putman. Sarah was the first person I met at the Smithsonian almost 13 years ago. I was starting an internship in the endocrine lab, and she was training me over my first few weeks. In the very beginning I was intimidated and so afraid to make a mistake. Hello… this was THE SMITHSONIAN! The big leagues! I was terrified that I would mess up or everyone would find out I didn't know how to do something or worse, think that I didn't belong there. But Sarah squashed all of my fears in the best way possible. While training me in the lab, she always told me, "You're going to mess up, maybe not today, maybe not tomorrow… but you will, and it will be okay because whatever mistake you make, remember that myself and almost everyone here has already made that same mistake." She helped me realize that it was *okay* to make mistakes, that it was part of learning. She encouraged me to ask questions, even when I was too scared of feeling stupid, because it's easier to ask a "dumb" question and get a simple answer than to guess the answer incorrectly and then have a much more difficult problem to solve. She didn't make me feel bad for not knowing something. Of course, this isn't to say Sarah was any type of a pushover. As my training went on my "dumb" questions were increasingly met with the famous Sarah expression. If you knew Sarah, you know exactly what I'm talking about. The look was equal part concern and intrigue with just a hint of sass. She gave this expression when she thought you *should* know the answer to your question, or if you had proposed something so wildly outrageous, she wasn't even sure how to respond. I was both amused by and afraid of this expression. Sometimes, many years later, I would pose a question to Sarah, knowing full well what was coming my way. This was then of course followed by a loud cackle of laughter from us both. All of this to say, Sarah completely changed the way I thought about learning, teaching, and mentoring. I took all of the lessons she taught me and applied them when starting in a new field for my PhD. But maybe more importantly, I carry her approach and lessons with me when mentoring my own students and interns. Sarah taught me how to be an empathetic and respectful mentor while still maintaining high expectations for my students (with a side of humor of course) to help them succeed. I owe so much of my own success to the confidence she helped me build at the very beginning. I wish I could tell this to her now, and not just because I know I'd likely get my favorite expression in response. I feel so privileged to have known her as a mentor, peer, and friend. So, from this mere poop apprentice to the ultimate Poop Sleuth, thank you, and I hope I've done you proud. We miss you. *Pterodactyl sound*

BIOGRAPHY

Morgan Maly is from DeForest, WI, a small town north of Madison. She began her college career at Colorado College in Colorado Springs, CO. After two years, she transferred to the University of Wisconsin-Madison where she earned her Bachelor of Science in Biology and a Certificate in African Studies. The next few years were spent volunteering and interning across the country from Alaska to Tennessee and California to Virginia. Her most influential internship was at the Smithsonian National Zoo & Conservation Biology Institute (NZCBI) where she interned with Dr. Adrienne Crosier learning non-invasive reproductive and adrenal hormone techniques. Morgan went on to earn a Master of Science in Animal Science and Minor in Biotechnology from North Carolina State working with Dr. Crosier at NZCBI studying adrenal and reproductive hormones in pubertal cheetahs. After her MS she gained experience in cheetah proteomics working with Dr. Diana Koester at NZCBI and cheetah genetics working with Drs. Jesus Maldonado and Klaus Peter Koepfli at the Center for Conservation Genomics at NZCBI. From there Morgan enrolled at NC State to work with Drs. Reade Roberts and Matthew Breen on collaborative research with Drs. Adrienne Crosier and Carly Muletz-Wolz at NZCBI. She is expected to graduate in summer of 2023 with a PhD in Genetics.

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I often joke that I feel like a child of divorced academic parents because I belonged to and bounced between multiple labs and institutions during this degree. While balancing work and relationships in all of these labs can be challenging ("when are you coming back here again?", "has anyone seen the samples I left on this shelf 6 months ago?"), I mostly consider it a privilege. This dissertation is truly built on community and collaboration. I would like to say my biggest thank you to my academic "parents", Drs. Adrienne Crosier and Carly Muletz-Wolz at the Smithsonian National Zoo & Conservation Biology Institute and Drs. Reade Roberts and Matthew Breen at NCSU. I would like to thank Adrienne, for signing up for another wild academic ride with me that once again not only crossed state, but international lines (and in a global pandemic, no less)! Such simple projects we embark on. ;) The knowledge and network I have built around cheetah and zoological research would not have been possible without you and I am so grateful for your guidance, encouragement, and for taking me on as a newbie-know-nothing intern all those years ago. To Carly, who although was not an "official" member of my committee, was every bit as important. These projects would never have been possible without you. Thank you so much for welcoming me into your lab and setting me up to be a successful molecular ecologist. I am so impressed by your enthusiasm and dedication to your science, but also your ability to make time for the important things outside of work. And of course, your statistical knowledge is second to none! I also thank Reade and Matthew for seeing this wayward grad student with minimal funding and big dreams and still saying "yes". I know it hasn't been easy or straightforward, but I am so grateful for your willingness, guidance, and belief in me. I've learned so much from both of you. Reade, thank you for always helping me see the forest for the trees. My ability to share my science through effective story telling has improved exponentially because of you! Matthew, thank you for all the laughs and life chats, especially when things got tough and frustrating. The world of conservation can be so tough!

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NZCBI will always hold a special place in my heart, a community of like-minded individuals with a shared mission to save species working together and lifting up those around them. Sometimes it still feels like a dream that I've belonged to this community for so long. I'd like to give a giant thank you to the PIs at NZCBI who helped me along the way with advice, guidance, and support, especially Drs. Budhan Pukazhenthi, Jesus Maldonado, Klaus Peter Koepfli, Rob Fleischer, Nucharin Songsasen, and the late Dave Wildt. They are an integral part of what makes this community so spectacular to be a part of. To Dr. Bec Gooley, thank you for rescuing me when I needed it most, that project would not have been possible without you. To Nancy McInerney, thank you for taking me under your wing when I first set out on my cheetah genetics journey and giving me training I needed to succeed. And of course to the rest of the NZCBI family, the staff, postdocs, graduate students and interns past and present, especially Diana Koester, Katie Edwards, Megan Brown, Jessica Kordell, Jennifer Nagashima, Mia Keady, Morgan Bragg, Molly Corder, Karen Holm, Lilly Parker, Matt Krcmarik, Steve Paris, Niki Boisseau, Parker Pennington, Lex Oestmann, Justin Cooper, Mike Byron, Craig Fergus, Kyle Rhodes, Sarah Macey and many more who have come and gone from all departments across NZCBI. Another special thank you to my intern at the very beginning of it all, Jaelyn Current! And to my microbe writing and programming group that gave me the structure and support we all so badly needed while being scattered across states during the pandemic and beyond, you are the best, we should have started it years ago.

During my PhD, I was lucky enough to join another lab at a third institution, though it ended abruptly far too soon (thanks Covid). Therefore, I also owe a great deal of gratitude to Drs. Laurie Marker and Anne Schmidt-Küntzel in welcoming me to the Cheetah Conservation Fund facility and genetics lab in Namibia. I'd also like to thank Julia Zumbroich for her help in the lab and for being so supportive in March of 2020 when the world slowly collapsed into pandemic chaos and I was uncertain of when, or if, I would get home. And to Tayla Green who was so positive and supportive during my entire time at CCF from helping me find allergy medication to identifying a gigantic spider in my room at 11pm, you are so kind and so patient and CCF was lucky to have you!

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are my rock, and I would not have made it through this without you. I can't wait to see what's next for us. ☺

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CHAPTER 1: Literature Review

In situ Cheetah Populations

The cheetah (*Acinonyx jubatus*) is a charismatic member of the *Felidae* family and the only living member of the genus *Acinonyx*. There are currently four recognized cheetah subspecies including three found in Africa, *A. jubatus hecki*, *A. jubatus soemmeringii*, and *A. juabatus jubatus* and the only living Asiatic cheetahs, *A. jubatus venaticus* [1]. *A. jubatus venaticus* are Critically Endangered and found only in a small area of Iran [2].

The remaining subspecies of cheetahs are listed as vulnerable by the International Union for Conservation of Nature (IUCN). Having been extirpated from 90% of their historical habitat, fewer than 7,100 cheetahs remain in the wild [2]. Primary threats to their survival include habitat loss and fragmentation, conflict with humans, and the illegal pet trade [2, 3]. Due to their specialized speed, cheetahs typically live in open grassland savannas where chasing prey is easier but can be successful in scrub forest habitat as well [2]. Cheetahs are diurnal hunters, primarily to avoid direct competition with larger apex predators including lions, leopards, and hyenas [4, 5]. However, due to decreasing habitat and increasing overlap with humans, some cheetahs are opting for hunting at night to avoid detection by people, putting them at risk for interacting with larger predators [6]. The typical prey for cheetahs includes small to medium ungulate species, primarily impala (*Aepyceros melampus*), Thomson's gazelles (*Gazella thomsoni*), Grant's gazelles (*Gazella granti*), springbok (*Antidorcas marsupialis),* and blesbok (*Damaliscus lycic phillipsi*) [7, 8]. Cheetahs will also hunt small mammals including springhares and ground-birds [8-10]. Due to habitat loss and human encroachment, 77% of the cheetah's current range is located outside protected areas [2] where cheetahs have increased access to livestock and less natural prey which often results in livestock predation and increased conflict [8, 11].

***Ex situ* Cheetah Populations**

Cheetahs are a popular attraction at zoos and conservation facilities. Cheetahs in these managed facilities provide a unique opportunity to engage and educate the public about conservation issues and ways they can support initiatives to protect wild cheetahs and their habitat. In addition to providing outreach, *ex situ* populations also serve as a safety net against extinction. Thus, it is important to maintain a self-sustaining *ex situ* population that is healthy and genetically variable. In order to reach this goal, there is a strong focus on basic biology and conservation research. Studying cheetahs in the wild is difficult and costly because cheetahs are elusive and far ranging. Females live alone, except when raising cubs, which means collecting data on a large sample size is challenging. Utilizing *ex situ* populations for systematic biological research initiatives enables thoughtful collection of scientific information on a threatened species, data that would be otherwise impossible to collect. Studying *ex situ* cheetahs increases sample sizes and gives researchers the ability to control more variability while having details of individual life history including birth date, lineage, illnesses, diets, and reproductive events. Cheetahs in facilities accredited by the Association of Zoos and Aquariums (AZA) in North America are managed as one population via the cheetah Species Survival Plan (SSP). There are roughly 330 cheetahs currently in the SSP. The SSP maintains a pedigree of all cheetahs in the population and monitors aspects of nutrition, reproduction, and health, and uses a handbook of guidelines for all participating institutions [12]. As part of the handbook, the SSP employs various condition checks to monitor the welfare of cheetahs. These include fecal score and body score condition ratings to monitor digestive health and weight. Facilities also keep medical and keeper records noting activities, symptoms, behaviors, and feed intake to assess the quality of life of their animals. The veterinary and pathology advisors of the SSP maintains a database with disease concerns for the population. For cheetahs these include gastrointestinal (GI)-related diseases like gastritis and infectious diarrheal conditions, inflammatory diseases in the kidney and liver, and neurodegenerative disorders including myelopathy [13]. Many of these diseases are specific to managed cheetahs and not seen in the wild. Gastritis specifically is unique to captive cheetahs, and accounts for up to 95% of death and euthanasia across *ex situ* populations [14-16]. Some hypotheses for the difference in prevalence of gastritis between wild and managed cheetahs include activity of *Helicobacter* [14, 15, 17], chronic stress from living in managed facilities [18-20], and differences in diet [21-25]. While there has been extensive, though controversial research on the association with *Helicobacter* [13, 16-19, 26-28] and how to improve *ex situ* cheetah management [20, 29-31], there still exists a paucity of information on managed carnivore diets. Many *ex situ* carnivores in AZA facilities consume commercially available diets made of ground raw lean meat from horse, beef, or pork [12]. Some facilities add neck or knuckle bones and/or whole prey items such as rabbits, chickens, rodents, or locally available wildlife (white-tailed deer, moose, geese, etc.), typically no more than one-two times per week. Very few facilities within the AZA feed diets of entirely carcass or whole prey. In contrast, many European zoos feed supplemented meat and carcass chunks from animals including horses or cattle. However, feeding of only supplemented meat or commercially available raw meat diets may not result in optimal nutrition for either diet type [32]. With limited knowledge on the exact nutritional compositions of wild felid diets and with access and welfare limitations in supplying wild-type diets, most commercially available non-domestic felid diets are based on data from domestic cats [33-38].

Domestic cat diet, gut microbiome, and animal-based fiber

All felids are obligate carnivores with physiological and metabolic adaptations that require diets high in protein and low in carbohydrates and are able to obtain all essential nutrients from animal proteins and fats [39, 40]. Despite these unique adaptations, many domestic cats are fed dry or wet extruded commercial diets that are high in carbohydrates [41]. Moreover, most commercial carnivore diets add and measure crude dietary fiber based on carbohydrate-derived fiber. Common plant-based additives include cellulose, fructooligosaccharides, pectin, and inulin [42-44]. Carbohydrates are fermented in the GI-tract by microbes, which produce short-chain fatty acids (SCFA) as by-products. SCFAs are important in gut health and contribute to epithelial cell growth and as a substrate for bacterial metabolism [45]. They are an energy source for colonocytes, promote blood flow and motility in the colon, and aid in competition-based control of pathogenic bacteria [46-50]. These plant-based fibers are added primarily to act as a fecal bulking agent [51], decrease fecal pH [52], manage weight [53] and diabetes [54, 55]. Adding dietary fiber or changing the ratio of proteins to carbohydrates are also associated with changes in the domestic cat gut microbiome [42-44, 53, 56, 57].

The domestic cat microbiome is dominated by five core phyla, Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, and Proteobacteria [58]. Core genera include *Prevotella*, *Bacteroides*, *Collinsella*, *Blautia*, and *Mehasphaera* [58]. Unlike herbivores and omnivores, cats do not rely on the gut microbiota for energy production [59]. Though cats do not rely on the gut microbiome for energy, it is still important for metabolism and digestive functions [60, 61]. Many studies show benefits of adding plant-based fibers to domestic cat diets [55, 62-64]. While cat species in the wild, and free-roaming domestic cats, do not consume high amounts of carbohydrate-based fiber, they do consume many indigestible parts of the animals they hunt, including the fur, skin, connective tissue, and bones [23]. Feral cats acquired 2% Nitrogen Free Extract (a measurement of carbohydrate as counted by what is not fat, crude fiber, protein, mineral, or moisture) from consuming entire carcasses [65]. These low-to-non-digestible parts of the carcasses may provide fermentable and non-fermentable substrates as a source of dietary fiber in obligate carnivores [24] and wild felids meet their fiber requirements by consuming them [65]. Animal-derived dietary fiber is also capable of producing substantial amounts of SCFA [24]. Collagen, which includes glucose, galactose, and glycosaminoglycans components [66] from connective tissues may be an important part of the animal-based fiber portion of the diet [23, 24]. Bacteria specified for utilizing cartilage at a much more rapid pace over plant-based substrates were also identified in the fecal inoculum of cheetahs [24]. Because the obligate carnivore diet is higher in protein, more protein makes it to the large intestine where it can be fermented by the gut microbiome. Protein fermentation by bacteria produces putrefactive compounds including ammonia, phenol, indole, p-cresol, and biogenic amines [48, 67]. Many of these are negatively associated with GI dysbiosis or disease in humans [68]. However, carnivores require greater protein intake in their diet and may have evolved to deal with the consequences of increased hindgut protein fermentation [24]. In domestic cats fed diets higher in protein, an increase in *Novosphingobium spp* was identified. This genus can utilize indoles and phenols for growth suggesting these species may help decrease the concentration of protein fermentation byproducts in cats with high protein fermentation [69]. Further, cheetahs fed whole rabbit diets had similar SCFA concentrations in their feces, but lower putrefactive indole and phenol compared to cheetahs fed chunk supplemented beef diets [23]. Likewise, a toxic metabolite of indole, indoxyl sulphate showed a dramatic decrease in the same cheetahs fed the whole rabbit diet. These toxic metabolites have been associated with kidney inflammation and renal failure in humans [70]. *Ex situ* cheetahs similarly suffer from chronic kidney failure [13, 16]. All these taken together suggest felids, cheetahs included, may benefit from consuming the low to indigestible parts of the animals for not only their gut health, but other aspects of overall health.

Cheetah gut microbiome

Initial insights on the cheetah gut microbiome include studies of both captive and wild cheetahs. Studies on wild cheetahs identified the same five phyla found in domestic cats, Firmicutes, Fusobacteria, Bacteroidetes, Proteobacteria, and Actinobacteria [71, 72]. Differences among cheetahs were identified based on relatedness of individuals, sex, and age [72]. The authors also noted microbial community differences between wild Namibian cheetahs and those kept in Namibian facilities, including a higher potential for pathogenic bacteria and disease-associated functional pathways. Interestingly, a study on captive cheetahs at a European zoo found very different abundances of main phyla compared to the wild studies, including a complete lack of any Bacteroidetes [73]. Part of this may be due to true differences in the gut flora of captive and wild cheetah populations due to environment and diet, or it may also be due to variations in the sampling, processing, and analyzing of the data. A similar study by the same group in Europe determined that the captive cheetah gut microbiome is relatively stable over time, except during times of GI distress [74]. Additional microbiome and metabolomic research in *ex situ* cheetahs includes a recent study using targeted metabolomicsthat found evidence of antibiotic transfer from one cheetah to another via grooming using targeted metabolomics [75]. Other than these few examples, there is little published information on cheetah gut microbiomes, particularly in response to managed diets or dietary supplementation. Domestic cat microbiome studies support age, sex, and health status as factors impacting the gut microbiome, particularly when inflammatory bowel disease or acute and chronic diarrhea is present. Significant microbial differences have been identified in domestic cats with GI disease [76-78], including a decrease in species richness in cats with acute or chronic diarrhea [79], and changes in the gut microbiota accompanying improvement in diarrhea symptoms [80]. However, given the long history of domestication and other evolutionary differences, it remains unclear how results obtained in domestic cat studies predict large felid biology.

Sampling the gut microbiome in non-model organisms

The gut microbiome plays a crucial role in animal health, and understanding these interactions is essential for wildlife care, medicine, and conservation [81-85]. This is particularly important for rare and endangered species, where the health of each individual can impact population viability. Current practices for studying wildlife gut microbiomes include invasive methods, such as collecting rectal swabs, and non-invasive methods, such as fecal collection as a proxy for the GI-tract. For most species, non-invasive methods are preferred. The current gold standard is to collect "fresh" fecals immediately after they are deposited, followed by immediate freezing of the sample. This can be difficult in remote field locations or when trying to track species that are elusive and solitary. Even within managed facilities, collecting fresh samples can be challenging with limited staff, shy, or dangerous animals. The deposit "event" is not always witnessed, and few facilities have staff or students to constantly observe animals until a defecation event. Many animals are too dangerous to share an enclosure space with and so collection requires an additional animal care staff member to relocate the animal to another location so the feces can be accessed.

To determine the best time window for sample collection, fecal microbiota time series studies have been conducted on a range of species, including domestic cats [86], minks [87], cattle [88], horses [89], giraffes [90], and springboks [90]. With the exception of the domestic cat study, these studies have identified shifts in microbial diversity or community composition over time and at various time points. In general, there is not a predictable pattern across studies and no "one time point fits all". This can be attributed to environmental factors (UV exposure, humidity, temperature) and species-specific factors (fecal shape and size). Currently, no information exists on the stability of non-domestic felid fecal microbiota post-defecation. Given the extensive interest in managing these carnivores in managed facilities, the lack of information about diet and nutrition, and the increase in GI and inflammatory diseases, gut microbiome studies are becoming more popular. However, it is important to determine appropriate fecal sampling to not only identify fecal stability for ease of sampling, but also to ensure that the samples collected are the most appropriate in order to form valid conclusions regarding the microbiome and associations with traits, diet, or disease.

Objectives and goals

The overall aim of this dissertation was to determine the best sampling methods for non-invasive cheetah gut microbiome investigations, and then to investigate the relationships between diet, the gut microbiome, and GI health in managed cheetahs. Specific questions include: 1) How fresh do fecal samples need to be to represent the cheetah gut microbiome in a common managed facility setting? (Chapter 2); 2) How fresh do fecal samples from the native habitat of cheetahs need to be for fecal microbiota sampling? (Chapter 3); 3) How different are two distinct populations of cheetahs living on different continents, and do we see any evidence of dietary influence on their microbiomes? (Chapter 3); 4) Is there a correlation among diets fed in managed facilities and presence of GI symptoms in cheetahs? (Chapter 4); 5) What is the relationship between diet and the cheetah gut microbiome? (Chapter 4); 6) What other traits are associated with the cheetah gut microbiome? (Chapter 4); and 7) What is the relationship between GI symptoms in cheetahs and the gut microbiome? Can we identify biomarkers for symptomatic cheetahs? (Chapter 4).

In this dissertation, I use one of the largest non-domestic felid microbiome samplings to date to characterize the gut microbiome of the cheetah and to understand the links between diet, host traits, and GI health in zoo-managed facilities. The ultimate goal of this dissertation is to provide data that may be used to help improve the health and welfare of cheetahs in managed facilities. More broadly, results of this dissertation will enhance the understanding of the relationships between diet, animal fiber, the gut microbiome, and GI health in carnivores.

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CHAPTER 2: Comparing Stability of Fecal Microbiota by Time and Weather Conditions in a Carnivore, the Cheetah (*Acinonyx jubatus*)

Abstract

Gut health and its relationship to gut microbiota is an important consideration in the care and well-being of managed endangered species, such as the cheetah (*Acinonyx jubatus*). Non-invasive fecal sampling as a proxy for gut microbiota is preferred and collecting fresh fecals is the current gold standard. Unfortunately, even in managed facilities, collecting fresh samples from difficult to observe or dangerous animals is challenging. Therefore, we conducted a study to determine the terminal collection timepoint for fecal microbial studies in the cheetah. We longitudinally sampled eight freshly deposited fecals every 24 hours for five days and assessed bacterial relative abundance, diversity, and composition changes over time. Our data indicated that fecal samples up to 24 hours post-defecation provided accurate representations of the fresh fecal microbiome. After 24 hours, major changes in community composition began to occur, which related to increases in precipitation. By 72 hours, individual cheetah fecal microbiota signatures were lost. Our findings suggest that cheetah fecal samples should be collected with 24 hours of defecation, especially if precipitation occurs, in order to provide a more biologically accurate representation of the gut microbiome, and we provide visual characteristics that can aid researchers in approximating time since defecation. These data provide guidelines for researchers investigating cheetah and other large felids and carnivores where the ability to collect fresh fecal deposits is limited.

Introduction

The role of the gut microbiome in animal health has been of increasing interest in wildlife medicine (Gillman, McKenney, Lafferty, & Moore, 2022; Sugden, Sanderson, Ford, Stein, & St Clair, 2020) and conservation (Bahrndorff, Alemu, Alemneh, & Lund Nielsen, 2016; Redford, Segre, Salafsky, Martinez del Rio, & McAloose, 2012). Understanding the native microbiomes of wildlife is essential to identify crucial taxa necessary for metabolic functions and overall health (Amato, 2013). This understanding is particularly relevant for rare and endangered species for which the health of each individual may be critical for population viability. A major challenge for examining gut microbiomes in these animals is the ability to collect non-invasive samples, such as fresh feces, to best represent the gastrointestinal tract. For fecal microbiome studies, the gold standard is to collect fresh fecal samples and immediately freeze them at -80°C (Choo, Leong, & Rogers, 2015; Fouhy et al., 2015; Gorzelak et al., 2015). While fresh samples are optimum for fecal sampling, they are often difficult to collect due to challenges posed by defecation timing and frequency, factors that are difficult to predict without continual observation of animals, which is impossible in most free-ranging populations.

Investigations into the temporal stability of the fecal microbiome after defecation are necessary to determine the time window for best collection. Time series studies where freshly voided samples are experimentally subsampled over time have become more prevalent in a wide range of species including domestic cats (Tal, Verbrugghe, Gomez, Chau, & Weese, 2017), minks (Lafferty, Gillman, Jeakle, Roell, & McKenney, 2022), cattle (Wong et al., 2016), horses (Beckers, Schulz, & Childers, 2017), and giraffes and springboks (Sebastian Menke, Meier, Sommer, & Bunce, 2015). With the exception of the domestic cats (Tal et al., 2017), these studies identified shifts in microbial diversity or community composition over sampling time points. Most fecals for captive wildlife studies are collected in the morning during routine enclosure maintenance and were thus deposited within the previous 24 hours of defecation. However, consistent fresh sampling is not feasible in the wild without intensive effort, and even then, may still be difficult in certain species. Fresh sampling is also difficult for managed animals that are dangerous and/or sensitive to human disturbance in facilities. Identifying a timepoint beyond which the fecal microbiome shifts and no longer represents the host gut microbiome is key. Doing so will ensure that the samples collected are most appropriate to form valid conclusions regarding the microbial ecology of fecal samples.

Here we studied the temporal stability of microbiota in cheetah fecals over time. Previous research shows that between 40-60% cheetahs under human care suffer from gastrointestinal diseases, such as chronic gastritis, that lead to death or euthanasia (Munson, 1993; Munson et al., 1999; Munson et al., 2005; Terio et al., 2018). Due to the prevalence of these diseases, there is a growing interest to better characterize and understand the role of the cheetah gut microbiome in overall individual health. While previous cheetah gut microbiome studies utilize rectal swabs (Becker, Hesta, Hollants, Janssens, & Huys, 2014; Becker, Janssens, Snauwaert, Hesta, & Huys, 2015; S. Menke et al., 2014; Wasimuddin et al., 2017), fecal samples are easier to obtain and are non-invasive. However, there are no data for the fecal microbial stability of non-domestic felids, including cheetahs. In domestic cats, fecal microbiota did not change in any community richness or composition measures examined when comparing fresh frozen samples to those stored at room temperature for up to four days (Tal et al., 2017). However, as the domestic cat samples were in tubes and not exposed to a natural sampling environment, those data may not be a good predictor of non-domestic felid sampling in the wild.

In this study, we examined the stability of the fecal microbiome in captive cheetahs in a US-based facility. Our objective was to determine for how many days post defecation the fecal microbiome remained representative of the microbiome of a fresh sample and maintained individual cheetah signatures. The goal was to establish timelines to aid the design, implementation, and interpretation of future cheetah, felid, and other large carnivore gut microbiome studies both *ex situ* (in managed populations) and *in situ* (from field sampling of wild populations). With *ex situ* sampling, it is easier to collect a sample less than 24 hours after deposit, however, it still requires a great deal of time and effort to acquire a fresh (<1 hour) sample. For *ex situ* cheetah research, it is important to know whether the next morning sample collection is representative enough of a fresh sample. For *in situ* sampling, it is even more difficult to find and collect fresh samples. Often it is more likely to find deposited feces and use some general characteristics to approximate fecal age (color, moisture, texture, mucous layer, insect activity, etc.) (Nardi et al., 2022). Identifying the best time window for fecal collections that can serve as a proxy for gut microbiome structure will strengthen our ability to make inference about the role of the gut microbiome in cheetah health *ex situ* and *in situ*.

Materials and Methods

Sample Collection

Sample names are given to each cheetah fecal as Aju short for *Acinonyx jubatus*. Eight fresh fecals were collected from two adults (individuals Aju 2 (5.6 years old) and Aju 6 (6 years old) and six juveniles (individuals Aju 1, 3, 4, 5, 7, and 8: all age ~ 1 year) living at the Smithsonian National Zoo & Conservation Biology Institute (NZCBI) in Front Royal, VA. Smithsonian NZCBI met required animal management and husbandry guidelines put forth by the Cheetah Species Survival Plan(Plan, 2009). The cheetahs were housed in 2000 m^2 enclosures with free access to both indoor and outdoor areas. Cheetahs were fed a commercial carnivore beef-based diet (Nebraska Premium Canine Diet, North Platte, NE, USA) and were also given one whole rabbit (1x weekly) and horse neck bones (2x weekly). Water was available *ad libitum*. Fresh fecal samples were collected in June and July between 8:30 am and 10:30 am from cheetah habitats within 30 minutes of deposit (all defecation events were witnessed by author MM), over the course of 20 days. The fresh fecal samples were placed in an experimental plot outside of but adjacent to the cheetah enclosures with full environmental exposures to allow for ease of access. A piece (approximately 1 inch in length) was removed from one end of each fecal sample with a sterile scalpel and a subsample was taken from the core of the fecal piece. This core sample was considered Day 0. The remaining feces was placed back in the experimental plot. Twenty-four hours after the fresh collection at Day 0, a new one-inch section was removed from the feces and another subsample was taken from its core. This process continued for each fecal sample for 5 days (Day 1 – 5) after the initial fresh collection. The collection date and maximum daily rainfall, temperature and humidity were recorded for all subsamples from each of the eight fecals (Supplemental Table 1). Maximum temperature and humidity levels did not vary across days, so only rainfall was analyzed. Upon collection, subsamples were placed immediately on ice and transported from the experimental plot to a -80°C freezer until processing.

Sample DNA Extraction and Library Prep

DNA was extracted from 0.25 g frozen core feces using the QIAamp PowerFecal DNA Kit (#12530-50, Qiagen, MD) following manufacturer's instructions. For each batch of sample extractions, an empty negative control was included to identify extraction contaminants. Following extractions, DNA concentrations were measured via fluorometric quantification (Qubit4 Fluorometer, ThermoFisher Scientific, MA).

Fecal bacterial DNA were amplified following a previously published two-step polymerase chain reaction (PCR) protocol with dual-index paired-end Illumina sequencing (Keady et al., 2021). The amplicon PCR reaction amplified the V3-V5 region of the 16S rRNA gene using universal primers 515F-Y (5'-GTGYCAGCMGCCGCGGTAA-3') and 939R (5'-CTTGTGCGGGCCCCCGTCAATTC-3') (Muletz Wolz, Yarwood, Campbell Grant, Fleischer, &

Lips, 2018). PCR reactions for each sample were performed in duplicate, including the negative extraction and PCR controls. The amplicon duplicates were pooled and then cleaned with magnetic beads, indexed with i5s and i7s, cleaned again with magnetic beads, quantified using fluorometric quantificaitons (Qubit4), and pooled as specified in (Keady et al., 2021). The target band for the 16S rRNA library (~578 base pairs) was isolated and removed using a QIAquick Gel Extraction Kit (#28704, Qiagen, MD) and diluted to 4 nM. All samples were sequenced using an Illumina MiSeq (v3 chemistry: 2 x 300 bp kit) at the Center for Conservation Genomics, Smithsonian National Zoo & Conservation Biology Institute.

Sequence Data Processing

Demultiplexed Illumina Miseq sequencing reads were imported into R version 4.0.3 (R CoreR. C. Team, 2022Team) using RStudio (v 2022.12.0+353). We utilized R package "dada2" version 1.16.0 (Callahan, McMurdie, & Holmes, 2017; Callahan et al., 2016) to merge paired ends, remove chimeras, and filter out low quality reads (maxEE > 2). Filtered and merged sequences from two sequencing runs were combined to generate amplicon sequence variants (ASVs) and assign taxonomy using the Ribosomal Database Project (RDP 16S training set (set 16, release 11.5) (Cole et al., 2007; Wang, Garrity, Tiedje, & Cole, 2007). A phylogenetic tree was built using Quantitative Insights Into Microbial Ecology 2 (vQIIME2-2020.8) (Bolyen et al., 2019) using FastTree (Price, Dehal, & Arkin, 2009). We imported the ASVs, taxonomy table, phylogenetic tree, and metadata into a 'phyloseq' object (McMurdie & Holmes, 2013) for processing. Putative contaminant sequences (n = 3) were removed using the combined Fisher method with a threshold of 0.1 in the R package "decontam" (v 1.18.0)(Davis, Proctor, Holmes, Relman, & Callahan, 2018). After contaminants were removed, we filtered out singleton ASVs (ASVs that occur in only one sample), ASVs classified as Cyanobacteria, negative control samples, and low sequence count (< 8,000 reads) samples. After quality control and filtering, the sequencing depth variation (max/min) was 4.89-fold change (max = 42,284, min = 8,948). Seven subsamples were removed due to low sequence coverage (empty boxes in Table 1).

Statistical Analysis

Statistical analyses were performed in RStudio version 2022.12.0+353 (R. Team, 2022) for R (v4.2.2) (R CoreR. C. Team, 2022Team). Significance for all analyses was set to $p < 0.05$. Analysis pipelines for characterizing microbial structure and composition were based on previous research in our laboratory (Bragg, Freeman, Lim, Songsasen, & Muletz-Wolz, 2020; Keady et al., 2021; Muletz-Wolz et al., 2017; Muletz-Wolz, Fleischer, & Lips, 2019). For comparisons across sampling days, we conducted three metrics of microbial diversity which included the changes in relative abundance, alpha diversity (within sample variation), and beta diversity (between sample variation).

Differential abundance of phyla and ASVs across sample days

Differential abundance testing was performed using package "DAtest" to identify the most appropriate test given our data set (Russel et al., 2018). At the ASV level, the phyloseq object was prefiltered to reduce the false discovery rate (min.samples = 5, min.reads = 10) and resulted in 160 taxa to be tested. The function testDA() was performed on the prefiltered ASV phyloseq object where the differential abundance was measured between sample days and paired by Fecal ID. The top ranked three tests were linear model (lmc), log linear reg.2 (llm2), and log linear reg (llm). After running the top three tests, ASVs were selected if they appeared in two of the three test results. We calculated post-hoc comparisons using lsmeans() between all days and output was subset to only include the comparisons between fresh (Day 0) samples and all other days (ex. Day 0 vs. Day 1, Day 0 vs. Day 2, etc) for further analysis. ASVs that were differentially abundant (after FDR correction) between fresh, and all other days were visualized using a heatmap. Heatmaps were created using the plot_heatmap() function in the R package "phyloseq" (McMurdie & Holmes, 2013). Differential Phyla were calculated, analyzed, and visualized similarly as the ASV level except the tax_glom() function was used to merge all taxa of the same Phylum before testing and there was no prefiltering of the original phyloseq object before running the differential tests.

Influence of sample day on fecal microbiota

Alpha diversity metrics included species richness (SR) and Faith's phylogenetic diversity (PD) over time (Sample Day) and by individual fecal (Fecal ID). Species richness is the number of unique ASVs in a sample and PD measures the amount of biodiversity based on the phylogenetic relationships of the taxa and the total tree branch length of ASVs in a sample (Faith, 1992). Faith's PD was calculated for each subsample with the R package "picante" (Kembel et al., 2010). Using the "stats" R package (Version 4.2.1) (R. C. Team, 2022), we performed linear models, with SR or PD as the response variable and sample day and fecal ID as fixed effects. SR and PD distributions met assumptions of normality (Shapiro-Wilk) and homoscedasticity (Levene). Post hoc tests were assessed using "emmeans" package (Lenth, 2023) in R with Tukey p adjustments.

To identify shifts in community composition between subsamples across sampling days we measured Bray-Curtis (abundance-weighted composition), Jaccard (presence-absence composition), and unweighted UniFrac (presence-absence with inclusion of phylogenetic relationships of taxa) distances. We used PERMANOVAs (Anderson, 2017) in the package "vegan" (Oksanen et al., 2022) where Bray-Curtis, Jaccard, and unweighted Unifrac distances were the response variable and Sample Day and Fecal ID were the explanatory variables. *Post hoc* analyses were performed in the package "pairwiseAdonis" (Martinez Arbizu, 2020) using the pairwise.adonis2() function and *p*-

values adjusted using the Bonferroni correction. To identify whether dispersion of community composition differed between sample days we used PERMDISP from the package "vegan", betadisper() function (Oksanen et al., 2022). The Bray-Curtis and unweighted UniFrac distance values between samples were also used in a sample-wise microbial network to identify connections between samples. We created an igraph microbiome network using the make_network() and plot_network() functions in the "phyloseq" package. Maximum ecological distance between two samples was set to the default value 0.7, which allowed all samples to appear in the network but minimized over-connectedness.

Influence of environmental factors on microbial community structure and composition

We were interested in how rainfall may influence the fecal microbial composition between Day 0 and subsequent days, particularly in the first few days (Day 1, Day 2, and Day 3). We subset all Day 0 vs Day 1, 2, or 3 pairings, their pairwise beta distance measures (Bray, Jaccard, Unweighted Unifrac), and the environmental variable (amount of rainfall between sample days). Pairwise day comparisons were placed into either a low or high group for rainfall (Table 2). We binned rainfall into high and low groups due to the variation in rainfall amounts and our hypothesis that high rainfall would lead to microbial compositional changes. To determine rain groups, the cumulative rainfall (inches) for each pairwise day comparison (ex. Day 0 vs Day 1) was calculated for each sample. Then the median cumulative rainfall value was calculated for each pairwise comparison (Day 0 v Day 1, Day 0 v Day 2, Day 0 v Day 3) across all samples (Aju1-8). The rainfall level for each Fecal ID subsample was classified into "low" or "high" values based on the pairwise median value. Pairwise Fecal ID subsample cumulative rainfall values were considered "low" if they were less than the median cumulative rainfall value for that comparison day group and "high" if they were equal to or greater. For example, the median cumulative rainfall for the Day 0 vs Day 3 comparison was 0.35

inches and the maximum cumulative rainfall for Fecal ID Aju1 in the Day 0 vs Day 3 comparison was 0.72 inches. This classifies the Aju 1 Day 0 vs Day 3 data point into the High Rain Group. We performed ANOVAs where the community composition measure (Bray-Curtis, Jaccard, or Unweighted Unifrac) was the response variable and the predictor was time (Day Comparisons) and rainfall amount (Low vs. High).

Results

Relative and differential abundance of phyla and ASVs across sample days

We obtained 984,381 high-quality sequences from 41 samples (Table 1). The average number of sequences per sample was 24,009 (range 8,648 – 42,284). We found 268 ASVs from five phyla including Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria, and Actinobacteria (Figure A.1). Fresh subsamples (Day 0), considered the most representative of the cheetah gut microbiome, were dominated by Firmicutes (63% mean relative abundance, 83 ASVs), Bacteroidetes (18%, 14 ASVs), Fusobacteria (13%, 8 ASVs), and with Actinobacteria (3.5%, 6 ASVs), and Proteobacteria (2.5%, 7 ASVs) present in lower amounts.

We identified four Phyla (Fusobacteria, Proteobacteria, Bacteroidetes, and Firmicutes) and 15 ASVs that were differentially abundant in at least one of the subsequent days (Day 1 – Day 5) compared to Day 0 (Figure 1). Fusobacteria were differentially abundant in all days compared to Day 0, Bacteroidetes differed between Day 0 and Day 2, Firmicutes differed between Day 0 and Day 3, and Proteobacteria differed between Day 0 and Days 3 and 4 (Figure 1). Fifteen bacterial ASVs were differential abundant (Figure 2, Table A.2), with three *Bacillus* ASVs and one *Clostridium sensu stricto* ASV lowest in Day 0 and then showing increases with sample day, while all other differential

ASVs, five *Bacteroides*, one *Eisenbergiella*, one *Clostridium XIVa*, and four *Fusobacterium* ASVs were highest in Day 0 and decreased with sample day (Figure 2).

Influence of sample day on fecal microbiota

Over time, bacterial communities in cheetah feces remained similar in richness and diversity but differed in composition. Among individuals, bacterial communities differed in richness over time and composition differed until Day 3 when individual identities could no longer be distinguished.

For alpha diversity measures, SR and PD were similar among sample days, but differed among individuals (SR Sample Day ANOVA: $F_{5,28} = 1.751$, $p = 0.156$; PD Sample Day ANOVA: $F_{5,28} = 1.535$, $p = 0.211$; SR Fecal ID ANOVA: $F_{7,28} = 3.709$, $p = 0.006$; PD Fecal ID ANOVA: $F_{7,28} = 3.78$, $p = 0.005$). At Day 0, bacterial species richness was 74.38 +/- 14.54 and phylogenetic diversity was 4.25 +/- 0.36, and this remained similar over time. Post hoc analyses of alpha diversity bacterial species richness among individuals showed that Aju 2 differed from Aju 3 and Aju 7 in species richness (Tukey $p.adj < 0.036$) and Aju 2 differed from Aju 3 and Aju 5 in Faith's PD (Tukey $p.adj < 0.011$). For beta diversity measures, microbial composition differed among both sample days and fecal IDs (PERMANOVA Sample Day: Bray-Curtis Pseudo-$F_{5,28} = 2.91$, $R^2 = 0.182$, $p = 0.001$; Jaccard Pseudo- $F_{5,28} = 2.367$, $R^2 = 0.170$, $p = 0.001$; unweighted UniFrac Pseudo- $F_{5,28} = 2.22$, $R^2 = 0.175$, $p = 0.004$; Figure 2) (PERMANOVA Fecal ID: Bray-Curtis Pseudo- $F_{7,28} = 5.314$, $R^2 = 0.466$, $p = 0.001$; Jaccard Pseudo- $F_{7,28} = 4.267$, $R^2 = 0.429$, $p = 0.001$; unweighted UniFrac Pseudo- $F_{7,28} = 3.515$, $R^2 = 0.386$, $p = 0.001$; Figure 2). We focused exclusively on post hoc comparisons of fresh (Day 0) versus all later sampling days as our aim was to determine if fecal samples collected later resembled fresh samples. Community composition at Day 1 remained similar to fresh samples (Bray Curtis, Jaccard, unweighted UniFrac $p.adj > 0.05$). By Day 2 composition shifted and remained distinct from Day 0 fresh samples for the rest of the experiment until Day 5 (Bray Curtis, Jaccard, unweighted UniFrac *p.adj* ≤ 0.05). Community composition differed among individuals at Days 1 and 2 (Bray Curtis, Jaccard, unweighted UniFrac *p.adj* ≤ 0.05), but by Day 3 individual fecal ID no longer differed (Bray Curtis, Jaccard, unweighted UniFrac *p.adj* > 0.05). By Day 1, dispersion in community composition began to increase in all measures (PERMDISP: Bray-Curtis $p = 0.016$, Jaccard $p = 0.007$, unweighted Unifrac $p = 0.003$; Figure 2), and the communities remained more dispersed in most measures thereafter (Figure A.2).

We built microbial networks based on distances calculated using Bray-Curtis (Figure 4) measures to better visualize differences over time. Day 0 (fresh) samples are closely connected in the center of the network, except for samples from Aju 7 and Aju 8. Samples from fecal ID Aju 7 and Aju 8 were loose stool samples (compared to normal, healthy fecal samples for all others) and were generally well connected to each other. Fecal ID Aju 2 and Aju 6, the only adults, exhibit multiple close connections between sample days within their own series. However, for samples Aju 1, Aju 3, and Aju 5, the subsequent post-fresh (Days 1-5) samples cluster together but unconnected to their respective fresh samples (Day 0s).

Influence of environmental factors on microbial community structure and composition

Rainfall was a source of concern on the exposed fecals during the study as some subsamples experienced large amounts of rain between sampling that may have influenced the fecal bacterial communities (Figure 4). We tested if a high amount of rainfall between sampling days resulted in a larger shift in microbial communities (pairwise Bray, Jaccard, unweighted UniFrac distances) over time. The amount of rainfall influenced changes in community composition across all days in abundance-weighted bacterial composition (Bray-Curtis Rain Group ANOVA: $F = 4.832$, $df = 1$, $p =$

0.042; Figure 5), but not presence-absence measures (Jaccard Rain Group ANOVA: F = 2.570, df = 1, p = 0.127 and unweighted UniFrac Rain Group ANOVA: F =3.503, df = 1, p = 0.079). The comparison Day was not significant in any of the models ($p \geq 0.103$).

Table 2.1. Subsample collection for each fecal ID. Day 0 represents the fresh subsample. The complete series (Day 0 – 5) were collected for all samples, but some samples did not yield adequate sequences for analyses (empty boxes). All feces were collected from juveniles, unless noted.

Fecal Sample ID	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Aju 1	💩	💩	💩	💩	💩	💩
Aju 2 (adult)	💩	💩	💩	💩	💩	💩
Aju 3	💩	💩	💩	💩	💩	
Aju 4	💩	💩	💩	💩	💩	💩
Aju 5	💩	💩	💩	💩	💩	
Aju 6 (adult)	💩	💩	💩	💩	💩	💩
Aju 7 (loose stool)	💩	💩	💩	💩		
Aju 8 (loose stool)	💩	💩	💩			

Table 2.2. Group rain data for linear test using pairwise community composition values between Day 0 and Days 1-3.

Day Comparisons	Total # of comparisons	Cumulative Rainfall Range (inches)	Rain Cumulative Daily Median (inches)	# in Rain Group High	# in Rain Group Low
Day 0 v Day 1	8	0 – 0.34	0.03	4	4
Day 0 v Day 2	8	0 – 0.71	0.235	4	4
Day 0 v Day 3	7	0 – 0.72	0.35	4	3

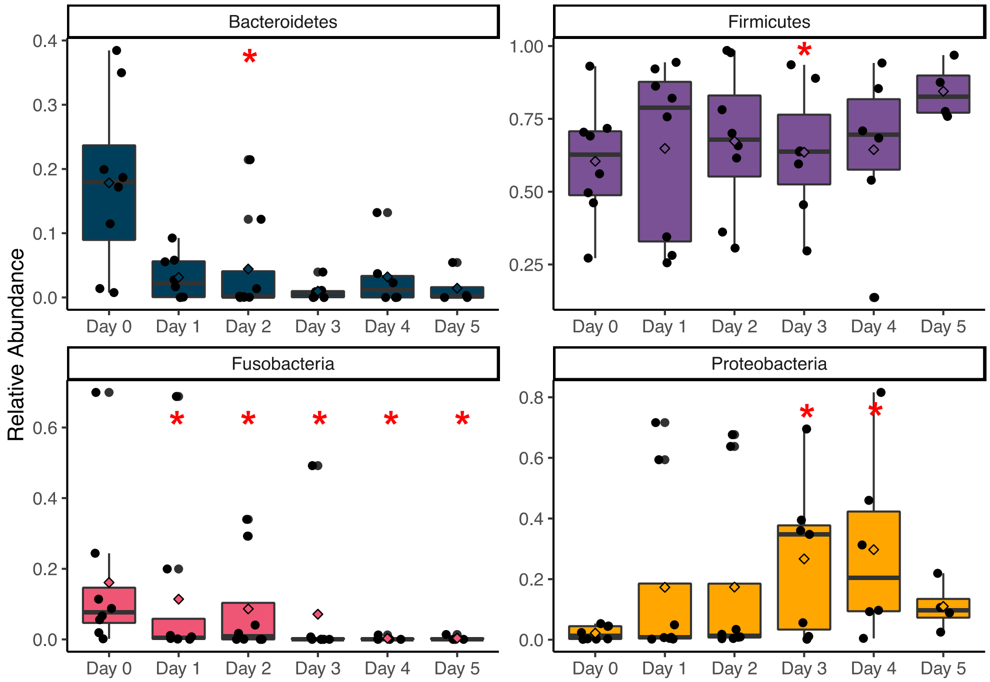

Figure 2.1. Box plots of differentially abundant phyla across sample days. Red asterisks indicate which days are different from Day 0 relative abundance ($p < 0.05$ in at least two of the following: linear model, log linear reg.2, and log linear regression in DAtest).

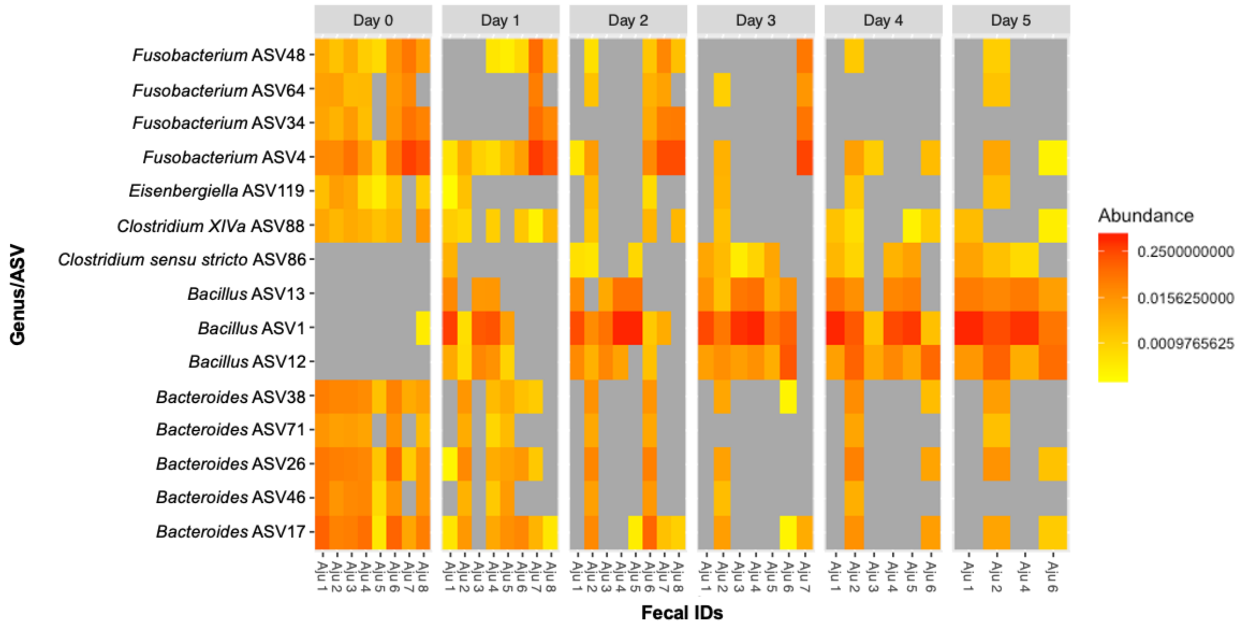

Figure 2.2. Heat map of differentially abundant ASVs across sample day. Differentially abundant ASVs are listed by Family membership and labeled with Genus and ASV number in rows and the columns are samples from each Fecal ID grouped by Sample Day. Color indicates abundance of the ASV in each sample from lowest (yellow) to highest (red) abundance, where gray indicates absence.

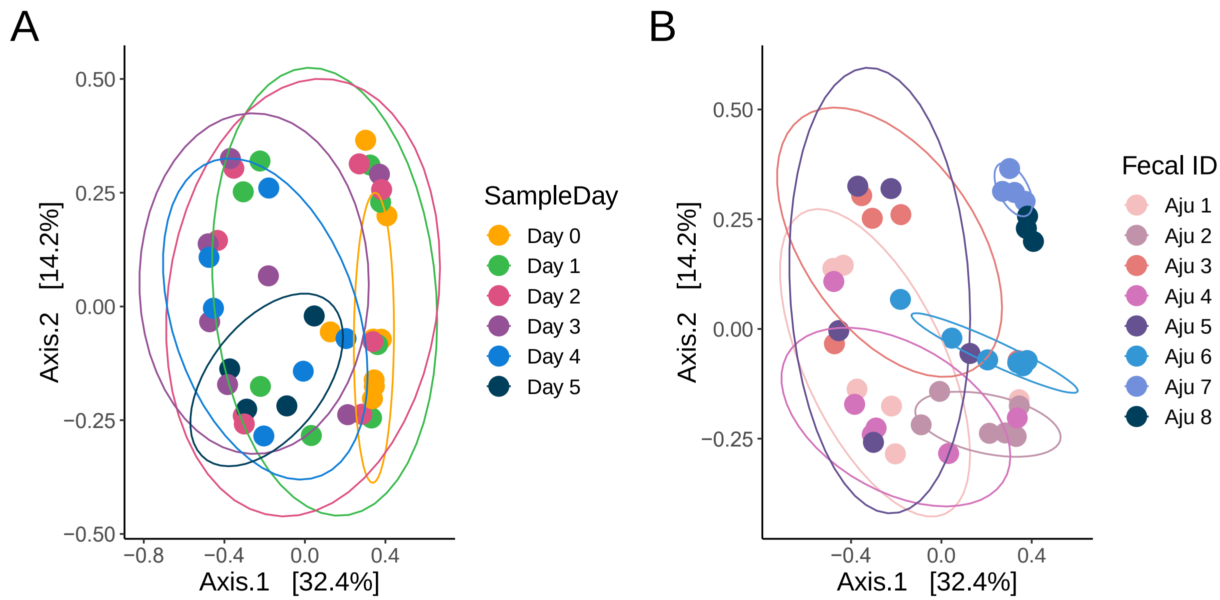

Figure 2.3. Principle coordinate analyses (PCoA) of Bray-Curtis distances for all samples color-coded by **(A)** Sample Day, where color indicates sample day (80% confidence ellipses by Sample Day) and **(B)** Fecal ID, where color indicates fecal IDs (80% confidence ellipses by Fecal ID, Aju 8 only had three samples, so no ellipse was drawn). Aju 2 and Aju 6 are adults, and the others are juveniles. Aju 7 and Aju 8 were experiencing loose stools and the others had normal fecal consistency.

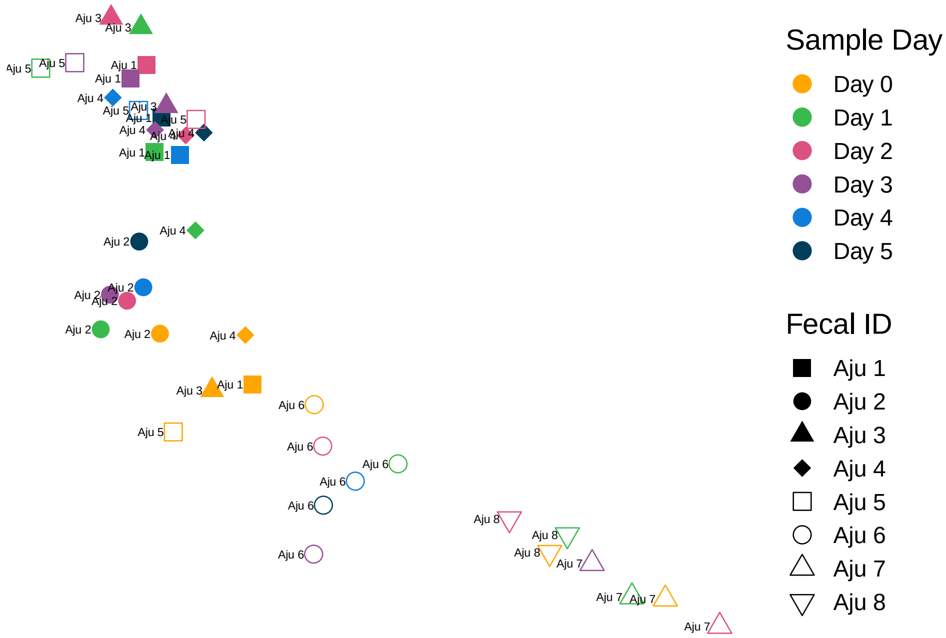

Figure 2.4. Microbiota network showing connections between samples (nodes) using Bray Curtis dissimilarity. Samples are identified by sample day (color) and fecal ID (shape). Lines connect samples that have a pairwise Bray Curtis less than 0.7. The length of the connecting line and the distance between two samples indicates the strength of the association, where shorter lines and distances are stronger associations.

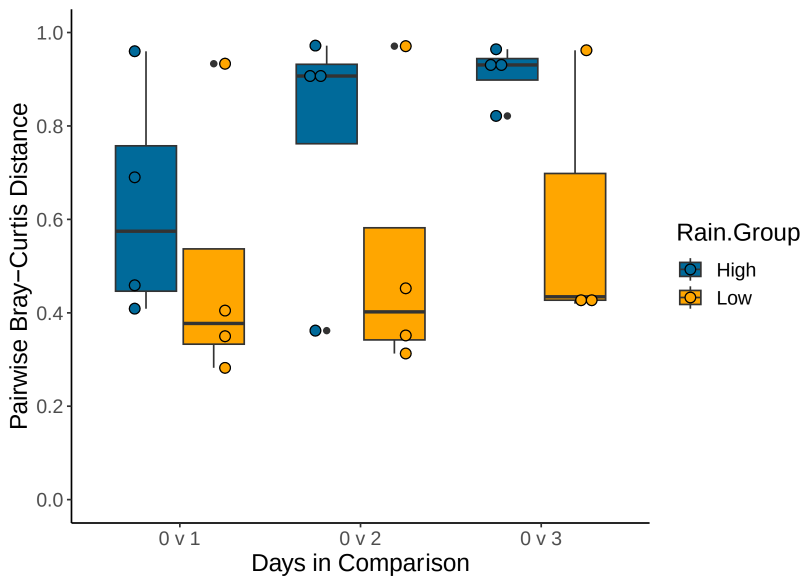

Figure 2.5. Box plot of pairwise community composition values calculated using Bray-Curtis. Colors indicate amount of cumulative rainfall groupings (high in blue vs low in yellow) across three different Day comparisons (Day 0 v. Day 1, Day 0 v. Day 2, Day 0 v. Day 3). Individual sample points are depicted with appropriate colored circle, and black points indicate outlier values. Amount of rainfall (High vs Low) was a significant predictor of pairwise distances (ANOVA: p = 0.042).

Discussion

The gut microbiome of wildlife species is important for their health and survival. Fecal samples are frequently used as a proxy for the gut microbiome because they are non-invasive and easier to collect. Here we performed time series analyses by subsampling freshly deposited cheetah fecals (Day 0) left to natural conditions for up to five days post-defecation (Days 1-5). We investigated the effects of time (sample day) and individual (fecal ID) on microbial relative abundances, diversity, community composition and dispersion. We also investigated how an environmental factor, rain, influenced the microbiota. We found that overall, fecal microbiota species richness and diversity remained similar, but that community composition and relative abundance of particular phyla and ASVs differed as early as Day 1 from fresh fecal samples. We explored the hypothesis that rainfall may explain some of these differences in composition and identified that samples that experienced higher amounts of rainfall had larger shifts in microbial composition changes. In general, our data suggest that for fecal microbiome studies, collecting a fecal within one day (24 hours) of deposit is acceptable. Within the first 24 hours while we observed small shifts in Firmicutes and increased dispersion in the community suggesting the beginning of changes to the community, the communities overall did not significantly change until 48 hours post-defecation. Our findings indicate microbial community shifts are likely through changes in endemic bacterial members and colonization of environmental microbes, and that biological signatures of individual animals can be detected in the earlier days, but not in the later stages of degradation. Other factors should be considered in the future for fecal microbiome stability such as environmental conditions, life stage of the host, and health (e.g., fecal consistency) of the host.

We generally demonstrate consistency in core Phyla across studies of the fecal microbiome of *in situ* and *ex situ* cheetahs. Our most representative samples of the cheetah gut microbiome, Day 0 subsamples, had relative abundance similarities with wild cheetah data for Firmicutes (63% this study vs 56.2-68.5%), Proteobacteria (2.5% vs 4.2-6.3%), and Fusobacteria (13% vs 18.1-18.4%, (S. Menke et al., 2014; Wasimuddin et al., 2017), respectively) and similar relative abundances of Actinobacteria with captive cheetahs in Belgium (3.5% vs 4.3%, (Becker et al., 2014)). Interestingly, we found the relative abundance of Bacteroidetes to be higher (18%) than previous studies in wild Namibian cheetahs (5.8% and 6.5% (S. Menke et al., 2014; Wasimuddin et al., 2017), respectively), and in captive cheetahs in Belgium (< 1%). Bacteroidetes are a dominant Phyla in domestic cats (Handl, Dowd, Garcia-Mazcorro, Steiner, & Suchodolski, 2011; Ritchie, Burke, Garcia-Mazcorro, Steiner, & Suchodolski, 2010; Tun et al., 2012) but are rare in other carnivores (Becker et al., 2014; Ley, Lozupone, Hamady, Knight, & Gordon, 2008; Schwab & Ganzle, 2011). This may be due to the high amount of starch and plant-based fibers found in domestic cat kibble (including beet pulp) compared to carcass diets of wild carnivores. Bacteroidetes have the ability to breakdown complex carbohydrates into short-chain fatty acids in the large intestine which are then utilized by the host

(Thomas, Hehemann, Rebuffet, Czjzek, & Michel, 2011; Tremaroli & Backhed, 2012). We hypothesize that we detected higher relative abundance of Bacteroidetes in fecal samples of our captive cheetah samples for similar reasons. The primary dietary source of the current study cohort was a commercially available beef diet that includes beet pulp as a bulking agent, which contains a mix of insoluble and soluble plant-based fiber and cellulose (de Godoy, 2018). Based on the dietary descriptions from the other studies, it seems likely the added source of plant-based carbohydrates may be the reason for the increased relative abundance of Bacteroidetes in the current study.

Relative and differential abundance of phyla and ASVs across sample days

Of the four Phyla that were differentially abundant across sample day, only Fusobacteria was different from Day 0 at all subsequent time points. Fusobacteria and Bacteroidetes exhibited the greatest abundance in Day 0 subsamples, indicating they were likely cheetah gut derived and subsequently decreased with exposure to the environment. The decline in Bacteroidetes between fresh (Day 0) and Day 1 subsamples may be related to the specific members of this Phyla being obligate anaerobes that cannot persist in aerobic conditions for long time periods outside of their host (Ley et al., 2008). Unlike Fusobacteria and Bacteroidetes, Firmicutes abundance was more consistent over time, with only Day 3 differing from Day 0. Proteobacteria abundance increased over time, suggesting an initial low contribution of microbes endemic to the host cheetah followed by an increase due to either proliferation of endemic microbes or colonization of new ones once in the external environment. Generally, fresher samples were characterized by greater abundance of Bacteroidetes and Fusobacteria, while more decayed samples were more likely to have greater abundance of Proteobacteria. At the ASV level, all four ASVs that were largely absent in Day 0 samples belonged to the phyla Firmicutes. Three ASVs belonged to the genus *Bacillus*, where two of these ASVs could not be identified at the species level. Previous research suggests *Bacillus* is a common genus in soil (Saxena, Kumar, Chakdar, Anuroopa, & Bagyaraj, 2020) as many species within the genera are capable of fixing atmospheric nitrogen (Yousuf et al., 2017). The third *Bacillus* ASV was *Bacillus infantis,* which has been previously isolated in a newborn human with sepsis (Ko et al., 2006) and from garden soil (Saggu & Mishra, 2017). The fourth ASV to increase over time, belonged to the Family *Clostridiaceae* in the genus *Clostridium sensu stricto*. Clostridia are primarily found in the environment (Popoff, 2015). A small proportion of *Clostridium sensu stricto* species are associated with human and animal pathogens (Prescott, MacInnes, & Wu, 2016). Additionally, many *Clostridium sensu stricto* species are also associated with plants (Li et al., 2023). These four ASVs likely arose from environmental sources as they were not present in the majority of fresh samples.

Influence of sample day on fecal microbiota

Cheetah fecal bacterial species richness and diversity did not vary over the time course of this study. This outcome is similar to that reported for domestic cat fecal samples stored at ambient temperature for four days (Tal et al., 2017) and captive mink fecals sampled for five days (Lafferty et al., 2022). We expected species richness to first decrease as the community switched from an anaerobic (inside the host gut) environment to an aerobic (outside the body) environment followed by an increase as environmental bacteria began to colonize the excreted feces. In general, we observed evidence for this pattern, but also that the number of bacterial taxa remained stable and did not increase. Our data are contrary to previous studies in herbivores, where species diversity was highest in fresh samples followed by a decline in diversity over time in horses (Beckers et al., 2017) and springbok and giraffe (Sebastian Menke et al., 2015). This may be due to inherent differences in host microbiota between carnivores and herbivores. The carnivore gut microbiome is less diverse than herbivores due to their simpler gut and high protein diet, comprising different core bacteria that may have different stability once exposed to the environment post-defecation (Ley et al., 2008).

In the first 24 hours after defecation there were minor shifts in Phyla relative abundance and some small changes in microbial compositional dispersion. However, large scale significant changes did not occur until Day 2. After Day 2, subsamples deviated and were no longer representative of the original fresh sample. By Day 3, fecal subsamples had changed so drastically, the individual fecal ID could no longer be identified as shown in the PCoA and microbial network analyses (Figures 3 and 4). Similarly, large shifts in community composition over time were not observed in studies of other fecal microbiota time series analyses including up to 12 hours in bats (Fofanov et al., 2018), multiple days in domestic cats (Tal et al., 2017) and springbok and giraffes (Sebastian Menke et al., 2015), or a week or more in humans (Dominianni, Wu, Hayes, & Ahn, 2014; Lauber, Zhou, Gordon, Knight, & Fierer, 2010). The variation in fecal community composition stability across all these species should be noted as host demographics (age, sex, reproductive and health status, etc.) digestive tract type (a complex system in an herbivore vs a simple system in a carnivore), diet, and environmental factors all likely play a role. Taken together, these data indicate that fecal microbial communities are robust within at least a 24-hour time period and are less susceptible to perturbations than first assumed.

For researchers collecting feces in the field, it is extremely challenging to collect fresh fecals, especially from species that are dangerous or elusive. Identifying a time point cut off for fecal collection as a proxy for gut microbiota is useful for captive and field researchers alike. However, it can be difficult to ascertain the age of fecals if the defecation event was not witnessed. Throughout this collection we discovered characteristics that may be helpful in these such situations. Fresh feces are warm, soft, high in moisture and insect activity. By 24 hours, the fecal is no longer warm and has formed a thin surface crust around the outside. While the fecal has lost some moisture and insect activity is reduced, there is still considerable moisture inside the fecal, almost a taffy like consistency.

The inside consistency and overall fecal color were the biggest differences between the 24-hour and 48-hour fecals. At 48 hours the fecal appeared lighter in color and the inside of the fecal was not as moist, resulting in a more crumble-like texture. These are some characteristics that may help researchers identify a fresher fecal both in captive habitats and in the wild. Further research is needed to determine whether microbial biomarkers exist (e.g., (Muletz-Wolz et al., 2021)) and when they appear or disappear over time when fecals are exposed to the natural habitat of wild cheetahs.

Two additional patterns in fecal microbial diversity and composition were observed that hint at age and gut health as drivers of microbial variation. Samples from the two reproductive-aged adult females in our study (Aju 2 and 6, approximately six years old) exhibited greater microbial diversity and less compositional shifts over time relative to samples from healthy juveniles approximately one year of age. Little is known about age differences in fecal microbiota of cheetahs, but our data agrees with previous studies in domestic cats showed detectable differences between the fecal microbiota of adults and kittens (Bermingham et al., 2018). While the juveniles in the current study were weaned, they were still pre-pubertal and may have a transitory microbiome between cub and adult. The two juvenile individuals that exhibited GI distress and diarrhea (Aju 7 and 8) during sample collection had species richness on the lower end of the juvenile scale and were also compositionally similar to each other but distinct from all other individuals. A previous three-year longitudinal study on cheetah fecal microbiomes reported decreased species richness, shifts in microbial membership, and greater variation over time in a cheetah that became ill with vomiting and diarrhea during the study (Becker et al., 2015). These data also agree with previous reports in domestic cats finding fecal microbial differences between healthy cats and those with GI inflammation in domestic cats (Honneffer, Minamoto, & Suchodolski, 2014; Janeczko et al., 2008) and other mammals (Bragg et al., 2020; Keady et al., 2021). Interestingly, both the adults and GI-unhealthy sample series were more consistent over time and these factors would be of interest for future studies.

Influence of environmental factors on microbial community structure and composition

Larger amounts of rainfall between sampling time points caused larger shifts in microbial community composition. This agrees with current literature where moisture or rainfall impacted microbial composition and diversity in fecal deposits in wolves (Wu et al., 2017), cowpats (Wong et al., 2016) and giraffe and springbok (Sebastian Menke et al., 2015). Just as the drying out of the fecal itself is likely to impact microbial diversity and composition, additional added moisture from the environment is also likely to have an effect. When samples had been exposed to precipitation, by Day 5 many subsamples had started to grow fungus on the outer parts of the feces. The relationships between fungi and bacteria are beyond the scope of this study, but it cannot be understated the influence that these new fungi may have on the bacteria in the fecal (Deveau et al., 2018). In addition, the physical aspect of rain breaking apart the feces, disturbing the soils around the feces, or causing water runoff to pass by the feces are all likely to contribute to microbial shifts over time.

While fresh samples will always be preferred, based on our data we believe collecting cheetah feces up to 24 hours post-deposit is acceptable for fecal microbial research. We predict that these recommendations will hold for other large carnivores, particularly the large cats of the Family *Felidae*, though empirical studies are needed to confirm this prediction. If samples are collected within the first 24 hours, it is unlikely there will be large differences in microbial diversity or community composition and structure, and community signatures distinguishing individual hosts should be retained, especially in adults. These findings will greatly reduce the resources needed and create a safer, more passive sampling strategy for both *ex situ* and *in situ* cheetah gut microbial ecology. *Ex situ* samples may be collected during routine daily enclosure cleaning instead of requiring extra time to observe animals for defecation or using a more invasive rectal swab technique. Additionally, *in situ* collections are more feasible with a longer sampling period and again may use alternative methods to trapping and rectal swabs. However, rainfall should be considered, as heavy rain between defecation and sampling may increase microbial community shifts. If fecal samples need to be collected past 24 hours post-deposit, we recommend additional studies on the effects of age, sex, location, and GI health on cheetah fecal microbiota to build a more thorough understanding of microbiome stability over time.

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CHAPTER 3: Fecal Microbiota is More Stable and Diverse in Captive Namibian Cheetahs (Acinonyx jubatus) Compared to Cheetahs in the US

Abstract

The relationships between gut microbiota and animal health is an important consideration that is increasingly influential in the management of wild and *ex situ* endangered species, such as the cheetah (*Acinonyx jubatus*). To better understand this relationship, fresh fecal samples are currently required as a non-invasive alternative for the gut microbiome. Unfortunately, fresh samples are challenging to collect, especially in the wild. This study had two main aims: the first was to determine the optimal collection time point for cheetah feces after deposit in their native environment of Namibia as a guide for wild cheetah fecal microbiome studies; and the second was to characterize and identify discernable alterations in cheetah fecal microbiota between two *ex situ* cheetah populations from two locations (Front Royal, VA, USA and Otjiwarongo, Namibia) and consuming different diets (commercial diet + carcass supplements and carcass only, respectively). Fresh fecal samples were collected from 16 cheetahs (Virginia, n = 8; Namibia n = 8) and analyzed for bacterial content using 16S rRNA gene sequencing. First, we found no difference in bacterial richness, diversity, or community composition from fresh fecal material compared to subsequent samples from decomposing fecals over a four-day sampling period in Namibia. Second, fresh cheetah samples in semi-wild conditions in Namibia had higher number of bacterial taxa, more phylogenetically diverse bacteria and were compositionally distinct (Bray-Curtis dissimilarity, Jaccard, and unweighted UniFrac measures) from cheetahs in zoo-managed conditions in the USA. We also found few differences in predictive functions of the fecal microbiota between the populations, where only one disease-related pathway was higher in the USA samples. Overall, our findings suggest that fecals in dry season conditions (no recorded rainfall) in Namibia, may be usable for up to four days after defecation for microbial ecology studies, but in rainy season or high humidity

conditions we recommend shorter time periods (≤ 24 hours) based on our previous research. Secondly, there are major differences between *ex situ* Namibian and US populations, and we suggest further investigation into the influence of diet and population on the gut microbiota and health of cheetahs.

Introduction

Microbiome studies are becoming an essential part of conservation biology. There is a plethora of evidence supporting the role of gut microbiomes in wildlife health and survival [1-6]. Many microbiome studies on non-domestic animals focus on *ex situ* individuals because it is easier to control experimental variables and ensure timely and accurate sample collection. However, to obtain a better understanding of the complex dynamics between hosts and their gut microbiomes it is also important to study animals in their natural habitat [7]. The current gold standard for non-invasive gut microbiome studies is to collect fresh fecal samples to characterize fecal microbiota as a stand-in for gut microbiota. Unfortunately, collecting fresh feces in the wild is quite difficult for many elusive, dangerous, or far-ranging species, including the cheetah.

Due to the difficulty in collecting fresh fecal samples, more studies have started investigating the temporal stability of fecal microbes post-defecation. These studies aim to characterize the shifts that occur in the fecal microbiota once they are exposed to the environment and to identify the timepoint after which the fecal microbiota no longer represent that of a fresh sample. Many studies found evidence for shifts in microbial diversity or composition within five or less days of excretion [8-11], including our previous study on cheetahs in the USA that demonstrated changes within one day post excretion when in moist conditions (Chapter 2). Other studies saw no changes at all in their study period for up to four days [12]. Because of the large variation in timeline of fecal microbial changes, there is no general rule for how fresh is ‘fresh enough’ in fecal microbiome studies. It is often recommended that temporal experiments be performed for each new species, but we suggest that environmental conditions should also be considered (Chapter 2). Our previous study on fecal microbiome stability was with *ex situ* cheetahs in a US facility, but there is still no information on the fecal microbial stability of cheetahs in their native environment. Before we can compare fecal microbiomes of wild and *ex situ* cheetahs through non-invasive fecal collection, we need a better understanding of how long fecal samples remain stable in the arid and hot climate of Namibia, within the natural range of wild cheetahs.

While microbiome studies in the wild provide important clues for conservation, microbiomes are also relevant for *ex situ* wildlife. There has been recent concern for potential dysbiosis of managed animal microbiomes as gut flora can be shaped by captivity through biotic and abiotic factors such as diet [1, 13, 14], veterinary care [15, 16], exposure to humans and the built environment [17-20], reduced exposure to conspecifics [21], and higher density of animals that may not naturally be in proximity to each other [22, 23]. One pertinent example for managed cheetahs includes their high incidence of gastrointestinal (GI) diseases that contribute to the majority (40-60%) of deaths and euthanasia in captivity [24-27]. Given its involvement in GI health and inflammatory responses in many other species, there is a growing interest in characterizing the gut microbiome of *ex situ* cheetahs for comparison to those in the wild. Lower species diversity and increased temporal microbial variation have been reported in *ex situ* cheetahs suffering from GI distress, compared to those that are healthy [28]. These findings are similar to reports comparing healthy and GI-inflamed domestic cats [29, 30]. Previous studies in cheetah gut microbiomes suggest there may be population differences across wild Namibian [31, 32], European *ex situ* [33], and US *ex* *situ* (Chapter 2) cheetahs. These existing studies span a variety of collection, processing, and analytical methodologies, making it difficult to make direct comparisons.

Due to difficulty in collecting fresh fecal samples from the wild, it is difficult to compare the fecal microbiota of wild and *ex situ* cheetahs. Collecting samples from cheetahs in a managed facility within their native home range offers a next-best opportunity. Cheetahs at the Cheetah Conservation Fund (CCF) in Namibia live in large outdoor-only enclosures and are exposed to the same environmental elements as those in the wild. Cheetahs at CCF consume whole carcass diets more similar to wild diets than the commercial ground meat-based diets of most US-based facilities. In the absence of fresh wild cheetah samples, a comparison of fresh samples from CCF would provide a first glimpse as to how the gut microbiomes of cheetahs in their native environments may differ from that outside of their range countries.

In this study, we had two objectives. The first objective was to determine how many days after defecation the fecal microbiota remained representative of a fresh fecal in managed cheetahs living in Namibia as a proxy for wild cheetah fecal microbiome sampling. Our second objective was to investigate the differences in fresh fecal microbiota between cheetahs in managed facilities in the US compared to those in Namibia to understand the effects of environment and diet on the cheetah gut microbiome.

Methods and Materials

Sample collection

Fecal microbial stability

We collected eight fresh fecal samples from cheetahs living at the CCF in Otjiwarongo, Namibia. These cheetahs were wild born but brought into CCF for various reasons and deemed unfit for re-release. Cheetahs lived in outdoor-only enclosures with native trees and plants for cover. They were fed a raw diet of butchered donkey and horse carcasses with an added vitamin supplement (Predator Powder, V-tech, Midrand, South Africa) and fresh water was available *ad libitum*. Fresh fecals were collected over the course of four days between 9 am and 5 pm. All defecation events were witnessed by the author or staff. Fecals were removed from the enclosure within 30 minutes and placed in a nearby field used as the experiment plot to allow ease of access for continued subsampling. We removed a one-inch subsection from the end of the fresh fecal with a sterile scalpel and subsampled the interior core of the feces piece. The first subsample was considered Day 0 (fresh). The remainder of the feces was placed back in the experimental plot. We positioned a screen window over the top of the feces to prevent dung beetles, and other animals from taking the remaining feces, but allowing other natural environmental exposure processes to occur. Every twenty-four hours the subsampling procedure was repeated for four days (Day 1 – 4) or until the feces ran out (Table 1). After each collection, we placed subsamples in a -20°C freezer until processing. Samples were labeled using an abbreviation for Namibia, NAM, to make combining samples from the US more intuitive.

Comparison of fresh samples from two populations

To compare the microbial ecology of two cheetah populations we used only the fresh subsamples from the above Namibian (NAM) collections. The eight fresh NAM samples collected above were also used in this study. In addition, we used eight fresh subsamples from a similar and previously published study collected from the Smithsonian's National Zoo and Conservation Biology Institute (Front Royal, VA, USA) as described (Chapter 2). Samples from the US-based study were renamed from the Aju moniker (Aju1, Aju2, etc.) to USA (USA1, USA2, etc.) to indicate population origin when comparing with Namibian samples.

Sample DNA Extraction and Library Prep

DNA was extracted from 0.25 g frozen feces using the QIAamp PowerFecal DNA Kit (#12530-50, Qiagen, MD) following manufacturer's instructions. For each batch of sample extractions, an empty negative control was included to identify extraction contaminants. Following extractions, DNA concentrations were measured via fluorometric quantification (Qubit4 Fluorometer, ThermoFisher Scientific, MA).

Fecal bacterial DNA were amplified following a previously published two-step polymerase chain reaction (PCR) protocol with dual-index paired-end Illumina sequencing (Keady et al., 2021). The amplicon PCR reaction amplified the V3-V5 region of the 16S rRNA gene using universal primers 515F-Y (GTGYCAGCMGCCGCGGTAA) and 939R (CTTGTGCGGGCCCCCGTCAATTC)[34]. PCR reactions for each sample were done in duplicates, including the negative extraction and PCR controls, then pooled, and cleaned with magnetic beads. After the first clean up, samples were indexed with i5s and i7s, cleaned again, quantified, and pooled as specified in Keady et al.[35]. The target band for the 16S rRNA library (~578 base pairs) was isolated and removed using a QIAquick Gel Extraction Kit (#28704, Qiagen, MD) and diluted to 4 nM. All samples were sequenced using an Illumina MiSeq (v3 chemistry: 2 x

300 bp kit) at the Center for Conservation Genomics, Smithsonian National Zoo and Conservation Biology Institute.

Sequence Data Processing

Fecal microbial stability

Demultiplexed Illumina Miseq sequencing reads were imported into R version 4.0.3 [36](R core team, 2022) using RStudio (v 2022.12.0+353). We utilized R package "*dada2*" version 1.16.0 [37, 38] to merge paired ends, remove chimeras, and filter out low quality reads (maxEE > 2). Filtered and merged sequences from two sequencing runs were combined to generate amplicon sequence variants (ASVs) and assign taxonomy using the Ribosomal Database Project (RDP; [39]) 16S training set (set 16, release 11.5). A phylogenetic tree was built using Quantitative Insights Into Microbial Ecology 2 (vQIIME2-2020.8; [40] using FastTree [41]. We imported the ASVs, taxonomy table, phylogenetic tree, and metadata into a phyloseq object [42] for processing. We removed putative contaminant sequences using the combined Fisher method with a threshold of 0.1 in the R package "*decontam*" (v1.18.0; [43]. Three contaminant sequences were removed. After contaminants were removed, we filtered out singleton ASVs (ASVs that occur in only one sample), ASVs classified as Cyanobacteria, negative control samples, and low sequence count (< 71,010 reads) samples. After quality control and filtering, the sequencing depth variation (max/min) was 4.6 fold change (max = 330,101, min =71,010).

Comparison of fresh samples from two populations

For population comparisons, sequence reads from only the fresh Namibian (NAM) and USA (USA) samples were combined into a phyloseq object after filtering and merging and were assigned taxonomy together using Quantitative Insights Into Microbial Ecology 2 (vQIIME2-

2020.8; Bolyen 2018) using FastTree [41]. We split the phyloseq object into two based on extraction location for *decontam* to remove contaminant sequences separately. The number of contaminants removed were three and one from the fresh Namibian (NAM) and USA samples, respectively. After *decontam*, data were merged into a final clean phyloseq object. After cleaning, we compared sequencing depth between locations. We found sequencing depth variation (max/min) was 14.3-fold higher in the NAM samples (max =201,217, min =14,110). Based on current literature [44], we rarefied all samples to the lowest sequencing depth (14,110).

Statistical Analyses

Statistical analyses were performed in RStudio for R. Significance for all analyses was set to $p < 0.05$ and we adjusted p-values for multiple comparisons using Bonferroni. Analysis pipelines for characterizing microbial structure and composition were based on previous research in our laboratory [35, 45-47]. For both studies, comparisons across sampling days and between two populations, we conducted three metrics of microbial diversity which included alpha diversity (within sample variation), beta diversity (between sample variation), and the changes in relative abundance at the ASV and Phyla levels. For comparisons of fresh samples by population we also predicted functional pathways based on marker gene sequences using PICRUSt2 and linear discriminant analyses effect size using LefSe.

Fecal microbial stability

Fecal IDs NAM1 and NAM6 were omitted from this objective, due to an incomplete series collection (Table 1). Relative abundance was measured using the package *phyloseq* (ref) using the function tax_glom() at the phyla level and merge_samples() function by Sample Day. To find the most appropriate differential abundance test for our data, we used the package DAtest [48]. Before running DAtest, we prefiltered our data to reduce the false discovery rate (min.samples = 10, min.reads = 10) which resulted in 155 taxa for testing. We used the function testDA() on the prefiltered dataset and found the top three tests based on their AUC and FPR values. The tests we used on the full dataset collapsed to the phylum level (tax_glom() function) were MetagenomeSeq ZIG, EdgeR quasi-likelihood with TMM normalization, and EdgeR quasi-likelihood with RLE normalization. All three tests were performed on the unfiltered dataset, the phyla were considered differentially abundant if it was significant in two of the three tests.

Alpha diversity metrics included species richness (SR) and Faith's phylogenetic diversity (PD) over time (sample days). Species richness is the number of unique ASVs in a sample and PD measures the amount of biodiversity based on the phylogenetic relationships of the taxa and the total tree branch length of ASVs in a sample [49, 50]. Faith's PD was calculated for each subsample with the R package "*picante*" [51]. Using the "*lme4*" R package [52], we performed mixed effects linear models, with SR or PD as the response variable, sample day as a fixed effect, and fecal ID as the random effect. SR and PD distributions met assumptions of normality (Shapiro-Wilk) and homoscedasticity (Levene).

To identify differences in community composition between subsamples across sampling days we measured Bray-Curtis (abundance weighted taxa), Jaccard (presence-absence of taxa), and unweighted UniFrac (presence-absence with inclusion of phylogenetic relationships of taxa) distances. We used PERMANOVAs [53] in the package "*pairwiseAdonis*" [54] using the adonis2() function where Bray-Curtis, Jaccard, and unweighted Unifrac distances were the response variable, sample day was the explanatory variable and fecal ID was the random effect. *Post hoc* analyses were performed in the same package using the pairwise.adonis2() function and *p*-values adjusted using

Bonferroni. To identify whether dispersion of microbiota composition differed among sample days we used PERMDISP from the package *"vegan",* betadisper() function [55]. The Bray and unweighted UniFrac distance values between samples were also used in a sample-wise microbial network to identify connections between samples. We created an igraph microbiome network using the make_network() and plot_network() functions in the "*phyloseq*" package. Maximum ecological distance between two samples was set to the default value 0.7, which allowed all samples to appear in the network but minimized over-connectedness.

Comparisons of fresh samples from two populations

Relative abundance, microbial diversity, and composition

We measured species richness (SR) and Faith's phylogenetic diversity (PD) between the two populations as defined above. Using the "lme4" R package (Bates et al., 2015), we performed mixed effects linear models, with SR or PD as the response variable and Population (NAM, USA) as a fixed effect. SR and PD distributions met assumptions of normality and homoscedasticity (Levene). For beta diversity, we again used the same metrics as described above to identify microbial compositional differences between the two populations. We used PERMANOVAs [53] in the package "*pairwiseAdonis*" where Bray-Curtis, Jaccard, and unweighted Unifrac distances were the response variable and population (NAM, USA) was the explanatory variable. To identify whether dispersion of microbiota composition differed between the populations we used PERMDISP from the package *"vegan",* betadisper() function.

Enriched microbial taxa analysis

We used Linear Discriminant Analysis (LDA) Effect Size (LEfSe) [56] in the R package "*microbiomeMarker*" [57] to identify microbial taxonomies enriched in one of the two populations.

Minimum LDA score was set to 4 to detect microbial features significantly different between the two cheetah populations.

Predictive functional analysis using PICRUSt2

To identify potential functional relevance of the microbial community differences between the two cheetah populations we used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2, Version 2.5.1; [58]). We used the Kyoto Encyclopedia of Genes and Genomics (KEGG) database to assign predicted functions and metabolic networks of the bacterial communities [59]. Statistical analyses were performed in the Statistical Analysis of Taxonomic and Functional Profiles (STAMP v2.1.1) software [60] to identify significant functional KEGG groups at three classification levels between the two populations. We performed Welch's t-tests to compare functional groups between the populations and adjusted p-values using the Benjamini-Hochberg false discovery rate (FDR) method to account for multiple hypothesis testing. Results were reported as the mean ± standard deviation of the proportion of sequences assigned to the category by population (USA or NAM). Significance was set to $p < 0.05$.

Results

Fecal microbial stability

We obtained 4,386,995 high-quality sequences from 27 samples (Table 1). Individuals NAM 1 and NAM 6 were dropped from the degradation study because of low sequence yields in subsequent samples which led to non-consecutive sampling. The average number of sequences per sample was 162,481 (range 71,010 – 330,101). There were 525ASVs from eight total phyla including Firmicutes, Bacteroidetes, Fusobacteria, Actinobacteria, Proteobacteria and to a lesser extent (≤ 2 ASVS) Candidatus Saccharibacteria, Chloroflexi, and Deinococcus Thermus (Figure B.1). The six fresh subsamples are the best representatives of the cheetah gut microbiota and they consisted of 179 ASVs from five Phyla including Firmicutes (39.08% mean abundance, 105 ASVs), Bacteroidetes (31.94%, 34 ASVs), Fusobacteria (16.31%, 13 ASVs), Actinobacteria (9.65%, 14 ASVs), and Proteobacteria (3.02%, 13 ASVs). None of the phyla were differentially abundant across sample days.

Table 3.1 Sample collection summary for each fecal sample series. Some samples were small and did not have enough fecal matter to last four days (NAM4-NAM7), while others completed the series but subsamples did not yield adequate sequences for analyses (NAM1 and NAM6)

Fecal Sample ID	Day 0 (fresh)	Day 1	Day 2	Day 3	Day 4
NAM 1	💩				💩
NAM 2	💩	💩	💩	💩	💩
NAM 3	💩	💩	💩	💩	💩
NAM 4	💩	💩	💩	💩	
NAM 5	💩	💩	💩	💩	
NAM 6	💩		💩	💩	
NAM 7	💩	💩	💩	💩	
NAM 8	💩	💩	💩	💩	💩

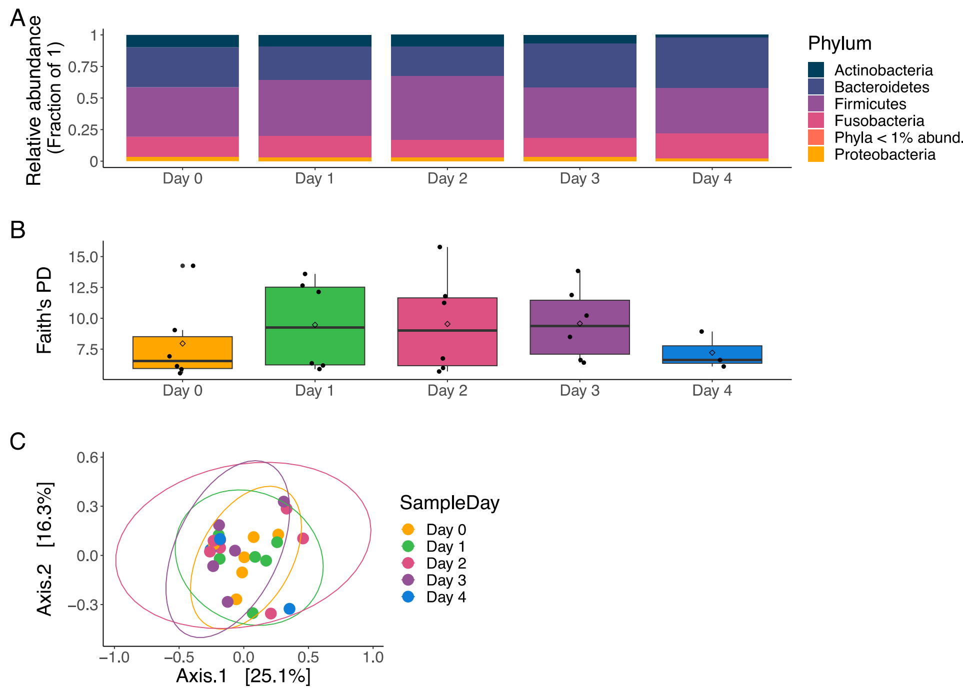


Figure 3.1 Namibian (NAM) cheetah fecal microbiota did not change from fresh sample day across any other sample day (colors) for **(A)** relative abundance, **(B)** alpha, or **(C)** beta diversity metrics. **(A)** Relative abundance of dominant bacterial Phyla in NAM cheetah feces across sample day. Phyla with < 1% relative abundance were grouped together. No phyla were differentially abundant across days. **(B)** Faith's phylogenetic diversity for NAM feces across sample day. PD did not change across sample days (GLMM: x^2 = 3.1438, *df* = 4, p =0.534). (C) Bray-Curtis dissimilarity by sample day. Overall model was significant (PERMANOVA Sample Day: Bray-Curtis Psuedo-$F_{4,22}$ = 0.9263, R^2 =0.14415, p = 0.026), but pairwise data show later days (Days 1-4) were not different from Day 0 (fresh).

Influence of sample day on fecal microbiota

Over time, bacterial communities were similar in microbial diversity but differed in abundance weighted microbial composition. Within samples, both species richness (Figure B.1) and Faith's phylogenetic diversity (Figure 1) remained similar across sample days. Bacterial SR was 163.67 ± 45.53 and PD was 7.96 ± 3.33 at Day 0 with minimal changes thereafter. Microbial composition did vary over time for abundance weighted composition (PERMANOVA Sample Day: Bray-Curtis Pseudo-$F_{4,22}$ = 0.9263, R^2 =0.14415, p = 0.026; Figure 1) but did not change over time in presence absence measures (PERMANOVA Sample Day: Jaccard Pseudo-$F_{4,22}$ = 0.4545, R^2 = 0.07632, p = 0.24, UniFrac Pseudo-$F_{4,22}$ = 0.4595, R^2 = 0.07711, p = 0.099; Fig B.1). Pairwise post hoc analyses focused on Day 0 (fresh) compared to subsequent days (Days 1-4). In our post hoc comparison of the abundance weighted composition by sample days, all of the later days (Days 1-4) were similar to Day 0 (post hoc Bray-Curtis *p adj* > 0.125 (Day 0 vs Day 3)). Dispersion of community composition were similar across sample day groups (PERMDISP: Bray-Curtis p = 0. 689, Jaccard p = 0.4528, unweighted Unifrac p =0.71). We built microbial networks based on distances calculated using Bray-Curtis (Figure B.2) and unweighted UniFrac measures to visualize compositional changes over time. Day 0 (fresh) samples were tightly associated with their subsequent day samples. Clustering seemed strongest within individual but, overall, most samples were generally overlapping in composition.

Comparisons of fresh samples from two populations

Relative abundance, microbial diversity, and community composition

For the fresh sample comparisons, after rarefying by lowest sequence sum, we obtained a total of 225,760 high-quality sequences from 16 samples. There were 259 ASVs from five phyla including Firmicutes, Bacteroidetes, Fusobacteria, Actinobacteria, Proteobacteria (Figure 2). Only Firmicutes was significantly different between the two populations (NAM 40.6% [114 ASVs] vs USA 58.9% [80 ASVs].

Alpha diversity measures were higher in the Namibian samples in both SR and PD (ANOVA; SR: $F_{1,14}$ = 34.62, p = 3.98e-05 and PD: $F_{1,14}$ = 20.34, p = 0.00049; Figure 3). Species richness and PD for were NAM 160 ± 39.4 vs USA 73.4 ± 13.6 and NAM 6.86 ± 1.86 vs USA 3.85 ± 0.33, respectively. Beta diversity measures also varied by population (PERMANOVA; Bray-Curtis Pseudo-$F_{1,14}$ = 5.1923, R^2 = 0.27054, p = 0.002; Jaccard Pseudo-$F_{1,14}$ = 9.3389, R^2 = 0.40014, p = 0.001; Unweighted UniFrac Pseudo-$F_{1,14}$ = 10.068, R^2 = 0.41832, p = 0.001; Figure 4). Dispersion of the samples within a group around the centroid were determined to be similar across populations (PERMDISP: Bray-Curtis p = 0. 4144, Jaccard p = 0.5658, unweighted Unifrac p =0.6836).

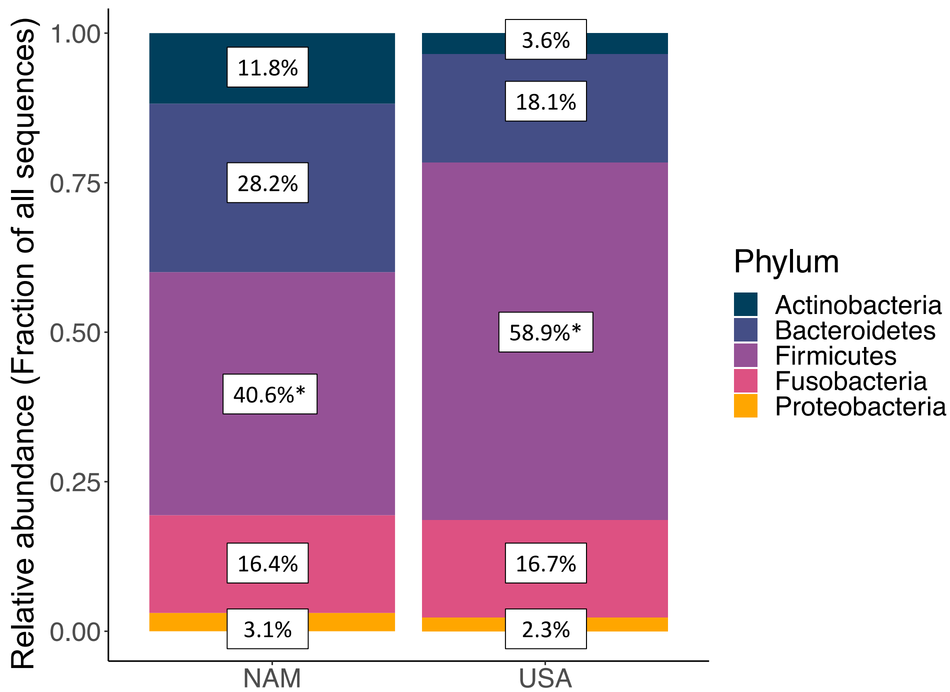


Figure 3.2 Relative abundances (fraction of total sequences) of phyla for NAM and USA cheetah populations. Asterisk denotes statistical differentiation ($p < 0.05$) between the two populations at the marked phyla.

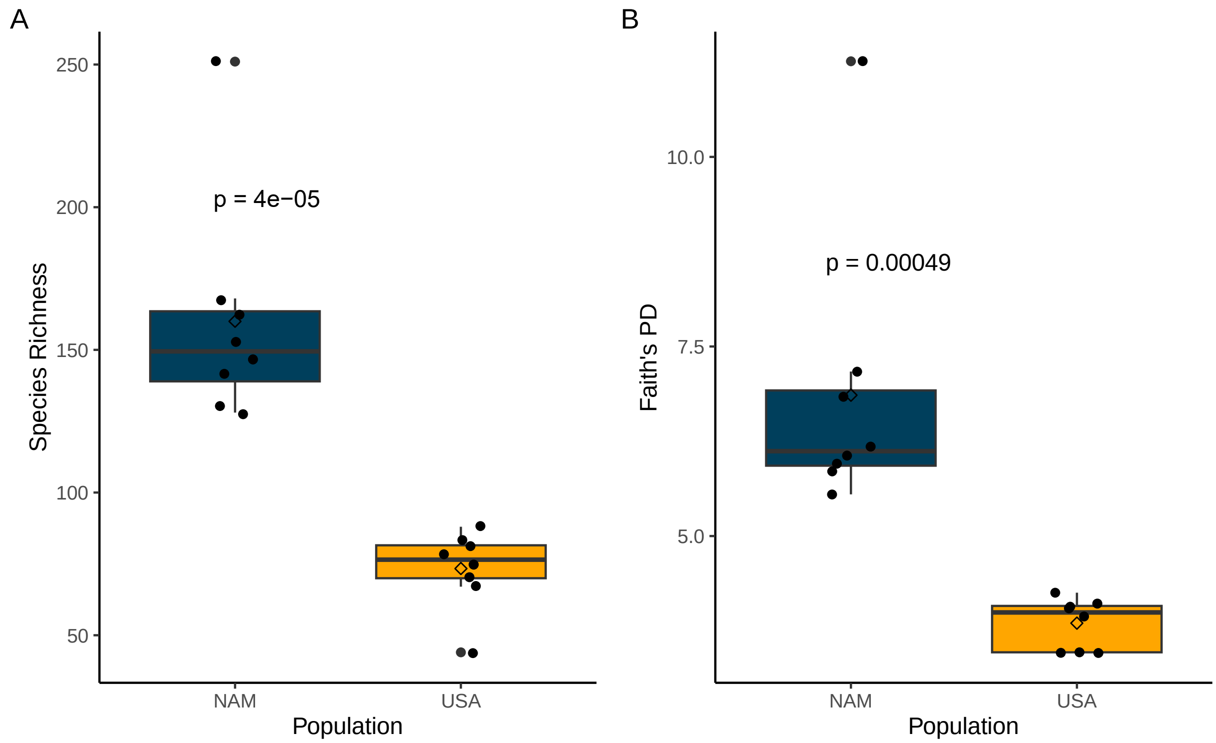


Figure 3.3 Box plots for **(A)** species richness and **(B)** Faith's PD of fecal samples grouped by population. USA samples were lower in both models (ANOVA).

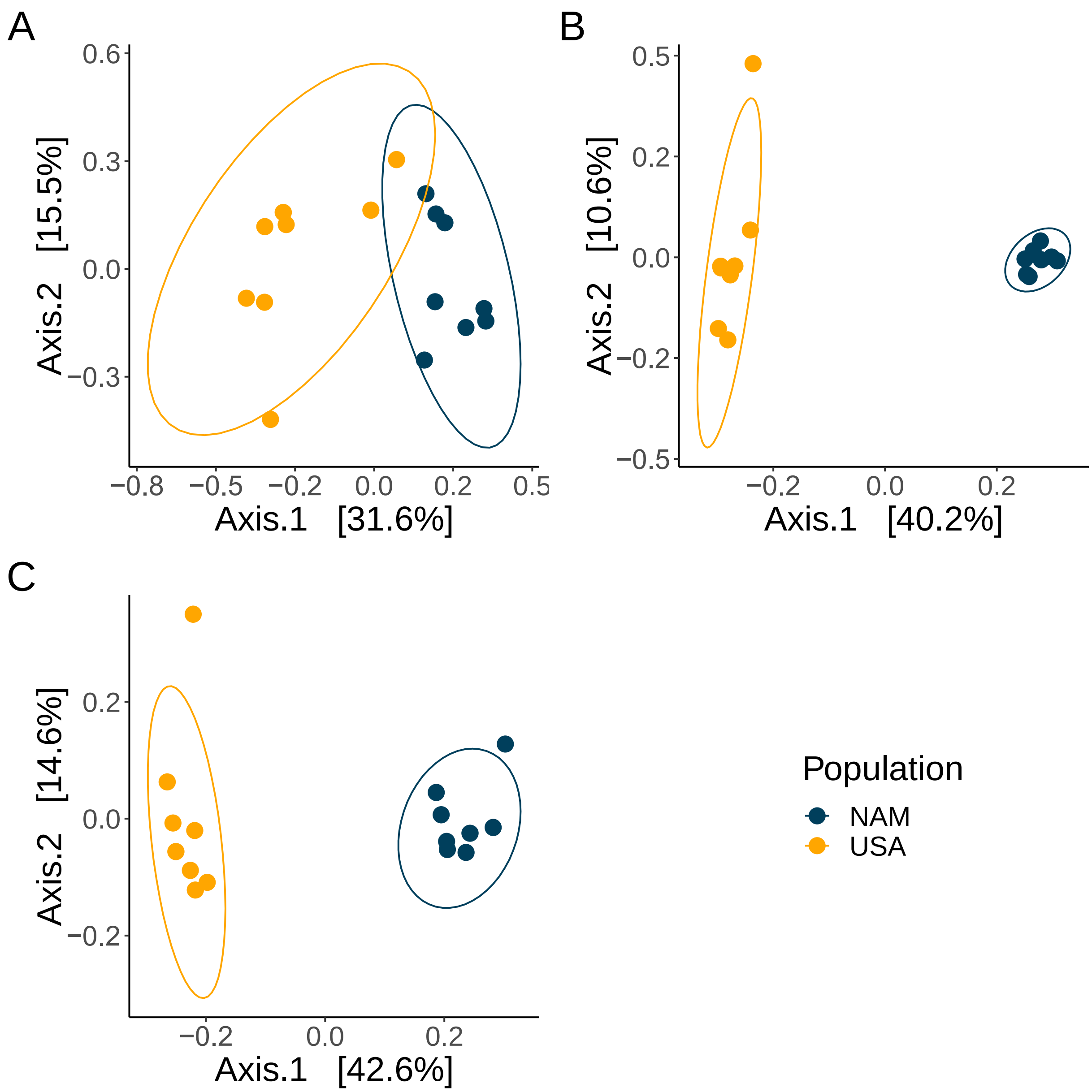


Figure 3.4 Principle coordinate analysis ordination plots for **(A)** Bray-Curtis dissimilarity, **(B)** Jaccard dissimilarity, and **(C)** Unweighted UniFrac distances with 95% confidence ellipses by population.

Enriched microbial taxa analysis

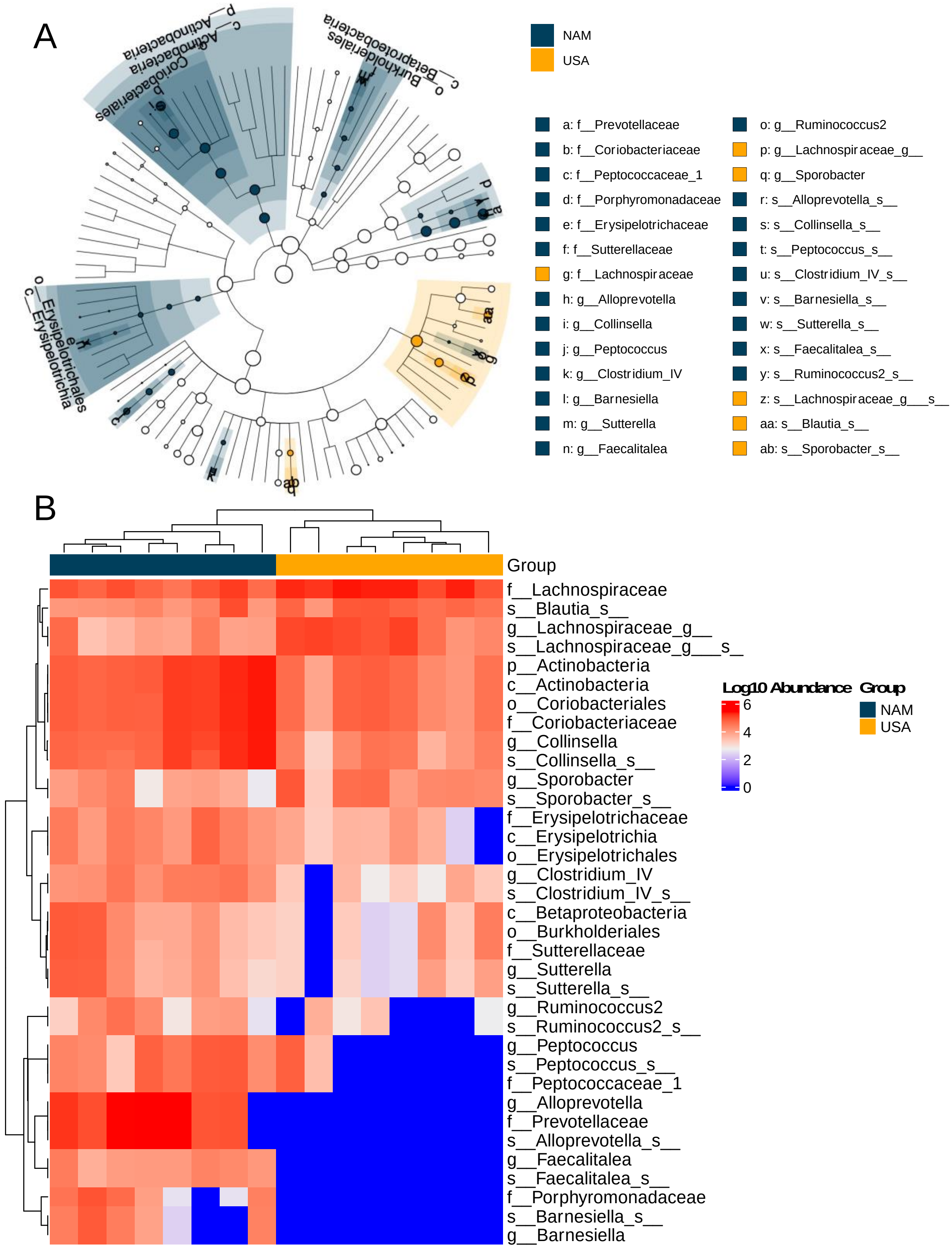
Overall, NAM samples had 127 ASVs not found in the USA samples, while the USA had 43 unique ASVs. The two populations shared a total of 89 ASVs. However, when these ASV counts were weighted by abundance 76% of the sequences were shared between the two populations. Even after weighting the abundance of the ASV sequences, the NAM population still had a greater number of unique sequences (17%) compared to the USA population (6%).

Using LefSe LDA we found 35 specific taxa that were more common in either the NAM (29 taxa) or USA cheetahs (6 taxa; Figure 5A). Hierarchical clustering of samples based on abundance of these 35 taxa cleanly delineates NAM versus USA samples (Figure 5B). Many of the taxa that were enriched in the Namibian samples belonged to one of three clades, Actinobacteria, Erysipelotrichia, and Betaproteobacteria. The six taxa that were enriched in the USA cheetahs, were also found in the NAM cheetahs, but at lower amounts. By contrast, eight of the taxa present in the NAM cheetahs were missing completely from all of the USA samples (Figure 5B). These eight taxa were all members of the Bacteroidetes (6) or Firmicutes (2) Phyla.

Predictive functional analysis using PICRUSt2

PICRUST2 predictive functional analyses indicated that for both populations the Level 1 KEGG Orthology (KO) category with the highest proportion of sequences among the samples are associated with metabolism (USA 78.27% ± 1.53% and NAM 78.89% ± 1.02). None of the level one KEGG Orthology (KO) categories differed between the two populations. For level 2 KO category comparisons, the proportion of sequences associated with parasitic infectious disease were higher in the USA samples ($p.adj$ < 0.047) though the total proportion of sequences attributed to this category for both groups was small (USA 0.02% ± 0.005% and NAM 0.006% ± 0.003%; Figure 6). Among the most specific categories, Level 3, there were no pathways that differed between the two cheetah populations.

Figure 3.5 Linear Discriminant Analyses Effect Size (LDA LefSe) of bacterial taxa between Namibian (NAM) and US (USA) *ex situ* cheetahs with LDA scores ≥ 4. **(A)** Cladogram depicting phylogenetic relationships of enriched taxa by population (blue = NAM, yellow = USA); **(B)** Heatmap of enriched taxa in rows with individual cheetah samples as columns. Samples are clustered by both taxa and sample similarities. Log10 Abundance is indicated by color where darker red is highly abundant (6) and dark blue is absent (0).



A
NAM
USA
a: f__Prevotellaceae
b: f__Coriobacteriaceae
c: f__Peptococcaceae_1
d: f__Porphyromonadaceae
e: f__Erysipelotrichaceae
f: f__Sutterellaceae
g: f__Lachnospiraceae
h: g__Alloprevotella
i: g__Collinsella
j: g__Peptococcus
k: g__Clostridium_IV
l: g__Barnesiella
m: g__Sutterella
n: g__Faecalitalea
o: g__Ruminococcus2
p: g__Lachnospiraceae_g__
q: g__Sporobacter
r: s__Alloprevotella_s__
s: s__Collinsella_s__
t: s__Peptococcus_s__
u: s__Clostridium_IV_s__
v: s__Barnesiella_s__
w: s__Sutterella_s__
x: s__Faecalitalea_s__
y: s__Ruminococcus2_s__
z: s__Lachnospiraceae_g___s__
aa: s__Blautia_s__
ab: s__Sporobacter_s__
B
Group
f__Lachnospiraceae
s__Blautia_s__
g__Lachnospiraceae_g__
s__Lachnospiraceae_g___s__
p__Actinobacteria
c__Actinobacteria
o__Coriobacteriales
f__Coriobacteriaceae
g__Collinsella
s__Collinsella_s__
g__Sporobacter
s__Sporobacter_s__
f__Erysipelotrichaceae
c__Erysipelotrichia
o__Erysipelotrichales
g__Clostridium_IV
s__Clostridium_IV_s__
c__Betaproteobacteria
o__Burkholderiales
f__Sutterellaceae
g__Sutterella
s__Sutterella_s__
g__Ruminococcus2
s__Ruminococcus2_s__
g__Peptococcus
s__Peptococcus_s__
f__Peptococcaceae_1
g__Alloprevotella
f__Prevotellaceae
s__Alloprevotella_s__
g__Faecalitalea
s__Faecalitalea_s__
f__Porphyromonadaceae
s__Barnesiella_s__
g__Barnesiella
Log10 Abundance
6
4
2
0
Group
NAM
USA

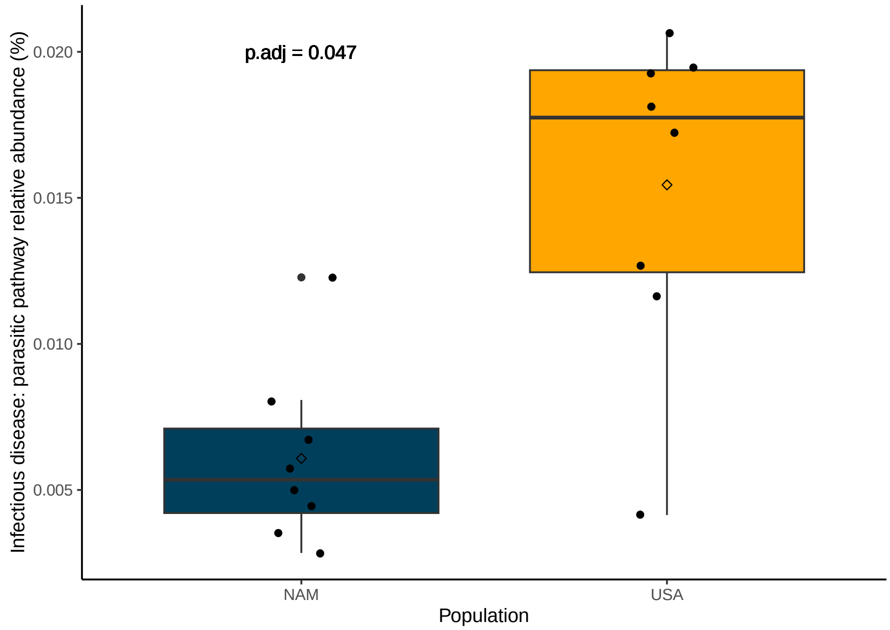


Figure 3.6 Mean proportions of sequences for the Level 2 KEGG Ortholog predictive functional category, Infectious disease: parasitic, of fecal microbial communities between fresh NAM and USA cheetahs using PICRUSt2. Welch's t-test with FDR corrected p-value of 0.047.

Discussion

Wildlife gut microbiomes are of increasing interest because of their roles in overall organismal health. Fresh fecal samples are regarded as the gold standard for gut microbiome studies but are difficult to collect from wild animals. Here we performed two studies to better understand 1) how fresh fecal microbiota change over time when exposed to the natural environment of the cheetah and 2) how *ex situ* cheetahs in a US facility compare to *ex situ* cheetahs living in more natural conditions as a stand-in for true *in situ* populations. In general, we found that the cheetah fecal microbiota was stable in the Namibian environment for up to four days post-defecation and that there were strong microbial differences between the fresh Namibian samples and the fresh US samples that may have functional metabolic and disease-related consequences.

Fecal microbial stability

Influence of sample day on fecal microbiota

In our first aim, we found that after four days exposed to the dry Namibian environment, cheetah fecals were similar in microbial diversity but differed in abundance weighted microbial composition. Fecal microbial membership and diversity did not vary over the duration of our study. These data are similar to findings previously reported in other carnivore species including *ex situ* US cheetahs (Chapter 2), domestic cats [12] and *ex situ* mink fecals [8]. Fecal microbial composition varied only in our abundance weighted measure, Bray-Curtis, which is less sensitive to rare or low abundant ASVs. This means changes in the voided fecals were likely due to more abundant ASVs. Despite the overall changes in abundance weighted community composition, pairwise post-hoc tests revealed all subsequent samples days (Day 1-4) were compositionally similar to Day 0. These patterns of compositional stability are similar to domestic cat samples in tubes at room temperature [12] but longer than previously reported in *ex situ* cheetah fecals sampled during a hot (max daily temperatures 80-91°F) and wet (max cumulative rainfall 0.82 inches) summer season in Virginia, USA (Chapter 2). In the US-based cheetah study, there were shifts in bacterial composition in abundance weighted and presence-absence metrics that occurred by Day 2 post-deposit. The hot (max daily temperatures 92-94°F) and arid (0 inches of rainfall) climate during the dry season in Namibia may aid in stabilizing the post-defecation shifts of the fecal microbiota. There was no precipitation during the sampling period for the current study compared to the US based study which experienced heavy rain during sampling. Similarly, giraffe fecal microbiota composition was stable in the dry Namibian environment until rained occurred later in the sampling series [9]. These data suggest that researchers sampling cheetahs in Namibia during the dry season can collect up to 4-day-old fecal samples as a proxy for studying gut microbiota, which greatly increases the odds of finding and collecting samples.

Comparisons of fresh samples from two populations

Fecal microbial relative abundances

We combined data from the fresh samples of the above Namibian project (NAM) with data from fresh samples at a US facility (USA) from a previous study (Chapter 2). NAM and USA samples consisted of the same main five Phyla previously reported for cheetahs [31, 32](Chapter 2). Four of the five Phyla were similar in abundance across both populations, only Firmicutes differed. Additionally, both populations exhibited greater proportions of Firmicutes compared to Bacteroidetes, as expected in carnivores [61], but NAM samples contained lower overall abundances of Firmicutes. The USA samples may have higher amounts of Firmicutes comparatively because the commercial diet they were fed contained powdered cellulose, which is a carbohydrate that can be converted into smaller sugar molecules by many members of the Firmicutes phylum [62, 63]. Another possibility may be body weight or condition as previous research in domestic cats has shown Firmicutes to be less abundant in obese compared to healthy weight individuals [64, 65]. We did not collect body weight or condition scores on the cheetahs in this study, but the NAM cheetahs were exercised regularly using a lure chase and their diets were monitored, so it is unlikely they would be considered obese, at least in comparison to the USA captive cheetahs. The NAM cheetahs may be less lean than their wild counterparts, however, which would explain the lower overall Firmicutes abundance in the captive NAM cheetahs in this study (40.6%) compared to that reported in wild cheetahs (56.2%, [32] and 68.5%, [31]; not statistically tested across studies). These data also agree with a study on bobcats, where those in zoos trended toward lower Firmicute abundance ($p < 0.1$) compared to wild bobcats [66]. The difference between the current study NAM samples and previously published wild cheetahs may also be a result of different experimental techniques including sample type, sequencing region, and bioinformatics methods in the other wild cheetah studies.

Fecal microbial diversity, composition, and enriched taxa

As expected, we discovered a high degree of variation in the fecal microbial diversity, community, and structure between the two populations. The higher diversity of the *ex situ* NAM fresh samples may be a result of the differences in diet between the two facilities (carcass meat for the NAM vs commercial ground meat as the primary diet for USA). There is evidence that carcass diets contain higher amounts of non-digestible elements which obligate carnivores, including the cheetah, may have evolved to utilize as a source of dietary fiber [67, 68]. This dietary fiber may lead to a healthier gut microbiome in the cheetah as higher gut microbial diversity is generally equated with a healthier GI system in many species including dogs [69] and cats [70]. In general, the *ex situ* NAM cheetahs in this study suffer less from overt symptoms often attributed to GI-related diseases, including gastritis, than the USA cheetahs (personal comm with facility veterinarians and facility health records). It seems likely that diet has a strong influence in the different microbial diversity measures between the two populations, but we were unable to control for all variables including cheetah age, sex, climate, and location. Therefore, a future study focusing on the role of dietary animal fiber from carcass feeding on gut microbiota diversity would be beneficial to cheetah management.

Bacterial community compositions differed between the two populations in all three measures of beta diversity indicating compositional differences were not driven entirely by rare or abundant species. Further, we found 35 taxa that were enriched in either NAM or USA samples. Six taxa were enriched in NAM samples but absent in the USA samples. One of these taxa, *Ruminococcus*, is likely from the diet as there is little evidence for this genus existing in plant or environmental sources and is hypothesized to be primarily host-associated [71]. While NAM cheetahs overall had fewer Firmicutes at the phylum level, they may have the enriched genus *Ruminococcus2* because of their horse and donkey carcass diet. *Ruminococcus* species are common in many ruminant and non-ruminant herbivores as they are excellent degraders of complex polysaccharides like cellulose (ref). The Namibian cheetahs are not fed the GI tracts of the herbivores, but it is possible that some of the microbes are transferred to other parts of the carcass while being processed onsite as the same knife is used for the entire butchering process (verbal communication with staff). *Ruminococcus2* and a second enriched Namibian taxa, *Barnesiella*, have been associated with reduced inflammation in asthma [72], allergic reactions [73], and rheumatoid arthritis in humans (*Ruminococcus2*, [74]. Interestingly, *Barnesiella* was also found to restrict the growth of an anti-biotic resistant bacteria in mice and humans [75]. The presence of these microbes may play a role in the fewer incidences of symptoms related to GI inflammation, including gastritis, in the Namibian cheetahs, but further research is needed to address their roles in the cheetah GI tract.

Predicted functional analysis using PICRUSt2

PICRUSt2 analyses showed approximately 78% of all sequences in both populations were associated with metabolism. This was expected given the microbes reside in the gut where host and microbial metabolic processes take place to utilize digestive material. Across all levels and categories, only parasitic infectious disease (KO Level 2), was different between the two populations. It was unexpected that the USA cheetahs had higher proportions of sequences related to parasite infection. Two factors often considered include age and medication use. Older age was identified as a risk factor for domestic cat endoparasite infection [76] but the USA cheetahs were younger (1-6 years old) compared to the NAM population (3-14.5 years old). The USA cheetahs also received monthly parasitic preventatives including Ivermectin for helminths and Frontline (Boehringer Ingelheim, Ingelheim, Germany) for ticks and fleas. In contrast, CCF cheetahs were regularly monitored for parasites and prescribed treatment only when necessary (personal communication with staff and veterinarians). It is important to note, that these sequences are based on predicted gene content of the bacteria found in the samples from the two populations and not direct sampling for parasites. These differences may be due to the presence of bacteria that simply carry genes that are known to be involved in pathways of parasitic infectious disease but are not a direct indication of parasite presence. The proportion of sequences was also a low percent for each of the populations so while the data are statistically significant, they may not be biologically relevant. A transcriptomic study is required to provide more concrete evidence of parasitic pathway activity. Additionally, two of the USA cheetahs were experiencing bouts of diarrhea and GI distress during the sampling period, but both of these individuals (USA7 and USA8) had the lowest percentages of sequences associated with this KO level 2 pathway for the USA samples (Figure B.3). It is unlikely these bouts of diarrhea were brought on by parasitic infection.

Some limitations from these studies include the dietary differences between the *ex situ* Namibian cheetahs in this study and those in the wild. The Namibian individuals in the current study live in similar conditions to cheetahs in the wild, but their diets were from domestic animals instead of natural prey. The natural prey for wild Namibian cheetahs includes small to medium antelopes like gazelles and impalas, small mammals (hares, rodents), and birds [77]. Additionally, the feedings of the *ex situ* NAM cheetahs were likely more consistent in quantity and frequency than those in the wild. Cheetah hunting success in the wild varies depending on sex and age, but they typically eat every 2-4 days [77]. The variation in diet type and frequency may alter the fecal consistency and subsequent fecal breakdown in the environment which may affect the stability of the exposed fecal microbiota. Second, fecals left in the experiment plot were covered with screens to prevent dung beetle and small animals from taking the fecals, as was a common occurrence in the area. The screens were a necessity because without them the fecals would have disappeared and disrupted data collection. We recognize that the screens may have offered some additional protection against larger insect and animal activity or ultra-violet exposure from the sun, both of which may have altered the microbial community more quickly [11]. Lastly, for objective two there were additional factors that may have influenced differences between the populations including a large difference in cheetah age ranges between the two populations (ranges, NAM: 3-14.5 years; USA: 1-6 years) and environmental conditions including but not limited to weather, habitat type, and medication usage.

In conclusion, our data suggest cheetah fecals from the dry season in Namibia are acceptable for microbiome collection for up to four days post defecation. We believe these patterns will apply to other large carnivores, including large cats of the *Felidae* family, under the same conditions, but we recommend additional studies to confirm these predictions. Samples collected within four days of deposit under hot and arid conditions are likely to have similar microbial diversity, composition, and structure to fresh samples. These data will provide greater access to cheetah and other large carnivore fecals and reduce costs for sampling efforts, offering an extended period for non-invasive fecal collection. Our study timeline terminated at four days, but it may be useful to sample less frequently over a longer period of time to identify changes beyond four days. These future studies may also help in assessing the age of fecals that were found in the field without witnessing the deposit event. Currently, because there were so few differences across four days, we were unable to identify age indicators. In the second aim we also identified differences in fecal microbiota diversity, composition, enriched taxa, and predictive functional relevance between two ex situ cheetah populations. Despite significant differences in taxa diversity and composition, there was only one predicted functional pathway that differed though we may have discovered more with a larger sample size. Nevertheless, many of the differentially abundant taxa between the two populations have known clinical relevance in the gastrointestinal tract and elsewhere. We suggest some of these differences are likely due to location and diet, but we recommend future studies to assess the effects of *ex situ* diet type on cheetah microbiomes and the metabolome.

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CHAPTER 4: The Relationship Among Diet, Gut Microbiomes, and Gut Health in Managed Cheetahs (*Acinonyx jubatus*)

Abstract

The health of endangered animals is critical for their future survival. Gut health and its relationship to gut microbiota is an important consideration that may be influential in the care and well-being of endangered species, such as the cheetah (*Acinonyx jubatus*). The cheetah is listed as Vulnerable by the International Union for Conservation of Nature (IUCN), with fewer than 7,100 individuals in the wild. The declining number of wild cheetahs emphasizes the importance of *ex situ* cheetah populations as insurance policies against extinction. Unfortunately, cheetahs in human care experience several health conditions, including gastrointestinal (GI) diseases, which pose significant risks to animal survival, welfare, and breeding success. In contrast, these often-terminal GI-diseases are rarely observed in wild cheetahs. We hypothesize this is due to the notable difference in diet and animal fiber between wild cheetahs and those living in zoos or conservation facilities. To identify the relationships among diet, gut microbiome, and GI health in managed cheetahs, we collected fecal samples from 95 individuals across five facilities consuming one of five diet types and analyzed bacterial content using 16S rRNA gene sequencing. From these data we provide evidence in cheetahs for 1) an association between diet type and GI symptoms, 2) diet type as a predictor of fecal microbiota, 3) additional effects of age on fecal microbiota, and 4) influence of fecal microbiota on GI health in cheetahs. In general, diet type explained the largest variation in fecal microbiota among samples. Specifically, we identified patterns that suggest two main important dietary factors: 1) whether cheetahs consumed carcass or commercial based diets as their primary source, and 2) what species they consume as their main diet. We found no significant influence of age, sex, or GI symptoms on alpha diversity but did identify additional influence of age on fecal microbiota beta diversity measures. Finally, while we did not find any correlations between GI symptoms and alpha

diversity, we did identify five ASVs that were associated with presence or absence of GI symptoms. In summary, this study represents one of the largest investigations into cheetah fecal microbiota to date. Evidence for the effect of diet on cheetah gut microbes and health is present, and we suggest further investigations into the effect of animal fiber, and species type and variety on the fecal microbiota of cheetahs and managed felids. Finally, with the known associations of gut microbiomes and digestive and overall health in numerous species, we recommend additional comparative studies using both microbial and metabolomic data with defined diagnoses of diseases of interest (gastritis, chronic kidney disease, etc.) in sampled cheetahs to reduce the number of confounding variables and identify important microbial biomarkers.

Introduction

Fewer than 7,100 cheetahs remain in the wild and their decline is primarily due to habitat loss and fragmentation, increased conflicts with humans, and the illegal pet trade [1, 2]. This drastic decrease in the wild population highlights the importance of maintaining captive breeding programs to protect the cheetah from extinction. Unfortunately, ex situ cheetahs suffer from high rates of gastrointestinal (GI) and renal-related diseases [3-6]. GI diseases, such as gastritis, are one of the leading causes of death or euthanasia in ex situ cheetahs [3-6]. Each loss has a significant negative impact on maintaining a self-sustaining population because cheetahs have very low genetic diversity from two historical population bottlenecks [7, 8].

In contrast to cheetahs in managed facilities, cheetahs in the wild rarely suffer from gastritis. Previous hypotheses for this difference include *Helicobacter* infection [3, 4, 9-12], chronic stress from captivity [13, 14] and differences in diet [15-18]. The answer is likely a combination of these factors, which is why it is important to have strong data for each. The literature has since shown that it is unlikely that *Helicobacter* alone is a direct cause of gastritis as many cheetahs in the wild are colonized by *Helicobacter* but have no signs of gastritis (symptoms or histological evidence) [5, 10]. Additionally, chronic stress in captivity has been shown to increase adrenal output and gland size [13] which may have negative effects on the cheetah immune system and ultimately gastric lining. However, it is important to note that cheetahs in the wild are also facing extensive stressors, especially those in areas being developed or with high densities of other top predators [19-21]. Lastly, the managed cheetah diet consists of primarily commercially available ground raw lean meat from beef, pork, or horse [22]. Conversely, wild cheetahs are consuming whole carcass diets, including the harder-to-digest parts of the animal such as the fur, skin, connective tissues, and bones. Recent evidence suggests these low to non-digestible parts of carcasses may play an important role in cheetah gut health through fiber-like effects, acting as substrates for the gut microbiota and providing fermentative substrates and physiological benefits [16, 17, 23].

Likewise, the effects of managed facilities and ex situ diets on the gut microbiome have been found across taxa. A study on non-human primates found that captivity and low fiber diet was associated with loss of wild gut microbiota and altered the gut microbiota to look more like modern human microbiome [24]. Black rhinos living in captivity had gut microbiota usually found in domestic livestock species, while wild rhinos did not [25]. Less direct comparisons have been made in the cheetah, but one study found that captive cheetahs living in Namibia under similar environments exhibited different gut microbial compositions to those in the wild [26]. Comparisons across studies on wild [26, 27] and captive [28, 29] cheetahs have been made, with many suggesting diets as a factor for differences in microbial composition. However, without direct and controlled environments, it is difficult to determine how much of an effect diet, particularly the varying amounts of animal fiber, has on the cheetah gut microbiome.

Here, we investigate the link between diet and cheetah gut microbiome diversity to better understand the effect of diet and animal fiber on cheetah gut microbiomes and health. We sampled ex situ cheetahs across five diets with varying amounts of animal fiber from five locations. From these data we investigated four major objectives; 1) identify a relationship between the five tested cheetah diets and GI symptoms, 2) characterize the effect of diet type on the cheetah fecal microbiome, 3) identify additional host-trait factors that may explain fecal microbiota variation, and 4) characterize the effect of microbial diversity on GI symptoms and identify bacteria associated with GI symptoms. Identifying associations of diet, gut microbiota, and GI health in the cheetah will increase our understanding of the effects of managed diets on cheetah health. This will provide valuable information to cheetah and other carnivore managers and improve our ability to maintain a captive breeding population for the long-term health of the cheetah population.

Methods

Experimental animals

We collected samples from 90 cheetahs of both sexes (male 38, female 52) and of a varying age range (0.61 – 14.5 years old) from four Association of Zoos & Aquariums accredited facilities (Fossil Rim Wildlife Center (FRWC, facility A) in Glen Rose, TX; Smithsonian National Zoo & Conservation Biology Institute (NZCBI, facility B) in Front Royal, VA; White Oak Conservation (WOC, facility C) in Yulee, FL; and Wildlife Safari (WS, facility D) in Winston, OR). We also collected eight samples from unknown sexes and ages at the Cheetah Conservation Fund (CCF, Facility E) in Otjiwarongo, Namibia. All US based institutions met required animal management and husbandry guidelines as outlined by the Cheetah Species Survival Plan [22].

Diets

An overview of diet types and classifications is shown in Table 1. In general, three of the four AZA institutions offered a commercial based diet from Central Nebraska (three commercial varieties in this study, Premium Beef Feline Diet and Super Lean Beef, Central Nebraska Packaging, Inc., North Platte, NE) or Milliken (Milliken Meat Products, Ltd., Ontario, Canada). Most cheetahs consumed only one type of commercial diet, but one cheetah ate a blend of the two beef diets. The remaining cheetahs consuming a beef diet ate exclusively the Premium Beef Feline Diet. Because the one individual eating the beef blend was still eating 100% beef, we kept her in the commercial beef category. Facility D fed exclusively carcass diets made of horse and rabbit and facility E fed exclusively carcass diets made of donkey and horse. Additional diet items (bones and carcass) were fed one to two times weekly across all commercial diets. All facilities provided access to water ad libitum.

Fecal collection

All fecal samples were collected with clean gloves within 24 hours of defecation (Chapter 2) and placed in an individual clean labeled fecal storage bag. Fecal samples were placed on ice until placed in a freezer (-20 or -80°C) and shipped on dry ice to NZCBI when necessary. The Namibian samples were extracted, and the first round PCR was performed at the CCF genetics lab before shipment to NZCBI to avoid the potential risk of sample thawing in transit to the US. Once at NZCBI all samples (feces and PCR amplicons) were stored at -80°C until further processing.

Table 4.1 Diet types offered at each of the five facilities, A-E and the number of cheetahs in the present study that consumed these diets. Please note, not all cheetahs at each facility received everything listed. For a more detailed view of what each cheetah at each facility was fed, please reference the metadata file from the publicly available data.

Facility	Location	Primary Diet	Diet Supplement	Diet Classification	n
A	Texas	Commercial Horse	Bones (horse)	Commercial Horse + Bones	20
			Carcass (deer)	Commercial Horse + Carcass	1
B	Virginia	Commercial Beef	Bones (cow or horse)	Commercial Beef + Bones	3
			Carcass (rabbit)	Commercial Beef + Carcass	21
C	Florida	Commercial Horse	Bones (horse)	Commercial Horse + Bones	5
			Carcass (deer, rabbit)	Commercial Horse + Carcass	19
D	Oregon	Carcass (horse)	Carcass (rabbit)	Carcass	18
E	Namibia	Carcass (horse, donkey)	NA	Carcass	8

Metadata collection

At the US facilities, for every fecal that was collected, a survey capturing metadata was completed by the facility cheetah keeper/management. Metadata included cheetah identification, demographic, diet, and health information (Figure C.1, Appendix C).

Sample DNA Extraction and Library Prep

For all samples, we extracted bacterial DNA from 0.25 g frozen feces using the QIAamp PowerFecal DNA Kit (#12530-50, Qiagen, MD) following manufacturer's instructions. To help identify extraction contaminants an empty negative control was included for every batch of extractions. Additionally, three extraction positive controls (Zymobiomics microbial community standard; #D6300, Zymo, CA) were dispersed across extraction batches to test our extraction efficiencies and identify any biases. After extractions, DNA concentrations were measured via fluorometric quantification (Qubit4 Fluorometer, ThermoFisher Scientific, MA).

We amplified fecal bacterial DNA following a previously published two-step polymerase chain reaction (PCR) protocol with dual-index paired-end Illumina sequencing (Keady et al 2021). First round amplicon PCR amplified the V3-V5 region of the 16S rRNA gene using universal primers 515F-Y (5'-GTGYCAGCMGCCGCGGTAA-3') and 939R (5'-CTTGTGCGGGCCCCCGTCAATTC-3') [30]. Negative and positive PCR controls (Zymobiomics microbial community DNA standard; #D6305, Zymo, CA) were included as well. PCR reactions were performed in duplicate for all samples, negative and positive controls. Following first round PCR, all reactions were cleaned with magnetic beads, indexed with i5s and i7s, cleaned, quantified, and pooled as specified in [31]. The target band for the 16S rRNA library (~578 base pairs) was isolated and removed using a QIAquick Gel Extraction kit (#28704, Qiagen, MD) and diluted to 4 nM. We sequenced all samples using an Illumina MiSeq (v3 chemistry: 2 x 300 bp kit) at the Center for Conservation Genomics, Smithsonian National Zoo & Conservation Biology Institute.

Sequence Data Processing

There were two datasets used depending on the question being assessed. The first dataset comprised only samples from the US, while the second comprised the same samples from the US with the addition of eight samples from Namibia. Separate datasets were used because there was limited metadata on demographic and health variables for the Namibian samples. Therefore, we restricted all demographic and health questions to the US only dataset.

Dataset 1: USA + Namibian samples

We imported demultiplexed Illunina MiSeq sequencing reads into RStudio 2022.12.0+353 [32] for R (v4.2.2). We used the R package "dada2" version 1.16.0 [33] to merge paired ends, remove chimeras, and filter out low quality reads (maxEE > 2). We combined sequences reads from the 87 US cheetahs and eight Namibian cheetahs into one phyloseq object after filtering and merging. All amplicon sequence variants (ASVs) were then assigned taxonomy together using the Ribosomal Database Project (RDP 16S training set (set 16, release 11.5)[34, 35]. The phylogenetic tree was built using Quantitative Insights Into Microbial Ecology 2 (vQIIME2-2022.11)[36] using FastTree [37]. The ASVs, taxonomy table, phylogenetic tree, and metadata were imported into a 'phyloseq' object using the phyloseq package [38] for downstream processing. We split the phyloseq object into two based on extraction location (USA vs. Namibia) to remove contaminant ASVs separately using the combined Fisher method (threshold = 0.1) in the R package "decontam" (v 1.18.0)[39]. The number of contaminants removed were one and five from the US and Namibian samples, respectively. At this point we assessed the positive controls and determined the values of the taxa to be acceptable

(Figure C.2). After removal of contaminant sequences, positive and negative controls, and samples with low reads (10,000 for US samples and 33,500 for NAM) were removed from the separate phyloseq objects. After filtering the two noncontaminant phyloseq objects were merged back together to filter out non-fresh Namibian samples and US samples without metadata, ASVs classified as Cyanobacteria, and singleton ASVs (ASVs occurring in only one sample). After filtering, we found a sequencing depth variation (max/min) of 13-fold change (max = 195,532 and min = 14,876). Based on recommendations by [40] we rarefied all samples to the lowest sequencing depth (14,876 reads).

Dataset 2: USA-only samples

For the USA-only samples, we followed the same pipeline as above, except we did not combine with Nambian samples, and we did not rarefy the data. In brief, we imported demultiplexed Illumina MiSeq sequencing reads into RStudio 2022.12.0+353 [32] for R (v4.2.2). We used "dada2" to merge paired ends, remove chimeras, and filter out low quality reads (maxEE > 2). Taxonomy was assigned to the to the ASVs using the RDP 16S training set (set 16, release 11.5). The phylogenetic tree was built using FastTree in QIIME2. The ASVs, taxonomy table, phylogenetic tree, and metadata were imported into a 'phyloseq' object for downstream processing. Contaminant sequences were removed using the combined Fisher method (threshold = 0.1) in decontam" (v 1.18.0). One contaminant sequence was removed and then we filtered out singleton ASVs (ASVs occurring in only one sample). ASVs classified as Cyanobacteria, negative control samples, and samples with low sequence counts (< 10,000 reads). After quality control and filtering, the variation (max/min) in sequencing depth was 3-fold change (max = 45,105, min = 14,876). In the final dataset there were 86 samples.

Statistical Analyses

We assessed four main objectives relative to cheetah diets, health, and the fecal microbiome. Our first aim was based purely on the metadata, to determine whether certain diet types have higher frequencies of GI-associated symptoms. Our following objectives used the microbial data to determine the effect of 1) diet type on cheetah fecal microbiota as a proxy for the gut, 2) host traits on cheetah fecal microbiota and finally 3) whether cheetahs with GI symptoms have lower microbial diversity or microbes associated with GI-related symptoms.

All statistical analyses were performed in RStudio version 2022.12.0+353 [32] for R (v4.2.2) [41]. Significance for all analyses was set to $p < 0.05$ and trends was set to $p < 0.1$. Analysis pipelines for characterizing microbial structure and composition were based on previous research in our laboratory [30, 31, 42].

Relationship between diet and GI-related symptoms

We collected metadata for each fecal sample asking whether or not the cheetah experienced any of the seven symptoms common in GI-disease, specifically, gastritis (Table 4.2)[43]. We then collapsed all symptom types into a binary variable, GI Symptoms, where the levels were yes or no. The observed symptom frequency was compared across the five diet variables (Table 4.2). Due to low sample numbers in some diet types, we used Fisher's exact test to determine independence of the diet types and frequencies of GI-symptoms (using Monte Carlo simulation with 10,000 replicates). To investigate the relationship between different diet types we performed pairwise Fisher exact tests, again using Monte Carlo simulation with 10,000 replicates, a confidence level of 0.95, and FDR p-value adjustment for multiple testing in the R package "stats" [41].

Table 4.2 GI-related symptom counts by diet type. All symptom counts were condensed into GI-symptoms or no GI-symptoms to create a contingency table for Fisher's exact tests. Note that individuals in the Commercial Beef + Carcass category had more than one symptom, so the sum of that column does not equal the total number of individuals with symptoms in that diet category.

	Carcass	Commercial Beef + Bone	Commercial Beef + Carcass	Commercial Horse + Bone	Commercial Horse + Carcass	Row Totals
Diarrhea	0	0	10	0	0	10
Vomiting	1	0	10	0	0	11
Loss of appetite	3	0	0	5	0	8
Poor hair/coat	0	0	0	3	0	3
Bloody stool	0	0	0	1	0	1
Weight loss	0	0	0	0	0	0
Excess salivation	0	0	0	0	0	0
GI-symptoms	4 (22%)	0 (0%)	10 (48%)	9 (37.5%)	0 (0%)	23 (27%)
No GI-symptoms	14 (78%)	3 (100%)	11 (52%)	15 (62.5%)	20 (100%)	63 (73%)

Diet and the fecal microbiome

We investigated the effect of diets on cheetah fecal microbial abundance, diversity, and composition. Using a data-informed approach we identified the best way to define the diet variable. Because there were two types of commercial diets fed in the US population, we used the USA-only data set (dataset 2) and subset only the cheetahs who consumed commercial diets. We then divided the commercial diets and supplement diet items into two separate categories each with two levels (commercial beef vs. commercial horse; bones vs. carcass). We tested alpha (ANOVA) and beta (PERMANOVA) diversity measures against a model with commercial diet, supplemental diet, and an Interaction term (Table S1). We found an interaction effect between the two diet variables (commercial diet and supplement diet type) in the beta diversity models (PERMANOVA: Table S1). Therefore, we kept the four unique commercial and supplement combinations as separate levels in the final model diet variable (Table 1). This left us with the new variable, diet type, which had 5 levels once cheetahs consuming carcass were added (commercial beef + bones, commercial beef + carcass, commercial horse + bones, commercial horse + carcass, and carcass, Table 1). The final model (see below) was run with dataset 2 which also included the eight samples from Namibia that consumed carcass diets. Relative abundance was measured in the R package "phyloseq" using the functions tax_glom() at the phyla level and merge_samples() by diet type. Differential abundance testing was used to calculate the differences in the relative abundances among the diet types. We used the package DAtest to identify the appropriate test based on our dataset [44]. For DAtest, we filtered the data at the ASV level to reduce the false discovery rate (min.samples = 15, min.reads = 15) which resulted in 163 taxa for testing. Within the package, we used the function testDA() on the prefiltered data and measured the differential abundance between diet classifications. The top three tests for our dataset were DESeq2 with manual geometric means (ds2), ANOVA multiplicative zero-correction and additive log-ratio normalization (aoa), Linear model multiplicative zero-correction and additive log-ratio normalization (lma). These three tests were run on the full dataset, using the tax_glom() function at the phylum level and any phyla that were differentially abundant in two of the three tests were selected. Differential phyla were analyzed using the same three tests identified for the ASV level and visualized via box plots. Alpha diversity metrics included species richness (SR) and Faith's phylogenetic diversity (PD) by diet type. Species richness is the number of unique ASVs in a sample and PD measures the amount of biodiversity based on the phylogenetic relationships of the taxa and the total tree branch length of ASVs in a sample [45, 46]. Faith's PD was calculated for each subsample with the R package "picante" [47]. We used the "stats" R package (Version 4.2.1) to perform linear models, with SR or PD as the response variable and diet type as the explanatory variable. SR and PD distributions met assumptions of normality (Shapiro-Wilk/qqplots) and homoscedasticity (Levene). Post hoc tests were assessed with "emmeans" [48] and Tukey adjustments. For shifts in community composition across primary and supplemental diet types we measured Bray-Curtis (abundance-weighted composition), Jaccard (presence-absence composition), and unweighted UniFrac (presence-absence with inclusion of phylogenetic relationships of taxa) distances. We used the package "vegan" [49] to perform PERMANOVAs [50] where Bray-Curtis, Jaccard, and unweighted UniFrac distances were the response variable and primary diet type was the explanatory variable. Post hoc analyses were performed in the package "pairwiseAdonis" [51] using the pairwise.adonis2() function and Benjamini Hochberg (FDR) adjusted p-values. We calculated PERMDISP in the package "vegan" using the betadisper() function [52] to determine whether dispersion of community composition differed across primary and supplemental diet types. The Bray-Curtis dissimilarity and unweighted UniFrac distance values among diet classifications were used in a microbial network to identify connections between samples. We created an igraph microbiome network using the make_network() and plot_network() functions in the "phyloseq" package [38]. Maximum ecological distance between two samples was set to the default value of 0.7.

We performed Linear Discriminant Analysis (LDA) Effect Size (LefSe) [53] in the R package "microbiomeMarker" [54] to identify microbial taxonomies enriched in the different diet classifications. Minimum LDA score was set to 4.

Predicted functional analysis of fecal microbial differences among diet types

To identify potential functional relevance in microbial differences among diet types we used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2, Version 2.5.1) [55]. We used the KEGG Ortholog (KO) database to identify and assign predicted KO pathways and metabolic networks. We then performed differential abundance tests of the KO pathway abundances at all three KEGG levels in ALDEx2 [56-58]. Data were center log ratio (clr) transform using the aldex.clr() function with default Monte Carlo sample conditions using the model matrix (pathways ~ Diet type). We performed a generalized liner model on the transformed data using the aldex.glm() function where "carcass" was chosen as the base group to make all other diet comparisons to because we were interested in how commercial diet types compared to a carcass-only diet. Results were reported for each diet group as clr estimates, standard error (SE), and Holm corrected p-values. Data was visualized using raw relative abundance values for each pathway level by diet type.

Effect of diet and host traits on fecal microbiota

Using the US-only database (Dataset 1), we investigated the relationships among host demographics (age and sex) and health traits (body condition score (BCS), fecal score (FS), GI symptoms) with the cheetah fecal microbial diversity and composition (Table C.2). Age was divided into four categories, the youngest two age categories were different for males and females given ages at puberty [59, 60]: 1) juvenile (0.6-1.5 years for males and 0.6-2 years for females) 2) young adult (>1.5-3.5 for males and >2 – 3.5 for females), 2) prime-aged adults (>3.5-10 years), and 3) geriatrics (>10 years). Sex and GI Symptoms each had two levels (male or female and no or yes, respectively). Sex had two levels, male and female. BCS was based on the Cheetah SSP scoring system [22] where 1 is emaciated and 9 is morbidly obese. A middle score of 4-6 is considered ideal. The BCS variable had six levels (3 (n = 1), 4 (n=14), 5 (n = 35), 6 (n = 19), 7 (n=7), and "Too young" (n = 10). The "Too young" individuals that were not scored because they were less than one year of age and still growing. GI-symptoms indicated whether any of the symptoms of interest (diarrhea, vomiting, loss of appetite, poor hair/coat) were present in the individual and the variable had two levels (no = 64 and yes = 22). Since diet type (from the previous aim), age, and sex were previously identified as contributing factors to gut microbiota, including in cheetahs [26], we kept them in the minimum model to explain variation. Because the influence of sex may be stronger post puberty, we also tested an interaction effect between sex and age. Similarly, because diet may influence GI symptoms, we tested an interaction between those variables as well. The final model, selected using Akaike information criterion (AIC), was an additive model that included diet type, sex, age group, and GI symptoms as the explanatory variables (response ~ diet type + sex + age group + GI symptoms).

We used this model to test the effects of these variables on cheetah fecal microbiota diversity and composition. For the four factors in our final model, carcass diet, male, adult, and no GI symptoms were set as the reference levels. We performed statistical tests similar to the previous section, with SR and PD and Bray-Curtis, Jaccard, and Unweighted UniFrac as the response variables for alpha and beta diversity measures, respectively. Dispersion of the communities were measured using PERMDISP in the package "vegan" using the betadisper() function (ref) and permutest () function for pairwise comparisons [49].

Effect of microbiota and host traits on GI health

The direction of the relationship between microbiota and GI health is unclear. Here we assess the effect microbial diversity and host traits have on whether cheetahs have GI symptoms. Our main questions were 1) do GI healthy cheetahs have higher gut microbial diversity and 2) are there specific ASV biomarkers that indicate GI disease?

For question one, we performed logistic regression in the R package "rms" [61] where GI symptoms (no, yes) was the response variable and the explanatory variables were SR or PD + age group + diet type, selected based on AIC criterion. Both models (SR and PD) passed goodness of fit tests ($p > 0.05$). Finally, we used Least Abundance Shrinkage and Selection Operator (Lasso) in the R package "glmnet" [62] for variable selection using presence/absence data of ASVs. Before analyses, to remove some background noise, ASVs were filtered so that only ASVs occurring in at least 15% of the individuals were used. We performed k-fold cross-validation to identify the best lambda value for our model using the function cv.glmnet(). We selected the largest value of lambda such that the error was within one standard error of the minimum (Figure C.3). Using this lambda value (0.089), the Lasso model identified twelve ASVs (ASV125, ASV 116, ASV36, ASV70, ASV32, ASV146, ASV64, ASV5, ASV611, ASV68) as important variables for GI symptoms. These twelve ASVs were identified and used in a logistic regression model in the R package "rms" along with the variable for age group, to account for the skewed number of GI symptom positive cheetahs across the age groups. Logistic regression model passed goodness of fit tests ($p > 0.05$).

Results

Relationship between diet and GI-related symptoms

In total, 27% (23 individuals) were experiencing GI symptoms at some point in the month prior to sampling while 73% (63 individuals) did not (Table 2). A majority of the GI symptoms were from the diarrhea (10 cheetahs) and vomiting (11 cheetahs) categories (only one cheetah experienced vomiting without diarrhea). Two diet types did not experience any GI symptoms at all in the month before sampling. Using dataset 2, we rejected the null hypothesis of independence between diet type and GI symptoms (Fisher's exact test: $p = 0.003$). This indicates a relationship between diet type and GI-related symptoms, though no particular base diet type was consistent in whether they had cheetahs with symptoms or not (commercial horse + bones vs commercial horse + carcass; commercial beef + bones vs commercial beef + carcass). In the pairwise Fisher's tests, the number of cheetahs with GI symptoms differed between commercial horse + carcass and commercial beef + carcass (*p.adj* = 0.007) and between commercial horse + carcass and commercial horse + bones (*p.adj* = 0.007). No other pairwise comparisons were significant (Table C.3).

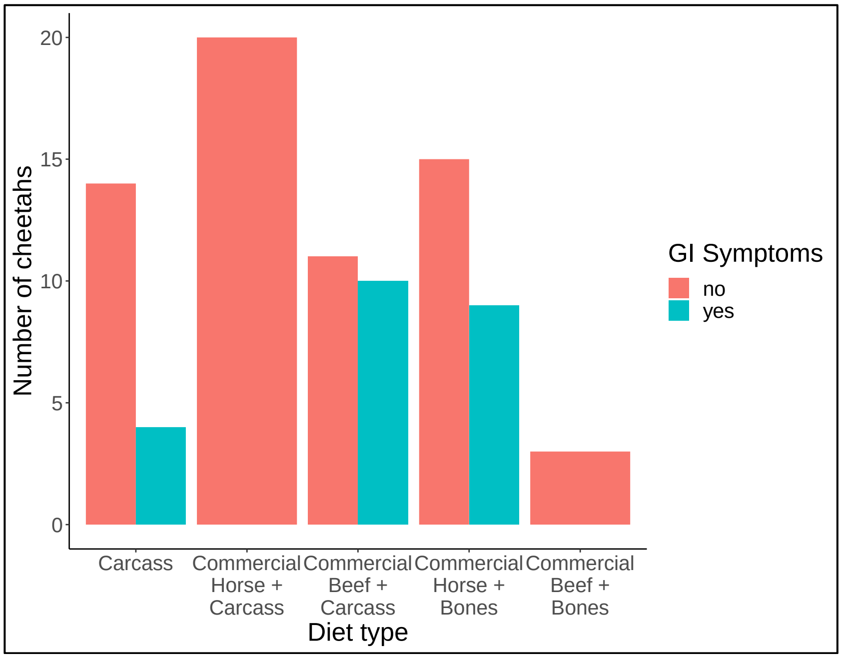

Figure 4.1 The number of cheetahs with and without GI symptoms by diet type.

Diet and the fecal microbiome

From dataset 1 (USA (n=87) and Namibian samples(n=8)) we obtained 1,412,975 high-quality sequences from 95 samples. Overall, we identified 771 ASVs from six phyla including Firmicutes (45.42%, 483 ASVs), Bacteroidetes (23.32%, 134 ASVs), Actinobacteria (14.17%, 56 ASVs), Fusobacteria (12.02%, 47 ASVs), Proteobacteria (5.06%, 50 ASVs), and Spirochaetes (<1%, 1 ASV) (Figure 4.2). Differential abundance testing determined five of these phyla differed across diet type (Figure 4.3, Table C.4).

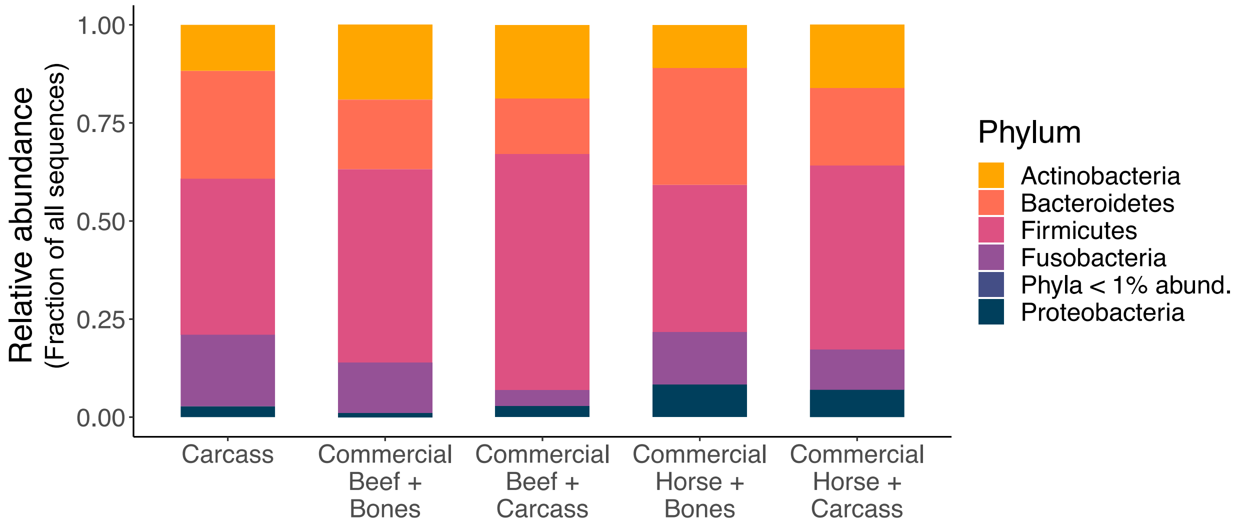

Figure 4.2 Relative abundance of phyla in cheetah fecals by diet type. Colors indicate phyla.

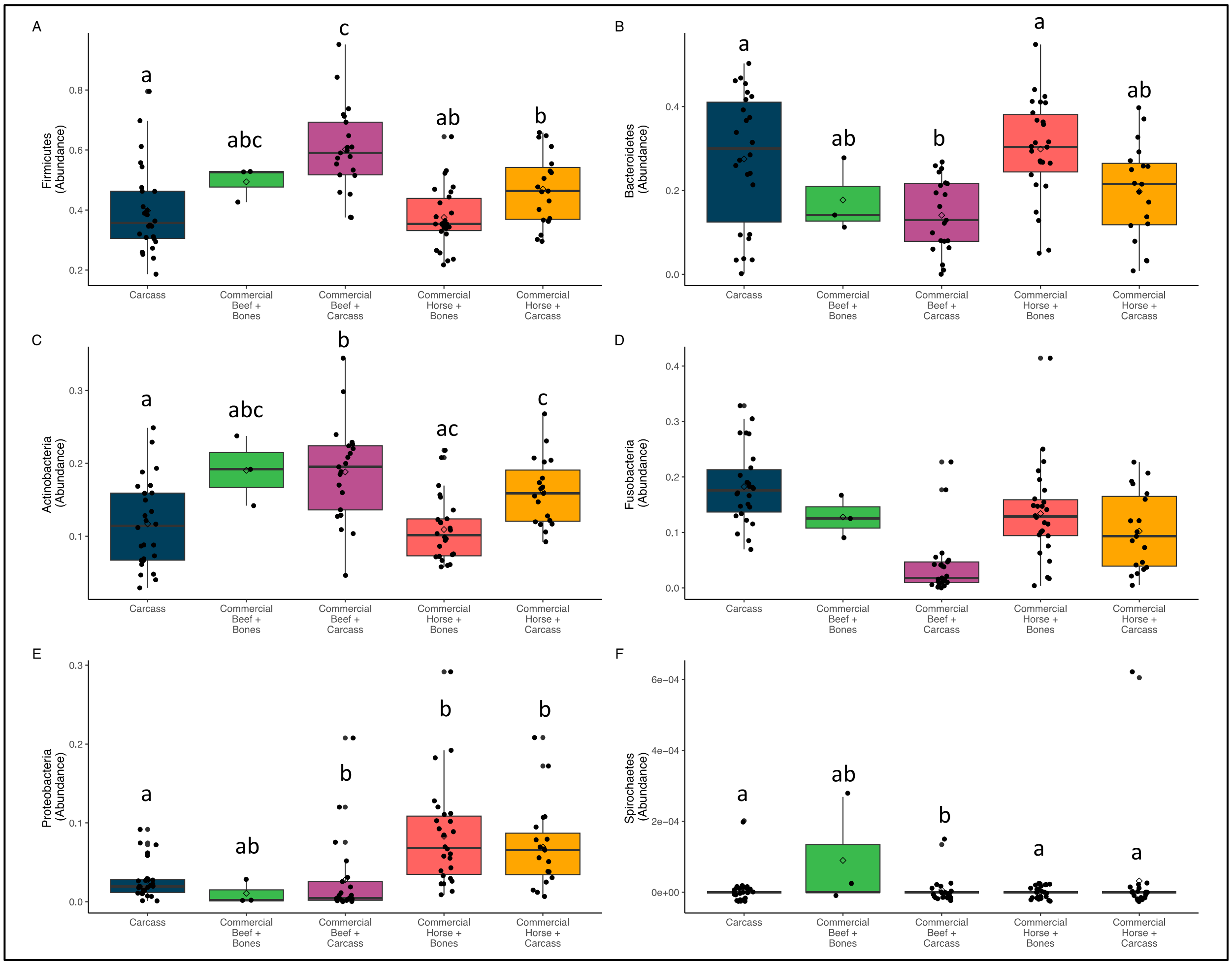

Figure 4.3 Relative abundance of the six phyla found in cheetah fecal microbiota by diet type (color). Different letters above boxes indicate pairwise differences (*p.adj* < 0.05 using DESeq2, ANOVA ALR, and Log LIMMA tests) among the groups, where diets with the same letter were not different. **(D)** Fusobacteria was not found to be differentially abundant across diet types.

Bacterial communities of cheetah fecals differed in diversity and composition but not species richness. Faith's PD, but not species richness, was influenced by diet type (PD Diet type ANOVA: $F_{4,90} = 10.2$, $p < 0.001$; SR Diet type ANOVA: $F_{4,90} = 1.746$, $p = 0.15$; Figure 4.4). Among the diet types, the bacterial communities in the carcass diet (5.63 ± 0.61) had greater PD than three of the commercial diet types, including commercial horse + bones (4.89 ± 0.56, Tukey *p.adj* = 0.0001), commercial horse + carcass (5.01 ± 0.53, Tukey *p.adj* = 0.0048), and commercial beef + carcass diets (4.65 ± 0.57, Tukey *p.adj* < 0.0001). The fourth commercial diet, commercial beef + bones, trended lower (4.76 ± 0.23, Tukey *p.adj* = 0.096) in PD compared to the carcass diet. No other pairwise diet type comparisons were significant (Tukey *p.adj* ≥ 0.25). For beta diversity measures, diet type also differed by microbial community composition (PERMANOVA: Bray-Curtis Pseudo-$F_{4,94}$= 7.36, R^2 = 0.247, p = 0.001; Jaccard Pseudo-$F_{4,94}$= 6.25, R^2 = 0.22, p = 0.001; Unweighted Unifrac Pseudo-$F_{4,94}$= 7.41, R^2 = 0.25, p = 0.001; Figure 4.5). Pairwise tests showed bacterial community composition differed across all diet type comparisons (*p.adj* ≤ 0.017). The bacterial community dispersion was also different across diet groups (PERMDISP: p < 0.001 for all three measures, Figure C.4, pairwise comparisons can be found in Table C.5) which may influence the significance of the PERMANOVAs. Microbial network analysis using Bray-Curtis dissimilarity showed clustering by primary diet type (commercial horse, commercial beef, and carcass) and country (USA, Namibia) (Figure 4.5). The Namibian samples were in their own unique cluster. For the US based samples, commercial horse + bones and commercial horse + carcass samples were between the carcass and two commercial beef-based diets. For the microbial network analysis using Unweighted Unifrac, there was one large cluster until the max distance was lowered to 0.5 (Figure 4.4) instead of 0.7 (Figure C.5). In the 0.5 map, all samples were more clustered than in the Bray Curtis plot, and Namibian samples were connected to the US samples, with the most connections to the carcass only diet group. The commercial horse-based diets continued to cluster between the carcass and commercial beef-based diets.

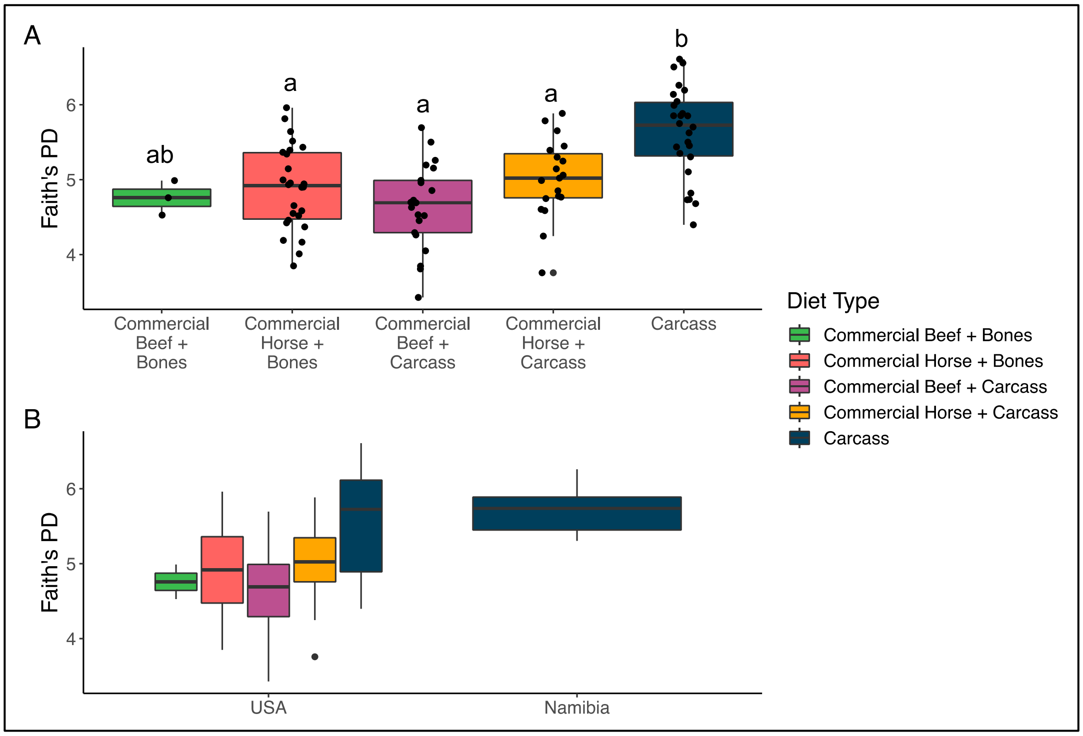

Figure 4.4 Faith's phylogenetic diversity (PD) of cheetah fecals by **(A)** diet type and **(B)** country of origin. **(A)** Letters above boxes indicate pairwise differences (*p.adj* < 0.05) among the groups, where diets with the same letter were not different. (B) Similar to (A) but diets (Carcass) were separated by country to show similarity in carcass diet group.

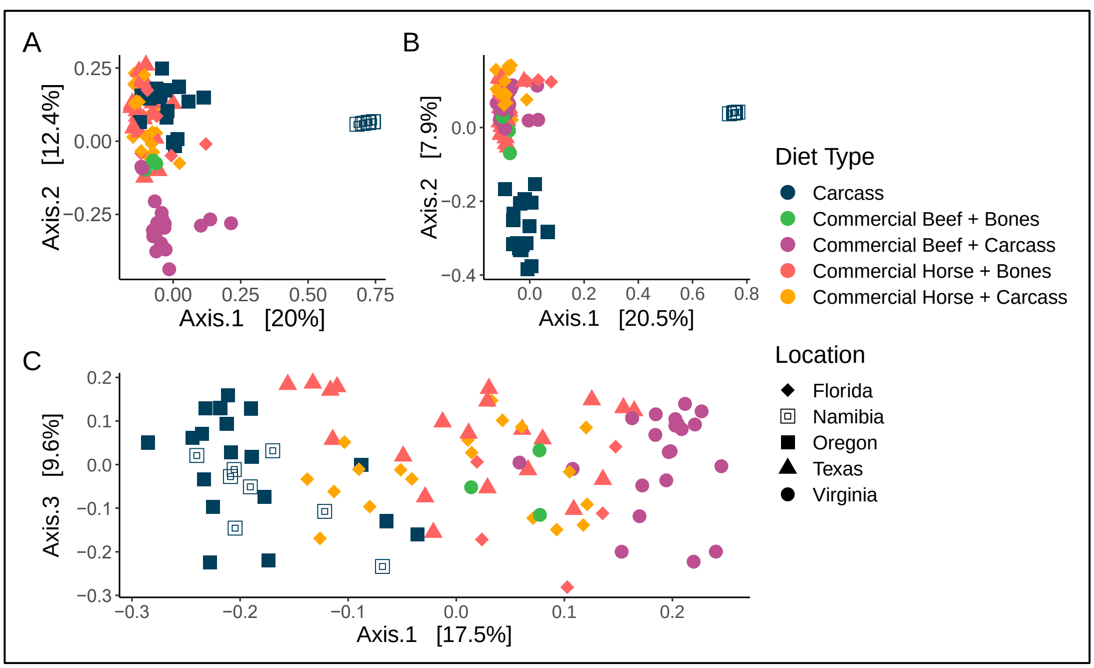

Figure 4.5 Community composition of fecal bacteria across diet type (color) and location (shape) for **(A)** Bray-Curtis, **(B)** Jaccard, and **(C)** Unweighted UniFrac measures.

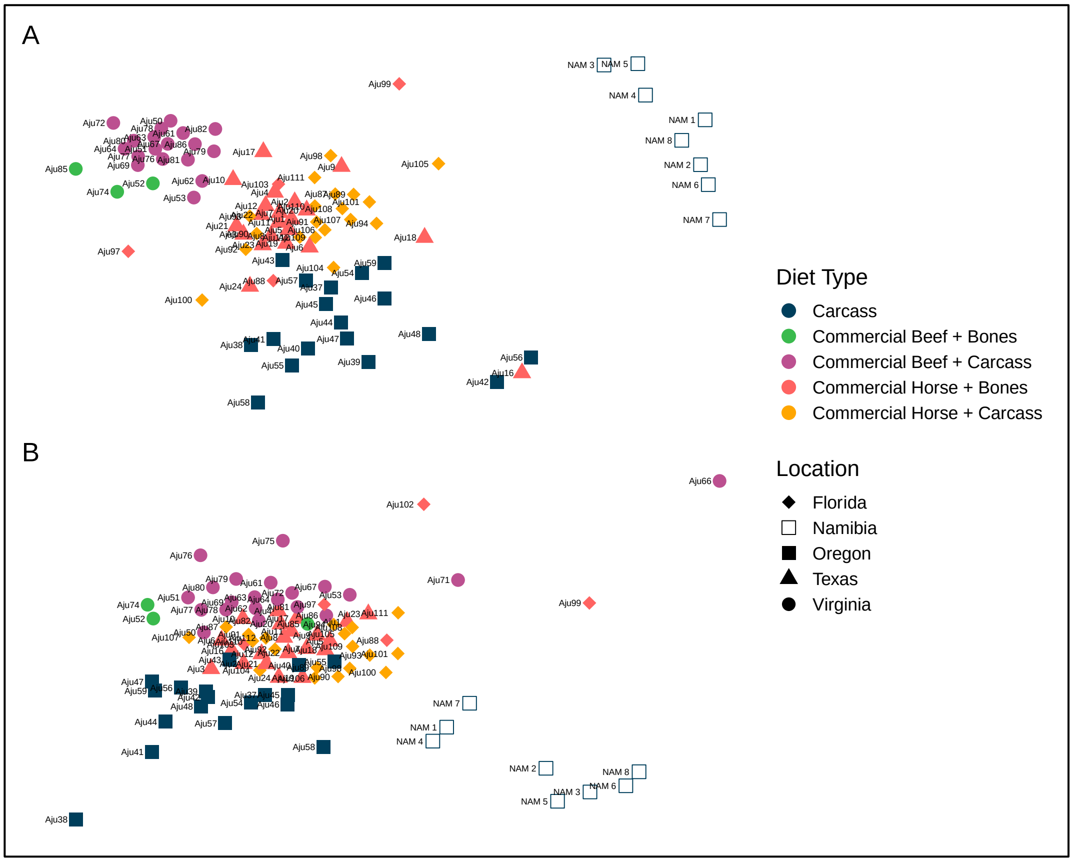

Figure 4.6 (A) Bray-Curtis and **(B)** Unweighted UniFrac microbial community composition networks. Samples are labeled by sample name (text), diet type (color) and location (shape). Maximum distance between samples (nodes) was set to 0.7 for Bray-Curtis and 0.5 for Unweighted UniFrac. The length of the connecting line and the distance between two samples indicates the strength of the association, where shorter lines and distances indicate greater community similarities.

LefSe LDA identified 64 taxa at various levels that were enriched in one of the five diet types (Figure 4.7). There were 16 taxa enriched in the carcass group, 12 in the commercial beef + bones group, 16 in the commercial beef + carcass, 12 in the commercial horse + bones, and eight in the commercial horse + carcass (Figure C.6). In general, most samples contained similar taxa, but at varying abundances. In a few cases, one or both of the commercial diets were lower in abundance than the carcass diets. Beef commercial diets had fewer *Faecalitalea* compared to the horse diets. Commercial diets of both horse and beef also had fewer *Prevotellaceae*, specifically *Alloprevotella*.

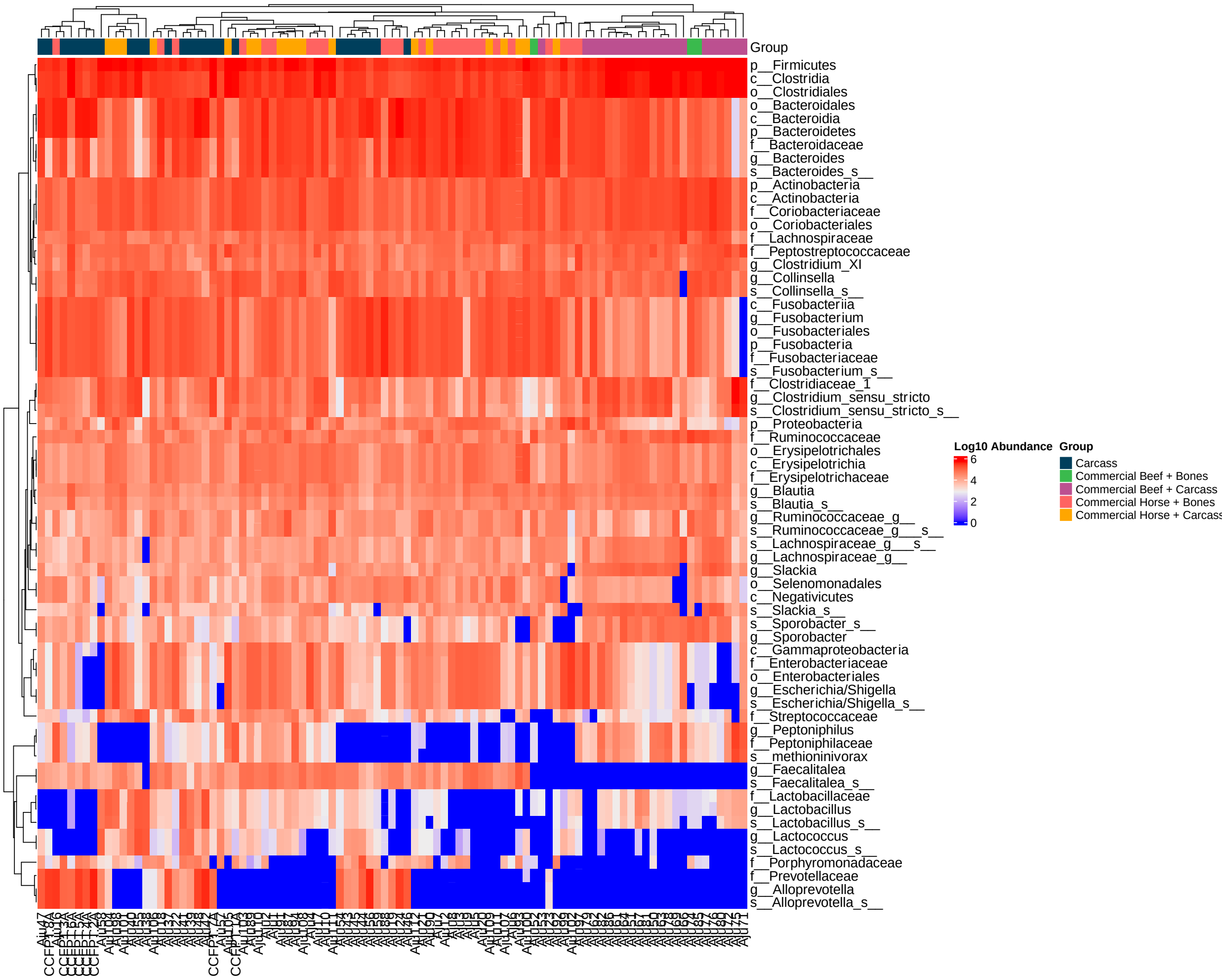

Figure 4.7 Heatmap of diet specific enriched taxa as determined by LefSe LDA. Each column represents one individual and diet type denoted by the color at the top of the map. Each row is a bacterial taxon. Abundance values are represented from less abundant (blue) to more abundant (red). LDA threshold was set to 4.

Predicted functional analysis of fecal microbiota among diet types

Functional profiles were inferred from the ASV dataset using PICRUSt2. During alignment of ASVs to the reference sequence database 73 were poorly aligned and therefore excluded from the rest of the PICRUSt2 pipeline. Using the KEGG database, we found 156 pathways in data set 1. At the broadest level, KEGG Ortholog Level 1, six pathway classifications were identified in the dataset, and four of them were differentially abundant (*p.adj* $\leq$ 0.03, Figure 4.8) between one or more of the diet types. The most abundant Level 1 category for all diet types was metabolism (range 74.1-80.9% of total sequences), which also differed among diet types (Figure 4.8A). For Level 2 classifications, only two of the 28 identified pathways were differentially abundant (*p.adj* ≤ 0.01) across diet groups (Figure 4.9). Finally, at the most specific level, KEGG Ortholog Level 3, 156 KO pathways were identified and 31 of them were differentially abundant (*p.adj* ≤ 0.045, Figure 4.10) between one or more diet types (Table C.6).

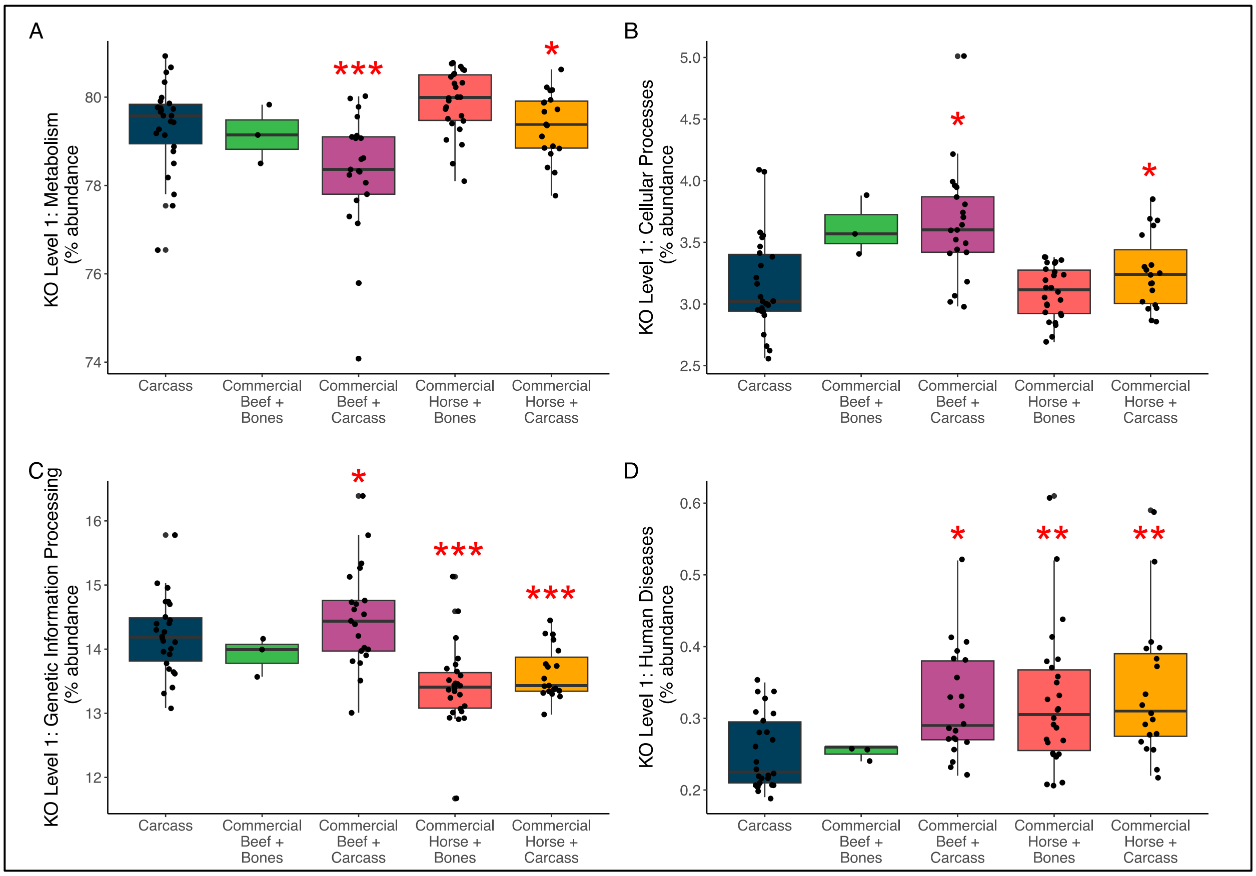

Figure 4.8 Differentially abundant Level 1 KEGG Ortholog pathways by diet type (color). All commercial diet types were compared to the carcass only diet in ALDEx2. Data are presented as percent proportion of sequences. Significance notation are for Holm *p*-adjusted values: * < 0.05, ** < 0.01, and *** < 0.001.

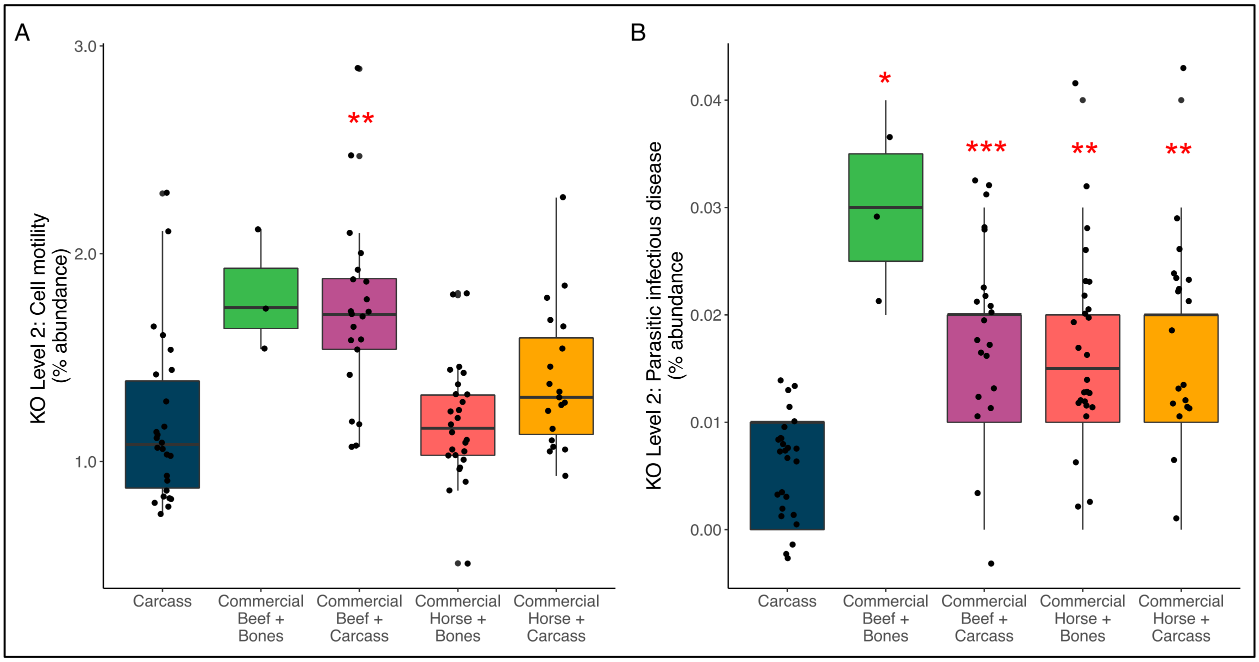

Figure 4.9 Differentially abundant Level 2 KEGG Ortholog pathways by diet type (color). All commercial diet types were compared to the carcass only diet in ALDEx2. Data are presented as percent proportion of sequences. Significance notation are for Holm *p*-adjusted values: * < 0.05, ** < 0.01, and *** < 0.001.

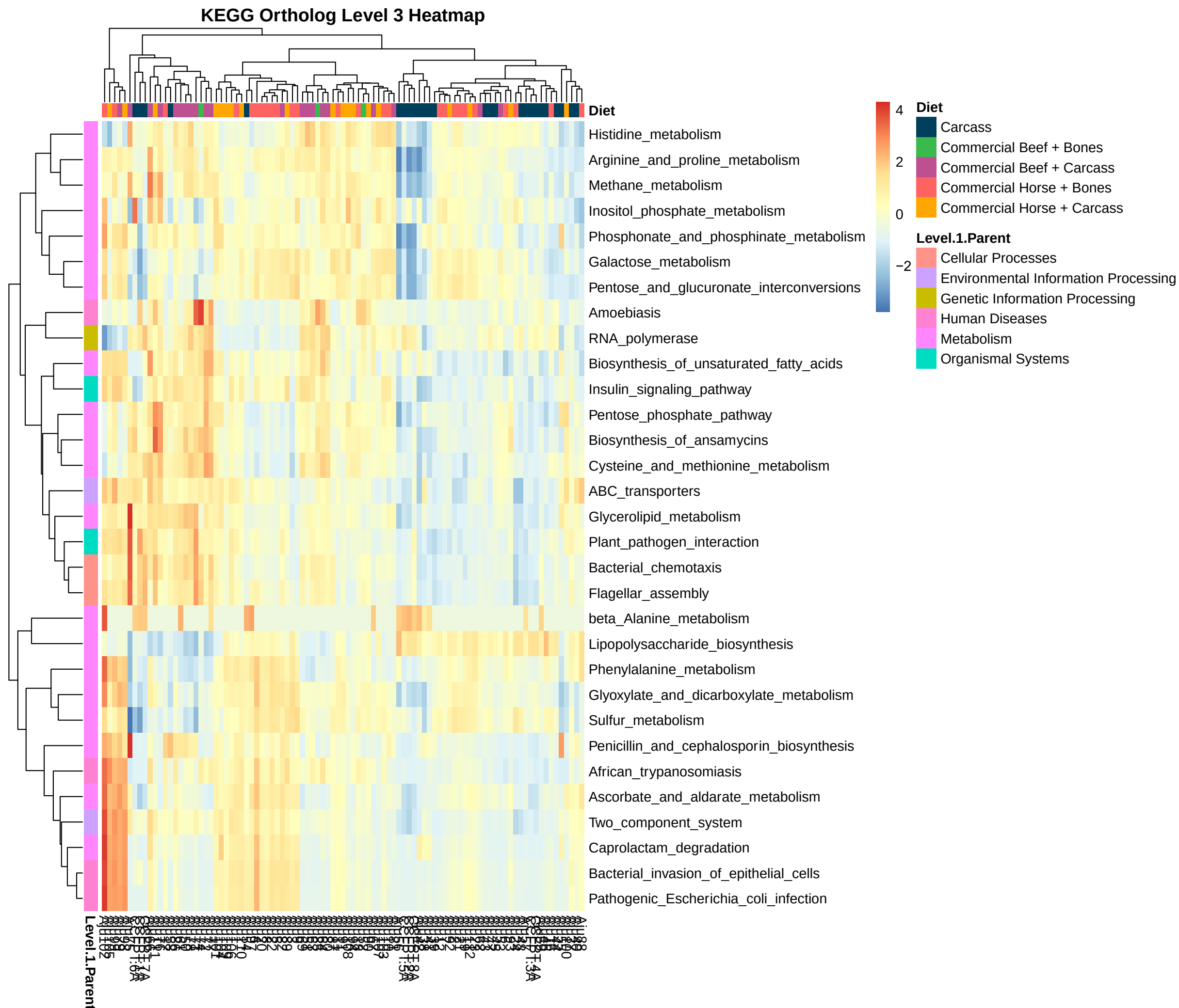

Figure 4.10 Heatmap of differentially abundant Level 3 KEGG Ortholog pathways. Each column represents one individual and diet type denoted by the color at the top of the map. Each row is a level 3 KEGG Ortholog pathway and parent Level 1 group denoted by color on the left side of the map. Abundance values were scaled by feature and represented from less abundant (blue) to more abundant (red).

Effect of diet and host traits on fecal microbiota

We obtained 2,321,021 high-quality sequences from 86 samples. One additional sample was dropped from this portion due to missing metadata for body condition score, leaving 85 individuals for the downstream analyses. The average number of sequences per sample was 26,989 (range 14,876 – 45,105). Overall, within the US samples we found 590 ASVs from six phyla including Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, Actinobacteria, and Spirochaetes.

To identify factors impacting the gut microbiota, all groups were compared to the reference group of male adult cheetahs eating the carcass diet without GI symptoms. Fecal bacterial diversity trended different by diet for SR ($F_{9,76}$ = 1.747, R^2 = 0.07, p = 0.09) and by diet and sex for PD ($F_{9,76}$ = 3.401, R^2 = 0.20, p = 0.001) (Figure 4.11). There were three factors that showed a suggested trend for explaining variation in species richness. Cheetahs consuming a commercial beef + carcass diet (106.59 ± 6.9 species, t = -1.712, p = 0.091) and cheetahs with GI symptoms (108.72 ± 5.6 species, t = -1.746, p = 0.08) resulted in ~12 and ~ 10 fewer species, respectively, than the reference group of male adult cheetahs eating a carcass diet with no GI symptoms (118.46 ± 6.2 species). In the PD model, diet type explained the most variation in the cheetahs. All diet types except commercial beef + bones decreased the bacterial PD compared to carcass ($p \leq 0.007$), noting that the commercial beef + bones group had relatively few samples. Females had a lower estimate PD (5.44 ± 0.14 PD) compared to the reference group (5.68 ± 0.20, t = -1.684, p = 0.096), but the difference was not statistically significant.

Fecal bacterial composition was best explained via diet and age group across all three measures, with diet explaining a higher proportion of variation than age (Figure 4.12; Bray Curtis PERMANOVA for Diet: Pseudo-$F_{4,76}$= 7.21, R^2 = 0.26, p = 0.001; Jaccard PERMANOVA for Diet: Pseudo-$F_{4,76}$= 6.18, R^2 = 0.23, p = 0.001; Unweighted UniFrac PERMANOVA for Diet: Pseudo-$F_{4,76}$= 6.89, R^2 = 0.25, p = 0.001; Bray Curtis PERMANOVA for Age Group: Pseudo-$F_{3,76}$= 1.36, R^2 = 0.04, p = 0.04; Jaccard PERMANOVA for Age Group: Pseudo-$F_{3,76}$= 1.29, R^2 = 0.04, p = 0.03; Unweighted UniFrac PERMANOVA for Age Group: Pseudo-$F_{3,76}$= 1.4, R^2 = 0.04, p = 0.05). For Bray Curtis and Unweighted UniFrac, there was a trend for sex in explaining community variation (Bray Curtis PERMANOVA for Sex: Pseudo-$F_{1,76}$= 1.53, R^2 = 0.01, p = 0.07; Unweighted UniFrac

PERMANOVA for Sex: Pseudo-$F_{1,76}$= 1.71, R^2 = 0.02, p = 0.06). Dispersion of community composition were similar for sex and GI-symptoms (PERMDISP: $p \geq 0.23$ all measures) but not for diet or sex (PERMDISP: $p \leq 0.001$). While age was important in the model, diet was the main driver of microbial community composition, particularly commercial vs. carcass and horse vs. beef (Figure 4.12). Within the Bray Curtis PCoA, axis 1 was best explained by the separation between beef and horse-based diets. Location was a confounding variable, but overall, all three facilities feeding horse based were on the right side of the plot. Axis 2 of the same plot was best defined by the amount of animal fiber, or base diet type (commercial vs. carcass). Cheetahs eating medium animal fiber diets (commercial of either type + bones or carcass) clustered near the top part of the plot, while the diet high in animal fiber (carcass) clustered near the bottom.

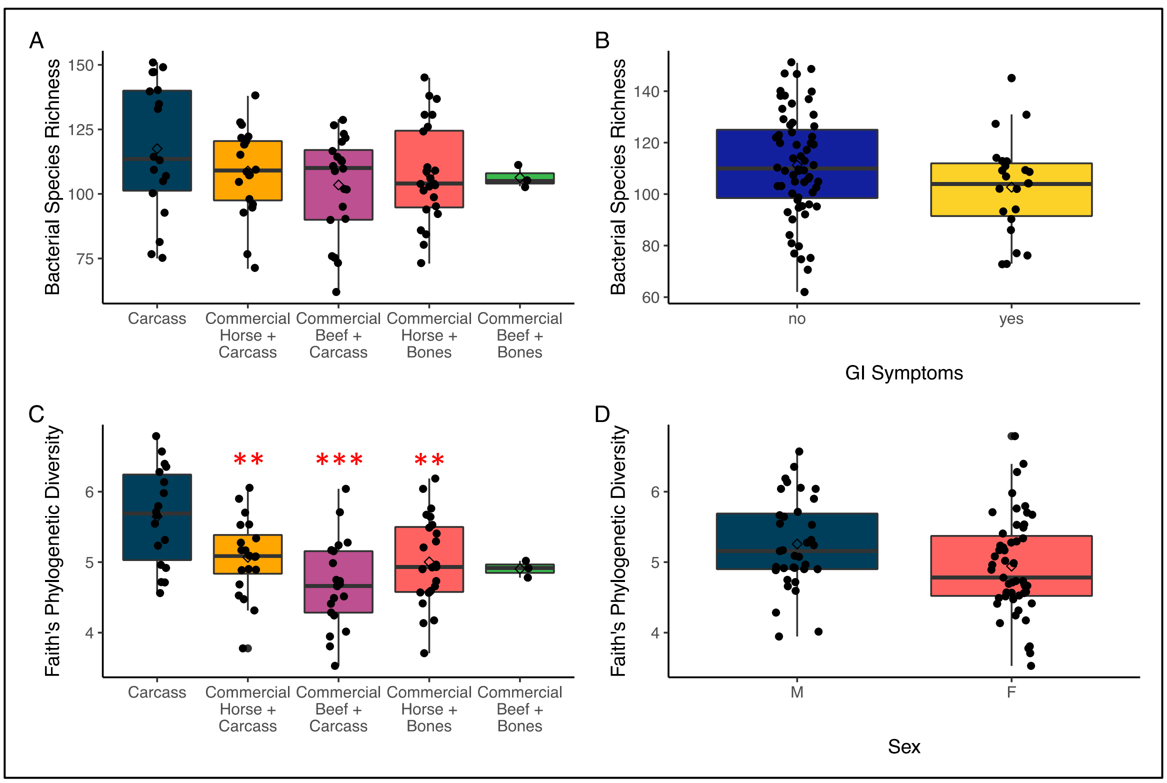

Figure 4.11 Variables of influence on species richness and Faith's phylogenetic diversity in the US only cheetah data set. The SR model trended significant (p = 0.09) and was impacted by **(A)** diet type (commercial beef + carcass lower than carcass, p = 0.09) and **(B)** GI symptoms (p = 0.09). PD was significant (p = 0.001) by **(C)** diet type where asterisks denote diet types different from carcass and **(D)** sex showed a statistical trend (p = 0.096).

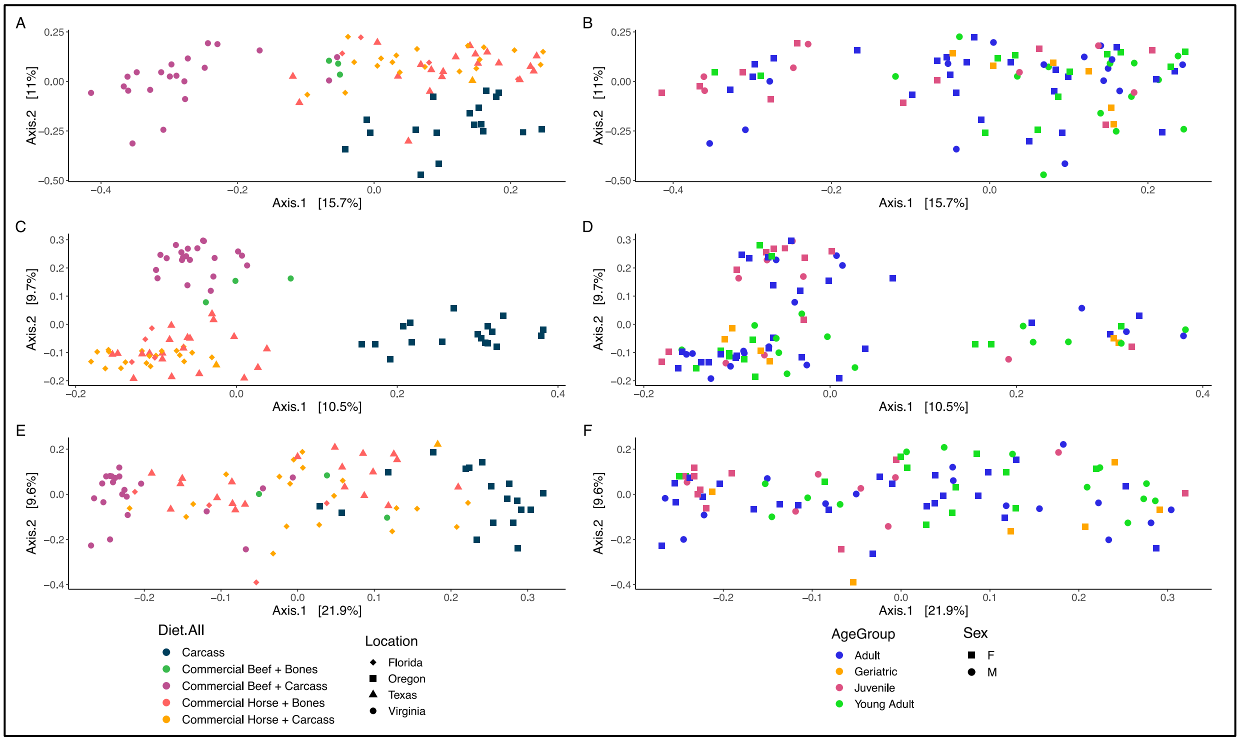

Figure 4.12 Principal coordinate analysis plots for cheetah fecal microbiota by host traits for **(A and B)** Bray-Curtis, **(C and D)** Jaccard, and **(E and F)** unweighted UniFrac measures. Samples are identified by **(A, C, and E)** diet (color) and location (shape) and **(B, D, F)** age group (color) and sex (shape). Diet and age group were significant predictors ($p < 0.05$) of microbial community composition in all three measures and sex trended statistically significant ($p < 0.1$) for Bray-Curtis and Unweighted UniFrac.

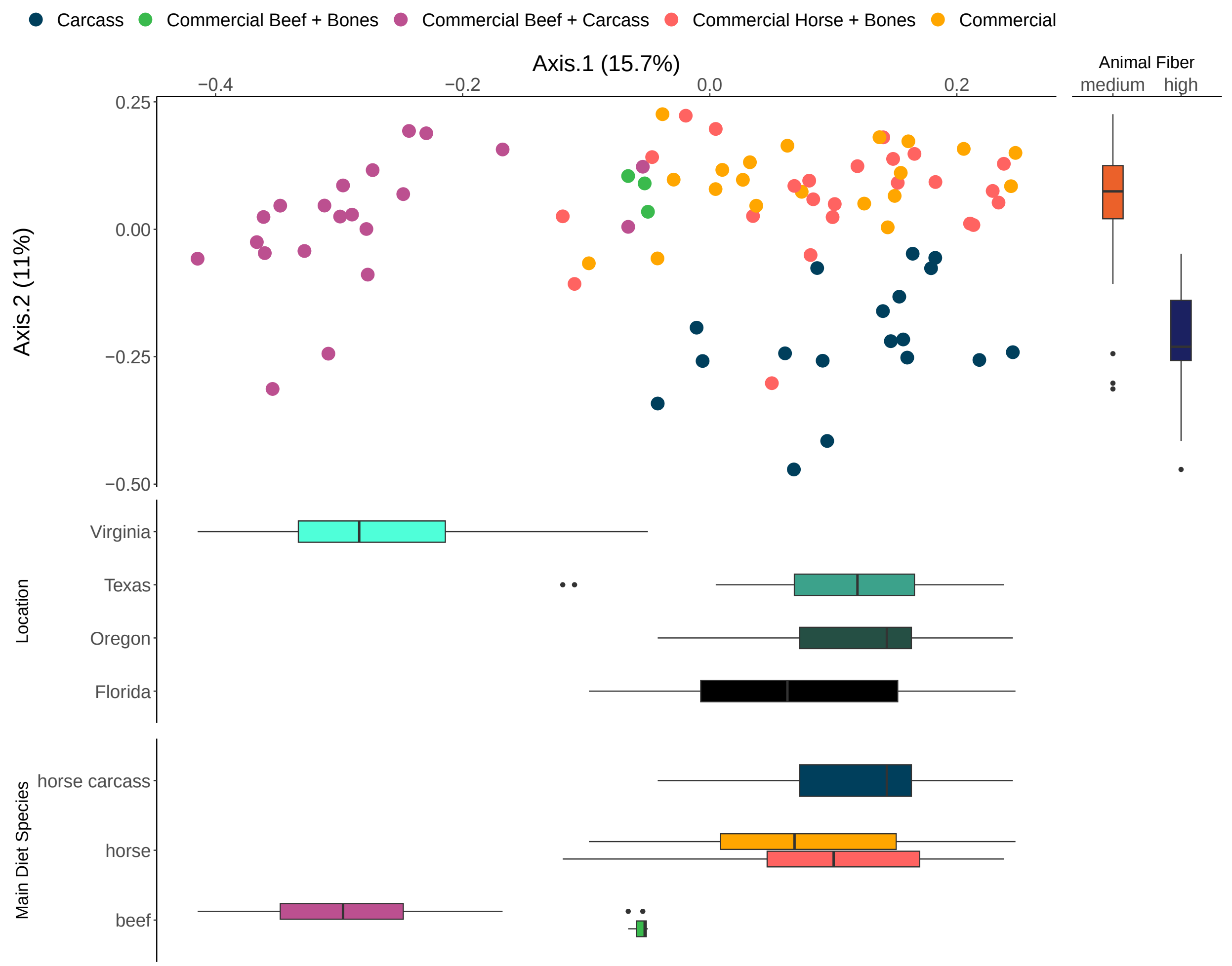

Figure 4.13 Location, Main Diet, and Animal Fiber variable boxplots by Bray-Curtis principal coordinates analysis Axis 1 or 2 values. PCoA plot: color indicates diet type. Box plots were built based on Bray-Curtis axis 1 or axis 2 values for each category.

Effect of microbiota and host traits on GI health

We found no association between GI symptoms and microbial diversity (SR lrm: Wald Z = -1.60, p = 0.11, Table C.7; and PD lrm: Wald Z = -0.39, p = 0.7; Table C.8) when holding age group and diet type constant. However, when SR and diets were held constant, being a juvenile increased the chance of having GI symptoms compared to the reference group (male adults eating carcass diets) (AgeGroup=Juvenile lrm: Wald Z = 2.21, p = 0.03).

We identified five ASVs with significant associations with GI symptoms. Three of the ASVs (ASV36: *Lactobacillaceae Lactobacillus*, ASV70: *Enterococcaceae Enterococcus*, and ASV68: *Lachnospiraceae Blautia*) decreased the chance of having GI symptoms, while two of the ASVs (ASV125: *Camplyobacteraceae Campylobacter* and ASV611: *Lachnospiracea Roseburia*) increased the risk. Presence or absence of each of the five ASVs, listed by Genus, for all individuals was visualized in Figure 4.14.

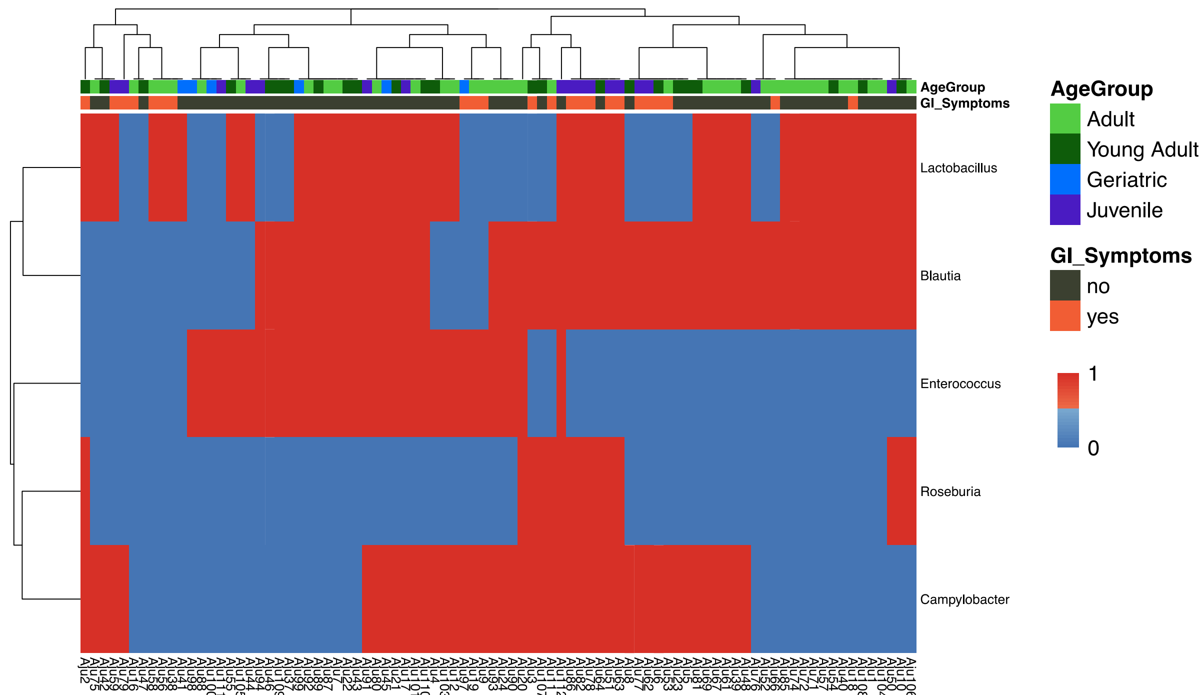

Figure 4.14 Presence/absence heatmap of ASVs with significant associations with GI symptoms in cheetahs. Individual cheetahs are listed as columns and denoted with color coded bars at the top of the columns by GI symptom status (bottom bar) and age group (top bar). ASVs are listed by Genus and color inside the rectangle indicates whether it was present (red) or absent (blue) from each individual sample.

Discussion

Understanding the role of the gut microbiome and its effects in managed endangered animals is important for their health, welfare, and even the survival of entire species for those with few wild individuals left. Cheetahs, like other obligate carnivores, require a specialized diet of animal-based proteins. Carnivore diets in managed facilities are typically ground lean meat that satisfy required protein, fat, and other nutritive requirements but lack in the low to non-digestible animal fibers found in whole prey or carcass diets of wild felids. Many cat species suffer from GI and inflammatory related diseases on commercial diets in managed facilities. The animal-based fibers may be important for maintaining gut microbial homeostasis and in part, play a direct role in GI or inflammatory health in these carnivores. We investigated the relationships between diet, the gut microbiome, and GI symptoms in managed cheetahs. Here we provide evidence in cheetahs for 1) an association between diet type and GI symptoms, 2) diet type as a predictor of fecal microbiota, 3) additional effects of sex, diet, and GI symptoms on fecal microbiota, and finally 4) influence of fecal microbiota on GI health in cheetahs.

Relationship between diet and GI-related symptoms

We identified a relationship between diet type and GI symptoms in our data. Among the pairwise comparisons, commercial horse + carcass had fewer GI symptoms than either the commercial horse + bones and the commercial beef + carcass. Fewer GI symptoms in the commercial horse + carcass diet was unexpected based on data from an international survey on cheetah diets and GI health parameters [18]. This survey found that feeding horse was the only factor that increased the odds of chronic gastritis and vomiting [18]. Cheetahs in the current study in the commercial horse + carcass group also consumed rabbit and occasional pieces of white-tailed deer carcasses as part of their regular diet. This included the bones and muscle meat, which have been shown to decrease the odds of vomiting (limb bones) and chronic gastritis or non-specified GI disease (muscle meat) in the same international survey [18]. Additionally, rabbit is known to decrease concentrations of putrefactive compounds and lower the rate of diarrhea [16]. It seems likely the rabbit and deer may offer protective benefits against symptoms, especially when compared to the high symptom rate of cheetahs consuming commercial horse + bones. Commercial horse + carcass symptom prevalence did not differ from the carcass diets indicating similarly low levels of GI symptoms in cheetahs consuming the carcass diets. Likewise, we expected carcass diet to have fewer GI symptoms compared all groups given the benefits of animal-based fiber [16, 17, 23] and feeding of muscle meat and long bones [18]. It may be likely that carcass diets did not differ in the presence of GI symptoms compared to other groups, due to the low sample number and reduced power. Symptoms were recorded only if they occurred within one month prior to sample collection. This may have missed any symptoms just outside this time window. Additionally, these were keeper reported symptoms and were not cross-checked with facility medical records. While we believe keepers fill out metadata surveys to the best of their abilities, we also understand this was an extra added duty to their already very demanding schedule and some symptoms could have been forgotten or omitted by accident if records were not kept or verified before sending the metadata. Overall, our GI symptom rate was 27%, which is lower than the previously published rates for gastritis 55-95%[3, 4, 6]. It is important to note that while we chose gastritis-related symptoms, we did not measure or confirm positive gastritis diagnoses as it requires invasive veterinary diagnostic techniques and not all participating facilities had access to these techniques. Therefore, our presence of GI symptoms is not directly comparable to previously published data on gastritis prevalence in managed cheetahs. Additionally, we sampled from large facilities that were part of the cheetah breeding coalition centers (BCCs) and overall, they tend to have fewer incidences of GI diseases [22]. Among the types of symptoms that were present in our data set, vomiting and diarrhea were the top reported and both were recorded together in all cases except one individual. This also aligns with previous data in cheetahs where reports of diarrhea and vomiting were strongly associated with each other [18]. However, due to low sample numbers, we collapsed symptom data into one variable and did not test associations of particular symptoms with diets, but this may be of interest for future work and diagnostic purposes. It would also be beneficial to test gastritis diagnoses with diet type to look for direct associations between animal fiber and disease prevalence.

Diet and the fecal microbiome

All cheetahs regardless of diet exhibited the same main six bacterial phyla. The dominant phyla across all diet types were Firmicutes. Spirochaetes was less than one percent of the total sequences in the samples which matches that of healthy domestic dogs and cats [63]. In general, the relative abundances of most of the phyla in the current study were more similar to previously published wild cheetah data [27] than European *ex situ* cheetahs [28]. An exception was the phylum Bacteroidetes, which was higher among all diet types in the current study compared to the European study and two wild cheetah studies [26, 27]. We expected carcass fed cheetahs to have lower Bacteroidetes based on previous data in cheetahs and carnivores in general [64]. Interestingly, the cheetahs fed a diet of commercial beef had Firmicutes to Bacteroidetes ratios more similar to the wild cheetahs [26, 27] compared to both the carcass fed and commercial horse fed cheetahs. The higher Firmicutes in the commercial beef diets may be due to the beet pulp in the diet, which can be fermented by Firmicutes. Bacteroidetes may be higher in the horse carcass and horse commercial diets due to increased levels of protein in the diet. If more protein reaches the hindgut, it can support bacteria that ferment proteins, much like many Bacteroidetes do [65]. Actinobacteria was also differentially abundant across diet types, highest in the commercial beef + carcass diet followed by the other commercial diets. Actinobacteria varied widely between two wild cheetah studies [26, 27], with one [27] being similar to cheetahs in the current study. The ratio of Actinobacteria to Fusobacteria was most pronounced in the commercial beef + carcass diet. This again could be due to the diet as the same ratio was higher in domestic kittens who consumed moderate protein and moderate carbohydrate diets [66].

Data suggests diet is a strong driver of microbial diversity, community structure and composition. This outcome is similar to that reported for other carnivores such as red wolves [67], dogs [68], domestic cats [69-71], and other non-domestic felids [26, 36, 72, 73]. As expected, carcass diets had greater Faith's PD. We predicted carcass diets would be higher in microbial diversity based on the known benefits of consuming animal fiber on gut fermentation processes [23], and the less uniform nature of carcass diets versus processed commercial foods. While carnivores have rather simple guts with lower species richness and diversity than omnivores and herbivores [64], the increased presence of animal-based fermentable substrates would likely foster a more hospitable environment for bacteria that metabolize these less-digestible substrates in the large intestine compared to more digestible diets that may be broken down in the proximal regions of the digestive tract [17]. Likewise, carcass diet bacterial community compositions were different from all other commercial diet types in abundance and presence/absence measures. Interestingly, all diet types were different from each other in composition. The data repeatedly showed two specific patterns in relation to the diet. The first was a difference between commercial based or carcass-based meat as the primary diet type. This pattern was particularly evident in the presence absence data shown by axis 2 in Jaccard (Figure 4.5B), axis 1 unweighted Unifrac (Figure 4.5C), and the microbial cluster of the unweighted Unifrac data (Figure 4.6B). This pattern can also be seen in the clustering of the microbial network for abundance data (Figure 4.6A). Carcass diets were enriched for Fusobacteria (Figure 4.7). The phyla Fusobacteria abundances were higher in lean compared to obese domestic cats [74]. We could not test BCS in this diet model using the Namibian samples because they did not have host trait metadata, however when testing only the US samples, BCS was not a strong predictor and was not in the final model. At lower levels, *Fusobacteriales*, and *Fusobacterium* were also enriched in the carcass diet cheetahs. *Fusobacteriales* abundances were higher in dogs fed a high fat compared to high carbohydrate diet [75], and *Fusobacterium* levels were higher in domestic dogs and cats fed a raw meat diet compared to those fed a commercial extruded diet [76] and in domestic cats fed high protein diets [71, 77]. The suggested reason for the elevated *Fusobacterium* is due to their ability to ferment amino acids to produce butyrate, a beneficial short chain fatty acid [78]. Though we did not have crude dietary analysis for each of the diets in this study, it may be likely that this was the case for cheetahs eating a strict carcass diet with less carbohydrate additives than the commercial diets, which use them as bulking agents. *Lactobacillus*, a common member of the GI tract in cats [79], was also enriched in the carcass compared to the commercial diets. A specific strain of *Lactobacillus* performed as a probiotic in domestic cats, altering the microbiota of the GI in healthy cats and providing systemic benefits and immunomodulatory effects [80]. Indeed, some *Lactobacillus* species are used as veterinary probiotics [81]. Carcass diets were also enriched for another lactic acid producing genus of bacteria, *Lactococcus*. Lactic acid producers are generally considered beneficial microbes in animal GI tracts as they produce growth inhibition substances that can prevent adherence, proliferation, and colonization of pathogenic bacteria in the gut [82-84]. Some of these enriched bacterial groups may explain the greater diversity and unique microbial composition of the carcass samples in comparison to the cheetahs consuming commercial diets. Additionally, both the commercial diet types had lower abundances of *Prevotellaceae*, including *Alloprevotella* compared to the carcass diet. *Prevotellacea* were found as part of a healthy gut microbiome in domestic cats [85]. This family was also higher in domestic kittens fed wet food diets compared to dry diets higher in carbohydrates [69].

The second pattern suggested by our data, was the influence of primary prey species (horse versus beef) on microbial composition. Abundance weighted data, Bray-Curtis, axis 2 shows a clear separation between horse (carcass and commercial) and beef (Figure 4.12, axis 1). Within the presence absence data with calculated phylogenetic distances, unweighted UniFrac, there was also a separation between horse and beef diets (Figure 4.5, axis 1). To our knowledge, this is the first evidence of a dietary species effect on cheetah, and felids in general, fecal microbiota. Previous studies have investigated the nutrient digestibility and fecal characteristics in domestic cats and other large non-domestic carnivores consuming horse and beef-based diets, but not fecal microbial changes [86]. The previous study found greater fecal output but lower fecal scores when the cats consumed the horse-based diets. They also found increased total tract apparent dry matter (DM) digestibility but lower organic matter (OM) and crude protein (CP) digestibility when fed the beef-based diets. The authors suggested the main drivers of these differences were likely the different fiber sources added to the two diets. The beef-based diet contained moderately fermentable beet pulp while the horse-based diet contained nonfermentable cellulose [86]. These dietary plant-based fiber differences may also reflect fecal microbial changes in the horse and beef commercial diets in the current study, as they used the same carbohydrate additives for each diet type as the Vester et al study [86]. This may also explain why the commercial horse diets clustered closer to the horse carcass samples in the Bray Curtis and unweighted UniFrac measures since they have less fermentable carbohydrates (similar to the horse carcass). However, within the predicted functional pathway data, the horse based commercial diets were also high in carbohydrate metabolism pathways compared to the carcass. There may also be an effect of dietary species, beyond the plant dietary fiber additives as there are inherent differences in host-based microbial communities between horses and cattle [87]. While the GI tracts are typically omitted from the diets for carnivores, the variation in cattle and horse host-microbes (skin, oral cavity, etc.), would likely affect their predators. Likewise, many captive carnivores, including cheetahs, have gut microbiota unique to their wild counterparts [26, 28, 72, 88, 89]. These differences are commonly attributed to diet and environmental factors, including homogenous environment and diet in managed facilities [90]. Wild felids, while perhaps specialized to catch a few specific types of animals, like cheetahs and impala or Thomson's gazelle, do not consistently consume the same one or two species in their diet [19, 91, 92]. Therefore, wild felid gut microbiomes exhibit more variation than those in managed facilities

[72]. A similar effect may be seen in the current study, but instead of a wild diet of variety and a captive single species diet, both are specialized to one or two different species which may amplify the unique microbial communities between the cheetahs on the separate diet types. Interestingly, the commercial beef + bones diets tended to cluster closer to the commercial horse diets compared to the commercial beef + carcass samples. This may be because the bones the cheetahs consume were from horses or it may also be coincidence of the few samples in this category. These relationships were also found in the early preliminary tests when identifying the best dietary grouping variables. Commercial type, in particular, was different in microbial composition and that was why we did not combine them into one "commercial diet" group.

Predicted functional analysis of fecal microbiota among diet types

Overall, most of the predicted functional pathways were related to metabolism. This was not surprising as these samples were from feces, as a proxy for the large intestine, where a large amount of bacterial metabolism takes place. In general, the patterns observed in the bacterial composition data were mirrored in the predicted functional outputs, suggesting differences again by carcass vs. commercial and horse vs. beef, but particularly between commercial horse and commercial beef diets. Among the carcass diets and commercial diets, all differentially abundant pathways were higher in the commercial diets. Four pathways were carbohydrate metabolism pathways (galactose metabolism, glyoxylate and dicarboxylate metabolism, inositol phosphate metabolism, and pentose and glucuronate interconversions). These pathways were likely higher in the commercial diets due to the common addition of beet pulp, cellulose, and other carbohydrates. The fifth metabolism pathway that was higher in cheetahs eating commercial diets, classified as "other amino acids", phosphonate and phosphinate metabolism. The ability to break these carbon-phosphorus bond molecules down and use them as food and a phosphorus source within bacteria is limited to mostly

Actinobacteria (check keg link in next sentence). C-P compounds are also common in antibiotics and herbicides which may be used in different amounts at the various facilities. While we did not collect information on herbicide application at each facility, we do know many facilities use it in their exhibits. The commercial diets also had higher abundances of African trypanosomiasis. This is a protozoan parasite that causes sleeping sickness. It is unlikely the cheetahs in this study from the US were carrying this parasite, but there may be bacteria that are commonly found with African trypanosomiasis present in the study cheetahs, or genes that are part of the parasitic infection pathway that may be present in other bacteria. Finally, two-component system pathways were higher in the commercial diets. This pathway is a common bacterial mechanism utilized to sense their surroundings and make adaptive responses [93]. It may be coincidental that these were higher in the commercial diets, or it may relate to changes in pH, osmolarity, and nutrient availability on a commercial based diet with weekly (or less) dietary supplements of bone or carcass (rabbits) instead of commercial every day. This is purely speculative and requires further transcriptomic studies comparing microbial gene expression by dietary differences among cheetahs.

The second repeated pattern involves the differences seen between the horse and beef diets. No pathways were significantly different in the beef + bones diet, again likely due to low sample size and reduced statistical power. Within the beef + carcass diet, there were 15 pathways higher and one that was lower. Eight of the higher abundance pathways were metabolism related. One of these, cysteine and methionine metabolism, was also increased in domestic cats with acute [94] and dogs with chronic [95] diarrhea. These pathways have also been documented in human patients with Crohn's disease [96]. Similarly, this diet type had higher abundance of glycerolipid metabolism, which was again more abundant in domestic cats with diarrhea [94]. Ten of the cheetahs in this diet type were reported to have both vomiting and diarrhea in the month prior to sample collection. It is likely these samples have contributed to these findings, though whether the bacteria were responsible for the symptoms or vice versa cannot be determined here and requires further investigation. This diet type also had higher abundances of bacterial chemotaxis and flagellar assembly (both members of cell motility) and ABC transporters (membrane transport). These are important bacteria pathways that help with cell movement and the ability to move substrates across membranes. They may be higher in these cheetahs, particularly those with the vomiting and diarrhea symptoms that were treated with a bismuth subsalicylate, which has antibiotic properties. These pathways may have helped those bacteria with increased motility and transport of toxins survive and therefore they were in greater abundance. While many of these pathways in the beef + carcass diet group may have been significant because of GI changes in the symptomatic cats, there were other pathways that were higher in abundance involving amino acid, carbohydrate, energy, and lipid metabolism pathways that may be more true reflections of the primary diet species but need further work to validate. The one pathway that was lower in the commercial beef + carcass diet was lipopolysaccharide (LPS) biosynthesis which involves the production of the large molecules that make up the outer membrane of Gram-negative bacteria. Conversely, within both commercial horse diets, there were only three metabolism related pathways in increased abundance. One was caprolactam degradation, involved in xenobiotics biodegradation and metabolism, which was also found to be higher in domestic cats with diarrhea [94]. No cheetahs in either of these groups were reported to have diarrhea. Two pathways of concern that were elevated in the commercial horse diets were "bacterial infectious disease pathway, "bacterial invasion of epithelial cells" and "pathogenic *Escherichia coli* infections". These may be related as pathogenic *E. coli* can invade epithelial cells. These are concerning because invasion of epithelial cells, regardless of which bacteria is responsible, initiates an inflammatory response by the host. While these pathway abundances were statistically higher in the commercial horse fed cheetahs, it should be noted that the overall percentages of sequences associated with these pathways was quite small ("bacteial infectious disease pathway" range 0-0.1%and "pathogenic *Escherichia coli* infections" range 0-0.26% of sequences). If these levels should be deemed biologically relevant remains to be determined and requires further investigations.

In general, using the KEGG Orthology database, we found functional pathways that differed among the diet types, with all of them being more abundant in one or more of the commercial diets, compared to the carcass diet. Additionally, there were signatures found only in the commercial beef or the commercial horse diets. All of these findings demonstrate that diet impacts gut microbiota in complex ways and highlight areas of interest for future work assessing the impacts of prey species and dietary animal fiber on cheetah gut microbial functional pathways.

Effect of diet and host traits on fecal microbiota

The addition of sex, age group, and GI symptoms with diet type in the final model when assessing the US-only data set, showed diet was still the strongest driver of fecal microbial diversity and community composition. The other variables that contributed to fecal microbial diversity were sex and presence of GI symptoms. The trend for GI symptoms to decrease SR agrees with data in domestic cats and dogs with acute and chronic diarrhea [94, 95, 97-99]. In our cheetah data, females barely trended lower in PD compared to the males. A previous study in wild cheetahs found no differences in sex for rectal microbial alpha diversity [26]. In humans, studies show higher alpha diversity in females compared to males after puberty until middle age [100, 101]. Likewise, sex (trend $p < 0.1$) along with age group ($p < 0.05$) also explained variation among cheetah fecal microbial communities when metrics of beta diversity were compared. Wasimuddin et al. identified alpha diversity changes across age groups, where diversity and richness were higher in older compared to younger cheetahs, but did not see differences in beta diversity measures for either age or sex (unweighted and weighted UniFrac)[26]. Data in domestic cat research suggests age-related bacterial community compositional differences [69, 85, 102, 103]. In one domestic cat study the community composition did not differ across age groups from 7 months to 14 years, but the number of core taxa decreased with age [85]. A second study found genera that changed by age, diet, or an interaction of both diet and age [69]. Pronounced gut microbial changes were reported in domestic cat kittens through weaning and dietary transitions [103]. The youngest cheetahs (~6 months of age) in the current study were post-weaning and post major dietary transitions so we may have missed some of these more predominant shifts. These juvenile cheetahs were post-weaning but pre-pubertal which may play a role in their gut microbiome differences from the pubertal (young adult) and sexually mature (adult) cheetahs [104, 105] as data in other species, including humans and mice, indicate the importance of sex hormones in shaping the gut microbiome [104, 106, 107]. Further, domestic cat studies show mixed results for geriatric changes in the fecal microbiome [108, 109]. The influence of both sex and age in the managed cheetah populations is of great interest for future research as it likely has large impacts on diet digestibility, reproductive success, overall health, and welfare. Ultimately, a better understanding of these interactions may lead to data driven dietary recommendations that differ by sex and life stage.

Effect of microbiota and host traits on GI health

In our current study, neither SR nor PD predicted GI status in cheetahs, though SR was close to being a trend ($p = 0.11$). We expected to find a stronger association between GI symptoms and measures of microbial diversity. Gut microbial diversity is lower in dogs [95, 99], and domestic cats [94, 97] with symptoms GI distress, including diarrhea. The results from the current study could be explained by the small sample size for individuals who had GI symptoms, making associations more difficult to detect. Diet, as a strong driver of microbiota composition, may have masked any small alpha diversity changes in relation to GI health in these samples. While we did not find any associations of overall microbial diversity measures and GI symptoms, we also tested whether presence or absence of particular bacteria were associated with GI health. We identified three ASVs associated with lack of GI symptoms, *Lactobacillaceae Lactobacillus*, *Enterococcaceae Enterococcus*, and *Lachnospiraceae Blautia*. *Lactobacillus* species are lactic acid producing bacteria and therefore may be important for GI health in cats [102]. *Enterococcus* are also lactic acid producing bacteria and specific strains have potential as a probiotic in cats [110]. Though it should be noted other Enterococcus species may be more detrimental and have been associated with antimicrobial resistance in cats and dogs [111, 112]. Members of the *Lachnospiracea* family are generally considered beneficial microbes as they ferment diverse plant polysaccharides to SCFA[113, 114]. Ramadan et al., found increased *Lachnospiracea* with improved fecal scores in cats that previously had chronic diarrhea after therapeutic dietary response [115]. Within the *Lachnospiracea* family, *Blautia* is also often identified as a beneficial microbe in many mammals [116] and has positive dietary associations in cats [66, 117]. We also identified two ASVs associated with presence of GI symptoms, *Campylobacteria Campylobacter* and *Lachnospiracea Roseburia*. Domestic cats and dogs are considered reservoirs for many *Campylobacter* species [118, 119]. *Campylobacter* species are commonly found in agricultural animal GI tracts [120, 121] and some are infectious pathogens in human enteritis [122], but their role in cats and dogs is still debated. They are occasionally associated with diarrhea in dogs and cats [123] but other studies have found no associations of *Campylobacter* and diarrhea [124]. In cheetahs, the pathogenic species *Campylobacter jejuni* may have played a role along with *Helicobacter pylori* in a fatal case of gastritis and enterocolitis [125]. Other studies in non-domestic felids in zoos have not found *C. jejuni* [126-129]. Some zoos have reported it in cheetahs with gastritis, which usually recover after antibiotic treatment [125]. Another study identified it in a healthy leopard tested by PCR [128]. Zingre et al suggest cheetahs in managed facilities may be more susceptible to *Campylobacter* infections compared with other felids [125]. It is unknown what the cause of the vomiting and diarrhea was in the ten individuals in the current study, but it is a possibility *Campylobacter* may have played a role. The role of *Roseburia* in cheetahs with GI symptoms is even less clear. It belongs to the *Lachnospiracea* family, which as mentioned above, are usually considered beneficial microbes. Our data is contradictory to previous data where domestic cats with diarrhea have lower *Roseburia* abundance compared to healthy cats [94]. This may be a true difference in the association of *Roseburia* with cheetahs and domestic cats, or a coincidental finding. Overall, the taxa we find correlated with GI symptom status are largely consistent with previous associations identified in other species, with the exception of *Roseburia*. Further analyses with a larger sample size and cheetahs with GI symptoms on varying diet types may determine whether these correlations are associated with diet, GI symptoms, or their interaction.

This current study is not without limitations. The first is that this was an observational study in managed cheetahs among facilities that each feed uniform diets and therefore there is a nested effect of location. Notably, we were unable to control for location because the commercial beef diet types were fed only in one location. While facility and environment likely effect the gut microbiomes of these cheetahs as seen particularly between the US and Namibian cheetahs, other diet types fed at multiple locations overlapped in some aspects of beta diversity. Future studies would benefit from an experimental design with more control over diet types at each facility. Likewise, while we identified animal fiber (commercial vs carcass) and diet species (horse vs. beef) as important drivers of microbial diversity and composition, there were many other variables to consider. Some we could control for (age, sex, GI symptoms) and others we could not (location, hygiene, human interactions, etc). We recommend a future study to perform diet trials at the same facility and match participating cheetahs by age, sex, and GI health as best as possible to remove some of the additional variation. Cheetahs fed commercial diets that were supplemented with bone or carcass were done so typically once or twice per week. The fecal sampling strategy was not based on which days these items were given. While overall the cheetah gut microbiome is relatively stable over time [29], cat and dog gut microbiomes are known to be plastic and change with diet, specifically where gut microbiome shifts require maintained dietary interventions [130]. There may be slight, but significant changes in the gut microbiota the day after consuming bones or carcass items that then decrease over time until the next feeding of said items. If this is the case, this may confound our findings among the commercial diet types. Therefore, we also recommend diet trials with longitudinal sampling to identify fluctuations with increasing bone or carcass items in the cheetah diet. Further, many facilities cannot provide entirely carcass-based diets, so it may be relevant to identify how much supplemental bone and carcass is necessary to provide animal fiber benefits to cheetahs consuming commercial diets. It may be that commercial diets will override any potential changes due to feeding bones or carcass, much like is seen in domestic cats when dry kibble is fed, even when wet canned or raw meat is also part of the diet [85]. Finally, we analyzed GI symptoms in the current study because many facilities in our study did not have access to gastric biopsies, which are required for veterinary diagnosis of gastritis. This may have influenced our ability to find associations with GI health in cheetahs, specifically in the case for gastritis. Even when associations were found, the direction of the relationship was not clear. Because cheetahs can be diagnosed with gastritis based on histological pathology of the gastric lining but present no symptoms, we may have also missed some microbial association specific to gastritis. It may be of use to study the fecal microbiome of cheetahs with diagnosed gastritis via biopsy with and without symptoms to identify useful bacterial community differences and biomarkers of disease.

In summary, this is one of the largest investigations of cheetah fecal microbiota to date. We identified a correlation with diet types fed in managed facilities and presence of GI symptoms, where cheetahs consuming commercial horse + carcass diets had significantly less symptoms than cheetahs consuming commercial horse + bones and commercial beef + carcass diets. We also determined, that as predicted, cheetahs consuming carcass diets had higher fecal microbial diversity compared to commercial diets. Diet effects on the fecal microbiota were also evident in all measures of beta diversity, and signatures of animal fiber and prey species were noted. We found no significant effects of age, sex, or GI symptoms on the fecal microbiota diversity but did find an association with age and microbial composition in managed cheetahs. Finally, we did not find any correlations between GI symptoms and microbial diversity measures, though we did identify ASVs that may increase or decrease the risk of these symptoms. The evidence for the effect of diet on cheetah gut microbes and potentially health was present, and we suggest further investigations into the effect of animal fiber, species type and variety on the fecal microbiota of cheetahs and managed felids. Finally, with the known associations of gut microbiomes and digestive and overall health in numerous species, we recommend additional comparison studies using both microbial and metabolomic data with defined diagnoses of diseases of interest (gastritis, chronic kidney disease, etc.) in sampled cheetahs to reduce the number of confounding variables when looking for microbial biomarkers. Evidence for dietary influence of the cheetah gut microbiome has the potential to be used as a preventative tool for remedying not only gastritis and GI related diseases, but some of the other inflammatory or neurological ones as well.

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Chapter 5: Epilogue and Future Directions

In my dissertation, I added valuable knowledge to help further the field of microbial ecology in carnivore conservation management. In Chapter 2, I determined that fecals up to 24 hours old are viable for microbial ecology studies even under extremely warm and humid conditions. I also show that there may be differences in the stability of fecal microbiota based on age and fecal consistency. In Chapter 3, I determined that fecals were viable for a longer period, up to 4 days post-defecation, in the dry season of the cheetah's natural range habitat in Namibia. I also saw initial differences between two populations of cheetahs consuming different diets that showed possible functional pathway differences. In Chapter 4, I found a relationship between cheetah diets and presence of GI symptoms. I determined that diet was a major driver of fecal microbiota diversity and composition in cheetahs. Sex, age, and GI symptoms did not contribute significantly to microbial diversity, but age was a predictor of microbial composition. Lastly, while GI symptoms were not associated with microbial diversity, I did identify five ASVs, identified at the genus level, that had strong associations with the absence or presence of GI symptoms. Overall, these data suggest fecal microbiota data, when collected appropriately, can elucidate important information regarding the care and management of an endangered carnivore.

Many carnivores in human care, domestic cats included struggle with GI-related, inflammatory, and neurological disease not frequently seen in wild or free-roaming populations. While animal (including human) health is multifaceted, the strong impact the gut microbiome has on these systems should not be ignored. Of the factors under our control as care providers, diet is a major one. Diets for non-domestic felids are poorly understood and heavily derived from knowledge about domestic cats. While domestic cats serve as better models than humans, mice, or even dogs, they may not be a perfect model for some of the larger, more distant members of the Felidae family. Some wild cat species also face extreme depletion in genetic heterogeneity that may play a role in the relationship between gut microbial ecology and animal health. Additionally, many domestic cats are spayed or neutered, and so reproductive hormones are usually not considered in many diet studies. Therefore, it is beneficial to conduct species-specific studies in these non-domestic animals to better understand their specific relationships with diet, gut microbiomes, and health.

Within this dissertation I determined that diet explained a large proportion of the variation in cheetah fecal microbiota. Two patterns emerged from these data that suggest the amount of animal fiber (carcass vs commercial) and species of the dietary meat (horse vs. beef) were important. While facility was definitely a confounding factor in this research, I hypothesize that these patterns are real. In general, while researching the background for these projects I was surprised by how little research has explored the role of animal fiber for carnivores, despite the fact that initial studies on the fermentative power of animal fiber in cats and cheetahs came out ten years ago [1-4]. I also noted that most domestic cat research is heavily based on comparing dry, wet, and raw meat diets, but usually all of the same general "prey" type (e.g., chicken). Additionally, much of the non-domestic felid research uses wild counterparts as a direct comparison for "normal" or healthy. While characterizing the microbiomes of wild felids is important for both *in situ* and *ex situ* conservation, I hypothesize that it is of greater importance to expand these studies beyond the microbiome and focus on the transcriptome, proteome, and metabolome of the wild felids rather than just their microbes. This is for two main reasons. The first is that we can never truly replicate the diets of these cats in managed facilities. The felids span five continents, their prey species are diverse, and some of their prey are themselves endangered. Second, because we cannot replicate the diet of the wild cats, it seems very unlikely that we will be able to replicate the same microbiomes, especially if microbiota differences are associated with prey species as suggested in my data. Managed cats also face evolutionary mismatches, including environmental differences of weather and climate (as seen in Chapter 3) and increased exposure to humans and built environments that can shift their microbiome. However, I hypothesize that characterizing the functional aspects of the wild felid microbiomes will provide a better understanding of the microbial impact on GI and overall health. While we may not be able to replicate the microbes acquired from consuming Thomson's gazelles and impalas of the Serengeti, perhaps we can promote an environment where microbes with similar functions thrive, thereby providing the same beneficial impacts to the cheetah. Part of this, I believe, starts with increasing the amount of animal-based dietary fiber. The health and nutrition benefits have already been established, though future research is definitely required. The second important aspect of improving managed carnivore diets includes providing more variety. Previous studies have shown that feeding a single source diet of supplemented beef chunks or whole rabbits does not provide adequate nutrition in cheetahs, and this is likely true for their microbes as well. While microbiomes in cheetahs have been shown to be relatively stable on a specific diet type, we do not know if the benefits provided from one bone or one rabbit per week are sustained or if there is fluctuation based on diet. Is one bone and/or rabbit each week enough to maintain a population of beneficial microbes that can utilize the non-digestible sources of animal fiber? Or do those microbes decrease in abundance in between feedings when commercial ground meat dominates the diet? Offering a greater variety of diet may translate into a larger variety of microbes or the ability for those beneficial microbes to maintain functional populations and mimic communities in wild cats. Offering a greater variety at all facilities may also help with picky eaters in managed cat populations or help prevent GI upset when moving to a new facility that uses a completely different diet. Working closely with the SSP and animal managers, I also understand that feeding a variable diet of approved species is both expensive and time consuming. However, the benefits that a variable diet could provide regarding behavior, stress reduction, and improved microbiomes and overall health likely outweigh the negatives. Perhaps it would benefit many of these commercial food suppliers to rethink what a commercial diet might look like. Maybe it can include ground meat, cartilage, and bones? Suppliers could increase the options they provide and process the carcasses they purchase differently to meet the demands of managed carnivores (sell whole limbs, heads, shanks with fur/skin, etc). It is evident that many commercial retailers already offer many of these items as many facilities provide knuckles, necks, and limbs as supplemental dietary items. Regardless of the path towards change, there is a definite need for more research in this field, and it needs to include collaborations from microbial ecologists, animal biologists, nutritionists, and veterinarians.

Future directions

As a PhD student in my third year, starting the most important sample collection time period in late 2019 and early 2020 when the COVID-19 pandemic began, it is an understatement to say this dissertation ended up quite different from what I had planned and hoped it would be. I had planned to include wild samples, *ex situ* Namibian samples, and more *ex situ* samples from US-based facilities feeding low fiber, commercial only diets. I also had planned to perform a diet trial of increasing animal fiber via whole rabbits in increments to characterize changes in the fecal microbiota. Both of these projects were prevented by pandemic-related travel restrictions and institutional policies. Therefore, here I suggest ways to continue and improve this research through future work.

Fecal microbiota sampling

My dissertation found collection time points for two specific climate types. While the US-based study was performed in the hot humid summer of Virginia, I hypothesize that the 24-hour time point should hold up if collecting in similar or colder and drier conditions. I would also suggest decreasing the time points between samplings. Twenty-four hours was chosen for ease of access to the samples (to avoid sampling in the middle of the night) but under certain conditions, there may be changes earlier than 24 hours. This may be important in areas of even higher temps and humidity. Second, based on the information in Chapter 2, I would suggest further investigations into the stability of younger cheetahs and those experiencing abnormal fecal consistency. With only two samples from both of these categories, I was unable to statistically test the effects of age or fecal consistency so it would be worthwhile to determine if there were truly inherent differences in the fecal microbiota of these groups and how that impacts how quickly the microbiota changes when it is exposed to the environment. This also highlights the need for increased research into age and health studies. I attempted to look at this in Chapter 4, but again with small data samples and an overarching effect of diet it was difficult to discern the effects of age and health. I did find age to be impactful on the microbial composition (chapter 4), but the effects would likely be easier to see if diet was uniform. Additionally, identifying not just the microbial changes, but the true metabolic changes across both age and fecal consistency would provide greater detail into the physiological changes occurring over life stage and GI health changes. Having previously studied hormonal pubertal onset in cheetahs, I think a great next project would be to determine the microbial and metabolic changes in young cheetahs as they develop. Is there something we could do during these important stages of development that might improve their reproductive, GI, or overall health later in life?

Similarly, for the Namibian collection in Chapter 3, I did not find changes within the four days of sampling but could not continue longer because there was not more fecal material left in the samples. To expand on this study, I suggest increasing the time period between sampling to identify changes in microbial ecology and in fecal characteristics. For *in situ* research, it can be quite difficult to age a fecal sample especially after the first 1-2 days post-defecation when most of the moisture is gone. Sampling for a longer duration of time may provide bacterial or fungal biomarkers that indicate the age of the feces, and that the microbiome has shifted. Field researchers could then perform these diagnostic tests to age the feces and determine whether it is appropriate for microbial ecology research. Additionally, I did not perform any sampling during the wet season. Partly due to the season I was able to travel, but also in general, fecal collection is more difficult in the wet season as there is greater insect activity (many of which take the fecals) and faster degradation of fecal material. If one was to perform field sampling in the rainy season, I would also recommend performing this time series first.

Influence of diet on the gut microbiome

As mentioned earlier, I did find evidence of dietary influence on the fecal microbiota in Chapter 4, but this could not be completely teased apart from a facility effect, because the commercial beef diet types all came from the same facility. As for the impacts of animal fiber, there were not enough cheetahs consuming the commercial beef + bones diet to make realistic recommendations based on the predictions of only three samples, so it was difficult to determine the true differences between the commercial beef + bones and commercial beef + carcass. However, some differences in microbial composition did exist between commercial horse + bones and commercial horse + carcass. It may be of interest to determine how much whole prey is necessary to add to commercial-based diets to improve the fecal microbiota and function and how this differs based on "prey" species (commercial horse vs. commercial beef). This would be of great interest for facilities that need to require primarily commercial-based diets, but that can also add whole prey items. The diet trial I originally proposed would have started with commercial diets only and progressively increased the amount of whole prey and sampling daily. With this dataset, I also wanted to determine how long the benefits of a whole prey feeding last in the microbiome. For example, if cheetah A and cheetah B are assigned to the commercial beef + rabbit diet and cheetah A is fed his weekly rabbit the day before sampling, but cheetah B had his weekly rabbit five days ago, how different will their fecal microbiota look even though they are technically eating the same diet? In chapter 4, we sampled cheetahs at one time point, but for those on a mixed diet (commercial + bones or commercial + carcass), it was not recorded what had been fed the day prior to the fecal collection. In general, I was interested in an overall view of the microbiome, but these small differences in when samples were collected may have influenced the data. Overall, future research on the influence of diet on the cheetah microbiome would benefit from performing more controlled dietary trials. Based on the results of chapter 4, there are three main areas of continued research I would recommend: 1) species of the diet, 2) amount of animal fiber/carcass, and 3) variety of both in diet. The two would be more straightforward studies. Most controlled cat studies use a Latin squares crossover design. This is more challenging with non-domestic endangered wildlife, because as discussed in this dissertation, cheetahs have management and health challenges and we do not want to cause issues or stress by subjecting them to changes, especially if it might upset their digestive tract. However, if there were enough healthy adults at one facility, I would suggest the Latin squares crossover design, to first test the effect of prey species using commercial diets and then the effect of carcass, providing washout periods between diets to allow the cheetahs to adjust. Samples would be collected after the new diets have been established but could also be collected daily during the transition period to identify when changes occur. Due to the added plant-based fiber differences of the two commercial diets used in my study, I would also suggest selecting commercial diets that use the same carbohydrate additives to avoid this as an additional confounding factor. Something I am curious about is the difference in carcass types. In my dissertation the carcass diets were higher in microbial diversity as expected and unique in composition and structure with many beneficial differences in their predicted microbial functions (chapter 4). However, this is not the same as suggesting that cheetahs fed horse carcasses have the ideal microbiome. Some studies suggest a diet based on horse is associated with GI health concerns [5]. It would be of great interest to feed cheetahs on a carcass diet of beef or another source to see how their microbes and metabolomes compare. Some of the cheetahs in this study were listed as consuming white tail deer (almost all of them in the commercial horse + carcass group). The frequency of deer was not regular as it depended on donations. Given the lack of GI symptoms in this group, this might be worth further investigation. The benefits of rabbit are suggested [1, 2, 6], however the authors also mention that neither rabbit nor beef chunks alone provide proper full nutrition for cheetahs. Which brings me to the last objective (variety in diet). This one may be more difficult to design but should be based on results from the first two objectives. If after controlling for additional variables (facility, age, health, sex), the species of the diet and carcass/animal fiber content are important, as suggested in chapter 4, then performing a diet experiment increasing the number and types of species and non-commercial dietary items would be warranted. This might include diets of commercial only, carcass only, and mixed diets of two or three combinations of diet types (commercial of pork, horse, beef + whole rabbits (2-4 times weekly) + bones (beef/horse) + heads (boar/calf/etc) + wild sourced meats if available (deer/turkey/moose/elk, etc)). If the results of chapter 4 stand, I would expect diets of more variety to lead to more diverse fecal microbiota and metabolomes more similar to those of wild cheetahs.

Relationships among GI symptoms, disease, and the fecal microbiota

Though we found evidence of a correlation between diet type and GI symptoms in Chapter 4, we did not find any associations between GI symptoms and microbial diversity or composition measures. I hypothesize that this was due to the low number of individuals with symptoms in our study and because I had to combine all symptom types into a binary "yes/no" variable. We also collected fecal score (FS) and body condition scores (BCS) as measures of GI and overall health. Despite their common use in domestic cats and other felid studies, including cheetahs, fecal scores were dropped from our dataset even before model selection due to low variability (most were 2s and 3s). BCS were also not that variable and were dropped from the model during AIC selection. Additionally, using GI symptoms or subjective measures (FS, BCS), instead of true disease diagnostics may mask the true differences in gastritis positive cheetahs compared to those without. We were unable to use diagnostic confirmed cases because not all participating facilities have the technology and ability to do so. As part of my original research plan, I had intended to build a machine learning model to predict biomarkers of gastritis so those facilities without veterinary diagnostic capabilities would have an alternative, non-invasive option. To accomplish this goal, future research should focus on using positive and negative confirmed diagnosis of gastritis or other diseases of interest (e.g., chronic renal failure) to first identify true differences in the microbiota or other datasets (e.g., metabolome) associated with those diseases. It may also be of interest to compare cheetahs with histopathology positive diagnoses of gastritis but no clinical signs, and those with positive diagnoses and clinical signs. One of the difficult aspects of understanding and diagnosing gastritis is that some affected cheetahs exhibit no clinical signs. FS and BCS may be better suited for monitoring but less effective in the early stages of model development.

Comparative carnivore studies

While this dissertation focused exclusively on cheetahs and heavily referenced domestic cat studies, it is likely that many of the findings in this dissertation apply to other felids and possibly other carnivores. While studies exist in various felids, it would be beneficial to have more comparative research to avoid the caveats of sample types, sample processing, sequencing, or data analysis variation that exists when trying to compare across studies. These dietary issues are not unique to cheetahs, and many other managed felids and carnivores will benefit from increased studies on diet type, animal fiber, variety, and health. Comparative studies may also help inform species-specific questions, like why do cheetahs suffer more from gastritis compared to lions and tigers even if they are eating the same diet? There may be nuances that comparative studies can help us identify, especially if we delve deeper beyond who is there. Metabolomic comparative studies will be especially informative to understand the physiological impacts of the microbial communities from similar diets in various cat species. Additionally, some cat species diets may be easier to replicate in managed facilities. *Ex situ* and wild bobcat microbiomes did not vary, and the authors suggested that while they had a small sample size, if real, this lack of difference was likely due to their ability to provide a varied diet similar to that of wild bobcats (including carcasses of small mammals and birds) [7]. The microbiomes of larger felids are more varied between *ex situ* and *in situ* populations [8, 9]. Some cat diets may be easier to replicate or are more similar to domestic cats. Understanding the dietary differences across felids may help us improve the health and welfare of these individuals in our managed facilities. In general, the carnivore gut is largely assumed to be simplistic compared to omnivores and herbivores [10]. While in comparison this may be true, there are likely smaller scale variations we are missing by grossly overgeneralizing carnivore GI systems. A recent study has shown greater variability within carnivore microbiomes compared to herbivores, despite their reduced diversity [11]. This likely plays a role in the unique health challenges facing various felid and carnivore species in our facilities and highlights the need for more comparative research to inform specialized dietary needs, possibly even within species.

The feasibility of these studies depends on the animal management and veterinary team, budget, and of course the willingness of participating cheetahs to try new things. The pickiness of cheetahs cannot go without mentioning. Many managers have told me that they try to feed more carcass items, but their cheetahs do not want to eat them, or will only eat them if they are skinned or cut up and all the beneficial animal fiber aspects are removed (likely because these cheetahs are used to commercial ground meat diets). To avoid picky eaters, it may be especially beneficial to increase variety in the food of young cheetahs to prepare them for a life of varied diets. I know many veterinarians reading this will be concerned about the potential risk of pathogens in carcass meat and the GI distress changing a diet may cause. Nutritionists will be concerned about cheetahs getting the proper nutrients. Animal biologists and caretakers will be concerned about the behavior, welfare, and logistics of performing studies like these. All of these concerns are valid and worth discussing, which is why microbial ecology studies in animals require intensive communication and collaboration among an interdisciplinary group of scientists and veterinary healthcare professionals.

In summary, this dissertation provides a foundation for future work in cheetah and feline microbial ecology research. Here we provide evidence that samples are viable for at least 24 hours (longer in dry/arid conditions) to help increase access to samples for greater sample numbers. We also found evidence for a correlation between diet type and GI symptoms, contributing to the concern over dietary discrepancies between ex situ and free-ranging cheetahs. We also determined diet is an important predictor of microbial diversity and community composition in managed cheetahs, highlighting the need for continued research on animal fiber, species of the diet, and variety of diet in these felines. As research continues to develop it may benefit facilities to increase the amount of carcass/animal fiber in the cheetah diet as well as provide greater variety in their diet.

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APPENDICES

Appendix A

Table A.1. Weather related metadata for all fecal subsamples.

Fecal ID	Day	Sequence ID	Collection Date	Max Daily Rainfall (inches)	Max Daily Temperature (F)	Max Daily Humidity (%)
Aju 1	Day 0	aju1a	06.27.2019	0	80	62
Aju 1	Day 1	aju1b	06.28.2019	0.23	89	100
Aju 1	Day 2	aju1c	06.29.2019	0.48	90	100
Aju 1	Day 3	aju1d	06.30.2019	0.01	91	100
Aju 1	Day 4	aju1e	07.01.2019	0.1	88	96
Aju 1	Day 5	aju1f	07.02.2019	0	84	98
Aju 2	Day 0	aju2a	06.30.2019	0	91	100
Aju 2	Day 1	aju2b	07.01.2019	0.1	88	96
Aju 2	Day 2	aju2c	07.02.2019	0	84	98
Aju 2	Day 3	aju2d	07.03.2019	0	91	99
Aju 2	Day 4	aju2e	07.04.2019	0.06	91	100
Aju 2	Day 5	aju2f	07.05.2019	0.07	89	100
Aju 3	Day 0	aju3a	07.03.2019	0	91	99
Aju 3	Day 1	aju3b	07.04.2019	0.06	91	100
Aju 3	Day 2	aju3c	07.05.2019	0.07	89	100
Aju 3	Day 3	aju3d	07.06.2019	0.09	87	100
Aju 3	Day 4	aju3e	07.07.2019	0.03	90	100
Aju 4	Day 0	aju4a	07.10.2019	0	85	100
Aju 4	Day 1	aju4b	07.11.2019	0	87	100
Aju 4	Day 2	aju4c	07.12.2019	0.34	87	100
Aju 4	Day 3	aju4d	07.13.2019	0.01	88	100
Aju 4	Day 4	aju4e	07.14.2019	0	88	100
Aju 4	Day 5	aju4f	07.15.2019	0	90	100
Aju 5	Day 0	aju5a	07.10.2019	0	85	100
Aju 5	Day 1	aju5b	07.11.2019	0	87	100
Aju 5	Day 2	aju5c	07.12.2019	0.34	87	100
Aju 5	Day 3	aju5d	07.13.2019	0.01	88	100
Aju 5	Day 4	aju5e	07.14.2019	0	88	100
Aju 6	Day 0	aju6a	07.11.2019	0	87	100
Aju 6	Day 1	aju6b	07.12.2019	0.34	87	100
Aju 6	Day 2	aju6c	07.13.2019	0.01	88	100
Aju 6	Day 3	aju6d	07.14.2019	0	88	100
Aju 6	Day 4	aju6e	07.15.2019	0	90	100
Aju 6	Day 5	aju6f	07.16.2019	0	90	100
Aju 7	Day 0	aju7a	07.13.2019	0	88	100
Aju 7	Day 1	aju7b	07.14.2019	0	88	100
Aju 7	Day 2	aju7c	07.15.2019	0	90	100
Aju 7	Day 3	aju7d	07.16.2019	0	90	100
Aju 8	Day 0	aju8a	07.14.2019	0	88	100
Aju 8	Day 1	aju8b	07.15.2019	0	90	100

Aju 8	Day 2	aju8c	07.16.2019	0	90	100

Table A.2. Taxonomic classifications of the 15 differentially abundant (DA) ASVs between Day 0 and every other Day grouped by Family.

ASV	**Phylum**	***Family***	***Genus***	***Species***	**Days DA from Day 0**
ASV48	Fusobateria	*Fusobacteriaceae*	*Fusobacterium*	*NA*	Day 3, Day 4
ASV64	Fusobateria	*Fusobacteriaceae*	*Fusobacterium*	*NA*	Day 4
ASV34	Fusobateria	*Fusobacteriaceae*	*Fusobacterium*	*NA*	Day 3, Day 4
ASV4	Fusobateria	*Fusobacteriaceae*	*Fusobacterium*	*NA*	Day 3, Day 4, Day 5
ASV119	Firmicutes	*Lachnospiraceae*	*Eisenbergiella*	*NA*	Day 1, Day 2, Day 3, Day 4, Day 5
ASV88	Firmicutes	*Lachnospiraceae*	*Clostridium XIVa*	*glycyrrhizinilticum*	Day 3
ASV86	Firmicutes	*Clostridiaceae*	*Clostridium sensu stricto*	*NA*	Day 3
ASV13	Firmicutes	*Bacilliaceae*	*Bacillus*	*NA*	Day 5
ASV1	Firmicutes	*Bacillaceae*	*Bacillus*	*NA*	Day 2, Day 3, Day 4, Day 5
ASV12	Firmicutes	*Bacillaceae*	*Bacillus*	*infantis*	Day 3, Day 4, Day 5
ASV38	Bacteroidetes	*Bacteroidaceae*	*Bacteroides*	*NA*	Day 2, Day 3, Day 4, Day 5
ASV71	Bacteroidetes	*Bacteroidaceae*	*Bacteroides*	*NA*	Day 3
ASV26	Bacteroidetes	*Bacteroidaceae*	*Bacteroides*	*NA*	Day 2, Day 3, Day 4, Day 5
ASV46	Bacteroidetes	*Bacteroidaceae*	*Bacteroides*	*NA*	Day 3, Day 4, Day 5
ASV17	Bacteroidetes	*Bacteroidaceae*	*Bacteroides*	*NA*	Day 3, Day 4, Day 5

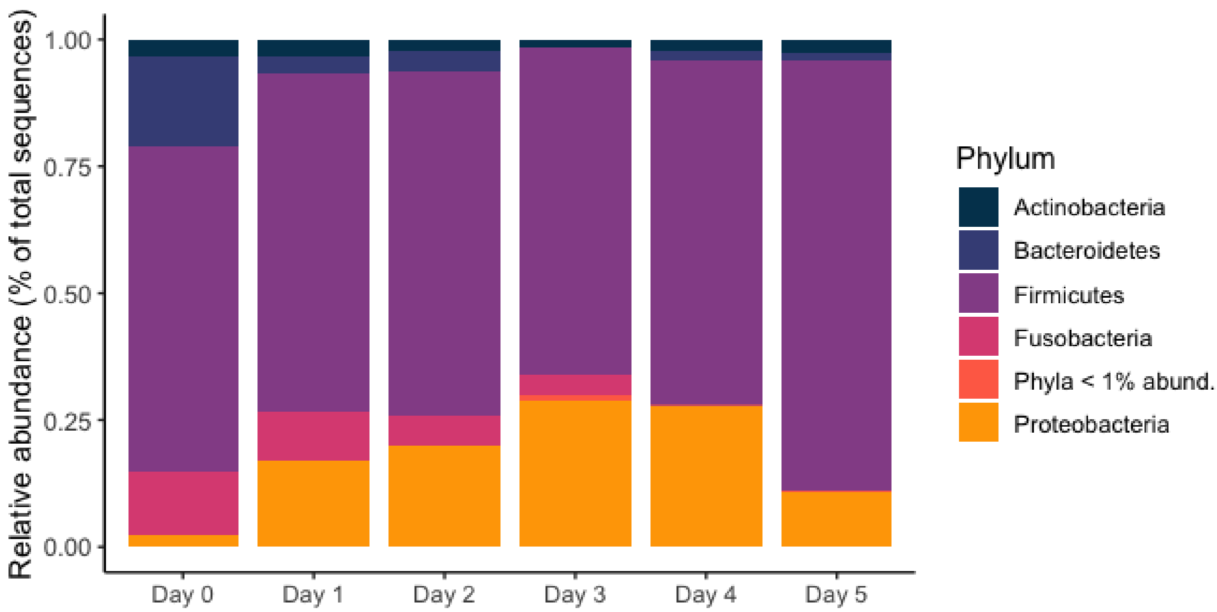

Figure A.1. Relative abundance of dominant bacterial Phyla in cheetah feces across sample days. Phyla with < 1% relative abundance are grouped together.

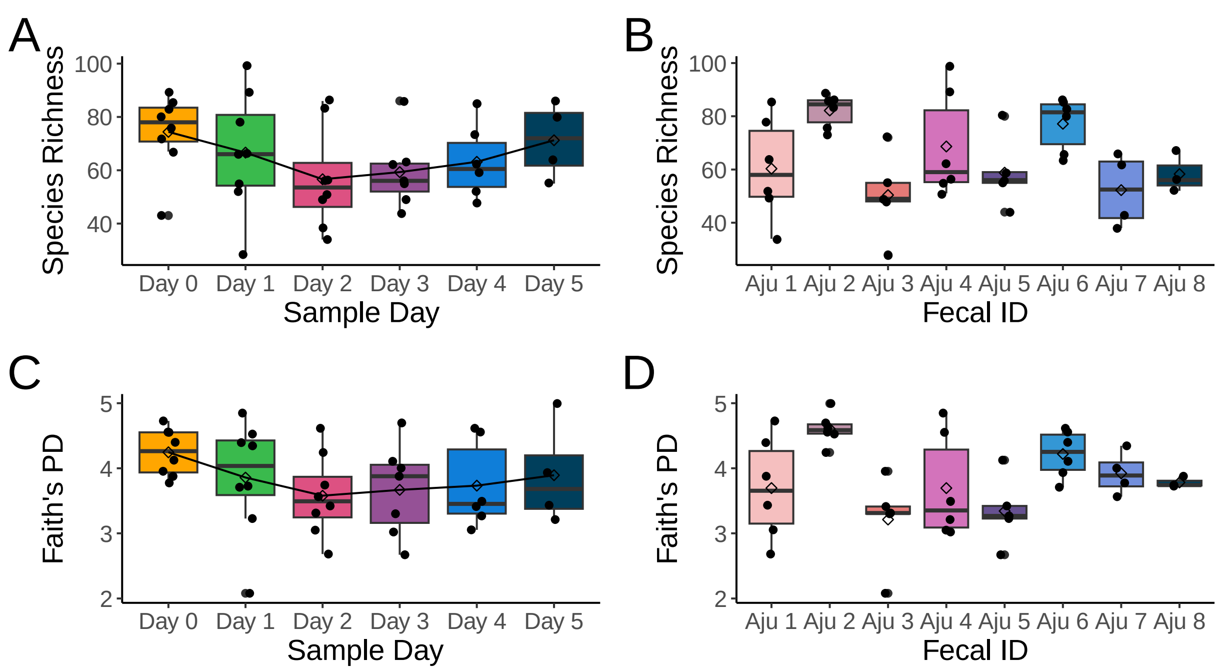

Figure A.2. Box plots depicting species richness and Faith's PD by Sample Day (A and C, respectively) and Fecal ID (B and D, respectively). Species richness and Faith's PD did not differ among Sample Day but differed among Fecal IDs (ANOVAs $p < 0.05$). Aju2 and Aju6 are adults, while the other individuals are juveniles. Aju 7 and Aju 8 were experiencing loose stools and the others had normal fecal consistency.

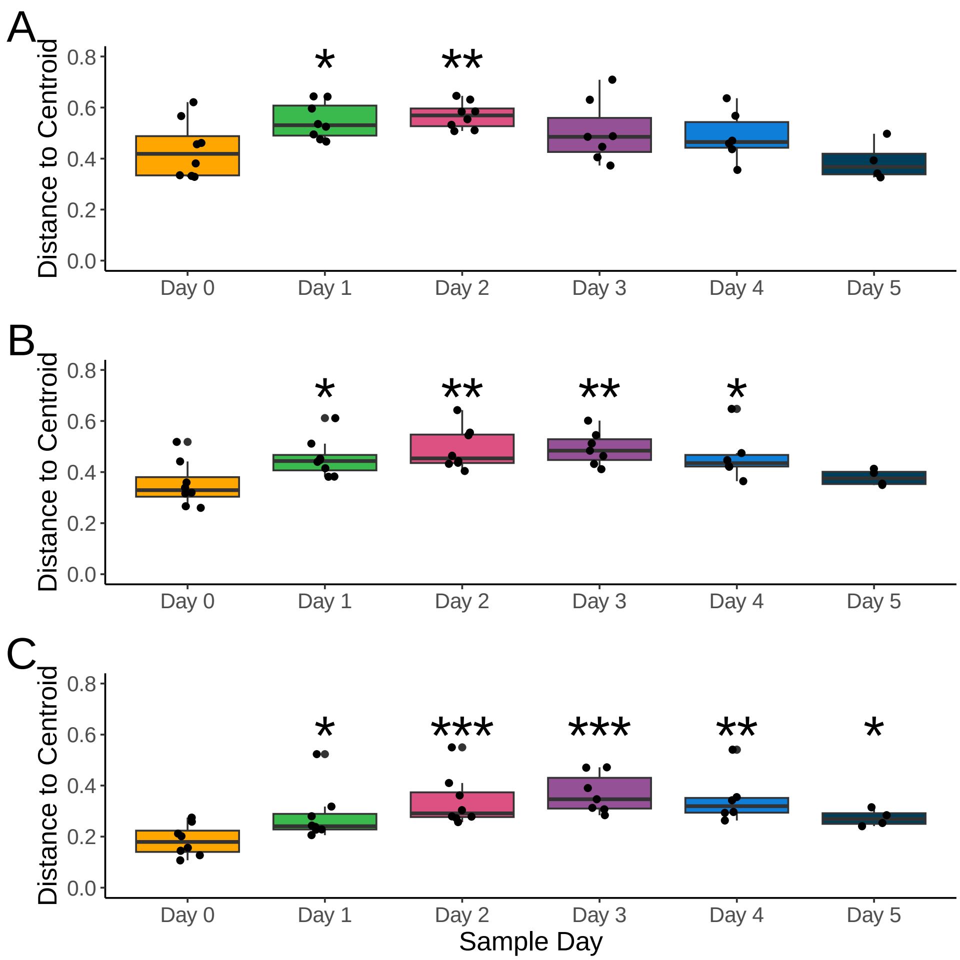

Figure A.3. Dispersion of composition among groups. Boxplots indicate the distance from the centroid for all subsamples within each Sample Day group for **(A)** Bray-Curtis, **(B)** Jaccard, and **(C)** unweighted UniFrac. Asterisks indicate significant difference in distances from the centroid compared to Day 0. Significance values are as follows: PERMDISP permutated p values, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Appendix B

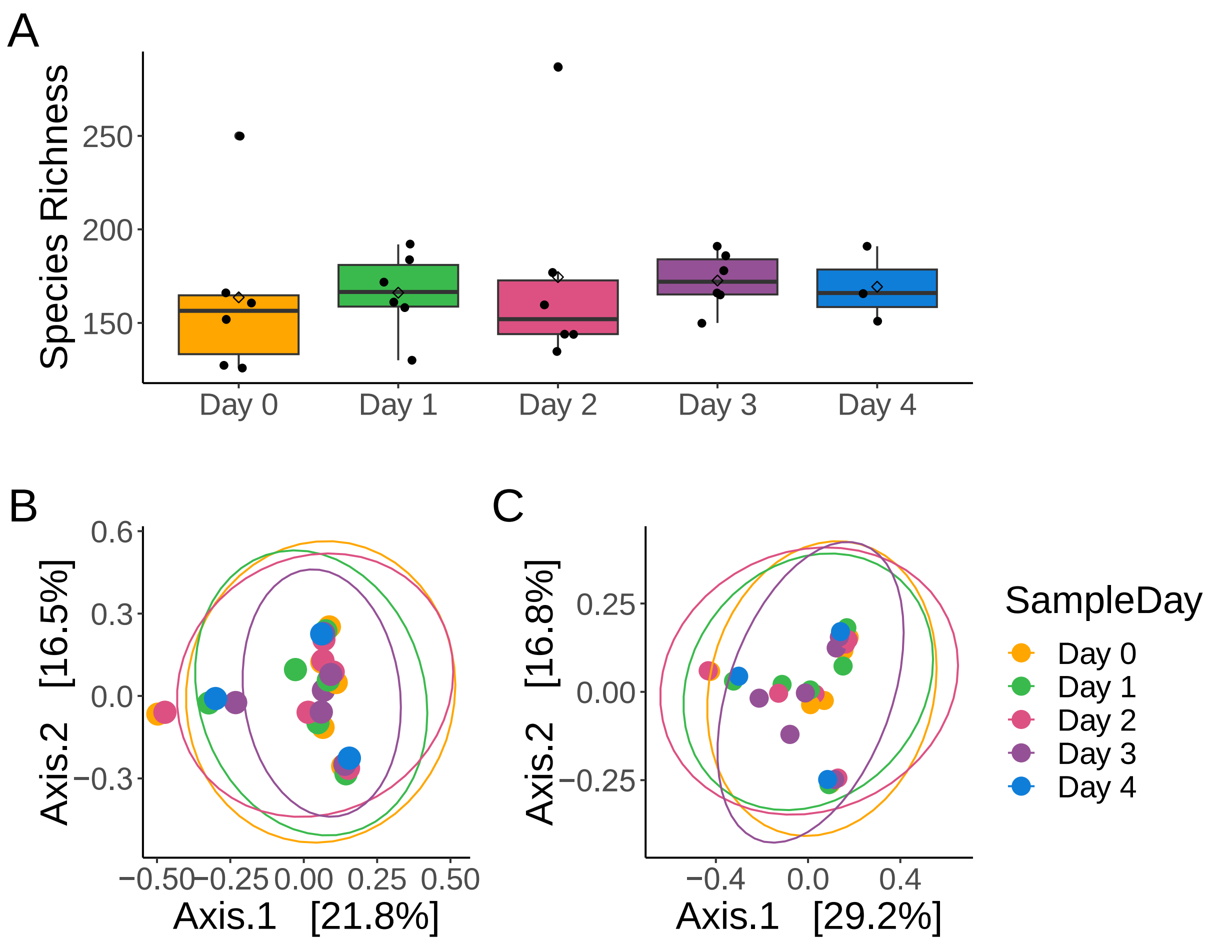

Figure B.1. (A) Alpha and **(B and C)** beta diversity measures for NAM fecal samples across sample day (colors). **(A)** Species richness (SR) did not differ by sample day (GLMM: x^2 = 0.6343, df = 4, p = 0.959). **(B)** Jaccard and **(C)** unweighted Unifrac distances did not differ by sample day (PERMANOVA Sample Day: Jaccard Pseudo-$F_{4,22}$ = 0.4545, R^2 = 0.07632, p = 0.24, UniFrac Pseudo-$F_{4,22}$ = 0.4595, R^2 = 0.07711, p = 0.099).

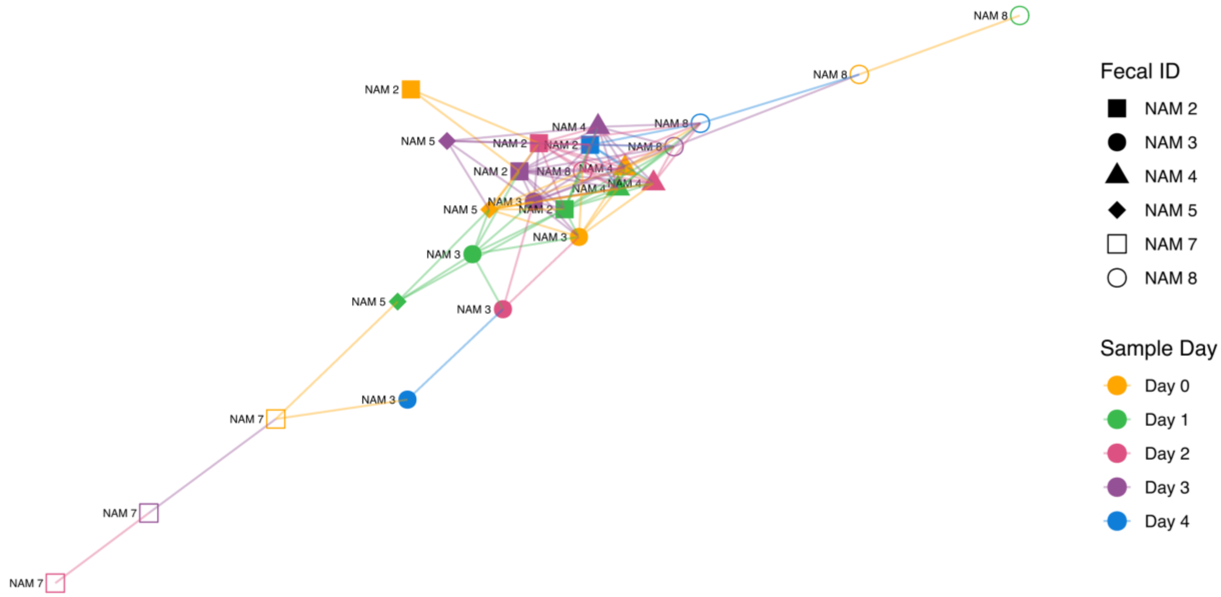

Figure B.2. Microbial network showing connections between samples (nodes) using Bray-Curtis dissimilarity values. Color indicates sample day and shape indicates fecal ID. Lines connecting samples have a pairwise Bray-Curtis distance less than 0.7. A shorter length of the line connecting two samples indicates greater compositional similarity.

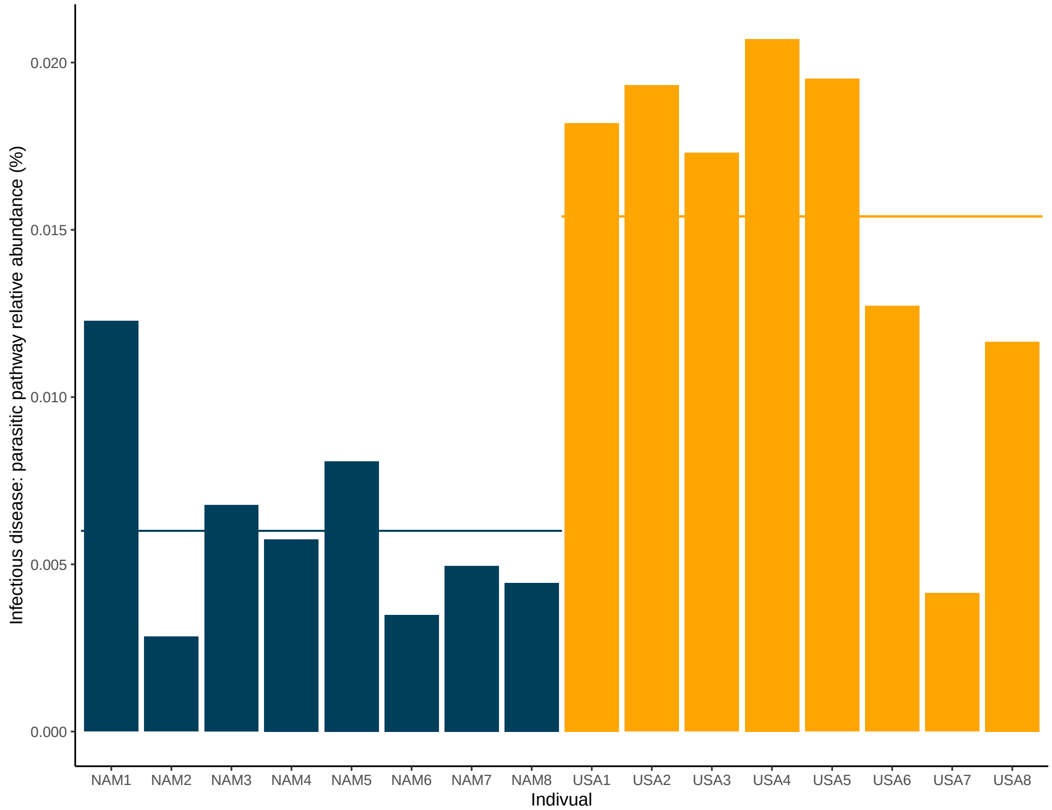

Figure B.3. Proportions of sequences for Level 2 KEGG Ortholog predicted functional pathway, parasitic infectious disease, for each individual. Color indicates population where and yellow is US (USA) cheetahs and blue is Namibian (NAM). Horizonal line indicates mean for each population. USA7 and USA8 were experiencing diarrhea at time of collection.

Appendix C

Table C.1 Interaction effects of primary diet and supplemental diet type between commercial diets in ANOVA and PERMANOVA models

ANOVAs					
lm(SR ~ Diet.Main*Diet.Supp); $F_{3,65}$ = 0.2742; p = 0.8438, Adjusted R^2 = -0.033					
	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**	
Intercept	106.333	10.96	9.699	2.96e-14	
Diet.Main – commercial horse	-0.413	11.60	-0.036	0.972	
Diet.Supp - carcass	-2.857	11.72	-0.244	0.808	
Interaction – commercial horse:carcass	5.787	13.03	0.444	0.658	
lm(PD ~ Diet.Main*Diet.Supp); $F_{3,65}$ = 1.56; p = 0.2076, Adjusted R^2 = -0.024					
	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**	
Intercept	4.904	0.35	14.168	<2e-16	
Diet.Main – commercial horse	0.063	0.37	0.172	0.864	
Diet.Supp - carcass	-0.223	0.37	-0.602	0.549	
Interaction – commercial horse:carcass	0.324	0.41	0.788	0.434	
PERMANOVAs					
Bray-Curtis ~ Diet.Main*Diet.Supp					
	Df	**Sum of Squares**	**R^2**	**F**	**Pr(>F)**
Diet.Main	1	2.29	0.16	13.35	0.001
Diet.Supp	1	0.42	0.029	2.46	0.007
Diet.Main:Diet.Supp	1	0.47	0.033	2.73	0.001
Residual	65	11.15	0.778		
Total	68	14.33	1		
Jaccard ~ Diet.Main*Diet.Supp					
	Df	**Sum of Squares**	**R^2**	**F**	**Pr(>F)**
Diet.Main	1	1.69	0.12	9.54	0.001
Diet.Supp	1	0.47	0.03	2.66	0.001
Diet.Main:Diet.Supp	1	0.41	0.03	2.32	0.002
Residual	65	11.49	0.82		
Total	68	14.05	1		
Unweighted UniFrac ~ Diet.Main*Diet.Supp					
	Df	**Sum of Squares**	**R^2**	**F**	**Pr(>F)**
Diet.Main	1	0.92	0.12	9.89	0.001

Diet.Supp	1	0.23	0.03	2.49	0.006
Diet.Main:Diet.Supp	1	0.34	0.04	3.62	0.001
Residual	65	6.04	0.8		
Total	68	7.52	1		

Table C.2. Sex, age, and body condition score (BCS) groups by diet types.

		Carcass	Commercial Beef + Bone	Commercial Beef + Carcass	Commercial Horse + Bone	Commercial Horse + Carcass	Row Totals
Sex	Males	9	1	7	10	8	35
	Females	9	2	14	14	12	51
Age	Juveniles	2	0	10	1	4	17
	Young Adult	8	0	2	10	5	25
	Adult	6	3	9	11	9	38
	Geriatric	2	0	0	2	2	6
Body Condition Score (BCS)	BCS 3	1	0	0	0	0	1
	BCS 4	3	0	0	8	3	14
	BCS 5	6	1	3	13	12	35
	BCS 6	5	0	6	3	5	19
	BCS 7	3	2	2	0	3	10
	BCS Too young	0	0	10	0	0	10
GI Symptoms	No	14	3	11	15	20	63
	Yes	4	0	10	9	0	23

Table C.3 Pairwise Fisher's exact tests for diet type and GI symptoms. *p*-values are FDR adjusted for multiple comparisons.

Comparison	***p*.adjust**
Carcass vs Commercial beef + bones	0.25
Carcass vs Commercial beef + carcass	0.465
Carcass vs Commercial horse + bones	0.465
Carcass vs Commercial horse + carcass	0.138
Commercial beef + carcass vs Commercial beef + bones	0.465
Commercial beef + carcass vs Commercial horse + bones	1
Commercial beef + carcass vs Commercial horse + carcass	0.007
Commercial horse + bones vs Commercial beef + bones	0.666
Commercial horse + bones vs Commercial horse + carcass	0.007
Commercial horse + carcass vs Commercial beef + bones	1

Table C.4 Relative abundance (%) of phyla across diet types (mean ± SD).

	Firmicutes	**Bacteroidetes**	**Actinobacteria**	**Fusobacteria**	**Proteobacteria**	**Spirochaetes**
Carcass	39.8 ± 14.6	27.5 ± 15.7	11.7 ± 6	18.3 ± 6.8	2.7 ± 2.4	< 1
Commercial beef + bones	49.4 ± 6.8	17.7 ± 8.8	19 ± 4.8	12.8 ± 3.8	1.1 ± 1.5	< 1
Commercial beef + carcass	60.2 ± 14.2	14.1 ± 8.7	18.8 ± 6.7	4.1 ± 5.7	2.8 ± 5.1	< 1
Commercial horse + bones	37.5 ± 10.1	29.9 ± 11.8	10.9 ± 4.3	13.4 ± 8.5	8.3 ± 6.4	< 1
Commercial horse + carcass	47 ± 11.8	19.7 ± 11.4	16.1 ± 4.6	10.2 ± 7.1	7 ± 5.3	< 1

Table C.5 Pairwise permutation test of bacterial community dispersion by diet type groups across all three beta diversity metrics. Diets are abbreviated (CH = commercial horse, CB = commercial beef, "+ B" = + bones, and "+ C" = + carcass). Permuted *p*-values < 0.05 are bolded.

Diet Comparison	Bray-Curtis	Jaccard	Unweighted UniFrac
CH + B vs CH + C	0.39	0.07	0.76
CH + B vs CB + C	0.48	0.09	0.53
CH + B vs CB + B	**0.02**	**0.002**	**0.03**
CH + C vs CB + C	0.97	0.89	0.73
CH + C vs CB + B	**0.03**	**0.001**	**0.004**
CB + C vs CB + B	0.11	**0.01**	0.15
Carcass vs CH + C	**0.001**	**0.001**	**0.001**
Carcass vs CH + B	**0.001**	**0.001**	**0.005**
Carcass vs CB + C	**0.001**	**0.001**	**0.001**
Carcass vs CB + B	**0.001**	**0.001**	**0.001**

Table C.6 Differentially abundant Level 3 KEGG Ortholog functional pathways across diet types identified by PICRUSt2 and analyzed using ALDEx2 glm. Carcass diet type served as the reference (intercept) and all other diets were compared to carcass. Holm adjusted *p*-values < 0.05 are bolded.

	KEGG Level 3 pathway	Intercept (Carcass as reference)	Commercial Beef + Bones	Commercial Beef + Carcass	Commercial Horse + Bones	Commercial Horse + Carcass
Cell motility	**Bacterial chemotaxis**	3.17 ± 0.09 t = 37.21	0.79 ± 0.26 t = 3.0 *p* = 0.55	**0.78 ± 0.13 t = 6.15 *p* < 0.001**	0.15 ± 0.12 t = 1.28 *p* = 1	0.41 ± 0.13 t = 3.1 *p* = 0.39
	Flagellar assembly	2.46 ± 0.10 t = 24.08	0.97 ± 0.32 t = 3.05 *p* = 0.48	**0.79 ± 0.15 t = 5.14 *p* < 0.001**	0.20 ± 0.14 t = 1.37 *p* = 1	0.47 ± 0.16 t = 3.0 *p* = 0.47
Membrane Transport	**ABC transporters**	3.29 ± 0.06 t = 54.17	0.35 ± 0.01 t = 1.88 *p* = 1	**0.36 ± 0.09 t = 4.0 *p* = 0.039**	0.18 ± 0.09 t = 2.04 *p* = 0.98	0.29 ± 0.09 t = 3.12 *p* = 0.41
Signal Transduction	**Two-component systems**	2.13 ± 0.07 t = 31.54	0.39 ± 0.21 t = 1.85 *p* = 1	**0.48 ± 0.1 t = 4.78 *p* = 0.002**	**0.4 ± 0.1 t = 4.23 *p* = 0.018**	**0.49 ± 0.1 t = 4.69 *p* = 0.003**
Transcription	**RNA polymerase**	3.62 ± 0.06 t = 59.57	0.34 ± 0.19 t = 1.82 *p* = 1	**0.36 ± 0.09 t = 3.91 *p* = 0.049**	0.06 ± 0.09 t = 0.73 *p* = 1	0.09 ± 0.09 t = 0.95 *p* =1
Infectious disease: bacterial	**Bacterial invasion of epithelial cells**	-4.07 ± 0.28 t = -14.56	-0.98 ± 0.87 t = -1.13 *p* = 1	0.21 ± 0.42 t = 0.51 *p* = 1	**2.46 ± 0.4 t = 6.23 *p* < 0.001**	**2.47 ± 0.43 t = 5.74 *p* < 0.001**
	Pathogenic Escherichia coli infection	-6.51 ± 0.67 t = -9.67	-0.32 ± 2.1 t = -0.16 *p* =1	1.61 ± 1.01 t = 1.59 *p* = 1	**5.94 ± 0.95 t = 6.24 *p* < 0.001**	**5.90 ± 1.04 t = 5.70 *p* < 0.001**
Infectious disease: parasitic	**African trypanosomiasis**	-5.05 ± 0.26 t = -19.42	2.86 ± 0.82 t = 3.53 *p* = 0.17	**2.16 ± 0.4 t = 5.52 *p* = 0.001**	**2.17 ± 0.37 t = 5.87 *p* = 0.001**	**2.27 ± 0.41 t = 5.64 *p* = 0.001**
	Ameobiasis	-4.85 ± 0.21 t = -23.64	2.48 ± 0.64 t = 3.89 *p* = 0.051	**1.25 ± 0.31 t = 4.06 *p* = 0.033**	-0.06 ± 0.29 t = -0.22 *p* = 1	-0.16 ± 0.32 t = 0.52 *p* = 1
Amino acid metabolism	**Arginine and proline metabolism**	3.17 ± 0.06 t = 54.95	0.33 ± 0.18 t = 1.87 *p* = 1	**0.45 ± 0.09 t = 5.19 *p* = 0.001**	0.29 ± 0.08 t = 3.51 *p* = 0.18	0.31 ± 0.09 t = 3.54 *p* = 0.16
	Cysteine and methionine metabolism	4.03 ± 0.06 t = 71.18	0.30 ± 0.18 t = 1.72 *p* = 1	**0.34 ± 0.08 t = 4.05 *p* = 0.035**	0.13 ± 0.08 t = 1.67 *p* = 0.99	0.21 ± 0.09 t = 2.42 *p* = 0.86
	Histidine metabolism	3.85 ± 0.06 t = 61.11	0.33 ± 0.20 t = 1.69 *p* = 1	**0.47 ± 0.09 t = 4.99 *p* = 0.001**	0.23 ± 0.09 t = 2.60 *p* = 0.81	0.27 ± 0.10 t = 2.81 *p* = 0.64
	Phenylalanine metabolism	2.24 ± 0.06 t = 38.80	0.18 ± 0.18 t = 0.98 *p* = 1	0.12 ± 0.09 t = 1.43 *p* = 1	0.41 ± 0.08 t = 5.02 *p* = 0.002	0.37 ± 0.09 t = 4.11 *p* = 0.032
Biosynthesis of other secondary metabolites	**Penicillin and cephalosporin biosynthesis**	-2.55 ± 0.28 t = -9.16	-0.57 ± 0.87 t = -0.66 *p* = 1	**1.56 ± 0.42 t = 3.74 *p* = 0.045**	**1.57 ± 0.39 t = 3.97 *p* = 0.022**	1.58 ± 0.43 t = 3.67 *p* = 0.059

Carbohydrate metabolism	**Ascorbate and aldarate metabolism**	1.76 ± 0.08 t = 22.06	0.32 ± 0.25 t = 1.27 *p* = 1	0.44 ± 0.12 t = 3.65 *p* = 0.085	**0.69 ± 0.11** **t = 6.14** ***p* < 0.001**	**0.68 ± 0.12** **t = 5.54** ***p* < 0.001**
	Galactose metabolism	3.89 ± 0.06 t = 67.49	0.27 ± 0.18 t = 1.49 *p* = 1	**0.39 ± 0.09** **t = 4.55** ***p* = 0.006**	**0.35 ± 0.08** **t = 4.35** ***p* = 0.014**	**0.37 ± 0.09** **t = 4.19** ***p* = 0.023**
	Glyoxylate and dicarboxylate metabolism	2.85 ± 0.06 t = 48.83	0.47 ± 0.18 t = 2.57 *p* = 0.86	**0.43 ± 0.09** **t = 4.93** ***p* =0.002**	**0.54 ± 0.08** **t = 6.50** ***p* < 0.001**	**0.51 ± 0.09** **t = 5.71** ***p* < 0.001**
	Inositol phosphate metabolism	1.32 ± 0.06 t = 23.58	0.22 ± 0.17 t = 1.28 *p* =1	**0.34 ± 0.08** **t = 4.04** ***p* =0.04**	**0.33 ± 0.08** **t = 4.23** ***p* =0.027**	**0.35 ± 0.09** **t = 4.09** ***p* =0.033**
	Pentose and glucuronate interconversions	3.12 ± 0.07 t = 45.98	0.57 ± 0.21 t = 2.69 *p* =0.81	**0.54 ± 0.10** **t = 5.28** ***p* < 0.001**	**0.60 ± 0.10** **t = 6.23** ***p* < 0.001**	**0.58 ± 0.10** **t = 5.3** ***p* < 0.001**
	Pentose phosphate pathway	4.46 ± 0.06 t = 78.26	0.32 ± 0.18 t = 1.8 *p* = 1	**0.35 ± 0.09** **t = 4.06** ***p* = 0.034**	0.17 ± 0.08 t = 2.08 *p* = 0.97	0.27 ± 0.09 t = 3.06 *p* = 0.46
Energy metabolism	**Methane metabolism**	2.68 ± 0.06 t = 46.96	0.38 ± 0.18 t = 2.15 *p* = 0.98	0.43 ± 0.09 t = 5.02 *p* = 0.001	0.28 ± 0.08 t = 3.41 *p* = 0.23	0.32 ± 0.09 t = 3.67 *p* = 0.12
	Sulfur metabolism	3.3 ± 0.06 t = 59.94	0.25 ± 0.17 t = 1.48 *p* = 1	0.21 ± 0.08 t = 2.58 *p* = 0.75	0.33 ± 0.08 t = 4.26 *p* = 0.021	0.28 ± 0.08 t = 3.36 *p* = 0.26
Glycan biosynthesis and metabolism	**Lipopolysaccharide biosynthesis**	3.81 ± 0.14 t = 27.69	-0.35 ± 0.43 t = -0.81 *p* = 1	**-1.30 ± 0.21** **t = -6.31** ***p* < 0.001**	0.08 ± 0.19 t = 0.44 *p* = 1	-0.22 ± 0.21 t = -1.04 *p* = 1
Lipid metabolism	**Biosynthesis of unsaturated fatty acids**	1.97 ± 0.06 t = 34.66	0.5 ± 0.18 t = 2.81 *p* = 0.69	**0.49 ± 0.09** **t = 5.72** ***p* < 0.001**	0.18 ± 0.08 t = 2.18 *p* = 0.94	0.26 ± 0.09 t = 3.02 *p* = 0.48
	Glycerolipid metabolism	2.59 ± 0.07 t = 37.69	0.27 ± 0.21 t = 1.25 *p* = 1	**0.58 ± 0.10** **t = 5.60** ***p* < 0.001**	0.20 ± 0.10 t = 2.07 *p* = 0.98	0.36 ± 0.11 t = 3.37 *p* = 0.22
Metabolism of oher amino acids	**Beta-Alanine metabolism**	-4.50 ± 1.10 t = -4.07	-6.65 ± 3.43 t = -1.95 *p* = 0.95	-4.80 ± 1.65 t = -2.92 *p* = 0.51	-6.06 ± 1.56 t = -3.90 *p* = 0.10	**-6.97 ± 1.70** **t = -4.12** ***p* = 0.043**
	Phosphonate and phosphinate metabolism	0.52 ± 0.09 t = 5.79	0.46 ± 0.28 t = 1.65 *p* = 1	**0.68 ± 0.13** **t = 5.06** ***p* = 0.001**	**0.62 ± 0.13** **t = 4.91** ***p* = 0.001**	**0.72 ± 0.14** **t = 5.20** ***p* < 0.001**
Metabolism of terpenoids and polyketoids	**Biosynthesis of ansamycins**	5.86 ± 0.07 t = 87.25	0.71 ± 0.21 t = 3.40 *p* = 0.23	**0.59 ± 0.10** **t = 5.88** ***p* < 0.001**	0.25 ± 0.10 t = 2.67 *p* = 0.77	**0.43 ± 0.10** **t = 4.17** ***p* = 0.02**
Xenobiotics biodegradation and metabolism	**Caprolactam degradation**	-1.44 ± 0.23 t = -6.30	-0.27 ± 0.71 t = -0.39 *p* = 1	0.99 ± 0.34 t = 2.91 *p* = 0.49	**1.73 ± 0.32** **t = 5.37** ***p* < 0.001**	**1.77 ± 0.35** **t = 5.05** ***p* < 0.001**
Endocrine system	**Insulin signaling pathway**	-0.15 ± 0.06 t = -2.53	0.35 ± 0.18 t = 1.93 *p* =0.99	**0.43 ± 0.09** **t = 4.86** ***p* = 0.003**	0.24 ± 0.08 t = 2.94 *p* = 0.535	0.29 ± 0.09 t = 3.27 *p* = 0.323
Environmental adaptation	**Plant-pathogen interaction**	1.38 ± 0.06 t = 22.79	0.30 ± 0.19 t = 1.59 *p* = 1	**0.40 ± 0.09** **t = 4.39** ***p* = 0.011**	0.16 ± 0.09 t = 1.93 *p* = 0.99	0.26 ± 0.09 t = 2.79 *p* = 0.63

Table C.7 Logistic regression model for GI symptoms predicted by Species Richness (SR), Age Group, and Diet. Model likelihood ratio test: LR x^2 = 32.35, df = 8, p < 0.0001 where reference levels were Adult and Carcass diet type.

	Coefficient	S.E.	Wald Z	Pr(>|Z|)
Intercept	1.8413	1.8224	1.01	0.3123
SR	-0.0260	0.0162	-1.60	0.1091
Juvenile	1.9720	0.8922	2.21	0.0271
Young Adult	-1.0801	0.8045	-1.34	0.1794
Geriatric	-0.9076	1.3770	-0.66	0.5098
Commercial Horse + Carcass	-9.1446	23.3064	-0.39	0.6948
Commercial Beef + Carcass	-0.1686	0.8955	-0.19	0.8506
Commercial Horse + Bones	0.8164	0.8004	1.02	0.3077
Commercial Beef + Bones	-8.5916	67.1852	-0.13	0.8982

Table C.8 Logistic regression model for GI symptoms predicted by Faith's phylogenetic diversity (PD), Age Group, and Diet. Model likelihood ratio test: LR x^2 = 29.72, df = 8, p = 0.0002 where reference levels were Adult and Carcass diet type.

	Coefficient	S.E.	Wald Z	Pr(>|Z|)
Intercept	0.1153	2.8412	0.04	0.9676
PD	-0.1961	0.5036	-0.39	0.6970
Juvenile	1.6630	0.8649	1.92	0.0545
Young Adult	-1.2913	0.7855	-1.64	0.1002
Geriatric	-0.7277	1.3005	-0.56	0.5758
Commercial Horse + Carcass	-8.9632	23.7318	-0.38	0.7057
Commercial Beef + Carcass	-0.0071	1.0127	-0.01	0.9944
Commercial Horse + Bones	0.8298	0.8288	1.00	0.3167
Commercial Beef + Bones	-8.6683	67.2295	-0.13	0.8974

Figure C.1 Metadata survey for individual fecal collection.

Cheetah Microbiome Metadata Survey

Please fill in one survey for every cheetah sampled. Alternatively, if you would like an excel version of this sheet to fill in (one cheetah per row), please email Morgan Maly (mamaly@ncsu.edu).

General Info

1. Cheetah studbook #:
2. Date of sample collection:
3. Please list the studbooks numbers of any other cheetahs sharing the enclosure space:
4. Most recent body condition score (BCS) and date of score:
5. Fecal consistency score of sample collected (based on Felid TAG rating scale, see attached):
6. If a fecal marker was used to indicate the individual please note here (ex. glitter):

Diet

1. Current primary diet, please be specific (e.g, Central Nebraska Premium Beef Feline, whole carcass horse, etc.):
2. Current carcass or whole prey items fed (e.g., whole rabbit, horse rib, beef knuckle, etc. or none):
3. Approximately how many times per week are carcass/whole prey items provided to the cheetah?

Health

1. Current medications (including supplements and preventatives like lysine, flea control, etc):
2. Has the cheetah presented any symptoms of interest in the last month (circle all that apply)? If yes, in the space after the symptom, please indicate the frequency of the symptom in the last month (including if it is a chronic condition).
    - Diarrhea If yes, frequency:
    - Vomiting If yes, frequency:
    - Loss of appetite/anorexia If yes, frequency:
    - Weight loss If yes, frequency:
    - Excess salivation If yes, frequency:
    - Bloody stool If yes, frequency:
    - Poor coat If yes, frequency:
    - Any other symptoms and frequency: ____________________________

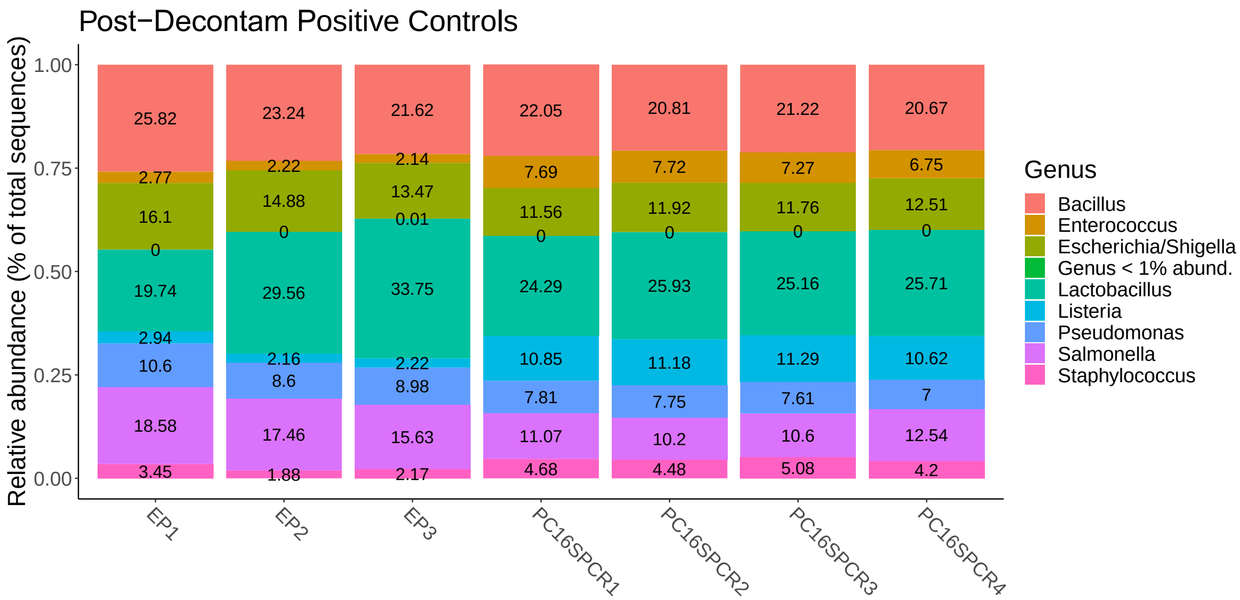

Figure C.2 Relative abundance percent values by genus for Zymobiomics extraction and PCR positive controls. Positive Zymo controls are listed for extraction (EPs, n = 3) and DNA standard PCR (PC16SPCRs, n = 4). Percent abundance values are listed over each corresponding genus (color). Values for Genus < 1% abundance are listed even though no color is shown for this category. Expected values for the standards was 12% for each of the following: *Bacillus subtilis*, *Enterococcus faecalis*, *Escherichia coli*, *Lactobacillus fermentum*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Staphylococcus aureus*.

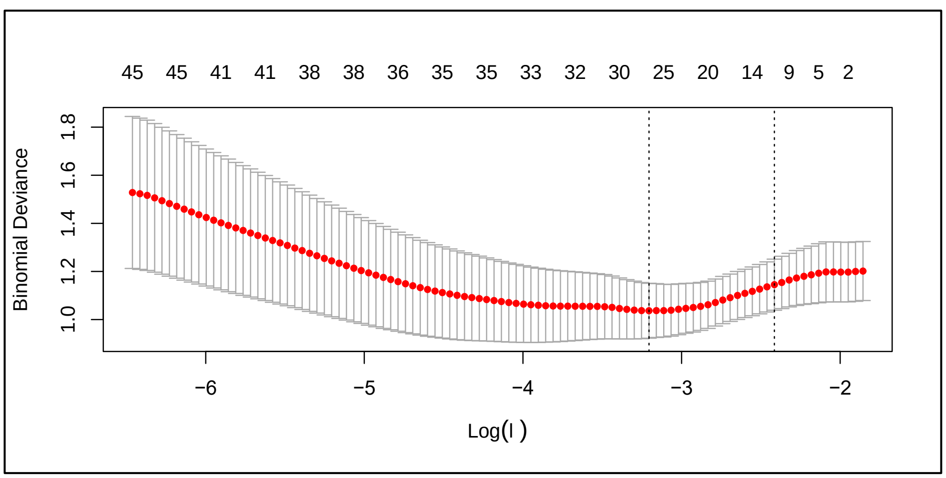

Figure C.3 Cross-validation error according to the log of lambda for lasso test. The dashed vertical line indicates the log of the optimal value of lambda that minimizes prediction error and provides the most accurate model.

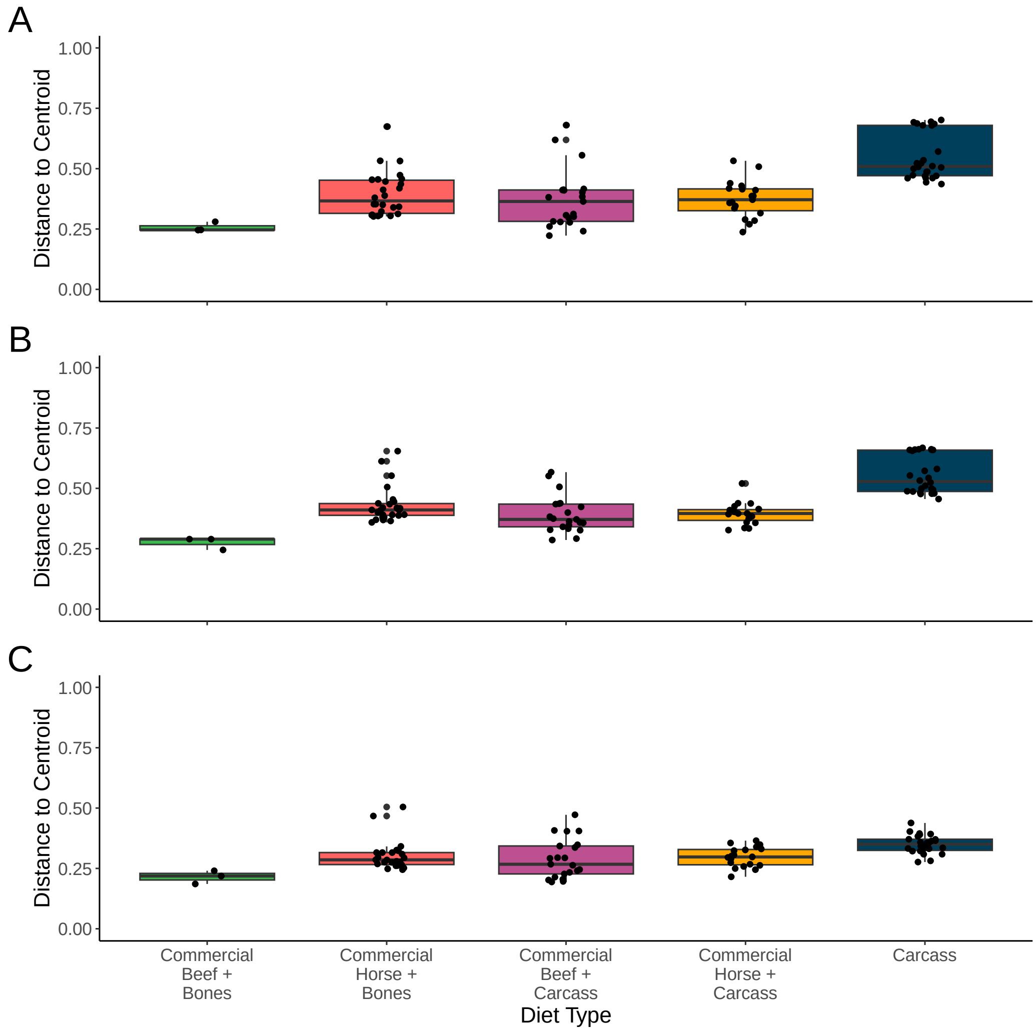

Figure C.4 Bacterial community dispersion as shown by diet groups. Dispersion is shown as distance to the centroid for **(A)** Bray-Curtis, **(B)** Jaccard, and **(C)** Unweighted Unifrac measures (PERMDISP: $p < 0.001$).

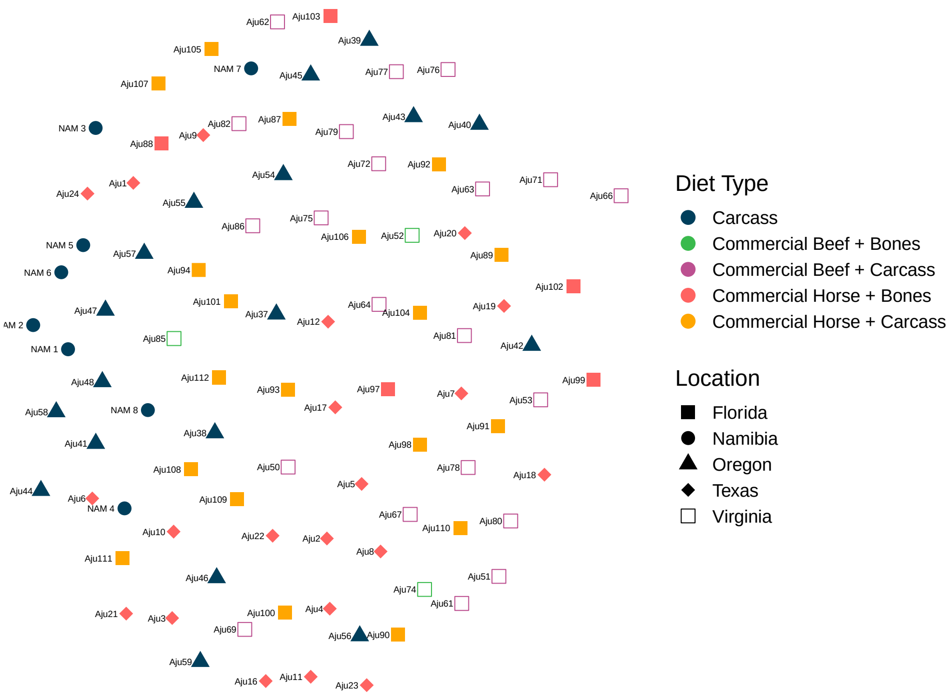

Figure C.5 Microbial network map of Unweighted UniFrac distances by diet (color) and location (shape). Max distance between samples (nodes) was set to 0.7.

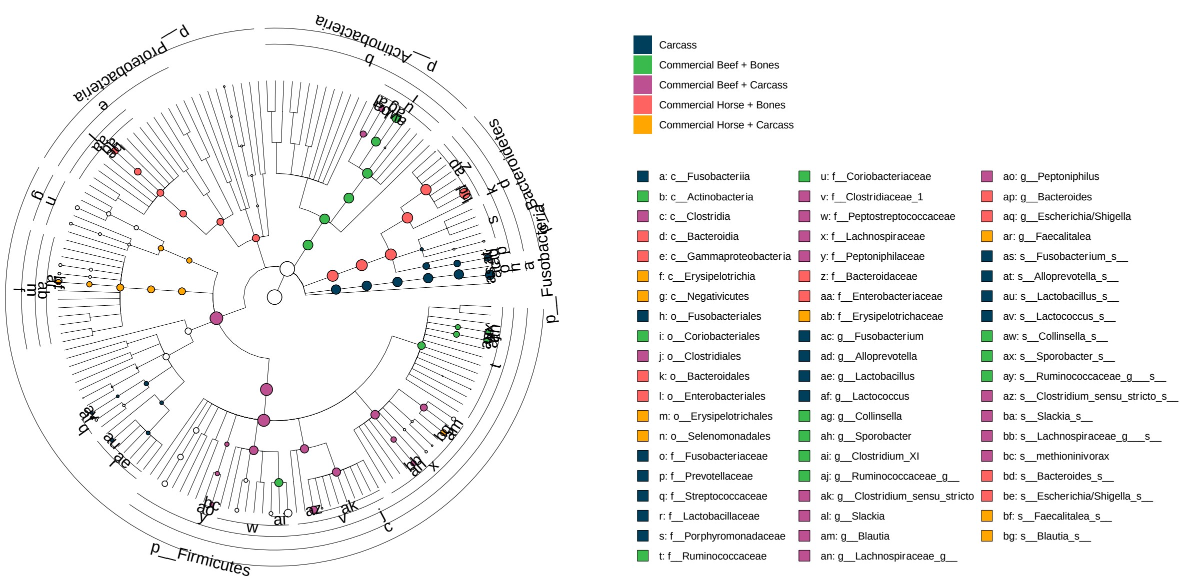

Figure C.6 Cladogram of diet specific enriched taxa as determined by LefSe LDA. LDA threshold was set to 4.