Associations Between Nutrition, Gut Microbial Communities, and Health in Nonhuman Primates

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Dedication

This dissertation is dedicated to

my incredible parents

Deborah Kay Clayton and Jerry Bruce Clayton,

who instilled a strong work ethic in me at a very young age,

taught me that every problem has a solution, and demanded that

I dream big.

Abstract

The primate gastrointestinal (GI) tract is home to trillions of bacteria that play major roles in digestion and metabolism, immune system development, and pathogen resistance, among other important aspects of host health and behavior. In 2009, the Human Microbiome Project was established with the goal of better understanding the role microbial communities play in health and disease. While the research community has made substantial progress in understanding the role microbial communities play in human health and disease, much less attention has been given to host-associated microbiomes in nonhuman primates (NHPs). My research is focused on developing a better understanding of the link between primate microbial communities and the establishment and maintenance of health. I have begun exploring host-associated microbiomes in NHPs, including red-shanked doucs (*Pygathrix nemaeus*) and mantled howling monkeys (*Alouatta palliata*), among other species. Some primate species, such as the red-shanked douc, fail to thrive in captivity due to health issues (e.g., gastrointestinal disease). Maintenance of many primate species in captive settings is hindered by critical gaps in our understanding of their natural diet and the enteric microbial adaptations that facilitate the digestive process. By comparing wild and captive animals within the same species, I hope to determine whether shifts in gut microbiota are linked with health in captivity. Microbes can act as indicators for health of the host, thus broad primate microbiome surveys may allow for the development of predictive biomarkers to improve nonhuman primate health and management.

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Chapter 1 The Gut Microbiome of Nonhuman Primates: Lessons in Ecology and Evolution

Summary

The mammalian gastrointestinal (GI) tract is home to trillions of bacteria that play major roles in host metabolism and immunity. While substantial progress has been made in understanding the role that microbial communities play in human health and disease, much less attention has been given to host-associated microbiomes in nonhuman primates (NHPs). Here, we review past and current research exploring the gut microbiomes of NHP. We begin by summarizing the development of methodological tools that allow us to examine the NHP gut microbiome and then discuss variation in gut microbiome composition and function across different NHP taxa. We conclude describing how the study of gut bacterial communities offers alternative views on primate nutrition and physiology and discuss how studying the gut microbiome of NHPs provides clues to understand primate ecology and evolution.

1.1 Introduction

All animals possess a microbiome: the collection of viruses, bacteria, archaea, fungi and protozoa colonizing animal surfaces and their genetically encoded functions. The relationship between animals and their associated microbiomes started from the moment pluricellular systems evolved in a biosphere where microbes, specifically bacteria, had already dominated for at least 2.5 billion years (Hooper & Gordon 2001; Ley et al. 2008). The sheer abundance of microbes in this environment made colonization of multicellular organisms by microbes almost inevitable as processes of evolutionary diversification and adaptive radiation shaped the tree of life as we recognize it today.

The primate gastrointestinal (GI) tract is home to trillions of bacteria that play major roles in important aspects of host health and behavior (Figure 1.1). To date, a basic understanding of the role microbial communities play in human health and disease has been established, however their function in nonhuman primates (NHPs) has yet to be investigated in depth. Nonhuman primates (NHPs) are the most biologically relevant research animal models for humans, and are unmatched in terms of their relevance compared to other animals used to model human diseases (Chen et al. 2012b; Stone et al. 1987). Given the mounting evidence for the role microbes play in the susceptibility to, development of, and prevention of many human diseases, it is of the utmost importance that we better understand the role microbes also play in nonhuman primate health and disease. Additionally, a better understanding of host-microbiome interactions in NHPs is critical to advance our understanding of the role microbes played in human evolution (co-evolution).

Recent research indicates a complex relationship between hosts and their microbiomes. Although microbes inhabit multiple host surfaces, including the oral cavity, the skin, the urogenital tract, currently the most is known about the microbiome of the GI tract (referred to as the GI microbiome or GIM in this review). The number of microbes in the GI tract surpasses the number of host somatic cells by a factor of 10 (Savage 1977), and the collective protein-coding genes of the GIM exceed those of the host by a factor of 150. As a result, hosts are likely to benefit from complementing their own genomes with those of their associated microbiome (Hooper & Gordon 2001; Ochman et al. 2010; Toft & Andersson 2010; Bäckhed et al. 2005). It is thus expected that the GIM plays a fundamental role in host physiological processes.

For example, the GIM allows hosts to recover energy from otherwise indigestible foods. Mammals do not possess the glycoside hydrolases, polysaccharide lyases and carbohydrate esterases required to breakdown the β-1,4 glycosidic linkages in complex plant polysaccharides (Bayer et al. 2008). Instead, the GIM is entirely responsible for breaking down and fermenting plant structural polysaccharides to yield energy-rich short chain fatty acids (SCFAs) (Hume 1997). These SCFAs can be absorbed by the host and utilized as an energy source. This function is critical for host nutrition. For example, non-human primates, who primarily depend on plant material as a main source of nutrients (Milton 1987), may obtain from 30 to 57% of their daily energy budget from SCFAs (Milton & McBee 1983; Popovich et al. 1997).

The GIM is also responsible for maintaining proper host innate and adaptive immune responses by establishing a tight spatial and functional relationship with the host's gut epithelia and associated lymphoid tissues (McFall-Ngai 2007; Lee & Mazmanian 2010; Round et al. 2011). The absence of a homeostatic GIM (dysbiosis) has been linked to susceptibility to infection, decreased lymphocyte and intestinal macrophage proliferation and low serum immunoglobulin levels (particularly IgA) (Larsen et al. 2010; Rautava & Isolauri 2002; Round & Mazmanian 2009; Bäckhed et al. 2004; Dicksved et al. 2008). Research suggests the presence of a an association between the gut microbiota and the development of a number of diseases, including obesity (Turnbaugh et al. 2006; Turnbaugh et al. 2009; Turnbaugh et al. 2008), diabetes (Giongo et al. 2011; Brown et al. 2011; Boerner & Sarvetnick 2011), Crohn's (Knights et al. 2013; Gevers et al. 2014), and Alzheimer's disease (Bhattacharjee & Lukiw 2013).

Because the GIM has such marked impacts on host physiology, a better understanding of the composition and function of the GIM in the context of animal biology provides an opportunity to assess the influence of these microbial communities in animal ecology and evolution. This review is aimed at: 1) summarizing the methodology that has set the foundation for gut microbiome research, 2) highlighting the variation in gut microbiome composition across NHP taxa, and 3) exploring how the study of the GIM can offer additional information on primate nutrition and physiology. The purpose of this review is to highlight the fundamental role of the microbiome and its importance in primatological,

and research to encourage microbiome approaches as useful, supplemental tools to study relevant issues in primate ecology.

1.2 Methodological approaches to gut microbiome research

With the recognition of the critical role the mammalian GIM likely plays in physiology, comes the need to characterize its diversity. Studies aimed to measure bacterial composition in the mammalian gut before the early 2000's relied mostly on culture-dependent methods (or in vitro isolation) to describe the diversity and functions of specific groups of gut microbes. However, to date, it is estimated that modern culturing methods can reproduce viable conditions for up to 20% of mammalian gut microbes (Eckburg et al. 2005; Savage 1977; Zoetendal et al. 2004) but less than 5% of existing bacterial species (Hugenholtz et al. 1998; Pace 1997; Rappe & Giovannoni 2003). Additionally, *in vitro* isolation of microbes does not necessarily reflect the complex interactions among the vast diversity of organisms in the GIM or their functional relevance.

After the realization that the cultivation methods available failed to recover the majority of microbial species due to biases imposed during culturing, came the breakthrough implementation of small subunit rRNA sequences for the molecular phylogenetic survey of microbes (Woese 1987; Woese et al. 1990). Since then, the use of molecular techniques to survey these microbes using DNA from environmental samples has increased our knowledge of microbial communities significantly. Specifically, molecular

methods to assess bacterial community composition exploit the hyper-variable regions of the "universal" 16S bacterial and archeal ribosomal RNA subunit (16S rRNA) as a phylogenetic marker. The 16S rRNA gene is composed of 9 hypervariable (V1-V9) regions, and the sequence dissimilarity among microbes in these regions allow researchers to assess bacterial diversity in a given sample and to identify organisms taxonomically (Pace 1997). Most microbial ecology studies assume that the sequences of two or more organisms sharing more than 97% 16S rRNA sequence similarity belong to the same species, known as an operational taxonomic unit (OTU). However, this delineation is arbitrary and may vary among studies adopting diverse thresholds (Forney et al. 2004).

DNA-based methods for profiling microbial diversity have progressed over time. Traditionally, sequence data were generated by obtaining 16S rRNA amplicons (via polymerase chain reaction) from extracted DNA, followed by cloning and Sanger sequencing (Frey et al. 2006; Ley et al. 2008). However, the cost-prohibitive nature of cloning and its limitations in terms of bias, sample size and coverage motivated the development of cloning-independent 16S rRNA gene fingerprinting techniques (Smalla et al. 2007). These methods describe the microbial composition of multiple communities simultaneously by detecting differences in 16S rRNA PCR amplicon size. For example, terminal restriction fragment length polymorphism (T-RFLP) (Liu et al. 1997) utilizes restriction enzymes to cut the amplicons from different microbes at different points and then separates the resulting fragments according to size via gel electrophoresis.

Denaturing/temperature gradient gel electrophoresis (D/TGGE) (Muyzer et al. 1993) also separates amplicons from different microbes based on composition and size while Automated Ribosomal Intergenic Spacer Analysis (ARISA) relies solely on amplicon size differences. These fingerprinting techniques have been used in a variety of microbial ecology studies of both free-living and host-associated bacterial communities (Kisidayová et al. 2009; Uenishi et al. 2007; Frey et al. 2006). However, while these techniques allow researchers to highlight differences in microbial community diversity and composition, additional analyses are required to pinpoint the microbial taxonomy associated with amplicons. Additionally, fingerprinting methods tend to provide information about only the most dominant taxa, and results from different studies cannot typically be compared (Hamady & Knight 2009).

In the past 10 years, the development of high-throughput sequencing techniques, such as 454-pyrosequencing and Illumina (and its two platforms, MiSeq and HiSeq), has allowed researchers to analyze the bacterial community composition of hundreds of samples simultaneously, while recovering a high number of 16S rRNA reads and a significant amount of sequence data. These sequence data provide improved sensitivity and diversity coverage and overcome many of the problems associated with cloning and fingerprinting techniques (Hamady & Knight 2009; Robinson et al. 2010). The principle of high-throughput sequencing relies on sequencing large numbers of 16S rRNA short-length sequences rather than the longer sequences obtained when analyzing the whole length 16S rRNA (Petrosino et al. 2009; Ronaghi 2001; Liu et al. 2007).

For both amplicon sequencing and fingerprinting techniques, additional challenges exist. Nucleic acid extraction methods may influence results via biases toward or against certain microbes (Yuan et al. 2012), and similar biases are introduced via the utilization of primers during the PCR necessary for generating amplicons (Hamady & Knight 2009). As a result, other sequencing-based techniques, such as shotgun metagenomics, have recently grown in popularity. Metagenomics is the sequencing of all DNA obtained directly from an environmental sample with the intent of assigning function to shotgun reads. It is not dependent on a PCR reaction, and surveys all of the genetic material in a sample (Tringe et al. 2005; Meyer et al. 2008; Riesenfeld et al. 2004). Metagenomics has been used to estimate the functional landscape of numerous microbial communities, including that of the mammalian GIM (Mongodin et al. 2005; Petrosino et al. 2009; Xu et al. 2013; Abubucker et al. 2012), but high costs compared to 16S rRNA analyses are still prohibitive for some projects (Figure 1.2).

1.3 Review of NHP microbiome studies

While the research community has made substantial progress in understanding the role microbial communities play in human health and disease, much less attention has been given to host-associated microbiomes in nonhuman primates. Beginning in the 1960's, with the pioneering work of Bauchop and Martucci on bacteria inhabiting the stomach of colobines (Bauchop 1971; Bauchop & Martucci 1968), there have only been a handful of studies attempting to portray the diversity of the bacterial communities associated to the

gastrointestinal tract of primates, from evolutionary, clinical and ecological perspectives. Here, we summarize the findings presented in these studies, organizing information by primate taxonomic group and highlighting the impact of the results on our understanding of primate ecology.

GIT structure, diet and gut microbiome patterns in diverse NHPs:

Strepsirrhines:

Of all extant (i.e., living) primates, strepsirrhines are our most distant relatives within the order primates (Yoder 1997; Charles-Dominique 1977). Examples of primitive characteristics of strepsirrhines that higher primates lack include a bicornuate uterus and toilet claws (Soligo & Müller 1999; Luckett 1976). Of the nonhuman primate microbiomes examined via culture-independent methods to date, strepsirrhines are the least represented. Microbiome-related studies focused solely on strepsirrhine primates have included only a limited number of species, including ring-tailed lemurs (*Lemur catta*) (Fogel 2015; McKenney et al. 2015), black-and-white ruffed lemurs (*Varecia variegata*) (McKenney et al. 2015), Coquerel's sifakas (*Propithecus coquereli*) (McKenney et al. 2015), Verreaux's sifakas (*Propithecus verreauxi*) (Fogel 2015), and pygmy lorises (*Nycticebus pygmaeus*) (Bo et al. 2010; Xu et al. 2013). Only the ring-tailed lemur, Verreaux's sifaka, and the pygmy loris have been sampled in the wild.

Lemurs (genera *Lemur*, *Varecia*, and *Propithecus*): *Lemur*, *Varecia*, and *Propithecus* are primate genera within the primate clade Lemuriformes. Lemurs are endemic to

Madagascar (Dolins et al. 2010), and their diets vary greatly across genera. For example, *Lemur catta* are considered generalists, while members of *Varecia* (frugivorous) and *Propithecus* (folivores) are considered specialists.

Ring-tailed lemurs are the best studied in terms of the gut microbiome. (Modesto et al. 2015; Villers et al. 2008; Bublitz et al. 2015; Fogel 2015; McKenney et al. 2015). In an effort to identify the major species of aerobic bacteria in the intestine, Villers et al. (2008) examined captive and wild populations of ring-tailed lemurs using culture-based methods. They found 14 species of bacteria shared by both captive and wild populations and eight species that were not shared. Additionally, more bacterial species were shared among wild populations than were shared by captive and wild populations, suggesting that differences in gut bacterial composition between captive and wild individuals are greater than those between wild individuals located at different field sites. Fogel (2015) also detected differences in the microbiome of wild and captive *L. catta* using high-throughput sequencing. The gut microbiome of wild individuals contained an increased abundance of *Firmicutes*, *Actinobacteria* and *Euryarchaeota* and a decreased abundance of *Bacteroidetes* and *Spirochaetes* compared to captive individuals (Fogel 2015). Ley et al. (2008) also utilized high-throughput sequence data for captive ring-tailed lemurs, identifying an enrichment of Verrucomicrobia. These distinctions appear to be a result of diet and individual host identities differing between environments.

In an effort to understand how anthropogenic activities affect lemur exposure to bacterial pathogens in Madagascar, Bublitz et al. (2015) used culture-based methods to test six species of wild lemurs in Ranomafana National Park for the presence of enteric bacterial pathogens, including Enterotoxigenic *Escherichia coli*, *Shigella* spp., *Salmonella enterica*, *Vibrio cholerae*, and *Yersinia* spp. (enterocolitica and pseudotuberculosis), which are all commonly associated with diarrheal disease in human populations in Madagascar. Bublitz et al. (2015) found that lemurs inhabiting disturbed areas of habitat tested positive for these bacterial pathogens while lemurs found in intact forests tested negative. Finally, Modesto et al. (2015) recently discovered a novel bacterial species within the genus *Bifidobacterium* in ring-tailed lemurs using culture-dependent methods. Bifidobacteria are normal inhabitants of the mammalian colon and thought to contribute to intestinal health in a number of ways, including pathogen inhibition, the production of vitamins, as well as immune system modulation (Mayo & van Sinderen 2010).

Other studies have been conducted to compare the gut microbiome among lemur genera (McKenney et al. 2015; Fogel 2015; Ley et al. 2008). McKenney et al. (2015) examined the microbiome of captive frugivorous black-and-white ruffed lemurs, generalist ring-tailed lemurs, and folivorous Coquerel's sifaka using high-throughput sequencing. Fecal microbiomes of both mothers and infants, from birth to weaning, were analyzed. The gut microbiome composition was species-specific, potentially due to the high fiber diet consumed by the sifaka. Specifically, the sifaka harbored the greatest microbial diversity, including four genera of cellulose degraders (Ruminococcaceae). Although ring-tailed

lemurs and black-and-white ruffed lemurs consumed similar diets and exhibited similar microbial diversity, their GIMs could be distinguished based on several bacterial lineages (McKenney et al. 2015). Additionally, distinct microbiome compositions were observed across life stages in each of the three lemur species studied.

In a similar way, Fogel (2015) examined the microbiome of wild ring-tailed lemurs and Verreaux's sifaka. Although the abundance of microbes differed between species, gut microbiome composition did not (Fogel 2015). However, inter-individual and seasonal (wet vs. dry season) variation in gut microbiome composition within each species was high.

Slow lorises (genus *Nycticebus*): Lorises are distributed throughout Southeast Asia in tropical, dry, montane, bamboo and lowland forests (Nekaris & Bearder 2007). Slow lorises (*Nycticebus* spp.) are one of the least studied NHP. Their small size, cryptic and nocturnal behavior has made them difficult characterize, and little of their ecology is understood. Nekaris et al (2010) characterized members of the genera *Nycticebus* and *Loris* with long tongues and a large caecum, which could be beneficial in processing the structural carbohydrates in gums and insect chitin. In fact, *N. pygmaeus* can be considered a specialized gumivore, targeting the exudates of trees of the genera *Sapindus*, *Vernicia*, *Saraca* and *Spondia* (Nekaris et al. 2010). Additionally, many of the insects consumed by *N. pygmaeus* are toxic and pungent (Nekaris et al. 2010; Bo et al. 2010).

Three studies have described the microbiome of lorises (Bo et al. 2010; Xu et al. 2013; Xu et al. 2014). Bacteroides is consistently the second most abundant phylum to Firmicutes found in mammals. In contrast, Bo et al. (2010) used clone libraries to find that the phylum Proteobacteria accounted for the second highest percentage (36%) of bacteria in the fecal microbiome of the wild pygmy loris (*Nycticebus pygmaeus*), and within the phylum Proteobacteria, *Pseudomonas* (13.79% of clone sequences) was the predominant genus. Because the authors found sequences closely related to *P. putida*, which are well known hydrocarbon-degrading bacteria (Gomez et al. 2011; Rentz et al. 2004), it seems that *Pseudomonas* plays a vital role in the digestion of plant materials. Other microbial taxa that might play a role in breaking down plant exudates such as *Acinetobacter*, *Alkalibacterium* (Phylum Proteobacteria), *Corynebacterium* (Phylum Actinobacteria), *Clostridium*, *Eubacterium* and *Bacillus* were also detected. Therefore, it appears that the gut microbiota of the pygmy loris is adapted to ferment sugars and degrade complex aromatic compounds. Both of these processes are likely important for the fermentation of plant exudates, which make up the majority of their diet (Starr & Nekaris 2013).

Contradicting the above study, Xu et al. (2013) used high-throughput sequencing to identify Bacteroidetes (41.9% of sequences) as the most dominant phylum in the pygmy loris (*Nycticebus pygmaeus*). This difference could be due to methodology. However, Xu et al. (2013) also found that the major genus represented in the phylum Proteobacteria was *Pseudomonas*, consistent with Bo et al. (2010). Xu et al. (2013) also found an

abundance of the phylum Verrucomicrobia, and specifically members of the genus *Akkermansia*. Members of the genus *Akkermansia*, such as *Akkermansia muciniphila*, have known roles in the gut, including mucin-degradation, and have been suggested to be protective (Everard et al. 2013; Belzer & de Vos 2012). Xu et al. (2013) also used metagenomics to observe that sequences involved in aromatic compound metabolism were overrepresented, specifically sequences in the benzoate degradation pathway. Finally, Xu et al. (2014) identified a novel gene (*amyPL*) coding for α-amylase, which is important for the breakdown of α-linked polysaccharides, such as starch and glycogen in the pygmy loris diet (Nekaris et al. 2010; Buisson et al. 1987).

New World monkeys (Ceboidea):

Compared to Old World monkeys and Great Apes, fewer culture-independent based microbiome studies have been published on New World monkeys. Of those, only a limited number of NHP genera have been represented, including lion tamarins (*Leontopithecus* spp.) (Carvalho et al. 2014), howler monkeys (*Alouatta* spp.) (Amato et al. 2013; Nakamura et al. 2011; Amato et al. 2014; Amato et al. 2015), and spider monkeys (*Ateles* spp.) (Hale et al. 2015).

Lion tamarins (genus *Leontopithecus*): Currently, there are four known species of lion tamarins (*Leontopithecus* spp.): golden lion tamarins (*Leontopithecus rosalia*), golden-headed lion tamarins (*Leontopithecus chrysomelas*), black-faced lion tamarins (*Leontopithecus caissara*) and black lion tamarins (*Leontopithecus chrysopygus*)

(Rylands & Mittermeier 2009). All are classified as endangered and are endemic to Brazil's Atlantic forest (Rylands & Mittermeier 2009).

Currently, only a single study focused on the gut microbiota of lion tamarins has been conducted (Carvalho et al. 2014). Noteworthy is the fact that Carvalho et al. (2014) utilized culture-dependent methods, and thus to date not a single study on the gut microbiome of any lion tamarin species using culture-independent methods has been conducted. Carvalho et al. (2014) screened for potentially pathogenic bacteria and fungi from the rectum, as well as the nasal and oral cavities, of both free-ranging and captive black lion tamarins (*Leontopithecus chrysopygus*) using a sterile swabbing technique followed by culture for microbial identification. In this study, there were no statistically significant differences in the proportion of bacterial groups between isolates from free-ranging and captive individuals (Carvalho et al. 2014). Overall, Carvalho et al. (2014) found Gram negative bacteria to be more frequent than Gram positive bacteria in the *L. chrysopygus* rectum, and of the rectal bacteria cultured, *Escherichia coli* was the most common, followed by *Serratia* spp.

Howler monkeys (genus *Alouatta*): Howler monkeys (*Alouatta* spp.) are often referred to as folivores because a majority of their diet is composed of leaves during some months of the year (Milton et al. 1980; Milton 1981; Milton 1998; Milton 1980). They are believed to rely primarily on microbial fermentation to break down the structural components of leaves and possibly plant secondary compounds (Milton 1998) in the

cecum and colon (Edwards & Ullrey 1999; Milton & McBee 1983; Ullrey 1986). Although the cecum and colon are somewhat enlarged, the howler monkey GI tract is relatively unspecialized compared to other folivorous primates.

A 2011 study examining gut microbial communities of wild and captive black howler monkeys (*Alouatta pigra*) with DGGE found clear differences in sulfate reducing and other hydrogenotrophic bacteria and archaea (methanogens) between captive and wild populations (Nakamura et al. 2011). The study showed that captive howlers had reduced diversity of hydrogenotrophic bacteria compared to their wild counterparts. In contrast, fecal samples of wild howlers showed greater diversity of sulfate reducing bacteria (Nakamura et al. 2011). Additionally, captive individuals had very similar hydrogenotrophic microbial profiles, which were dominated by a pectin degrader, *Lachnospiraceae pectinoschiza* (phylum Firmicutes), despite being rescued from different geographic locations (Cornick et al. 1994). This pattern suggests a strong role of a captive diet, rich in domesticated fruits, in shaping the howler gut microbiome over periods of months and years.

Another study utilizing high-throughput sequencing showed that a relationship exists between habitat quality and black howler monkey gut microbiome composition (Amato et al. 2013). Specifically, gut microbial diversity and richness was highest in howler monkeys inhabiting continuous, evergreen rainforest compared to fragmented rainforest and captivity. These patterns in microbial diversity appeared to match patterns in diet

diversity. Furthermore, gut microbial composition varied with howler habitat and diet (Amato et al. 2013). For example, captive black howler monkeys harbored higher relative abundances of *Prevotella,* which is likely related to higher levels of simple carbohydrates in the captive diet (fruits, cereal, and primate pellets) (Amato et al. 2013).

In a longer study that tracked black howler GIM composition in individuals over 10 months, Amato et al. (2014) determined that adult males, adult females, and juveniles each have a distinct microbiome composition, and that juvenile and adult female howlers may derive nutritional benefits from the GIM that compensate for the demands of growth and reproduction. Specifically, the microbiome of juvenile howlers was dominated by the phylum Firmicutes, including *Roseburia* and *Ruminococcus* while adult females had a higher than expected Firmicutes to Bacteroidetes ratio and were characterized by *Lactococcus* (Amato et al. 2014). These results indicate the potential for juvenile and adult female GIM's to produce additional energy and vitamins for their hosts. In fact, juvenile howlers exhibited high fecal volatile fatty acid (VFA) content relative to body size, suggesting that microbes greatly contribute to host energy balance (Amato et al. 2014). Although diets varied across howler age and sex classes as well, patterns in GIM composition were not correlated with patterns in diet. As a result, other mechanisms such as hormone shifts are likely to be responsible for GIM differences.

Finally, Amato et al. (2015) provide evidence that wild black howler GIMs vary with seasonal shifts in diet, and these microbial shifts may help howlers meet their nutritional

demands during times when the consumption of a less energetically favorable diet is the norm. During periods when howler energy intake was lowest, relative abundances of Ruminococcaceae were highest and relative abundances of Lachnospiraceae were lowest. Also, *Butyricicoccus* was most abundant when the howler diet was dominated by young leaves and unripe fruit (Amato et al. 2015). Not only did the GIM shift in composition, but also when energy intake was reduced howlers showed increased fecal VFA concentrations, indicating increased microbial energy production (Amato et al. 2015). Because howlers also showed little variation in their activity levels over the 10-month study period despite variation in diet, it appears that shifts in the GIM help compensate for seasonal reductions in howler energy intake.

Spider monkeys (genus *Ateles*): Spider monkeys (*Ateles* spp.), which are native to Central and South America, are commonly described as frugivorous primates, although their diet also includes leaves (González-Zamora et al. 2009). In captivity, spider monkeys are primarily fed a diet consisting of fruits and vegetables, as well as primate pellets (Hale et al. 2015).

Virtually nothing is known about the spider monkey GIM currently. Nevertheless, Hale et al. (2015) used fecal samples collected from captive black-handed spider monkeys (*Ateles geoffroyi*) to examine the effectiveness of five commonly used fecal preservation methods, including storage in −20 °C freezer, storage in −80 °C freezer, storage in 100% ethanol, application to FTA cards, and storage in RNAlater solution. While the goal of

the study was not to characterize the spider monkey fecal microbiome, Hale et al. (2015) did observe that Bacteroidetes and Firmicutes dominated the captive spider monkey microbiome. The increased abundance of Bacteroidetes detected could be an effect of captivity, as the microbiomes of most wild NHPs, including howlers, are dominated by Firmicutes.

Old World monkeys (Cercopithecoidea):

Of the nonhuman primate microbiomes examined via culture-independent methods to date, Old World monkey taxa are most represented. However, despite the high volume of studies conducted, investigations of gut microbiota in Old World monkeys have focused primarily on macaques, members of the subfamily Cercopithecinae, and of these studies, the majority have been limited to captive settings. Other Old World monkeys that have been studied from the perspective of their gut microbiota include baboons (*Papio* spp.), guenons (*Cercopithecus* spp.) also cercopithecines, and taxa belonging to the subfamily colobinae (*Colobus* spp., *Procolobus* spp., *Presbytis* spp. and *Rhinopithecus* spp.). Of these, *Colobus* spp., *Procolobus* spp., and *Rhinopithecus* spp. are represented by wild individuals.

Macaques (genus *Macaca*): The genus *Macaca* has the most widespread distribution of all NHPs, ranging from the tropics to temperate regions, with 22 species recognized (Thierry 2011). Macaques are considered omnivorous and feed on a wide range of foods, including fruits, seeds, roots, flowers, herbs and invertebrates (Sawada et al. 2011;

Thierry 2011). Some species, such as *Macaca mulatta* exhibit a commensal and sometimes parasitic relationship with humans taking advantage of crops and garbage from cities and villages (Else 1991). Macaques have simple stomachs, typical of caeco-colic fermenters with an enlarged colon or cecum and are able to adopt diverse food processing strategies (Sawada et al. 2011).

In one of the first microbial-based studies involving macaques, Bauchop (1971) analyzed the macaque (*Macaca mulatta*) gut-associated microbiota using culture-based isolation of bacteria from intestinal and stomach contents of sacrificed animals. *Lactobacillus* and *Clostridium* spp. (Firmicutes phylum) were the most abundant genera (Bauchop 1971). Other culture-dependent examinations of macaque gut microbial communities have reported the presence of *Lactobacillus* sp., especially in infant and young *Macaca fuscata* and *Macaca fascicularis* (Bailey & Coe 1999; Benno et al. 1987). In a study examining the response of the long-tailed macaque (*Macaca fascicularis*) gut microbiome to *Shigella* infection, Seekatz et al. (2013) identified *Lactobacillus* as a dominant member of the macaque gut microbiome. In fact, based on relative abundance calculations performed, *Lactobacillus* was the most abundant of all genera observed (Seekatz et al. 2013).

In another early study examining gut bacteria in wild and captive Japanese snow macaques (*Macaca fuscata*), Benno et al. (1987) found that wild and captive individuals differed in their microbial compositions, likely as a result of diet. The wild group mainly

fed on tree bark, while the captive group was fed a commercial diet (Y Benno et al. 1987). Benno et al. (1987) observed significantly higher total bacterial counts in captive macaques. Despite this, the ratio of anaerobic bacteria to aerobic bacteria was much higher in wild macaques. Most interestingly, a significant reduction of *Bacteroides* spp. was observed in wild macaques.

Wireman et al. (2006) used culture-independent methods to examine gut microbial communities in four male macaques (*Macaca fascicularis* and *Macaca mulatta*) for a period of eight months. The major findings of this study were that the macaque gut microbiome is dynamic, exhibits positive and negative correlations among certain bacterial taxa, and is dominated by the *Clostridium-Eubacterium*, *Lactobacillus*, and *Bacteroides* groups (Wireman et al. 2006). The diet consumed by the four macaques included in this study was kept uniform, and thus the inter-individual differences in gut microbiota composition observed were likely due to factors other than diet.

McKenna et al. (2008) used pyrosequencing to examine captive enterocolitis-affected rhesus macaques (*Macaca mulatta*) and reported differences in the numbers of taxa from the Bacteroides and Firmicutes phyla. Specifically, they found a higher prevalence of *Campylobacter* in the symptomatic animals than in healthy animals (McKenna et al. 2008). McKenna et al. (2008) also reported the abundance of *Treponema* and *Helicobacter* in *M. mulatta* from feces and tissue taken from different sites along the gastrointestinal tract (jejunum and colon). No diet data were collected, but it is possible

that captive diets are low in fiber and high in calories from lipids and sugars, which influenced gut microbiome composition.

In an investigation of the relationships between diet, microbiome, and host immune response, Ardeshir et al. (2014) examined breast-fed and bottle-fed rhesus macaques (*M. mulatta*) to see how these two different nursing practices influence gut microbiome composition, and the resulting effects on immune system development. Compared to bottle-fed macaques, breast-fed macaques had increased abundances of *Prevotella* and *Ruminococcus* and a decreased abundance of *Clostridium* (Ardeshir et al. 2014). In addition to major differences in gut microbiota, breast-fed and bottle-fed macaques had substantial differences in their immune systems, including the development of robust T_H17 cell populations exclusively in breast-fed macaques (Ardeshir et al. 2014). This study highlights both that diet strongly influences gut microbiome composition and that gut microbial communities strongly influence immune system development in early life.

Klase et al. (2015) examined how SIV infection status, and associated immunological effects, impact bacterial translocation in a macaque model. Previous investigations in both humans and NHPs have confirmed that bacterial translocation occurs with both acute human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infection. However these studies failed identify the translocating bacteria (Klase et al. 2015). Klase et al. (2015) found that while differences in gut microbiome composition of healthy vs. SIV-infected macaques due to infection alone were unremarkable, differences

in gut microbiome composition after the administration of antiretroviral therapy was substantial (Klase et al. 2015). A key finding in this study was the increased abundance of Proteobacteria observed in the tissues of SIV-infected macaques, which suggested Proteobacterial species preferentially translocate (Klase et al. 2015).

It has been previously established that an intimate link between the gut microbiome and obesity exists in mammals (Turnbaugh et al. 2006; Turnbaugh et al. 2009). In order to examine this link in a primate model, Ma et al. (2014) exposed Japanese macaques (*M. fuscata*) to both high-fat and low-fat diets and analyzed their fecal microbiomes over an extended period. In this study, the investigators established the role of diet in shaping the maternal gut microbiome, as well as the role of maternal diet during gestation and lactation in shaping the juvenile microbiome. The offspring's gut microbiome was negatively altered when the dam was fed a high-fat diet during pregnancy or lactation. Specifically, Ma et al. (2014) reported that non-pathogenic *Campylobacter* was far less abundant in juvenile macaques exposed to a high-fat diet early in life compared to those exposed to a low-fat diet. Additionally, the consumption of a low-fat diet by the offspring post-weaning only partially corrected the dysbiosis. These results suggest that maternal diet establishes the gut microbiota in offspring, which then affects offspring metabolism.

In the most recent study examining gut microbiota of macaques, Yasuda et al. (2015) examined the microbiome of ten different sites along the intestine in rhesus macaques (*M. mulatta*), and then compared the results to fecal microbiome. They determined that feces

are highly representative of the colonic lumen and mucosa microbiota (Yasuda et al. 2015). However, fecal samples are not representative of the small intestine, especially with respect to *Proteobacteria* since *Proteobacteria* are under-detected in feces (Yasuda et al. 2015). Considering that feces are the most commonly used biological material for microbiome studies, the findings of this study are invaluable.

Baboons (genus *Papio*): Baboons (*Papio* spp.) are large, omnivorous, terrestrial Old World monkeys found almost exclusively in Africa. Of the six recognized species: The Guinea baboon (*Papio papio*), chacma baboon (*Papio ursinus*), olive baboon (*Papio anubis*), yellow baboon (*Papio cynocephalus*), Kinda baboon (*Papio kindae*) and hamadryas baboon (*Papio hamadryas*), only the hamadryas baboon is native to both Africa and Arabia (Zinner et al. 2013). To date, only yellow baboons (*P. cynocephalus*) and hamadryas baboons (*P. hamadryas*) have had their gut microbiomes examined, and of these two species, only the yellow baboon has been studied in the wild.

In one of the first studies of baboon (*Papio* sp.) gut bacteria, Brinkley & Mott (1978) found the presence of Fusobacteria, which have also been found in humans (Eckburg et al. 2005). Using culture-based methods (anaerobic culture), they identified the predominant genera present in baboon feces, which were *Lactobacillus*, *Eubacterium*, *Streptococcus*, and *Bacteroides* (Brinkley & Mott 1978). In another study focused on baboon gut microbiota, Brinkley et al. (1982) used culture-dependent methods to isolate and characterize nine novel bacterial strains from feces and intestinal contents, all of

which were cholesterol-reducing bacteria (Brinkley et al. 1982). In study using culture-independent methods, Ley et al. (2008) found an enrichment of Spirochaetes in a hamadryas baboon (*Papio hamadryas*). Conversely, McKenney et al. (2014) did not report finding any Spirochaetes in their examination of microbial communities in captive hamadryas baboons. However, they did identify other phyla, including Proteobacteria, Firmicutes, and Bacteroidetes, of which Proteobacteria was most abundant (McKenney et al. 2014).

Ren et al. (2015) attempted to determine predictors of gut microbiome composition in wild yellow baboons (*P. cynocephalus*), including host-specific factors, such as identity, age and sex, as well as other factors, such as rainfall, natal social group, current social group and group size. Using fecal samples collected over a 13-year period, Ren et al. (2015) observed that wild baboons harbor gut microbial communities representative of what has been seen in other omnivorous primates. One notable finding was the high abundance of *Bifidobacterium*. Also, host age, diet and rainfall were most responsible for variation in the GIM. Lastly, these researchers identified two provisional enterotypes in adult baboons, both of which are different than enterotypes previously identified in chimpanzees (Moeller et al. 2012) and humans (Arumugam et al. 2011).

Tung et al. (2015) examined the relationship between social networks and gut microbiome composition in wild yellow baboons (*P. cynocephalus*). They found that contact rates directly explained the observed variation in gut microbiome composition

among wild baboons, as other potential confounding factors were controlled for, including diet, kinship, and overlapping geographic space. These results suggest that gut microbiome composition in wild baboons is influenced by social relationships (Tung et al. 2015).

White-eyelid mangabeys (genus *Cercocebus*): Currently, there are seven known species of white-eyelid mangabey (*Cercocebus* spp.): including the sooty mangabey (*Cercocebus atys*), collared mangabey (*Cercocebus torquatus*), agile mangabey (*Cercocebus agilis*), golden-bellied mangabey (*Cercocebus chrysogaster*), white-naped mangabey (*Cercocebus lunulatus*), Tana river mangabey (*Cercocebus galeritus*), and sanje mangabey (*Cercocebus sanjei*) (Rowe & Myers, 2011).

Currently, a single study focusing on the gut microbiota of white-eyed mangabeys has been conducted (Nakamura et al. 2009). Nakamura et al. (2009) aimed to better understand how methanogenic status is regulated in primates by using rectal swabs and DGGE to identify hydrogenotrophic microbial community profiles of Hamadryas baboons (*Papio hamadryas*) and sooty mangabeys (*Cercocebus atys*). The results of this study revealed that intestinal Archaea and Sulfur-reducing bacteria (SRB) are present simultaneously in baboons and mangabeys, and that the hydrogenotrophic microbial community profiles of these NHP species differ.

Guenons (genus *Cercopithecus*): Members of the genus *Cercopithecus* belong to the most diverse and numerous groups of African monkeys known as guenons (Bruorton et al. 1991; Jaffe & Isbell 2011). Guenons possess simple stomachs, globular caecums and engage in colonic fermentation. Diet composition is largely species-specific, but generally speaking the diet of most guenons includes seeds, flowers, insects and gums in various amounts (Lambert 2002; Jaffe & Isbell 2011).

To date, limited information exists regarding gut microbial communities of members of the genus *Cercopithecus*. However, both culture-dependent and culture-independent studies have been performed. For example, Bruorton et al. (1991) relied on cultivation techniques and electron microscopy to identify the gut microbiota of the blue monkey (*Cercopithecus mitis*). The authors identified a characteristic abundant population of rod-shaped bacteria in the stomach of the blue monkey through light microscopy, although taxonomic tests were not performed to confirm the identity to genus- or species-level (Bruorton et al. 1991). The study also identified a number of isolates capable of metabolizing cellulose.

Cercopithecines have also been involved in ecological studies where culturable bacteria were used as a tool to examine how gut bacteria found in different mammalian species occupying a given geographic area were related (Goldberg et al. 2008). In a study using *Escherichia coli*, a readily culturable bacterial species and known inhabitant of the mammalian colon, Goldberg et al. (2008) used red-tailed guenons (*Cercopithecus*

ascanius), as well as two other African species of NHP, black-and-white colobus monkeys (*Colobus guereza*) and red colobus monkeys (*Procolobus tephrosceles*), to investigate how anthropogenic change, such as forest fragmentation, affects bacterial transmission among NHPs, humans, and livestock (Goldberg et al. 2008). They determined that deforestation and agricultural land use increase interspecific bacterial transmission, as these disruptions in the ecosystem lead to an increase in ecologic overlap between wild primate populations, humans, and domestic livestock. Their results suggest that environmental contamination is the most likely source of interspecific bacterial transmission. Information gleaned from this and other similar studies allow for the calculation of risk factors associated with human encroachment, notably habitat disturbance, on wild NHP populations, and help with the establishment of conservation strategies aimed at protecting threatened NHPs.

Of the culture-independent studies performed on cercopithecines, most have focused on wild populations, but these studies have also included multiple other NHP taxa. In a recent study examining fecal microbial communities, McCord et al. (2014) used ARISA to analyze the fecal microbiomes of three African NHP species, including one species of cercopithecine, the red-tailed guenon (*C. ascanius*), and two species of colobine, the red colobus (*Procolobus rufomitratus*) and black-and-white colobus (*Colobus guereza*). As expected, fecal microbiomes were host species-specific, and thus could be readily differentiated based on host species alone (McCord et al. 2014). These differences

persisted in the face of habitat degradation, which led the authors to conclude that the gut microbiome of these cercopithecines was largely resilient to environmental disturbance.

In a 2010 study examining the species-specific differences in fecal microbiomes of three African NHPs, including the red-tailed guenon (*Cercopithecus ascanius*), only a few phyla were found in high abundance. These were Firmicutes (the largest portion of genomic sequences) and Bacteroidetes (second largest portion) (Yildirim et al. 2010). *Ruminococcus*, *Roseburia*, *Oscillibacter*, *Anaerovorax*, *Novosphingobium*, *Coprococcus*, *Parabacteroides*, *Blautia*, *Faecalibacterium*, *Subdoligranulum*, *Anaerotruncus*, *Anaeroplasma*, and *Dorea* were present in all individuals sampled. *Anaeroplasma*, a genus in the class Mollicutes was also present in all red-tailed guenon subjects examined (Yildirim et al. 2010). Mollicutes can colonize human and other hosts and may be implicated in disease (Szekely et al. 2010). Also, red-tailed guenons show an abundance of *Prevotella* (phylum Bacteroidetes). *Prevotella*, usually isolated from human oral cavities and feces and the rumen ecosystem, are proteolytic and saccharolytic, and have the capacity to ferment sugars, including glucose, lactose, maltose, mannose, raffinose and sucrose (Alauzet et al. 2007; Downes et al. 2009). This is consistent with the frugivorous profile of guenons (Lambert 2001; Lambert 2002) in which energy may be derived from lipid, sugar and protein calories. *Oscillibacter* (Phylum Firmicutes, class Clostridia) was the most abundant genus found in all guenon fecal samples examined. Walker et al. (2011) report that *Oscillibacter* and *Subdoligranulum* were enriched in the fecal samples of individuals under diets rich in resistant starch and non-starch

polysaccharides. Mondot et al. (2010), report that *Oscillibacter* and *Faecalibacterium*, also enriched in the fecal samples of guenons, are associated with healthy individuals under diets low in sugar and fat calories (Sokol et al. 2008). Other genera from the Firmicutes phylum found in the fecal samples of guenons, such as *Roseburia* and *Ruminococcus*, are associated with fermentation and production of H_2, CO_2 and VFAs (e.g., butyrate) (Kim et al. 2011; Nakamura et al. 2011). This could mean that despite being mostly frugivorous, the microbiota of guenons can also adapt to a diet high in fiber, or that fruits consumed by guenons are fibrous (Lambert 2002). Similarly, the ability of the microbiota to process fiber structural polysaccharides also represents an advantage when breaking down the exoskeleton of arthropods, which *C. ascanius* regularly consumes.

Colobines (subfamily Colobinae): Colobines are the only monkeys capable of forestomach fermentation, facilitated by their enlarged and multi-chambered stomach (Bauchop 1971; Caton 1999; Chivers & Hladik 1980; Lambert 1998; Kay & Davies 1994). Microbial fermentation and absorption of VFAs occur in the specialized saccus gastricus of colobines, and then digesta passes to the tubus gastricus (Kay & Davies 1994). This adaptation allows them to ferment plant material, absorb VFAs and ammonia and transform plant secondary metabolites present in leaves by increasing retention time in the stomach. Compared to their voluminous stomach, colobines have a relatively small midgut, which allows them to carry on extended fermentation of fibrous material, and thus more efficient absorption of VFAs compared to caeco-colic fermenters (Chivers

1994; Kay & Davies 1994). Because it is in the pancreas and liver where hydrolysis and digestion of protein ultimately takes place (Kay & Davies 1994), part of the foregut microbiota can be also digested, unlike the colonic microbiota of other primates (Lambert 1998; Ley et al. 2008). Also referred as "leaf-eaters", colobines are subdivided in Asian and African representatives with 7 and 3 genera respectively. Although both groups forage mainly on leaves and seeds, they also consume fruits, buds, bark, lichens, fungi and even soil (Fashing 2011).

Since the 1960s there has been increasing interest in exploring the gut microbiology of foregut fermenting colobines. One of the first attempts to show the bacterial communities in foregut contents used two NHP species, *Presbytis entellus* and *Presbytis cristatus* (Bauchop & Martucci 1968). They noted that bacterial fermentation occurs in the langur stomach, which is diverticular in form (Bauchop & Martucci 1968). Additionally, Bauchop and Martucci (1968) noted that bacterial fermentation of the leafy diet consumed by colobines results in the production of vital nutrients, such as volatile short-chain fatty acids required for survival of these primates.

Examination of both host genetics and the microbiome of snub-nosed monkeys, showed that the stomach microbiome of *R. roxellana* was dominated by Firmicutes (38.7%), Proteobacteria (28.9%) and Bacteroidetes (28.8%) (Zhou et al. 2014). They also detected similarities between the stomach microbiomes of *R. roxellana* and the stomach microbiome of both humans and cattle. Specifically, the stomach microbiome of *R.*

roxellana was more similar to that of the cattle rumen. However, *Akkermansia* (Phylum Verrucomicrobia) was the 5th most abundant genus present in the stomach of the golden snub-nosed monkey (*Rhinopithecus roxellana*), and *Akkermansia* was not recovered from the stomach of cattle or humans (Zhou et al. 2014). Finally, through their analysis of microbial function, they identified genes involved in the digestion of cellulose, including 27 cellulose genes, 17 1,4-β-cellobiosidase genes and 179 β-glucosidase genes. Collectively, their results suggest the presence of a strong diet-microbiome link in the stomach of *R. roxellana*.

Examining microbial diversity and functional capacity of the *Rhinopithecus bieti* fecal microbiome, Xu et al. (2015) found the microbiome of *R. bieti* was dominated by Firmicutes (39.36%), Bacteroidetes (27.60%), Proteobacteria (19.41%), and Actinobacteria (3.61%). Similar to the findings of Zhou et al. (2014), Xu et al. (2015) found that the fecal microbiome of *R. bieti* was closely related to that of cattle. Specifically, Xu et al. (2015) found that the glycoside hydrolase profile of the *R. bieti* fecal microbiome was most closely related to that of the cow rumen.

In the aforementioned 2010 study published examining the species-specific differences in fecal microbiomes of three African NHPs, Yildirim et al. (2010) studied bacterial diversity in fecal samples of colobines, including black-and-white colobus monkeys (*Colobus guereza*) and red colobus monkeys (*Procolobus tephrosceles*). As was seen in the McCord et al. (2014) study, fecal microbiomes were host species-specific, and thus

could be readily differentiated based on NHP species. Bacteroidetes, which have been retrieved from the human oral cavity and colon and are associated with the fermentation of simple sugars, were less abundant in the colobines compared to the guenons (Hardham et al. 2008; Ueki et al. 2006; Sakamoto & Benno 2006). Except for exhibiting lower levels of *Prevotella* (Phylum Bacteroidetes), the microbiomes of the two colobine species, *C. guereza* and *P. tephrosceles*, examined by Yildirim et al. (2010) were highly similar to that found in guenons (Yildirim et al. 2010). For example, as was the case in *C. ascanius*, *Oscillibacter* was the most abundant genus found in all *C. guereza* and *P. tephrosceles* fecal samples examined. Basically, the same genera of Firmicutes found in guenons were found in the two species of colobines examined. As with red-tailed guenons, Yildirim et al. (2010) found the following genera in all black-and-white and red colobus monkey samples: *Ruminococcus*, *Roseburia*, *Oscillibacter*, *Anaerovorax*, *Novosphingobium*, *Coprococcus*, *Parabacteroides*, *Blautia*, *Faecalibacterium*, *Subdoligranulum*, *Anaerotruncus*, *Anaeroplasma*, *Dorea*, as well as *Anaeroplasma*, a genus in the class Mollicutes. As previously mentioned, Mollicutes are also found in humans and have been suggested to be implicated in disease. Taken together, this suggests that Mollicutes, including human pathogens, are part of the normal primate gut microbiome. All Firmicutes bacteria found in the fecal samples of the two colobines examined by Yildirim et al. (2010) are known fiber fermenters. This mainly fibrolytic microbiota may support the highly folivorous diet of colobines (Kay & Davies 1994). However, the extent to which the fecal bacterial profiles in foregut-fermenting colobines faithfully reflect the population of fermenting bacteria may not be clear. The microbiota

profiles detected in colobines by Yildirim et al. (2010) may represent bacterial communities associated with late colonic fermentation of plant residues that could not be fermented in the saccus gastricus. Consequently, it does not represent the fibrolytic and fermentative microbiome of the foregut. This may also explain why the fermentative microbiota of the caeco-colic fermenting guenon was qualitatively similar to that found in colobines, and could mean that both species share bacterial lineages of colonic bacteria with similar ancestors that expanded or contracted to adapt to ecological constraints imposed by diet.

Apes (Hominoidea):

The genetic, behavioral and ecological similarities between chimpanzees, gorillas, orangutans and humans (Gagneux & Varki 2001; Rozen et al. 2003; Mitteroecker et al. 2004; Suddendorf & Whiten 2001) have motivated research on the exploration and characterization of their gut bacterial communities. Although the initial approach has been mainly focused on the way primate microbiomes resemble or differ from that of humans, from an evolutionary standpoint (Ochman et al. 2010; Moeller et al. 2012; Moeller et al. 2014), more recent reports have shown the strong influence of environmental forces in shaping great ape microbiomes and the way gut microbes could be contributing to primate foraging plasticity (Gomez et al. 2015; Degnan et al. 2012; Moeller et al. 2013).

Gorillas (genus *Gorilla*): *Gorilla* spp. display special dentition patterns and enlarged colons, which would allow them to process large amounts of dietary fiber, rich in plant secondary metabolites (Remis 2000; Remis & Dierenfeld 2004; Spears & Crompton 1996; Doran & McNeilage 1998). These observations also imply that gorillas are especially dependent on the gut microbiome for nutrient extraction, particularly when exploiting increased amounts of plant structural carbohydrates.

Few studies have explored bacterial diversity and influence of the gut microbiome in the feeding ecology of *Gorilla* spp. The first study exploring gorilla gut microbiomes using molecular techniques (Frey et al. 2006) showed that mountain gorillas at Bwindi impenetrable park (Uganda) (*G. b. beringei*, *n*=1, a silverback) harbor microbial taxa potentially associated with fiber processing and fermentation (*Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Bulleidia extructa*) as well as degradation of condensed tannins *(Eubacterium oxidorreducens*). Subsequent work using 454 pyrosequencing showed that the microbiomes of mountain (n=2) and western lowland gorillas (*G. g. gorilla*) (n=2) are more similar to each other than they are to that of chimpanzees, bonobos and humans (Ochman et al. 2010). However, efforts to capture variation in microbiome composition driven by ecological/dietary factors, based on monthly fingerprint screening of fecal samples (T-RFLP), and chloroplast sequence analysis from feces to detect difference in plant species consumed, were unsuccessful in these studies.

More recent reports have illustrated the importance of foraging constraints on the gut microbiome of gorillas. For instance, high-throughput sequences of 34 western lowland gorillas revealed significantly different gut bacterial communities and metabolomic profiles among groups with non-overlapping home ranges and evident distinctions in diet (Gomez et al. 2015). For example, there was an increased abundance of *Prevotella*, *Anaeroplasma* and metabolites associated with fiber and phenolic processing in gorillas consuming more leaves and herbaceous vegetation. In contrast, gorillas consuming more fruit showed increased abundance of *Lactobacillus,* taxa related to the Lachnospiraceae, Erysipelotrichaceae, and metabolites involved in lipid processing and fermentation of more soluble sugars. It has also been suggested that the gut microbiome of western lowland gorillas assorts into enterotypes; microbiome configurations characterized by increased frequency of specific genera (Moeller et al. 2015). The presence of these enterotypes in gorillas has been associated to both environmental (dietary) and evolutionary constraints on primate microbiome composition. Further support on the influence of ecological and dietary factors on shaping gorilla gut microbiomes points to increased patterns of shared taxa between sympatric chimpanzees and western lowland gorillas compared to allopatric individuals (Moeller et al. 2013). Authors hypothesized that these convergent microbiome patterns in sympatric gorillas and chimpanzees were caused by shared diets. Finally, recent culture-based studies suggest novel diversity in the gut microbiome of western lowland gorillas (new bacterial species, possibly gorilla specific) and prevalence of bacteria usually associated with disease in humans (Bittar et

al. 2014). These observations imply that strong evolutionary and ecological drivers on primate gut microbiomes that are yet to be fully explored.

Chimpanzees (genus *Pan*): Compared to gorillas, chimpanzees (*Pan* spp.) are characterized by a larger small intestine and smaller colon, which could reflect a more prominent role of fruit, more digestible resources, and less dependence on fibrous foods in their diets (Stumpf 2011; Milton 1987). Moreover, gut microbiome configuration in chimpanzees is closer to the patterns observed in humans under non-western diets (Moeller et al. 2014). In one of the first reports exploring the gut microbiome of wild chimpanzees (at Bossou, Guinea), using molecular fingerprint methods it was shown that captive and wild *P. t. verus* harbored significantly different gut microbial communities (Uenishi et al. 2007). Using complementary cloning techniques, the study also identified several taxa affiliated to the *Eubacterium*, *Clostridium*, *Ruminococcus Lactobacillus*, *Bifidobacterium* and *Prevotella*, in both captive and wild individuals. These are all taxa that could potentially have saccharolytic, fibrolytic and fermentative roles in the colonic ecosystem. Additional work in wild *P. t. schweinfurthii* from Tanzania, employing molecular fingerprinting techniques (T-RFLP), confirms most of these taxonomic patterns and reports marked interpersonal differences in the community profiles detected, just as it happens in humans. Analyses of the fiber digesting capabilities of captive *P.t. troglodytes,* coupled with molecular fingerprinting of their gut bacterial communities (DGGE) showed a shift in microbiome composition between diets high in fiber (26% neutral detergent fiber, 15% cellulose) and low in fiber (14% neutral detergent fiber, 5%

cellulose) (Kisidayová et al. 2009). This study reports blooms of *Eubacterium biforme* and increased production of short chain fatty acids from fecal innocula under high fiber diets. However, it also shows that digestibility of high cellulose substrates in the gut of captive *Pan* is limited.

The application of high throughput sequencing techniques to explore the gut microbiota of chimpanzees has allowed researchers to have a more complete view of specific evolutionary and ecological factors shaping gut microbiome composition in *Pan*. For instance, work exploring gut bacterial communities in *P. paniscus*, *P.t. troglodytes*, *P.t. schweinfurthii*, and *P. t. ellioti* (Ochman et al. 2010) suggested that bacterial communities within *Pan* spp. are species-specific, and different from those in other apes (*Gorilla* and humans). However, no diet correlates to bacterial community composition were reported. Other reports have shown that the gut microbiomes of *P. t. schweinfurthii* in Tanzania reflect the biogeographical and community affiliation patterns of the host (Degnan et al. 2012). This pattern could be hypothesized to arise from shared ecological factors (i.e. diet, social contact). Additionally, this biogeographical signal was found to persist for long times (nearly a decade), even after dispersal of individuals to other communities or ranges. The use of high throughput sequencing techniques to explore the gut microbiome of chimpanzees has also revealed that their gut microbiome assorts into enterotypes, analogous to those reported in humans (Moeller et al. 2012; Arumugam et al. 2011). However, despite increased abundance of *Prevotella* (a taxon usually linked to fiber and starch degradation) in all chimpanzees, compared to patterns seen in human enterotypes,

no associations between enterotypes and dietary factors in chimpanzee gut microbiomes were reported. Finally, reports on the effect of SIV infection on the gut microbiome of chimpanzees showed that infection can trigger increased abundance of potentially pathogenic taxa in the chimpanzee gut (i.e. *Staphylococcus*). No allusions to environmental factors on the gut microbiome of infected and uninfected chimpanzees were made on such reports.

Orangutans (genus *Pongo*): To date no studies examining microbial communities in wild orangutans have been conducted. Due to the primarily frugivorous behavior of orangutans, and diets with high caloric loads, an exploration of their gut microbiomes could offer important clues on how diet and other ecological factors could have contributed to shaping current gut microbiome arrangements in humans. The only culture-independent investigation of orangutan gut microbiota was part of a much larger study examining co-evolution of mammals and their respective gut microbial communities, and only included captive individuals (Ley et al. 2008). They observed that the gut microbiomes of captive orangutans, as is the case with most mammals, were dominated by Firmicutes and Bacteroidetes. The limited information generated as a result of this study does not offer much insight in terms of how gut microbes impact orangutan ecology, thus there is a critical need in the field to include orangutans in the systematic exploration of NHP gut microbiomes.

1.4 The use of microbiome research for the field of primatology

Understanding host-microbiome interactions from primates covering diversity of extant members of this order is of great importance to the field of primatology. Areas of primatology where studying the microbiome can be especially beneficial include primate health, evolution, behavior, and conservation (Figure 1.1). Some example expected impacts lying in these four core areas of focus are as follows.

Health: The gut microbiome is so intimately related to animal health that it is regularly referred to as an additional organ in the body. These bacteria are critical players in primate health and development: they protect from infection, aid in digestion, produce vitamins from the diet, and influence immune system development (Turnbaugh et al. 2006; Morgan et al. 2012; Petersen & Round 2014; Ley et al. 2005). When one's microbiome is composed of an unnatural mixture of bacterial species, this is referred to as dysbiosis. Dysbiosis may increase the risk of obesity, diabetes, cancer, among other diseases in humans, and is likely to reduce host fitness by alter the ability of the host to adapt to and digest different diets. Recent advances in human and primate microbiome research have fundamentally changed our understanding of primate immune and metabolic health (Knights et al. 2013; Muegge et al. 2011; Ridaura et al. 2013). Despite these studies, much more work is required in order to fully understand how the microbial communities impact primate health.

Health and pathogen resistance in nonhuman primates have direct links to human health, for example in the case of SIV. It is well established that HIV, the causative agent of

AIDS and arguably the most devastating human disease in our recent past, originated from related viruses of chimpanzees (*Pan troglodytes*) and sooty mangabeys (*Cercocebus atys*) (Hahn et al. 2000; LeBreton et al. 2007; Wolfe et al. 2005). Other notable examples of diseases shared by humans and nonhuman primates include, herpes B virus, monkeypox, polio virus, ebola virus, tuberculosis, malaria, and yellow fever, just to name a few (Chapman et al. 2005). Many of these diseases are highly pathogenic, such as Ebola virus, which was responsible for about 5,000 gorilla deaths in northwest Republic of Congo in between 2002 and 2003 (Bermejo et al. 2006). The increasing occurrence of diseases plaguing humankind, such as the AIDS epidemic, sheds light on the paramount importance of understanding infectious disease ecology and how to establish mechanisms for protecting both human and nonhuman primate populations (Chapman et al. 2005). In order to achieve this arduous task, a better understanding of the role microbial communities play in the maintenance of health must be established.

Given that microbes can act as indicators for health of the host, broad primate microbiome surveys could aid in the development of predictive biomarkers for certain diseases that affect both humans and nonhuman primates alike. Since humans are catarrhines, other catarrhines (nonhuman primates) are likely good animal models for understanding human health. Understanding what drives the structure and variation of their microbiota will help us understand our own.

Evolution: Nonhuman primates are of interest because of their importance to the evolution of the human species. Humans and chimpanzees have 98.77% nucleotide and 99% amino acid identity in common with one another. While all primates are more closely related to one another than they are to nonprimate mammals, there are substantial differences in appearance and behavior from species to species, including stark differences in diet. Currently, our understanding of how adaptations to diet have shaped primate evolution is based primarily on paleontological, ecological, morphological, and behavioral data. While these approaches have provided priceless information and formed the basis of our current understand, they have fail to address a critically important factor in digestive health and energy acquisition, which is gut microbial composition. The symbiotic relationship between host and gut microbes is a major determinant of feeding ecology and the adaptation to a specific diet since gut microflora provide the host with critical metabolic specializations, including digestive enzymes.

It is well established that microbial communities play key roles in animal health. An understanding of the evolution of these communities is essential to a better understanding of the microbial component of human evolution (Ochman et al. 2010; Yildirim et al. 2010). In the gastrointestinal tract, characterization of these populations will help to explain divergent adaptations in closely related species. The emerging field of metagenomics allows direct, unbiased interrogation of microbial populations (i.e., microbiomes), thus enabling the investigation of unique dietary differences in primate species that may reveal the role of microbial communities in primate evolutionary history

(Ochman et al. 2010; Yildirim et al. 2010). It is likely that these changes played a major role in the ability of the hominid ancestor to move from a forest (woody plant) diet to a savanna (grass) diet, as the gut microflora largely govern what types of foods can be digested by the host. In summary, the gut microbiota likely played an important role in primate specialization of diet and gut physiology, however this role has yet to be elucidated.

Behavior: It has been known for some time that there is a relationship between gut microbiota and nervous system function (Cryan & Dinan 2012). Some early studies identified changes in the gut microbiota associated with stress, and a role for the microbiota in modulating stress and stress-related behavior. A list of potential mechanisms by which the microbiota can affect CNS function has been generated and includes immune system activation, vagus nerve activation, generation of metabolites with neuroactive properties, and production of cell wall sugars that impact function of primary afferent axons (Cryan & Dinan 2012). Chronic inflammation due to bacterial infection has also been shown to exhibit a dramatic effect on nervous system function.

The microbiota-gut-brain axis is an emerging concept that suggests an intricate role for our microbes in the establishment and treatment of nervous system disorders. However, as an emerging area, this concept has been studied in a very limited fashion. With the availability of novel technologies to study the microbiota in a host, it is now possible to identify microbiome modulations that impact nervous system function.

While gut-brain communication is well established in other animal models, little is known about this form of communication in nonhuman primates, despite the fact that nonhuman primates are ideal models by which to study the microbe-gut-brain relationship in a natural context. By collecting longitudinal and cross-sectional gut microbiome samples while tracking feeding and social behavior of individual animals, a better understanding of how microbes influence primate behavior can be established.

Conservation: The Earth's biosphere contains a finite land mass. Rapid human population expansion has led to the need for more resources, many of which are taken from the Earth's remaining forests. For example, forests provide humankind with timber, medicine, and food (Costanza et al. 1998). The United States Census Bureau currently estimates the world population to number slightly over 7 billion. Human population expansion has and continues to take a massive toll on the environment (McNeill 2001). Deforestation poses a major threat to the world's remaining wildlife populations, especially nonhuman primates, and is a primary concern of conservationists (Thomas et al. 2004). Forest fragmentation presents major hurdles for primate populations, as fragmentation disrupts natural ranging patterns, which are necessary for primates to locate enough food to meet their daily energetic demands (Arroyo-Rodríguez & Dias 2010). Forest fragmentation also leads to pollution, destruction of watersheds, as well as numerous other problems.

The spread of infectious diseases represents a major threat to wildlife populations and humans alike (LeBreton et al. 2007; Wolfe et al. 2007; Wolfe et al. 2005). As primates struggle to move through their fragmented home ranges, primate interactions with humans and domestic livestock increase dramatically (Chapman et al. 2005; Goldberg et al. 2008; Goldberg et al. 2007). These interactions are problematic because humans and nonhuman primates are predisposed to similar pathogens and the potential for zoonotic transfer is drastically elevated with human-primate interactions compared to human contact with other nonprimate species (Chapman et al. 2005).

Nonhuman primates, such as howlers (*Alouatta* spp.), can act as sentinels for unhealthy shifts in their habitat ecosystems (Amato et al. 2013). However, it is currently unknown whether shifts in their gut microbiota accompany increased stress or other health issues related to habitat encroachment, and thus a better understanding of the cause-and-effect relationship occurring is critical if we are to utilize microbiome-related information to aid in the conservation of wild primates and their associated habitats. In addition, some endangered primate species fail to thrive in captivity due to gastrointestinal issues; through comparison of wild and captive animals within the same species it can be determined whether shifts in gut microbiota are linked with gastrointestinal health in captivity.

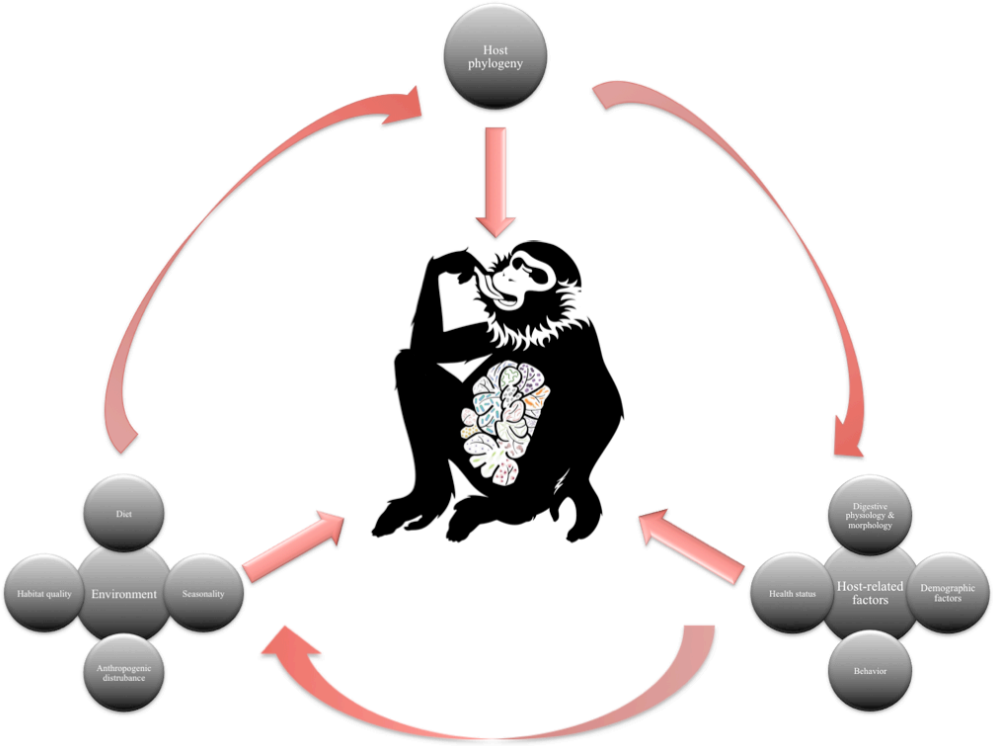

Figure 1.1 Dynamism of NHP microbiota profiles. NHP microbiotas originate from early evolutionary traits of their host-microbial system ancestors, but then are further shaped by host-related and environmental factors. In this way microbiotas conserve specific bacterial lineages from their ancestors but other bacterial groups can then be expanded or contracted according to constraints found in their surroundings.

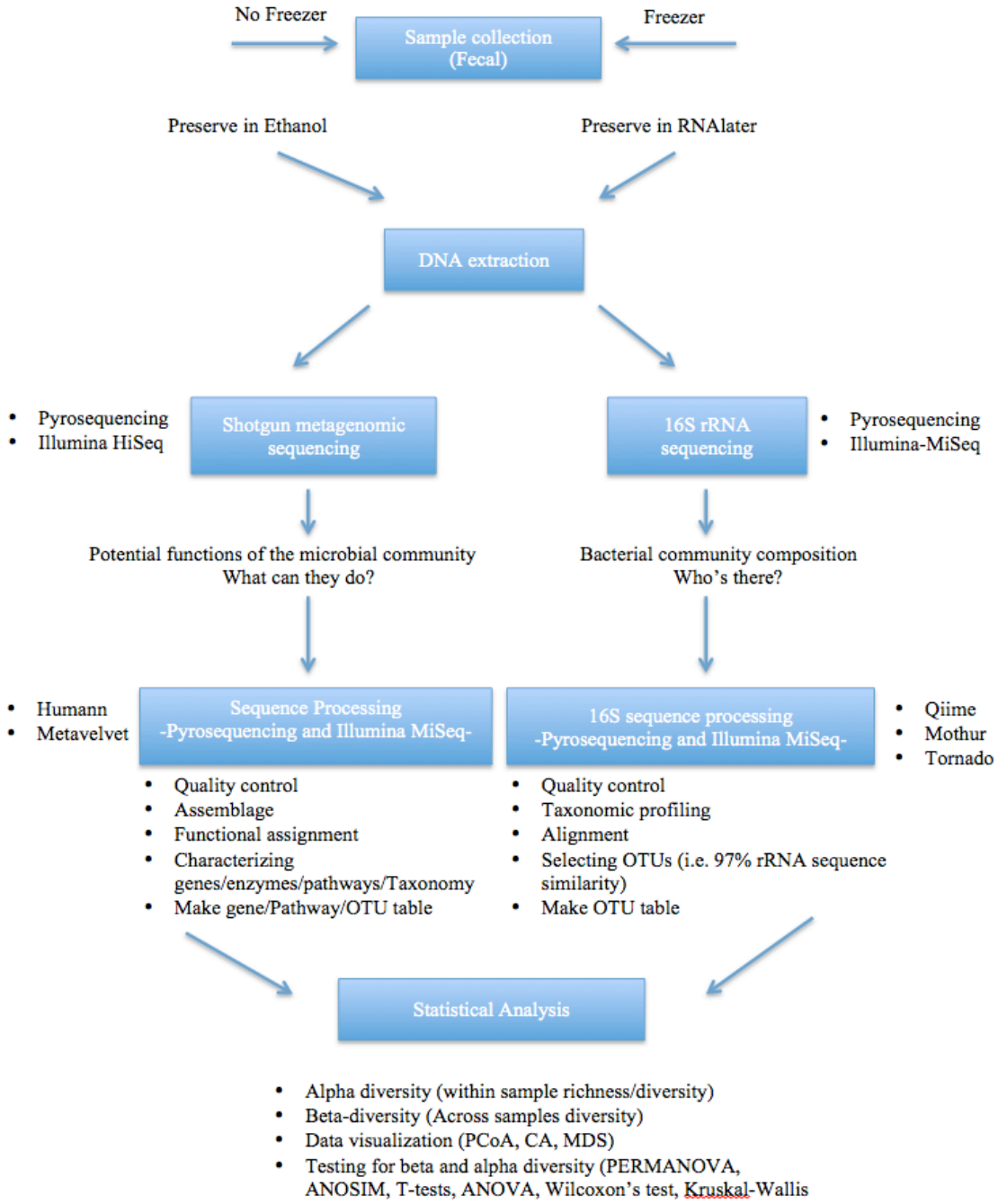

Figure 1.2 Overview of current methods used in microbiome studies.

Chapter 2 Associations Between Nutrition, Fecal Microbiome, and Health in Red-shanked Doucs (*Pygathrix nemaeus*): A Model for the Subfamily Colobinae

Summary

The red-shanked douc (*Pygathrix nemaeus*) is a species of Asian colobine, which inhabits east-central Lao People's Democratic Republic (PDR), central Vietnam, and northern Cambodia. It is an endangered primate that is of great concern to conservationists. Recovery efforts to restore red-shanked douc populations currently utilize conservation sanctuaries, anti-poaching laws, and captive breeding programs. However, maintenance of red-shanked doucs and other colobines as captive populations has been largely unsuccessful due to an inadequate understanding of their nutritional requirements. They are highly susceptible to gastric disorders when maintained on commercially prepared diets in captivity. Simply improving their diet in captivity is challenging or impossible due to critical gaps in our understanding of local fibrous vegetation and the enteric microbial adaptations that facilitate efficient extraction of key nutrients. This study examines the relationship between the douc diet and the specialized douc digestive capacity and fecal microbiota. An improved understanding of the douc gastrointestinal microbiota, the relationship between the douc's diet and microbiota, and the identification of biomarkers or microbial species that positively impact douc digestive capacity may benefit the maintenance of this rare species.

2.1 Introduction

One mechanism for the preservation of threatened species is the creation of sustainable captive populations that may act as a genetic repository that buffers against extinction.

The red-shanked douc (*Pygathrix nemaeus*) is a species of Asian colobine, which inhabits east-central Lao People's Democratic Republic (PDR), central Vietnam, and northern Cambodia. Because it is listed as endangered this primate is of great concern to conservationists (Nadler et al. 2003; Ngoc et al. 2008; Timmins & Duckworth 1999; Phiapalath et al. 2011; Heistermann et al. 2004). Recovery efforts to restore the red-shanked douc population currently utilize conservation sanctuaries, anti-poaching laws, and captive breeding programs. However, maintenance of red-shanked doucs and other colobines as captive populations has been largely unsuccessful due to an inadequate understanding of their nutritional requirements. They are highly susceptible to gastrointestinal (GI) disorders when maintained on commercially prepared diets in captivity. Improving their diet in captivity is challenging due to critical gaps in our understanding of local fibrous vegetation and the enteric microbial adaptations that facilitate efficient extraction of key nutrients.

Colobines are Old World monkeys and most are folivorous, and are anatomically, physiologically, and ecologically unique amongst the living primates (Davies & Oates 1994). They possess specialized GI systems similar to ruminants, including a sacculated stomach, allowing for the digestion and utilization of extremely high fiber diets (Chivers 1994; Lambert 1998). As primates with a primarily folivorous diet, they rely on a diet that is less nutrient dense than that consumed by non-folivorous primates. Colobines are capable of metabolizing plant cell wall biomass due to the cellulolytic microorganisms that colonize the compartments of their GI tract. These mutualistic microbial populations

are imperative to digestive processes such as the fermentation of polysaccharides and subsequent production of short-chain fatty acids, which are later assimilated and metabolized as an energy source (Jablonski 1998; Nijboer et al. 2006). The complex gut structure and GI-associated microflora of colobine primates together enables neutralization of digestive inhibitors and potential toxins present in plant materials, which constitute the majority of their natural diet (Jablonski 1998; Yildirim et al. 2010; Davies & Oates 1994). However, an understanding of specific microbial taxa residing in the GI tract of colobine primates, and their associated functions, is currently lacking.

Microbial fermentation of plant material is critical for many primates, as the host alone cannot digest and extract nutrients from most plant cell wall material. The microbial community composition of the distal gut is responsible for many physiological attributes in mammals, including the ability to digest and break down polysaccharides, which would otherwise be indigestible (Xu et al. 2007). These microbes have thus contributed to the evolution of mammalian digestive physiology, as they have removed the need for mammals to evolve these physiological abilities for themselves (Xu et al. 2007). This is particularly true for species such as the red-shanked douc, whose diet primarily consists of leaves and who rely heavily on microbial fermentation for the extraction of nutrients (Mark S Edwards & Ullrey 1999; Milton & McBee 1983; Duane E Ullrey 1986; Xu et al. 2007).

Although the digestive specializations of colobines have allowed them to thrive in their native habitat, the same specializations appear to challenge their survival in captivity. In fact, these primates are among the most difficult to keep in captivity and are rarely kept in zoological institutions (Nijboer 2006; Power et al. 2012; Agoramoorthy et al. 2004). To put this into perspective, we consider the fact that less than 50% of the captive doucs sampled in the current study survived more than one year after sample collection was performed. Nine captive individuals were sampled in this study between 2012-13, and as of August 2014, five were deceased. According to International Species Information System (ISIS) database accessed through the Zoological Information Management System (ZIMS) as of 2014, only 70 red-shanked doucs are housed in zoos worldwide. Of those 70, only one individual remains in a U.S. zoo and one in a European zoo (ZIMS, 2015). For comparative purposes, consider the hindgut fermenting lar gibbon (*Hylobates lar*), which is a primate species represented by more than 500 individuals in zoos worldwide, both in tropical and temperate countries (ZIMS, 2015). Foregut fermenting primates are notoriously hard to keep in captivity compared to hindgut fermenting species. The specialized dietary requirements of folivorous primates have long been overlooked (Edwards 1997), resulting in wasting, and eventual digestive-associated mortality in captive doucs and other colobines. Common conditions include gastric distress (vomiting & diarrhea), gastric amebiasis, bloat, and weight loss (Ensley et al. 1982; Heldstab 1988; Sutherland-Smith et al. 1998; Agoramoorthy et al. 2004; Nijboer 2006; Janssen 1994; Loomis & Britt 1983; Ruempler 1998a; Calle et al. 1995; Overskei et al. 1994; Shelmidine et al. 2013). Previous studies have focused on the effect of food

and nutrient intake in captive colobines (e.g., langurs) and the effects of altering fiber on their feces quality in captivity (Nijboer 2006; Nijboer et al. 2001; Nijboer et al. 2006; Nijboer et al. 2006). All previous studies on this matter have failed to address the symbiotic relationship between digestion of their unique food source and GI microflora; despite the importance this symbiosis has received in the literature (Ensley et al. 1982; Overskei et al. 1994; Edwards 1997; Bauchop & Martucci 1968). This study examines the relationship between the specialized douc digestive capacity and the douc GI microbiota.

2.2 Materials and Methods

Study site and study subjects, and sample material

Fecal samples (n = 111) were collected opportunistically immediately after defecation from captive (n = 27), semi-captive (n = 18), and wild (n = 66) red-shanked doucs (*Pygathrix nemaeus*) between 2012-2013. Nine captive and eighteen semi-captive red-shanked doucs were sampled. Fecal samples (n = 26) were collected from seven known individuals. Remaining fecal samples (n = 40) collected from the wild population originated from unknown individuals. One captive population was located at the Philadelphia Zoo in the USA while another was located at the Singapore Zoo in Southeast Asia. Doucs housed at the Endangered Primate Rescue Center (EPRC) in Ninh Binh, Vietnam served as the semi-captive population. Doucs inhabiting Son Tra Nature Reserve, Da Nang, Vietnam (16°06'—16°09'N, 108°13'—108°21'E) served as the wild population in this comparative study (Lippold & Thanh 2008) (Figure 2.1). Son Tra is

located only 10 km from the heart of Da Nang City, which is the third largest city in Vietnam. The nature reserve is comprised of 4,439 total ha and of those 4,190 ha is covered by both primary and secondary forests (Lippold & Thanh 2008).

Wild doucs served as a normal (control) group. Doucs housed at the EPRC served as an intermediate group, as the doucs there live a semi-captive lifestyle. For example, these doucs are fed exclusively plants but are not offered any supplemental dietary items, such as fruits, vegetables, or vitamin supplements, all of which are fed to doucs housed at traditional zoological institutions. Doucs housed at Philadelphia Zoo and Singapore Zoo served as the unnatural group. Doucs housed at these zoos live in artificial environments compared to their semi-captive and wild counterparts. It is important to note that doucs housed at the Singapore Zoo live in an environment intermediate between the EPRC and the Philadelphia Zoo, thus they were also considered a transitional population similar to the way EPRC-housed doucs were considered transitional between wild and Southeast Asia zoo-housed doucs.

Genomic DNA extraction

Total DNA from each fecal sample was extracted using the established method of Yu and Morrison (2004) with some modifications (Yu & Morrison 2004). Modification included two rounds of bead-beating in the presence of NaCl and sodium dodecyl sulfate, followed by sequential ammonium acetate and isopropanol precipitations; treatment of precipitated nucleic acids with DNase-free RNase (Roche); and DNA purification using the QIAmp®

DNA Stool Mini Kit (QIAGEN, Valencia, CA), according to manufacturer's recommendations with some modifications. DNA quantity was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc, Massachusetts, USA).

Bacterial 16S rRNA PCR amplification and Illumina MiSeq sequencing

The bacterial 16S rRNA gene was amplified using primers 515F and 806R, which flanked the V4 hypervariable region of bacterial 16S rRNAs (Caporaso et al. 2012). The oligonucleotide primers included Illumina sequencing adapters at the 5' ends and template specific sequences at the 3' ends. The primer sequences were: 515F (forward) 5' GTGCCAGCMGCCGCGGTAA 3' and 806R (reverse) 5' GGACTACHVGGGTWTCTAAT 3' (Caporaso et al. 2012). The 16S rRNA amplification protocol from the earth microbiome project was adopted to perform the PCR amplification (Gilbert et al. 2010). Each sample was amplified in two replicate 25-µL PCR reactions and pooled into a single volume of 50.0 µL for each sample. The amplification mix contained 13.0 µL of PCR grade water (MoBio, Carlsbad, CA), 10.0 µL of 5 PRIME HotMasterMix (5 PRIME, Gaithersburg, MD), 0.5 µL of each fusion primer, and 1.0 µL of template DNA in a reaction volume of 25.0 µL. PCR conditions were an initial denaturation at 94°C for 3 minutes; 35 cycles of 94°C 45 seconds, 50°C for 60 seconds, and 72°C for 90 seconds; and a final 10 minutes extension at 72°C. Following PCR, concentration of PCR products was determined by a PicoGreen assay. Next, samples were normalized and pooled, and size selection was performed using the Caliper XT (cut at 386 bp +/- 15%). Final quantification was performed via a PicoGreen

assay and assessment on a Bioanalyzer 2100 (Agilent, Palo Alto, California) using an Agilent High Sensitivity DNA chip. The PCR amplicons were sequenced at the University of Minnesota Genomics Center (UMGC) via Illumina MiSeq to yield 2x300 base paired-end reads (Illumina, San Diego, California).

16S Data analysis

Raw sequences were analyzed with QIIME 1.8.0 pipeline (Caporaso et al. 2010). The demultiplexed sequences from the UMGC encountered the following quality filter: 150 bp < length < 1,000 bp; average quality score > 25. Preprocessed sequences were then clustered at 97% nucleotide sequence similarity level. Two different OTU picking protocols were used to assign the operational taxonomic unit IDs (OTUs): Closed reference-based and open reference-based OTU picking pipelines, both of which used Greengenes 13_8 as the reference database (DeSantis et al. 2006). The QIIME scripts, parallel_pick_otus_usearch61_ref.py and pick_open_reference_otus.py, were used for each pipeline respectively, using USEARCH algorithm (Edgar 2010). Unmatched reads against the reference database were excluded from the downstream analysis. Taxonomy information was then assigned to each sequence cluster using RDP classifier 2.2 (Wang et al. 2007). The QIIME script, filter_samples_from_otu_table.py, was employed to remove the OTUs of chloroplast origin from the OTU table, which fall under the phylum of Cyanobacteria. All samples were rarefied to 31,500 reads for the downstream analysis. Alpha diversity analysis, beta diversity analysis, weighted and unweighted UniFrac

distance matrix (Lozupone & Knight 2005), and PCoA (principal coordinate analysis) plots were obtained through the wrapper scripts in QIIME and custom written R scripts.

The functional profiles of the microbial sample in this study were investigated using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al. 2013), a bioinformatics tool that is used to predict gene family abundances based on 16S rRNA surveys (i.e. the OTU table as the input). Within this pipeline, OTUs were normalized by 16S rRNA copy number, and metagenomes were predicted from the Kyoto Encyclopedia of Genes and Genomes (KEGG) catalogue (Kanehisa & Goto 2000).

Chemical analysis of diet components

One population of wild doucs was observed between January and August 2013 in Son Tra Nature Reserve, Da Nang, Vietnam. All occurrences of observed feeding behaviors were recorded. Identified plant parts ingested were recorded and reachable feeding trees were marked. The plant parts of specific trees that were prevalent in their diet and were available in sufficient quantities were sampled and dried to 95% dry matter (Barnett 1995). Samples were sent to the Biochemical Lab at The Agriculture and Forestry University in Ho Chi Minh City, Vietnam, for chemical analysis. Concentrations of crude protein, simple sugars, crude fiber, calcium, sodium, manganese, potassium and iron were determined on a dry matter basis, all of which follow AOAC methods 920.152, 973.18C and 974.06 (AOAC 2012). Additionally, all plants fed at the EPRC during a

two-week period in October 2012 were also sent for chemical analysis for comparison. Chemical compositions of zoo diets were also analyzed. Nutrients were compiled from the laboratory results and from both zoos on a concentration per dry matter basis. Wild and semi-captive nutrient contents were constructed by observed frequency. Zoo nutrient contents were assembled purely from diets as given to their specimens. No estimates of diet intake were attempted.

2.3 Results

Bacterial diversity of red-shanked douc feces: OTU-based analysis

For OTU-based analysis in this study, microbiome compositions across four douc populations were examined, including the USA zoo, Southeast Asia zoo, EPRC, and wild populations. To investigate fecal microbiome diversity open-reference OTU picking was performed. Amplicon sequencing of the 16S V4 region generated 8,376,833 sequence reads after quality control. The number of OTUs found in samples was used as the primary measure of bacterial diversity. Based on an OTU analysis at 97% sequence similarity level, the number of OTUs observed in this study after OTU picking was 74,219. Following OTU picking, OTUs with less than three sequences were removed, resulting in 39,401 OTUs. The number of OTUs present in each douc population correlated with lifestyle. Specifically, fecal microbiome diversity showed a steady decline from wild towards captive environments. The number of OTUs in the doucs decreased in a gradient-like fashion with the highest number seen in the douc population living under the most natural conditions (i.e., wild), and the lowest number of OTUs seen in the douc

population living under the most unnatural conditions (i.e., USA zoo). Overall, the wild doucs harbored the highest number of OTUs (4231.68 ± 584.37 OTUs) (i.e., greatest diversity), followed by the EPRC-housed doucs (2845.50 ± 494.98 OTUs), Southeast Asia zoo-housed doucs (2696.93 ± 417.00 OTUs), and USA zoo-housed doucs (2332.08 ± 180.30 OTUs) (Figure 2.2).

In addition to investigating overall OTU diversity (i.e., number of OTUs) amongst the four unique douc populations included in this study, alpha diversity (i.e., within-sample diversity) metrics were calculated, including Chao1 and Shannon diversity index. Chao1 estimated the number of OTUs to be 10103.71 ± 1218.11 for the wild population, 6425.89 ± 1099.87 for the EPRC population, 6011.62 ± 878.85 for the Southeast Asia zoo population, and 4930.31 ± 524.83 for the USA zoo population. Based on the results of the Chao1 analysis, the wild douc microbiome was the most diverse of the four populations investigated (Figure 2.3). Shannon diversity indices, which measure species evenness, were calculated for each douc population. Shannon diversity indices were different across the four douc populations (wild: 7.86 ± 0.34; EPRC: 7.07 ± 0.55; Southeast Asia zoo: 7.11 ± 0.53; USA zoo: 6.65 ± 0.53; ANOVA, $p=4.3\times10^{-18}$). Based on the calculated Shannon diversity indices, the wild douc microbiome was the most even of the four douc populations (Figure 2.3). The same pattern seen in previous measurements of diversity (i.e., the more unnatural the lifestyle the less diverse the microbiome) was not seen in analysis of Shannon diversity. In the case of Shannon

diversity, while the microbiome of the doucs housed at the EPRC was less even than the wild population, it was also less even than the Southeast Asia zoo population.

Following examination of within-sample diversity, beta diversity calculations were performed as a comparative measure used to investigate the relationship between samples. Similar to within-sample diversity analysis, microbiome compositions of the four douc populations were examined to see if significant differences between populations were present. This was done using unweighted unifrac distances (i.e., unweighted PCoA plots), as this analysis is most effective at detecting differences in community membership when considering abundance changes in rare lineages (Chen et al. 2012a). Analysis of similarity (ANOSIM) revealed that fecal microbiome grouped statistically by douc population (ANOSIM $R = 0.98$; $p = 0.001$), suggesting that each douc population had a unique microbiome. Overall, the results of this analysis indicated that microbiome composition was distinct for each of the four douc populations examined at the 97% OTU level and genus level (Figure 2.4).

Red-shanked douc fecal bacteria: Taxon-based analysis

Taxon-based analysis for this study was conducted using sequences that mapped to the Greengenes database. Phylum-level analysis revealed the fecal microbiome of the four douc populations included in this study differed substantially. The fecal microbiomes of wild, EPRC-housed, Southeast Asia zoo-housed, and USA zoo-housed doucs were dominated by the phylum *Firmicutes* (91.4%; 70.8%; 78.2%; 75.3%). *Bacteroidetes* was

found in very low abundance in both the wild (1.3%) and semi-captive EPRC population (2.8%). In contrast, *Bacteroidetes* was the second most abundant phylum found in both the Southeast Asia (13.4%) and the USA (19.1%) captive zoo populations. Additionally, the fecal microbiomes of the doucs housed at the EPRC contained two phyla, *Verrucomicrobia* (12.1%) and *Tenericutes* (5.8%), in much higher abundance than the other douc populations examined (Figure 2.5a).

Class-level analysis was performed to determine the relative abundance of bacterial classes. *Clostridia* dominated the microbiome of all douc populations examined (Figure 2.5b). Order-level analysis was also performed and the order found in highest abundance in all douc populations was *Clostridiales* (Figure 2.5c). Classification at the family level yielded differences in abundance of multiple families belonging to the order *Clostridiales*, including *Ruminococcaceae*, *Lachnospiraceae*, and Unclassified *Clostridiales*, across the douc populations examined. Specifically, *Ruminococcaceae* was most abundant in the USA zoo population (54.6%), followed by the wild (52.7%), Southeast Asia zoo (41.9%), and EPRC populations (21.2%). *Lachnospiraceae* exhibited a great deal of variation across douc populations as well, as this family was more abundant in the wild and EPRC populations (20.5%; 23.7%) than in the Southeast Asia and the USA captive zoo populations (15.5%; 9.1%). Other notable findings included a much higher abundance of *BS11* (6.3%) and *Prevotellaceae* (3.0%) in the USA zoo population compared to other douc populations. In fact, *BS11* and *Prevotellaceae* were virtually absent in all doucs aside from those housed at the USA zoo (Figure 2.5d).

In terms of genera, the wild douc microbiome was dominated by unclassified *Ruminococcaceae*, as were the Southeast Asia zoo and USA zoo populations (40.9%; 25.6%; 41.2%). However, the doucs housed at the USA zoo harbored approximately 16 percent more of this genus than did the doucs housed at the Southeast Asia zoo. Conversely, the microbiome of the doucs housed at the EPRC was dominated by Unclassified *Clostridiales* (18.0%), and contained almost equal amounts of Unclassified *Ruminococcaceae* (12.7%) and *Akkermansia* (12.1%) (Figure 2.6a). Furthermore, the genus *Akkermansia* was much more abundant in the EPRC population (12.1%) compared to the wild, Southeast Asia zoo, or USA zoo populations (1.9%; 0.2%; 0.1%) (Figure 2.6a; Figure 2.6b). While comparing the microbiome of the wild doucs to that of the semi-captive and captive populations, a few other genera were noticeably more abundant in the wild population, including *Oscillospira*, *Dorea*, and *Blautia* (Figure 2.6). On the contrary, there were genera that showed increased abundance in the captive populations when compared to the wild and semi-captive populations. Specifically, the Southeast Asia zoo and USA zoo douc microbiomes contained more of the following genera than did the wild and EPRC populations: *Ruminococcus*, Unclassified *S24-7*, and *Treponema* (Figure 2.6a). Lastly, when examining the relative abundance of two genera regularly focused on in microbiome studies, *Bacteroides* and *Prevotella*, we saw that *Bacteroides* was far more abundant in the Southeast Asia zoo population and *Prevotella* was far more abundant in the USA zoo population compared to the other populations examined in this study. In fact, *Bacteroides* accounted for 2.4 percent of the Southeast Asia zoo

population's microbiome compared to 0.8 percent of the other three populations combined and *Prevotella* accounted for 3.0 percent of the USA zoo population's microbiome compared to 0.4 percent of the other three populations combined (Figure 2.6a; Figure 2.7).

In addition to examining relative abundances of bacterial taxa between douc groups, we calculated and compared the *Firmicutes* to *Bacteroidetes* ratio for each douc group. The *Firmicutes* to *Bacteroidetes* ratio, which has been suggested as a measure of energy harvest capacity by microbial communities (Ley et al. 2006; Turnbaugh et al. 2006; Turnbaugh et al. 2009), was higher in wild population (4.64 ± 0.94) than in the EPRC population (3.78 ± 1.14), Southeast Asia zoo population (1.94 ± 0.81), and USA zoo population (1.43 ± 0.50) (Figure 2.8). In fact, there appears to be a relationship between lifestyle and the *Firmicutes* to *Bacteroidetes* ratio in red-shanked doucs, as we see the highest ratio in wild doucs, the second highest ratio in the semi-captive doucs, the third highest ratio in captive doucs in Asia, and finally the lowest ratio in captive doucs housed in the USA (i.e., gradient-like decrease based on how natural the environment of the douc population).

Chemical analyses of douc diets

The diets of the douc populations examined in this study, including wild, semi-captive (i.e., EPRC), Southeast Asia zoo, and USA zoo, were compared to determine what factors, if any, could have contributed to the differences in microbiome composition

observed (Table 2.1). Wild doucs were observed being very specific in the plant parts they chose for ingestion. They were observed feeding on 57 different plant species from 24 different families representing 313 feeding observations. Sixty-one percent of all identified plant parts observed being ingested were collected and sent for chemical analysis. Members of the EPRC douc population were offered 55 plant species over the course of one year (Tilo Nadler, personal communication). In contrast to the high diet diversity (i.e., number of plant species) consumed by the wild and semi-captive doucs, the captive doucs were fed far fewer plant species, and thus consumed a much less diverse diet. Specifically, the Southeast Asia zoo doucs were offered approximately 15 species of browse and the USA zoo doucs were offered only one species of browse over the course of one year (Figure 2.9).

Nutritional composition of wild, semi-captive, and captive (Southeast Asia zoo and USA zoo) douc diets differed (Table 2.2). Specifically, the semi-captive and captive (Southeast Asia zoo and USA zoo) douc diets (16.52%; 16.94%) contained more crude protein than did the wild douc diet (9.46%). On the contrary, the wild and semi-captive douc diets contained much more fiber (ADF and NDF) than did the captive diet (ADF = 46.76%, NDF = 53.67%; ADF = 23.20%, NDF = 35.60%; ADF = 14.04%, NDF = 19.31%). We examined the three douc diets for differences in the amount of three macrominerals: calcium, potassium, and sodium. The diet consumed by wild doucs contained more potassium than did the diets consumed by either semi-captive or captive doucs (0.96%; 0.76%; 0.25%). Additionally, the diet of captive doucs contained the most sodium,

followed by the diets of wild and semi-captive doucs, which contained equal amounts of the mineral (0.25%; 0.01%; 0.01%). In terms of micronutrient content, the semi-captive douc diet contained far more iron and zinc than either the captive or wild diet (iron = 337.32 mg/kg, zinc = 101.59 mg/kg; iron = 41.45 mg/kg, zinc = 13.43 mg/kg; iron = 26.47 mg/kg, zinc = 19.39 mg/kg). Of the three diets examined, the wild diet contained the least amount of iron and zinc (Table 2.2). The concentration of sugar was not available for all populations. The USA zoo had the highest amount of soluble sugars (7.90%) compared to wild (2.70%) and semi-captive (2.28%) populations (Table 2.2).

2.4 Discussion

In this study, the douc was used as a model system to study the relationships between dietary composition and microbial community activity within the gastrointestinal tract. We hypothesized that specific and unique microbial subsets play a critical role in the utilization of fibrous vegetation with natural toxicants, and that captive colobine primates lack the microbiota to maintain optimal health due to inadequate nutrient intake. We examined the fecal microbiomes of four red-shanked douc populations living three distinct lifestyles (wild, semi-captive, and captive).

GI microbiome composition is shaped by host genetics and environment, among many factors (Goodrich et al. 2014; David et al. 2014). Examining four populations of the same NHP species living in four distinct environments enabled assessment of the contribution of host genetics towards shaping the microbiome. 16S rRNA sequencing results revealed

that each douc population had a unique microbiome. The major environmental differences, to which each douc population is exposed, such as climate and diet, suggest that environment plays a fundamental role in shaping gut microbiome composition in wild and captive NHPs. Of the environmental factors that contribute to the establishment and maintenance of the gut microbiota, diet is likely the most influential, as studies have shown that changes in diet are directly associated with shifts in gut microbial community structure (Xu & Knight 2015; Muegge et al. 2011; Wu et al. 2011; Gophna 2011; David et al. 2014). Examples exist of species adapting to specific dietary niches in both wild (Amato et al. 2014) and captive (Kohl et al. 2014) settings via changes in their gut microbiota.

16S rRNA sequencing results revealed that captive doucs had a marked reduction in gut bacterial diversity when compared to wild and semi-captive doucs. Reduced bacterial diversity is often viewed as a negative indicator of health (Fujimura et al. 2010). Considering that doucs often fail to thrive in captivity, this was a salient finding. Not only was a reduction in diversity detected in captive doucs, but also a gradient-like decrease in diversity related to lifestyle was observed, as the level of diversity observed in the semi-captive doucs was intermediate between wild and captive doucs. Recent studies have shown that modern humans have lost a substantial portion of their natural microbial diversity (Moeller et al. 2014; Martínez et al. 2015; Clemente et al. 2015). Clemente et al. (2015) found that the microbiome of Yanomami Amerindians, an uncontacted human tribe who live seminomadic hunter-gatherer lifestyles in the Amazon, was more diverse

than semitransculturated Guahibo Amerindians and Malawians, which in turn had more diverse microbiomes than Americans living a westernized lifestyle. Our findings suggest the existence of a strong link between lifestyle, including level of dietary diversity, and gut bacterial diversity for NHPs, similar to what has been shown for humans.

The data generated in this study agreed with previous studies regarding the high abundance of Firmicutes present in NHPs. However, the same was not true for Bacteroidetes. Firmicutes and Bacteroidetes consistently dominate the microbiome of most studied primates (Frey et al. 2006; Kisidayová et al. 2009; Ochman et al. 2010; Wu et al. 2010; Yildirim et al. 2010; Uenishi et al. 2007; Xu et al. 2015). In fact, Firmicutes and Bacteroidetes make up more than 90% of all phylogenetic types of bacteria in humans (Eckburg et al. 2005; Turnbaugh et al. 2006). We found Bacteroidetes to be in very low abundance in both the wild populations and semi-captive EPRC population. Interestingly, this same trend was not true for the captive doucs, as Bacteroidetes was the second most abundant phylum found in both captive populations. Another interesting result was the increased abundance of Tenericutes observed in wild and semi-captive (i.e., EPRC-housed) doucs compared to the captive doucs. Tenericutes are a bacterial phylum associated with degradation of plant secondary compounds in woodrats (Kohl et al. 2014). This finding might be a function of the greater diversity and proportion of plants (i.e., browse) consumed by wild and semi-captive doucs compared to captive doucs. Looking at the differences in Tenericutes abundance between the two captive populations provides further evidence for this potential explanation, as a substantial

increase in abundance of Tenericutes was seen in the Southeast Asia zoo-housed doucs compared to the USA zoo-housed doucs, and the Southeast Asia zoo-housed doucs consume much more plant material in their diet than the USA zoo-housed doucs. While acknowledging differences in lifestyle were present between populations, the differences in microbiome composition observed at the phylum level were greater than expected considering that only a single NHP species was examined. With this in mind, the high degree of difference observed between douc populations suggests that lifestyle plays a major role in the development and maintenance of microbiome composition.

Following our phylum-level analysis, we examined the *Firmicutes* to *Bacteroidetes* (F:B) ratio, as this ratio is important in humans in terms of dietary energy extraction (Ley et al. 2008; Turnbaugh et al. 2006). Ley et al. (2006) found an increased presence of *Firmicutes* with a corresponding decrease of *Bacteroidetes* correlating with an overall greater energy harvest (Ley et al. 2006). Our results showed that the F:B ratio differed by douc population. We saw a higher F:B ratio in wild and semi-captive doucs compared to captive doucs. When examining this ratio in humans, Ley et al. (2006) found that the F:B ratio was not altered by a change in diet, which was an unexpected finding. However, our findings suggest the opposite, as it appears the decrease in the F:B ratio observed in this study was clearly associated with lifestyle, notably diet, as the doucs living under the most natural conditions (i.e., wild) had the highest ratio, followed by the doucs living under semi-natural conditions (i.e., EPRC), doucs living under semi-artificial conditions (i.e., Southeast Asia zoo), and doucs living under artificial conditions (i.e., USA zoo).

The higher F:B ratio in wild and semi-captive doucs compared with doucs living under semi-artificial and artificial conditions provides further evidence that lifestyle, including diet, is a major factor in shaping the microbial communities that reside within the douc gastrointestinal tract. Wild and semi-captive diets contained substantially more natural roughage than captive diets. Naturally this equates to diets much higher in fiber fractions (ADF, NDF). Due to the scarcity of high-quality food items, wild doucs ingest very fibrous plant parts such as bark, mature leaves, flowers, seeds and unripe fruit. In the semi-captive facility, the doucs are habituated and know that they will receive leaf meals, which provides them with a balance of fiber and soluble nutrients, making the ingestion of very fibrous items such as bark unnecessary. This can partially explain the higher reported NDF values in wild doucs when compared to semi-captive doucs. This relationship between F:B ratio and diet was expected, as our results show captive populations have diets lower in fiber fractions and higher in soluble carbohydrates, notably sugars, when compared to wild or semi-wild populations. Overall, the differences in the F:B ratio observed between populations living in natural versus unnatural settings, suggests the ratio is a predictor of overall gut health, as a higher ratio is associated with a higher fermentation efficiency and increased VFA production (Amato et al. 2014; Turnbaugh et al. 2006)**,** and doucs living under artificial (i.e., captive) conditions, which had a lower ratio, often suffer from a wasting syndrome (Crissey & Pribyl 1997; Lacasse et al. 2007).

Our more in-depth taxon-level analysis revealed substantial differences between wild, semi-captive, and captive doucs. Captive doucs exhibited increased relative abundances of Unclassified *S24-7*, *Ruminococcus*, *Treponema*, *Bacteroides*, and *Prevotella*, among others. Of the two captive populations examined, the Southeast Asia zoo population had a higher relative abundance of Unclassified *Lachnospiraceae* compared to the USA zoo population. Higher levels of *Lachnospiraceae* could be contributing to the better fecundity observed in this Southeast Asian zoo population compared to douc populations in Europe and the USA (Schwarz 2001), as this family may be protective against inflammation and aids in the breakdown of cellulose required by the host to remain in an energetically favorable state (Bajaj et al. 2012).

As previously mentioned, a major focus of this study was establishing a link between diet, including nutritional composition, and the douc microbiome. The relative abundance of two bacterial genera, *Bacteroides* and *Prevotella*, were of specific interest in this regard. *Prevotella*, which is involved in the digestion of simple sugars and carbohydrates (Purushe et al. 2010), was notably higher in the captive doucs. In a study examining the microbiomes of wild vs. captive *Rhinopithecus brelichi*, a colobine primate species with similar gut physiology to that of the douc, captive individuals had increased *Prevotella* compared to their wild counterparts (Hale 2014), which is in agreement with our findings. Although not available for the Southeast Asia zoo population, the percentage of sugars in the diets of wild, EPRC-housed, and USA-housed doucs were available. A clear relationship was visible between the amount of sugars in the diet and *Prevotella*

abundance, as the diet of USA zoo-housed doucs contained more than a threefold increase in the percentage of sugars compared to the wild and semi-captive douc diets. In addition, the fact that captive doucs harbored more *Bacteroides* than semi-captive or wild doucs was of particular interest. *Bacteroides* are found in higher abundance in humans who consume diets high in protein and fats (i.e., humans living a westernized lifestyle) (Wu et al. 2011; Yatsunenko et al. 2012). Unfortunately, we were not able to compare douc diets based on the percentage of fat they contained, however the percentage of protein for all diets was known, which showed the diet of captive doucs contained more protein than wild doucs. Interestingly, we see that captivity moves doucs in the same direction along the *Bacteroides* gradient as westernization does to humans. One possible explanation is that the same factors, including diet, that are causing the shift in humans are causing the shift in doucs.

An unexpected result found as a result of our taxon-based analysis, was the high abundance of *Akkermansia* and Verrucomicrobia found in the semi-captive doucs housed at the EPRC. Verrucomicrobia, which is the phylum *Akkermansia* belongs to, was the second most abundant phylum in the microbiome of the EPRC-housed doucs. Our Shannon diversity index calculations showed that the microbiome of the Southeast Asia zoo population was more even than the EPRC population, which was unexpected based on the results of all other measurements of microbial diversity (i.e., number of OTUs and Chao1) performed in this study. The overrepresentation of *Akkermansia* in the microbiome of semi-captive doucs could have contributed to the unexpectedly decreased

evenness observed. Members of the genus *Akkermansia*, such as *Akkermansia muciniphila*, are known for their roles in mucin degradation, and have been suggested to play protective roles in the gut (Everard et al. 2013; Belzer & de Vos 2012). Everard et al. (2013) showed that obese and type 2 diabetic mice had decreased abundance of *A. muciniphila*, and treatment with this microbe reversed high-fat diet-induced metabolic disorders. In our study, while *Akkermansia* was most abundant in the semi-captive doucs by far, this genus was also more abundant in the wild doucs than in both captive populations combined. Another study examining gut microbiome composition of a colobine primate, *Rhinopithecus brelichi*, showed that *Akkermansia* was more abundant in captive individuals when compared to their wild counterparts, which is different than what was seen in doucs (Hale 2014). The extremely high abundance of *Akkermansia* in the EPRC-housed doucs could be a population-specific finding. In other words, it is possible that the doucs housed at the EPRC harbor high levels of *Akkermansia* simply due to local infection and continued vertical and horizontal transmission. However, the higher level seen in wild doucs compared to captive doucs might suggest that the microbe is tightly linked to diet, as the diets of wild and EPRC-housed doucs contain much more plant diversity compared to those of the captive doucs.

While comparing the microbiome of the wild doucs to that of the semi-captive and captive populations, a few other genera were noticeably more abundant in the wild population, including *Oscillospira*, *Dorea*, and *Blautia*. Many of our results suggest the existence of a relationship between abundance of certain bacterial genera in the gut and

dietary patterns. A notable example of this diet-microbiome relationship seen in our analysis was with the genus *Oscillospira*, which has a known association with the consumption of plant material, including leaves and grass cuticles (Clarke 1979; Mackie et al. 2003; Yanagita et al. 2003; Zoetendal et al. 2013). In our study, *Oscillospira* was markedly increased in the wild doucs compared to the other douc populations. In addition, *Oscillospira* was more abundant in the EPRC and Southeast Asia zoo populations than in the USA zoo population. The observed differences in abundance of *Oscillospira* between douc populations was likely a function of the stark differences in dietary consumption between populations, and most importantly, the difference between the diversity and proportion of browse consumed by the different douc populations examined. Wild, EPRC, and Southeast Asia zoo populations all consume diets that contain a higher proportion and diversity of browse compared to the USA zoo population. In addition, wild, as well as EPRC and Southeast Asia zoo-housed doucs consume tropical browse, while the USA zoo-housed doucs consume temperate browse. While a high variety of plant species is bound to impact the gut microflora, the sheer differences in proportion of the diet which is browse is likely to be equally as much of a causative factor (de Menezes et al. 2011). Besides the proportion and diversity of browse species offered, there is a difference in the fiber contents of temperate versus tropical browse, which could also be contributing to the observed differences (Nijboer & Dierenfeld 1996). Previous studies examining the difference between ruminants reared on pasture versus grain diets showed that *Oscillospira* was more abundant in ruminants on pasture diets, which is the more natural diet (Mackie et al. 2003). Also, in an investigation of

wild vs. captive *Rhinopithecus brelichi*, wild individuals had increased *Oscillospira* compared to their captive counterparts (Hale 2014). Diets high in soluble carbohydrates contribute to a spike in VFA production, which lowers the luminal pH within the rumen and hindgut of ruminants (Gressley et al. 2011). If this dip in pH is constant, the gut community will shift to this environmental change and species such as *Oscillospira* will become less predominant (Zoetendal et al. 2013). This change in microbial community structure affects what nutrients are predominantly absorbed and the efficiency with which these are assimilated (Khafipour et al. 2009). One very different component in the diets of wild versus captive doucs is the inclusion of produce in the captive diets. Cultivated fruits are not at all similar to wild fruits (Oftedal 1997). They have been grown for human consumption to be lower in fiber and protein and higher in sugar as opposed to wild fruits which are, in general, the exact opposite (Schwitzer and Kaumanns 2003). Even though 17.8% of wild feeding observations were fruit, these observations were all unripe fruit. The consumption of unripe fruit by wild doucs is drastically different than that of ripe fruit fed to captive individuals, and thus its impact on the douc microbiome is different than cultivated fruits would be. This difference suggests that captive doucs should not receive any ripe fruit in their diets and that vegetables should be limited. There have been several studies linking fruit as a causal factor in langur wasting disease (Crissey & Pribyl 1997; Lacasse et al. 2007). Sugar content is higher in captive diets than in the wild, which will impact gut microbiome composition. This cause-and-effect relationship has also been recorded within ruminants and has also been supported by our results using a foregut-fermenting primate as a model species.

Captive breeding programs have historically been challenged to identify the optimal approaches by which to support primate species with an appropriate diet and environment. Digestive-associated morbidity and mortality remains a major hurdle for the successful management of red-shanked doucs and other colobines in captivity. Comparing the microbiomes of red-shanked doucs living under four very different conditions allowed us to examine how environmental factors, especially diet, influences microbiome composition in this folivorous primate, and, ultimately, how microbiome composition contributes to health status. Our results suggest the existence of a clear relationship between abundance of bacterial genera in the gut and dietary patterns. The fact that several genera, including *Oscillospira* and *Dorea* were highly abundant in wild doucs, yet not very abundant in the other populations, and other genera, such as *Clostridium* and *Blautia*, were highly abundant in douc populations living under natural (wild) and semi-natural (EPRC) settings, while not very abundant in douc populations living under semi-artificial (Southeast Asia zoo) and artificial (USA zoo) settings, indicates that these genera could be markers of better gut health, and consequently overall health, in these populations. Another likely contributor to diet-driven microbiome variation in this study is the difference in high fibrous food amounts in the diet between lifestyles, as we observed a trend towards high fiber diets and more stable microbiomes for colobine primates. These results suggest that measuring gut microbiome composition, including presence of wild-associated genera such as *Oscillospira*, *Dorea*, and *Blautia* and captivity-associated genera such as *Bacteroides* and *Prevotella*, could be a promising

new tool for assessing GI health in primates. Considering the critical role the gut microbiota play in the digestive process and in the overall maintenance of primate health, a better understanding of critical host-microbe interactions could inform conservation efforts for delicate and endangered primate species.

Table 2.1 Components of the douc diets in all four populations. Table is depicting the higher browse content given in Asian populations as opposed to American.

Dietary Groups	**Proportion of Diet (%)**			
	Wild	EPRC	Southeast Asia zoo	USA zoo
Leaves*	65.50	100.00	66.10	1.40
Flowers	5.30	0.00	0.00	0.00
Seeds	2.00	0.00	0.00	0.00
Fruit	17.80	0.00	6.60	0.00
Other plant parts**	9.40	0.00	0.00	0.00
Pellets	0.00	0.00	0.90	7.30
Vegetables	0.00	0.00	26.40	90.90
Cereals	0.00	0.00	0.00	0.40

*** Values for leaves may also include petioles or stems.**

****Other plant parts include pith, bark and leaf buds.**

Table 2.2 Nutrient content on a dry matter basis for each population. The following nutrients were measured: CP: crude protein (%), CF: crude fiber (%), SS: soluble sugars (%), ADF: acid detergent fiber (%), NDF: neutral detergent fiber (%), Ca: calcium (%), K: potassium (%), Na: sodium (%), Zn: Zinc (mg/kg) and Fe: iron (mg/kg).

Diet	C.P.	C.F.	S.S.	ADF	NDF	Ca	K	Na	Zn	Fe
Wild	9.46	22.28	2.70	46.76*	53.67*	0.49	0.96	0.01	19.39	26.50
EPRC	16.52	15.71	2.28	23.20**	35.60**	1.05	0.76	0.01	101.59	337.32
Southeast Asia zoo*	13.37	-	-	23.07	31.97	0.22	0.21	0.24	8.25	20.74
USA zoo	16.52	16.57	7.90	8.65	12.64	0.72	0.29	0.27	26.30	64.33

Diet also included a vitamin and mineral supplement, which was not included in the analysis.

NDF and ADF were not available from the laboratory analyses, therefore values for * were taken from Ulibarri (2013) and ** were taken from Otto (2005) in order for a comparison to be possible.

Data from Ulibarri (2013) was weighted to represent the proportion of plants part selected.

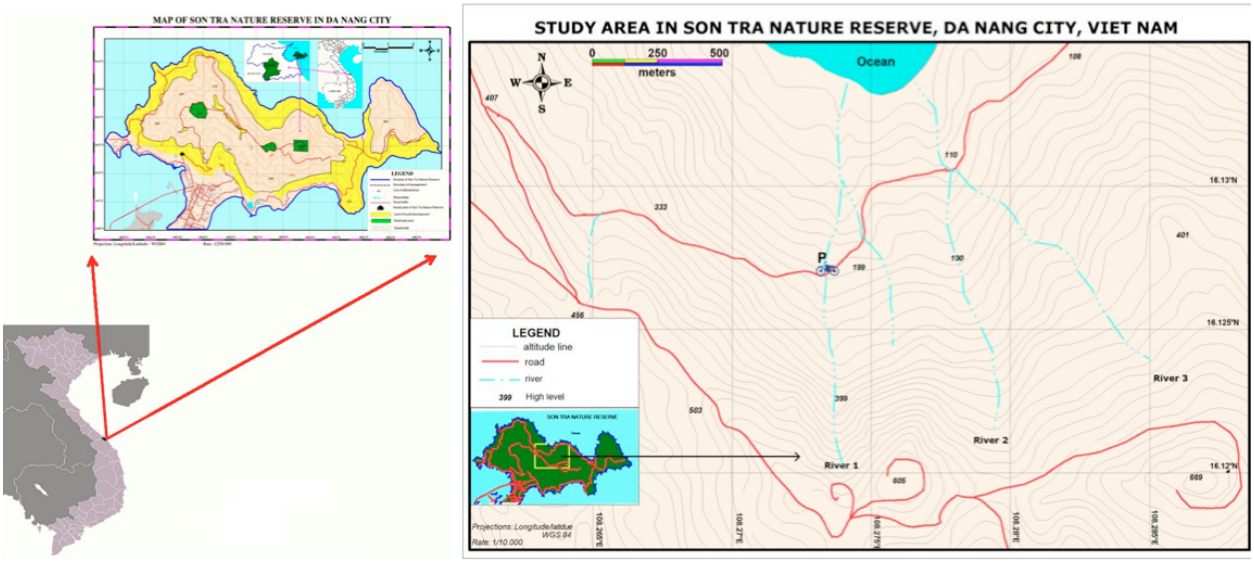

Figure 2.1 Study site. Red-shanked doucs (*Pygathrix nemaeus*) inhabiting Son Tra Nature Reserve, Da Nang, Vietnam (16°06'—16°09'N, 108°13'—108°21'E) served as the wild population for this comparative study (Lippold & Thanh 2008). Son Tra is located only 10 km from the heart of Da Nang City, which is the third largest city in Vietnam. The nature reserve is comprised of 4,439 total ha and of those 4,190 ha is covered by both primary and secondary forests (Lippold & Thanh 2008). Our study area was approximately 600 ha and is located on the north central region of the peninsula.

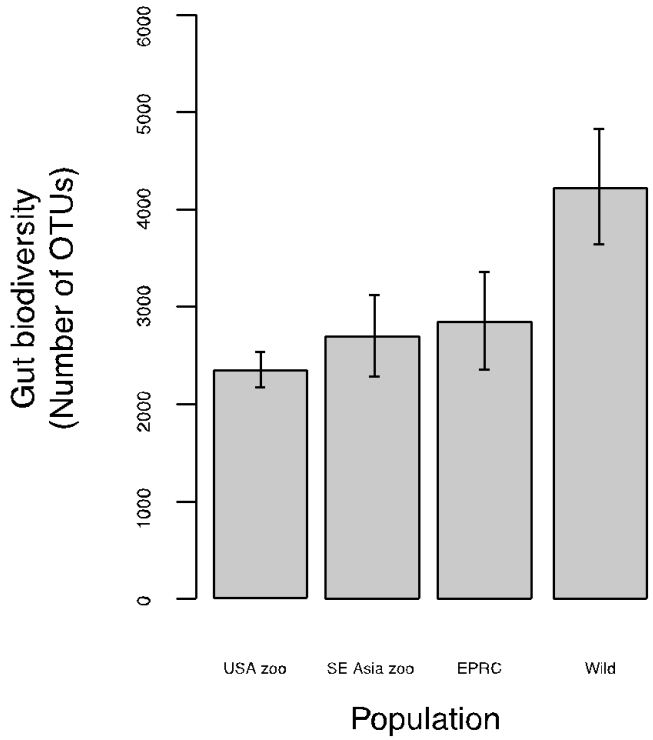

Figure 2.2 Diminished diversity in red-shanked douc microbiomes across populations. Numbers of OTUs per fecal sample in red-shanked douc populations. Bar plots of mean and spread of gut microbial biodiversity, as measured by the number of species-like operational taxonomic units (OTUs) in red-shanked douc gut microbiomes across populations using the total number of observed OTUs. These indicate a significant loss of biodiversity from the wild population to the semi-captive (EPRC) population, and again from semi-captive to captive in Southeast Asia, and again from captive in Southeast Asia to captive in the USA. Error bars correspond to standard deviation.

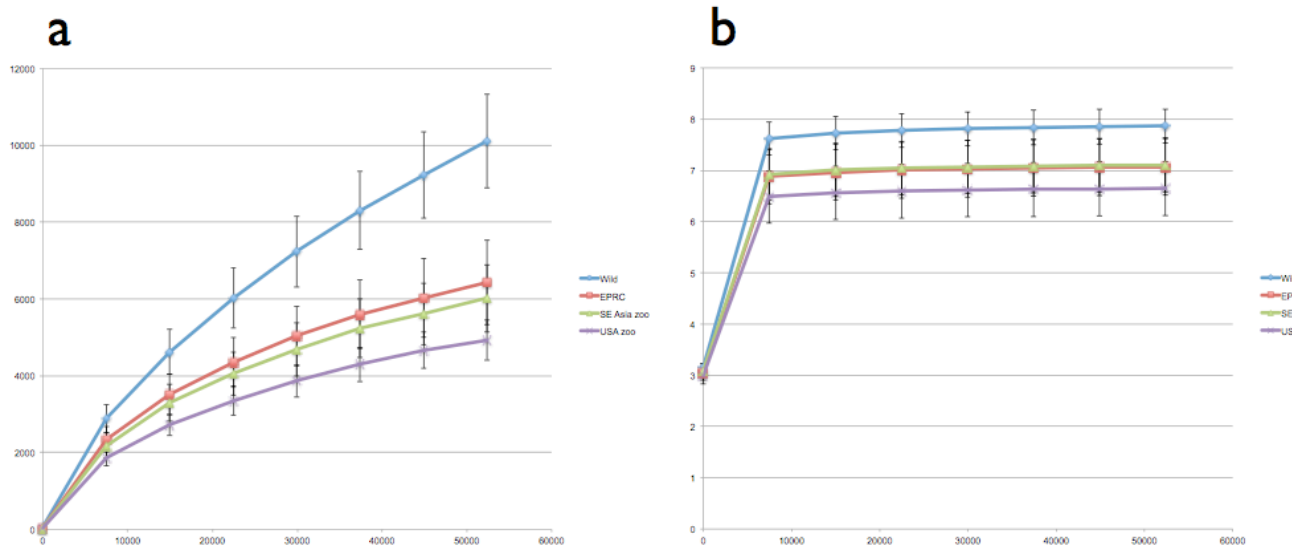

Figure 2.3 Rarefaction curves for different red-shanked douc groups. a) Chao-1 index and b) Shannon diversity. Blue (diamond): wild; red (square): EPRC; green (triangle): SE Asia zoo; purple (x): USA zoo.

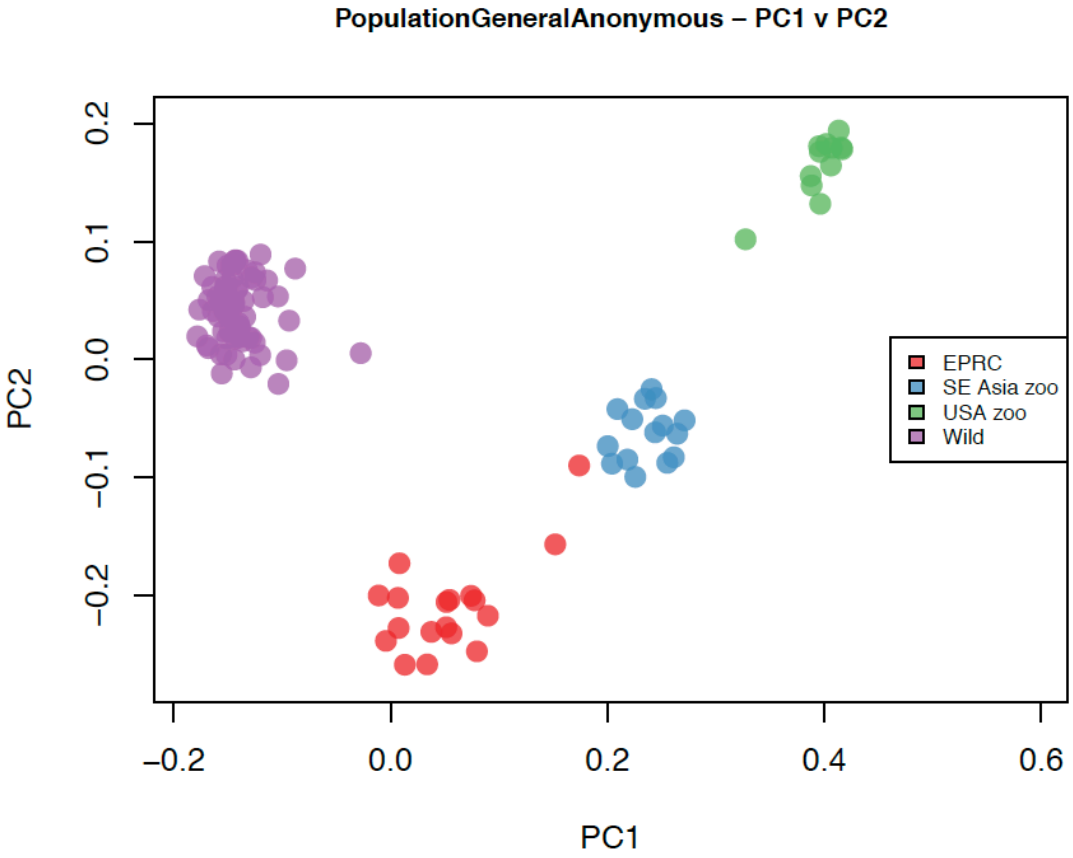

Figure 2.4 Principal coordinates analysis plot of genus-level unifrac distances (unweighted) among red-shanked douc microbiomes plotted by population. Microbiomes included are based on V4 16S sequences (R1 only). Colored dots correspond to the microbiomes of red-shanked douc populations. Red-shanked douc populations are represented as EPRC (Endangered Primate Rescue Center), Southeast Asia zoo, USA zoo, and wild. One captive population was located in a zoo in the USA while another was located in a zoo in Southeast Asia. Doucs housed at the EPRC served as the semi-captive population. Doucs inhabiting Son Tra Nature Reserve in Da Nang, Vietnam served as the wild population.

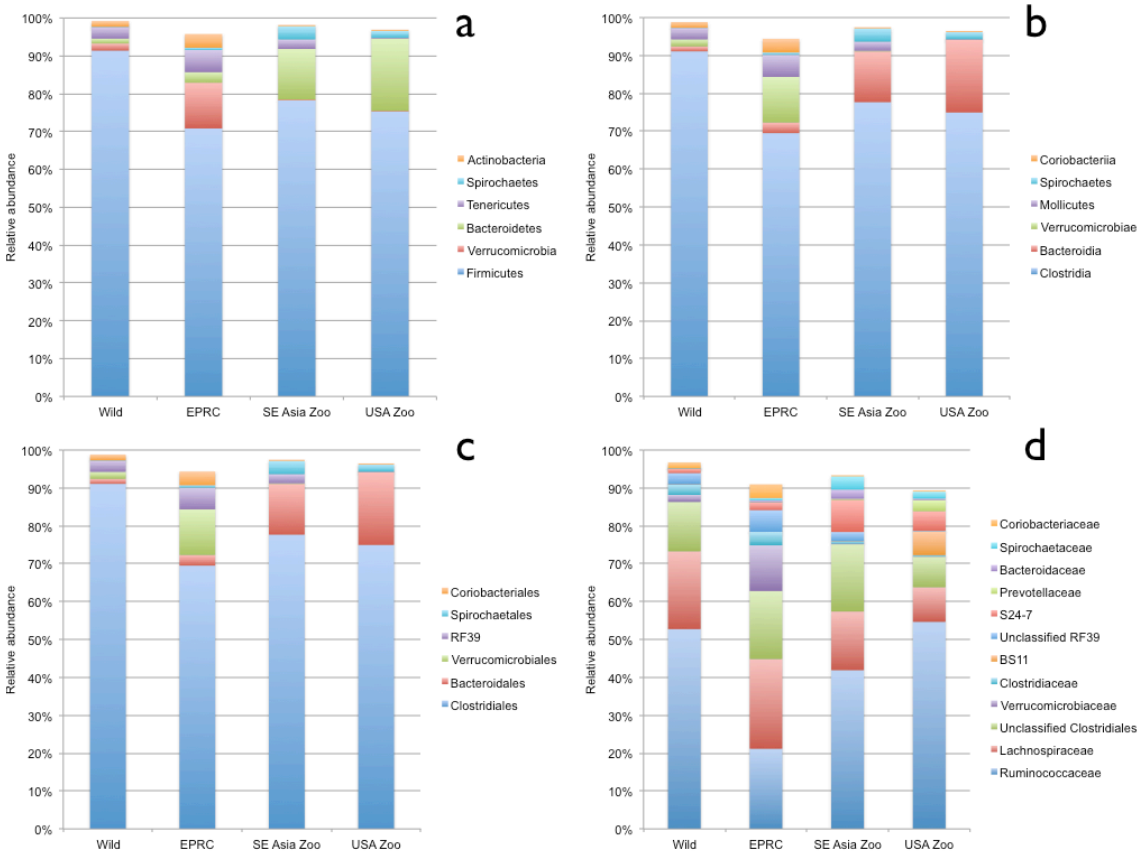

Figure 2.5 Taxa summary output by red-shanked douc population (Phylum to Family). a) Phylum, b) Class, c) Order, and d) Family. The remaining white space in the graph represents unclassified reads and low-abundance phyla, classes, orders, and families.

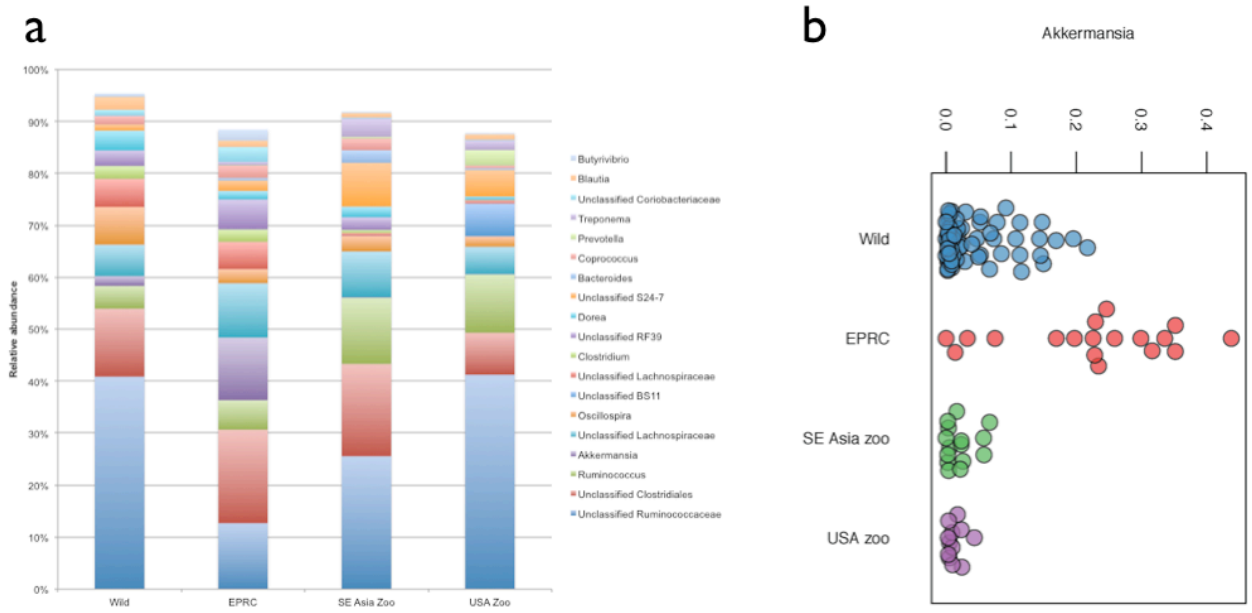

Figure 2.6 Differences in abundance of taxa at genus-level. Taxa summary output by red-shanked douc population (a). The remaining white space in the taxa summary graph represents unclassified reads and low-abundance genera. A beeswarm plot of the arc-sin square-root relative abundance of bacterial genus *Akkermansia*, which is a dominant member of the microbiomes of EPRC-housed doucs (b).

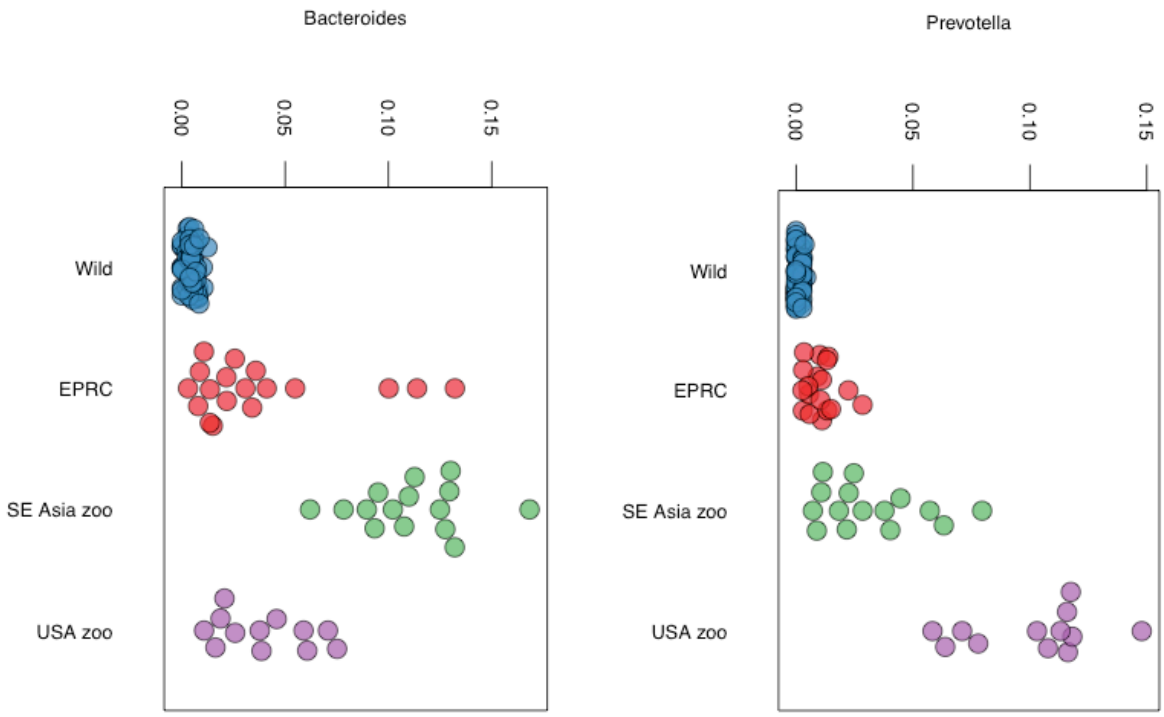

Figure 2.7 Differences in abundance of *Bacteroides* and *Prevotella*. A beeswarm plot of the arc-sin square-root relative abundance of bacterial genera *Bacteroides* and *Prevotella*, the two dominant modern human gut microbiome genera, shown in each red-shanked douc population. As populations move away from the wild state toward the captive state their microbiomes acquire these dominant human gut bacterial genera.

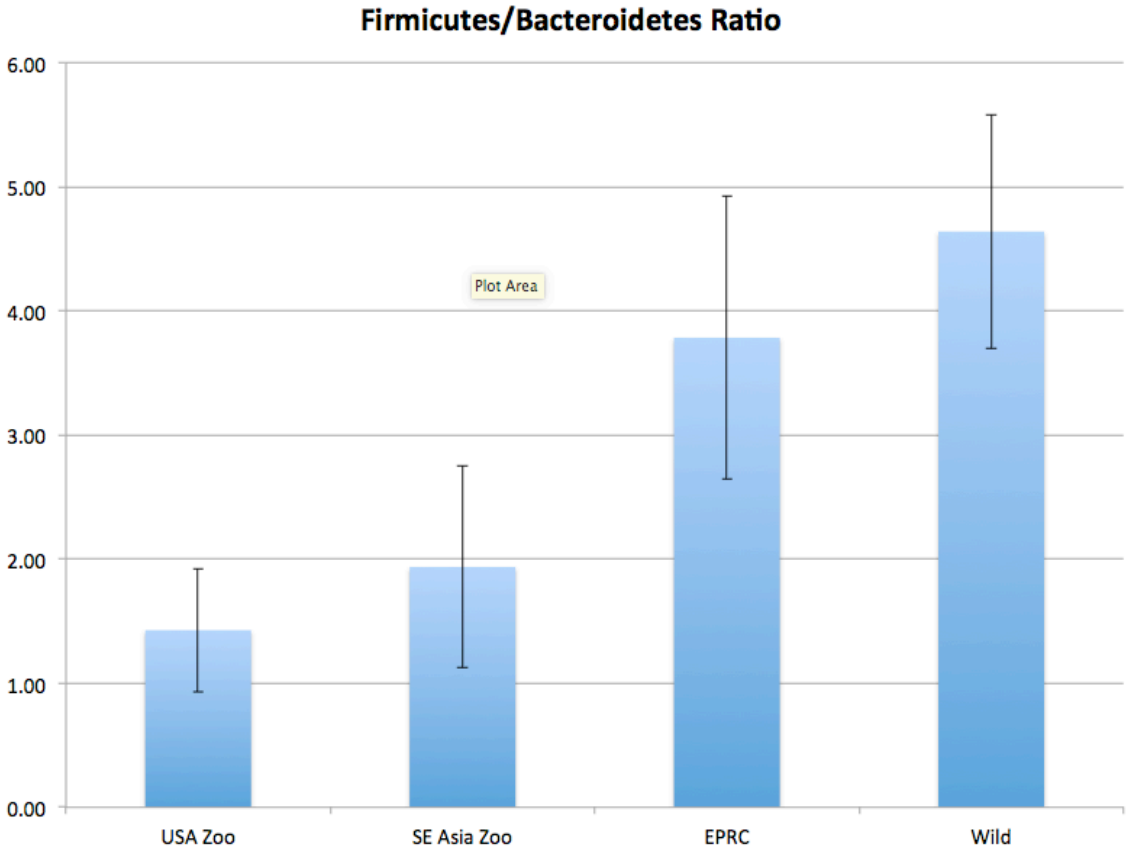

Figure 2.8 Firmicutes-to-Bacteroidetes ratio plotted by red-shanked douc population.

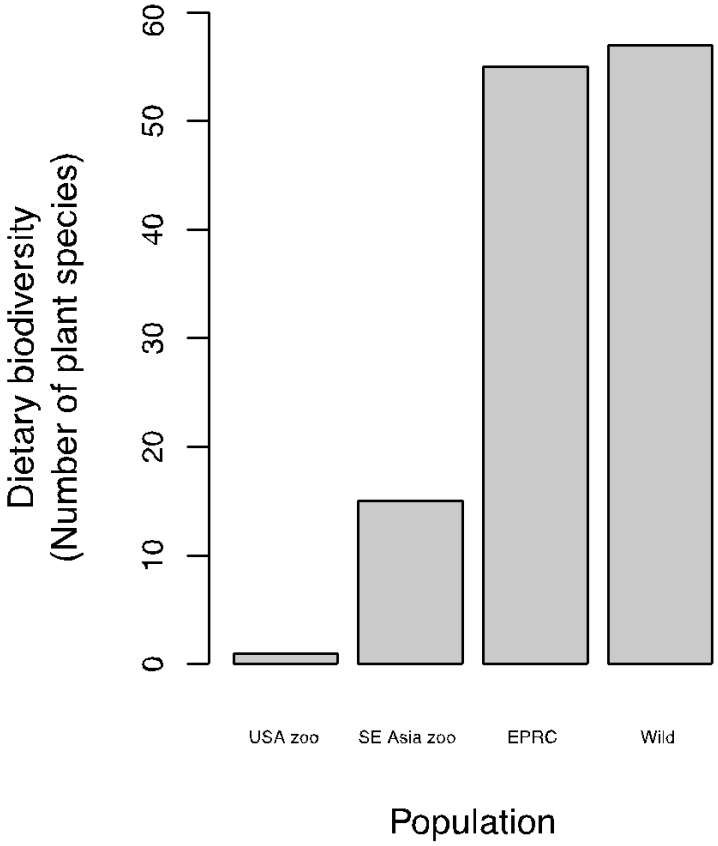

Figure 2.9. Diet diversity (number of plant species) consumed by each red-shanked douc population. Wild and EPRC-housed doucs consume many more plant species than does either captive douc population. Red-shanked doucs housed at the USA zoo consume the least number of plant species by far (n = 1).

Chapter 3 Captive primate dysbiosis converges toward the modern human microbiome

Summary

The primate gastrointestinal (GI) tract is home to trillions of bacteria that play major roles in metabolism, immune system development, and pathogen resistance. Microbiome composition is associated with metabolic and autoimmune human diseases such as obesity, diabetes, Crohn's disease, and ulcerative colitis, all of which have been on the rise globally in recent decades. Although there is increasing evidence that modern society and Westernized society in particular are associated with a dramatic loss of natural human gut microbiome diversity, the causes and consequences of this loss are challenging to study. Here we use nonhuman primates (NHP) as a model system for studying the effects of Westernization on the human gut microbiome. Using 16S rRNA gene sequencing in two model NHP species we show that although different primate species have distinctive signature microbiota in the wild, in captivity they lose their native microbial diversity and become infected with *Prevotella* and *Bacteroides*, the dominant genera in the modern human gut microbiome. We confirm that captive individuals from eight other NHP species in a different zoo show the same pattern of convergence, and that semi-captive primates housed in a sanctuary represent an intermediate microbiome state between wild and captive. Finally, in a meta-analysis including published human data we show that captivity causes the NHP gut microbiome to shift in the same direction that the human microbiome shifts from developing nations to the USA. These results demonstrate that captive NHPs may serve as a useful model

system for studying the effects of Westernization on the human gut microbiome, and that captivity causes the primate microbiome to lose diversity and converge along an axis of dysbiosis toward the modern human microbiome.

3.1 Introduction

Dysbiosis, which is a state of microbial imbalance, is often seen in human patients with inflammatory conditions affecting the intestines, as well as colorectal cancer (Yang & Jobin 2014). In a review article written by Petersen and Round (2014), three categories of dysbiosis are described, including loss of beneficial microbial organisms, expansion of pathobionts or potentially harmful microorganisms, and loss of overall microbial diversity. As noted by these authors, it is important to note that these three types of dysbiosis may all occur simultaneously, and are therefore not mutually exclusive (Petersen & Round 2014).

Lifestyle has a major impact on the pathophysiology of many diseases (Hold 2013). In addition, there is mounting evidence that an intimate interplay exists between the gut microbiota and the development of a number of diseases, including obesity (Turnbaugh et al. 2006; Turnbaugh et al. 2009; Turnbaugh et al. 2008), Crohn's (Knights et al. 2013; Gevers et al. 2014), diabetes (Giongo et al. 2011; Brown et al. 2011; Boerner & Sarvetnick 2011), non-alcoholic fatty liver (Henao-Mejia et al. 2012), kwashiorkor (Smith et al. 2013), atherosclerosis (Koeth et al. 2013), autism (Mulle et al. 2013), and Alzheimer's (Bhattacharjee & Lukiw 2013), among others. Thus, understanding how lifestyle impacts the development and maintenance of the gut microbiome is of the utmost importance. A few specific factors related to lifestyle have been linked to the development of dysbiosis, including antibiotic usage, stress, and diet (Myers 2004). Of these, dietary habits have been suggested to play one of the strongest roles in shaping the

gut microbiome (Myers 2004; Wu et al. 2011; Hildebrandt et al. 2009; Muegge et al. 2011). Furthermore, previous studies examining the relationship between dietary patterns and dysbiosis suggest a strong association between Western lifestyle and dysbiosis exists (Hold 2013; Myers 2004; Martinez-Medina et al. 2014). It is important to note that in westernized countries, the number one cause of morbidity and mortality are diet-related chronic diseases (Hold 2013; Cordain et al. 2005). Strikingly, greater than 50% of the adult population is affected by these diet-related diseases (Hold 2013; Cordain et al. 2005). The cause of dysbiosis in westernized countries is thought to be due mainly to diet, as the Western diet is evolutionarily discordant from the diet of ancestral humans (Hold 2013; Cordain et al. 2005). The Western diet is high in fat and animal protein (e.g., red meat), high in sugar, and low in plant-based fiber (Hold 2013; Myers 2004; Martinez-Medina et al. 2014). Consequences of living in a state of evolutionary discordance include development of disease, increased morbidity and mortality, and a reduction in reproductive success (Hold 2013; Cordain et al. 2005).

Nonhuman primates (NHPs) are the most biologically relevant research animal models for humans as our species is embedded in the extant primate radiation (Chen et al. 2012b; Stone et al. 1987; Carlsson et al. 2004). Compared to other animal models used to study human diseases, NHPs are unparalleled in terms of their relevance, both genetically and physiologically (Chen et al. 2012b; Stone et al. 1987; Carlsson et al. 2004). Rhesus macaques have been used to investigate how shifts in the microbiome relate to health status in order to serve as a model for what happens in human subjects (McKenna et al.

2008). In a comprehensive study conducted by McKenna et al. (2008), the gut microbiome was compared between captive healthy macaques, macaques with lentiviral infection (i.e., SIV), and macaques with chronic enterocolitis in an attempt to understand the relationship between host-microbiome interactions and the development of gastrointestinal disease.

In this study, we sought to explore the relationship between lifestyle and the development of gut microbial dysbiosis in NHPs. We examined the microbial communities of two NHP species, the red-shanked douc (*Pygathrix nemaeus*) and the mantled howling monkey (*Alouatta palliata*), both living under captive and wild conditions, an additional captive NHP population represented by eight different species, as well as previously published human populations living in the USA (i.e., western) and outside the USA (i.e., non-western, including Venezuela and Malawi). Both red-shanked doucs and mantled howling monkeys are folivorous NHPs, thus they consume a diet, which is nutritionally poor and difficult to digest when compared to diets consumed by non-folivores. These species are rarely housed in captivity, due in part to the challenge of replicating their wild diets. Our ability to sample from captive, semi-captive, and wild populations from the same species gave us a unique opportunity to study the relationship between lifestyle and disturbance of the native gut microbiota in primates.

3.2 Materials and Methods

Study subjects and sample material

Fecal samples (n = 297) were collected opportunistically immediately after defecation from two wild, one semi-captive, and three captive NHP populations located around the globe between 2009-2013. Ten different NHP species were examined in this study, including the Red-shanked douc (*Pygathrix nemaeus*), Mantled howling monkey (*Alouatta palliata*), Western lowland gorilla (*Gorilla gorilla gorilla*), Sumatran orangutan (*Pongo abelii*), De Brazza's monkey (*Cercopithecus neglectus*), Black-handed spider monkey (*Ateles geoffroyi*), White-faced saki (*Pithecia pithecia*), Blue-eyed black lemur (*Eulemur flavifrons*), Emperor tamarin (*Saguinus imperator*), and Geoffroy's tamarin (*Saguinus geoffroyi*). Of these, the red-shanked douc and mantled howling monkey represented the wild NHP species sampled, and the red-shanked douc represented the sole semi-captive NHP species sampled. Captive populations of both red-shanked doucs and mantled howling monkeys were also sampled. The remaining eight NHP species sampled consisted of all captive individuals housed at the Como Zoo in Saint Paul, MN (Table 3.1).

Genomic DNA extraction

Total DNA representing the fecal microbial communities was extracted from each fecal sample using the established method of Yu and Morrison (2004) with some modifications (Yu & Morrison 2004). The DNA extraction method employed two rounds of bead-beating in the presence of NaCl and sodium dodecyl sulfate, followed by sequential ammonium acetate and isopropanol precipitations. The precipitated nucleic acids were then treated with DNase-free RNase (Roche) and the DNA purified using the QIAmp®

DNA Stool Mini Kit (QIAGEN, Valencia, CA), according to manufacturer's recommendations with some modifications. DNA quantity was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, USA).

Bacterial 16S rRNA PCR amplification and Illumina MiSeq sequencing

The bacterial 16S rRNA gene was analyzed using primers 515F and 806R, which flanked the V4 hypervariable region of bacterial 16S rRNAs (Caporaso et al. 2012). The oligonucleotide primers included Illumina sequencing adapters at the 5' ends and template specific sequences at the 3' ends. The primer sequences were: 515F (forward) 5' GTGCCAGCMGCCGCGGTAA 3' and 806R (reverse) 5' GGACTACHVGGGTWTCTAAT 3' (Caporaso et al. 2012). The 16S rRNA amplification protocol from the earth microbiome project was adopted to perform the PCR amplification (Gilbert et al. 2010). Samples were amplified in duplicate and then pooled. Thus, each sample was amplified in 2 replicate 25 µL PCR reactions and then the PCR reactions for each sample were combined into a single volume resulting in 50 µL of amplicon for each sample. The amplification mix contained 13.0 µL of PCR grade water (MoBio, Carlsbad, CA), 10.0 µL of 5 PRIME HotMasterMix (5 PRIME, Gaithersburg, MD), 0.5 µL of each fusion primer, and 1.0 µL of template DNA in a reaction volume of 25.0 µl. PCR conditions were an initial denaturation at 94°C for 3 minutes; 35 cycles of 94°C 45 seconds, 50°C for 60 seconds, and 72°C for 90 seconds; and a final 10 minutes extension at 72°C. Following PCR, concentration of PCR products was determined by a PicoGreen assay. Next, samples were normalized and pooled, and size selection was

performed using the Caliper XT (cut at 386 bp +/- 15%). Final quantification was performed via a PicoGreen assay and assessment on a Bioanalyzer 2100 (Agilent, Palo Alto, California) using an Agilent High Sensitivity chip (Agilent). The PCR amplicons were sequenced at the University of Minnesota Genomics Center (UMGC) via Illumina MiSeq to yield 2x300 base paired-end reads (Illumina, San Diego, California).

Data analysis

Raw sequences were analyzed with QIIME 1.8.0 pipeline (Caporaso et al. 2010). The demultiplexed sequences from the UMGC encountered the following quality filter: 150 bp < length < 1,000 bp; average quality score > 25. Preprocessed sequences were then clustered at 97% nucleotide sequence similarity level. Two different OTU picking protocols were used to assign the operational taxonomic unit IDs (OTUs): Closed reference-based and open reference-based OTU picking pipelines, both of which used Greengenes 13_8 (DeSantis et al. 2006) as the reference database. The qiime scripts, parallel_pick_otus_usearch61_ref.py and pick_open_reference_otus.py, were used for each pipeline respectively, using USEARCH algorithm (Edgar 2010). Unmatched reads against the reference database were excluded from the downstream analysis. Taxonomy information was then assigned to each sequence cluster using RDP classifier 2.2 (Wang et al. 2007). The qiime script, filter_samples_from_otu_table.py, was employed to remove the OTUs of chloroplast origin from the OTU table, which fall under the phylum of *Cyanobacteria*. All the sequences were rarefied to 14,100 reads per sample for the downstream analysis. Alpha-diversity analysis, beta-diversity analysis, weighted and

unweighted UniFrac distance matrix (Lozupone & Knight 2005), and PCoA (principal coordinate analysis) plots were obtained through the wrapper scripts in QIIME and custom written R scripts. Published data used in our meta-analysis (Yatsunenko et al. 2012; Rollo et al. 2006; Tito et al. 2012; Muegge et al. 2011) were analyzed similarly.

3.3 Results

Lifestyle influences gut microbiome composition of multiple NHP taxa

Amplicon sequencing of the V4 region of the 16S rRNA gene was performed on numerous fecal samples collected from captive, semi-captive, and wild red-shanked doucs (n = 111), captive and wild mantled howling monkeys (n = 56), as well as from a captive NHP population, comprised of eight species, housed at a single zoological institution (n = 130). Microbiome composition was examined by lifestyle. Nonhuman primates (NHPs) included in this study were grouped by lifestyle, which resulted in three groups, including captive, semi-captive, and wild. Red-shanked doucs housed at a primate sanctuary in Vietnam represented the sole semi-captive population included in this study.

We calculated beta diversity, a measure of the ecological distance between each pair of microbiome samples. We examined microbiome compositions of six NHP populations to determine if significant differences between lifestyles were present. We focused on unweighted unifrac distances (i.e., unweighted PCoA plots), as this analysis has been effective for distinguishing highly divergent microbial ecosystems in past analyses

(Yatsunenko et al. 2012; Stahringer et al. 2012). Analysis of similarity (ANOSIM) revealed that NHP gut microbial communities grouped by lifestyle (ANOSIM R = 0.69; p = 0.001). This analysis confirmed previous findings that different lifestyles, including geography, diet, and other environmental exposures, have a profound effect on the composition of the microbiome (Figure 3.1).

Wild NHPs have distinctive and predictable signatures of native bacterial taxa

Based on our analysis of population-level microbiome variation, we hypothesized that the two wild NHP species would each have a signature fingerprint of native gut microbiota distinct from the other species, and that they would lose these taxa in captivity, causing distinct and predictable gut microbiome signatures in the different lifestyle groups. Using the random forests classifier we determined the most discriminative genus-level taxa between wild, semi-captive, and captive NHPs. We assessed the accuracy of the classifier using 10-fold cross-validation. In other words, we trained the classifier on 90% of the samples, used the discovered signatures to predict which populations the remaining 10% of samples belonged, and then repeated the process 10 times. This machine learning-based analysis revealed that individual primate populations have such distinct signature microbiomes that they can be identified from their microbiota with 99.6% accuracy (Figure 3.2). From this analysis we identified the bacterial genera most important for distinguishing the populations (random forests feature importance score ≥ 0.001). We found that wild NHPs possessed higher relative abundances of a variety of microbes, including *Collinsella*, *Tannerella*, Unclassified *Methanobacteriaceae*, *Marvinbryantia*,

etc., while captive NHPs possessed higher relative abundances of *Bacteroides*, *Prevotella*, *Parabacteroides*, *Ruminococcus*, *Clostridium*, *Mogibacterium*, *RF16*, Unclassified *Prevotellaceae*, Unclassified *Rikenellaceae*, *SMB53*, *Epulopiscium*, Unclassified *Peptostreptococcaceae*, Unclassified *Cerasicoccaceae*, *p-75-a5*, and *Helicobacter* (Figure 3.2). Compared to wild and captive NHPs, our population of semi-captive NHPs possessed higher relative abundances of a variety of microbes, including *Akkermansia*, Unclassified *Mogibacteriaceae*, Unclassified *Coriobacteriaceae*, *Atopobium*, *Pseudoramibacter*, and *Gyrocarpus* (Figure 3.2).

The two wild primate populations had large blocks of highly distinctive native microbiota (Figure 3.2). Wild doucs possessed higher relative abundances of a variety of microbes, including *Oscillospira*, *Dorea*, Unclassified *Lachnospiraceae*, *Atopobium*, *Pseudoramibacter*, *Adlercreutzia*, *Butyrivibrio*, *Dehalobacterium*, *rc4-4*, Unclassified *Peptococcaceae*, Unclassified *Mogibacteriaceae* and Unclassified *Christensenellaceae*, and lower relative abundances of *Collinsella*, *Dialister*, *Tannerella*, Unclassified *Methanobacteriaceae*, *Marvinbryantia* and *Methanosphaera*, when compared to wild howlers. This analysis allowed us to identify signature bacterial genetic diversity for both doucs and howlers. This analysis allowed us to identify signature bacterial genetic diversity for both doucs and howlers (Figure 3.3).

Captive primate dysbiosis driven by loss of diversity

To investigate gut microbial diversity including previously unknown taxa we performed open-reference OTU picking. The number of OTUs found in samples was used as the primary measure of bacterial diversity. Next, we calculated how many OTUs were associated with each lifestyle (captive, semi-captive, and wild) and tested for significant differences between lifestyles. Gut microbial diversity showed a steady decline the more unnatural the environment the NHPs were found. In other words, the number of OTUs in the NHPs decreased by lifestyle with the highest number seen in the NHPs living under the most natural conditions (i.e., wild), and the lowest number of OTUs seen in the NHPs living under the most unnatural conditions (i.e., captive). After dropping singleton OTUs present in only one sample, the wild NHPs (2544.3 ± 390.9 OTUs) harbored the highest number of OTUs (i.e., greatest diversity), followed by the semi-captive NHPs (2141.4 ± 293.0 OTUs), and captive NHPs (1967.9 ± 538.2 OTUs) (Figure 3.4) By this metric, wild NHPs had significantly higher diversity than captive or semi-captive (t-test $p = 2.1\times10^{-21}$, 1.9×10^{-5}, respectively), and captive and higher diversity than semi-captive (t-test $p = 0.040$). We repeated this analysis with the Chao1 estimator of the true number of OTUs (i.e., species richness) in our samples. Using the Chao1 estimator differences were significant between all three populations (t-test $p = 4.6\times10^{-39}$, 7.3×10^{-8}, 0.0099 for wild vs. captive, wild vs. semi-captive, and captive vs. semi-captive, respectively) (Figure 3.5).

Captivity in primates partially parallels westernization in humans

Due to the use of standardized methodologies across studies (i.e., sequencing of the V4 region), we were able to conduct a meta-analysis combining nonhuman primate populations with previously published samples from adult humans living both Western and non-Western lifestyles. These included humans living in urban environments in the United States (n = 129), in rural environments in Malawi (n = 21), and in underdeveloped environments in the Amazon rainforests of Venezuela (n = 34) (Yatsunenko et al. 2012; Rollo et al. 2006; Tito et al. 2012). For this comparative analysis, we calculated the unweighted UniFrac distance between all samples, a beta diversity measure used to compare samples based on phylogenetic information. The axis of convergence seen in Figure 3.1 continues toward non-westernized humans and finally westernized humans, suggesting that a similar loss of signature biodiversity seen in captive NHPs has taken place as humans adapted to modern society. Following this analysis, we plotted the same samples included in Figure 3.1 by dietary preference (herbivore vs. omnivore). This analysis showed clear clustering patterns associated with diet, as herbivores and omnivores formed separate clusters in the plot (Figure 3.6).

To better characterize the similarities between captive primates and westernized humans, we calculated relative abundance transformed with arcsine-square root, which is a popular transformation in microbial ecology that is used to make species distributions more normal. Higher relative abundances of both *Bacteroides* and *Prevotella*, the two dominant human gut microbiome genera, were seen in captive NHPs, compared to wild and semi-captive NHPs (Figure 3.7). In addition, a higher relative abundance of

Bacteroides was seen in westernized humans compared to non-westernized humans (Figure 3.7). The high relative abundance of *Bacteroides* seen in both captive NHPs and westernized humans is suggestive that captivity moves NHPs in the same direction along the bacteroides gradient as westernization does to humans.

3.4 Discussion

A major challenge in performing the meta-analyses necessary for both identifying key differences, if present, in microbiome composition between NHP species and for identifying key differences, if present, in microbiome composition between NHPs living different lifestyles is the presence of batch effects when combining microbiome datasets processed with different methods. To extract such meaningful patterns from primate microbiome data requires joint analysis of multiple wild populations of species with varied gut physiologies and occupying different dietary niches. Previous work has characterized gut microbiome variation in a number of specific groups of primate species, such as chimpanzees, African apes, and baboons (Moeller et al. 2014; Tung et al. 2007). However, these published data are generated using varied approaches to sample storage, DNA extraction, amplification, and DNA sequencing, impeding efforts toward large-scale meta-analyses. Quantitation of microbiome data requires application of consistent standardized methods to avoid batch effects. In this study, all NHP fecal samples were obtained by our group using the same protocols, were processed using comparable methods, and were sequenced at the same sequencing facility using the same method

(i.e., EMP method; V4 region) which resulted in wild, semi-captive, and captive NHP microbiome samples that were amenable to meta-analysis.

In this study, we used high-throughput DNA sequencing to examine the impact of lifestyle changes on the gut microbiota of various NHP taxa. We quantified taxonomic diversity of the gut microbiota of ten different primate species, two of which were represented by both captive and wild populations, and used state-of-the art computational methods to identify keystone microbial species that serve as biomarkers for different NHP species according to NHP phylogeny and population. We then identified in two species, doucs and howlers, what portions of their respective keystone microbial genetic diversities were lost in captivity. Next, we included an additional captive NHP population, represented by eight species, in our analysis, which allowed us to further examine how captive rearing impacts the gut microbiota. Finally, we conducted a meta-analysis using pre-existing human microbiome data to investigate the similarities between the effects of captivity on the NHP microbiome and the effects of westernization on the human microbiome.

Lifestyle influences gut microbiome composition of multiple NHP taxa

Examining multiple populations of NHPs living three distinctly different lifestyles (captive, semi-captive, and wild) allowed us to test the extent to which host genetics influence microbiome composition. Two main factors, host genetics and environment, are responsible for determining one's gut microbiome composition (Goodrich et al. 2014;

David et al. 2014). In humans, host genetics have been suggested to be the most influential factor in determining gut microbiome composition (Goodrich et al., 2014). The same as been suggested to be the case in NHPs, as Ochman et al. (2010) revealed a significant association between microbiome phylogeny and host phylogeny in higher primates. However, this study (Ochman et al. 2010), as well as other studies of NHPs (Moeller et al. 2014; Ley et al. 2008; Muegge et al. 2011), have included only a small number of individuals from each host taxonomic group and did not contain matched wild and captive samples from the same species. In contrast to previous studies, our study included both a large number of individuals, as well as matched wild and captive samples from two different NHP species (doucs and howlers). Because a previous study suggested host genetics to be the major determinant of microbiome composition in higher primates (Ochman et al. 2010), we wanted to assess the degree and significance to which the phylogenetic relatedness of NHP hosts impacts the taxonomic composition of the microbiota in other NHP species, including both New and Old world monkeys.

Our 16S rRNA sequencing results revealed a strong association between lifestyle and gut microbiome composition. In our examination of wild and captive red-shanked doucs as well as mantled howling monkeys and other captive NHPs, we found that although the two species sampled in the wild, doucs and howlers, have highly divergent gut microbiomes, their gut biodiversity converges toward the same configuration in captivity. The analysis of the intermediate “semi-captive” samples from doucs support this hypothesis. A selection of other captive NHPs from multiple individuals in eight

phylogenetically divergent species demonstrates that they have all converged to a similar gut microbiome configuration as the doucs and howlers, despite being housed in a different zoo. Ultimately, our results show that captivity causes convergence of gut microbiomes despite host genetic differences.

Each of the three lifestyles, wild, semi-captive, and captive, examined are associated with very different environmental conditions, notably diet composition. The strong link we observed between lifestyle and microbiome composition is suggestive that diet plays a fundamental role in shaping gut microbiome composition in wild, semi-captive, and captive NHPs. Previous studies have shown that changes in diet are directly associated with shifts in gut microbial community structure (Xu & Knight 2015; Muegge et al. 2011; Wu et al. 2011; Gophna 2011; David et al. 2014; Amato et al. 2014; Kohl et al. 2014; Ma et al. 2014). Considering that the diets of wild NHPs differ substantially from that of semi-captive and captive NHPs, it is highly plausible that changes in diet composition are largely responsible for the observed differences in microbiome composition of NHPs living different lifestyles and likely contribute to increased morbidity and mortality among captive primates.

Captive NHPs' diets are usually dramatically different from that of wild or even semi-wild NHPs. NHP species are regularly categorized by the dietary niche they occupy, such as folivore, frugivore, and omnivore. In captive settings, this results in NHPs being given very similar, if not identical diets (Kleiman et al. 2010). However, even if primates do

belong in similar feeding guilds, different feeding ecologies, morphologic and physiologic adaptations, and habitats all equate to different nutrient requirements (Kaumanns et al. 2000). The recommended diet of captive NHPs is corn and soy based. It is high in fat (5%) and protein (23%) while low in fiber (14%) (Anon 2014) compared to the diet of wild leaf-eating NHPs that has 0% fat, 10-13% protein and 23-54% fiber (Glander 1981). Unlike corn and soy, tree leaves contain plant secondary compounds (alkaloids, cyanide and phenolics for example) in addition to nutrients (Ehrlich & Raven 1964; Feeny 1968; Janzen 1969; Rhoades & Cates 1976; Rosenthal & Janzen 1979). In fact, the Golden Bamboo lemur consumes four times the lethal dose of cyanide every day (Glander et al. 1989). Given the complex and diverse nature of wild NHPs' diet, it is not surprising to see the results presented here.

Primate dysbiosis driven by loss of diversity

The gut microbiome is so intimately related to animal health that it has been likened to an additional organ in the body, one whose complexity science has only recently become able to measure. These bacteria play essential roles in primate health and development: they protect from infection, aid digestion, help extract and produce vitamins from the diet, as well as aid in training the immune system (Turnbaugh et al. 2006; Morgan et al. 2012; Petersen & Round 2014). Consequently, an unnatural mixture of bacteria, called dysbiosis, may increase risk of obesity, diabetes, cancer, Crohn's disease, and many other diseases in humans, and is likely to decrease or alter the ability of the host to adapt to and digest different diets.

Our 16S rRNA sequencing results revealed that captive NHPs had a marked reduction in gut bacterial diversity when compared to wild NHPs. Interestingly, the level of diversity observed in the semi-captive NHPs was intermediate between wild and captive NHPs, and thus a gradient-like reduction in diversity related to lifestyle was observed. Diversity is used as a measure of microbiome health, and a reduction in bacterial diversity is considered by many to be a sign of a compromised health status (Fujimura et al. 2010; Guinane & Cotter 2013; Gerritsen et al. 2011; Taur et al. 2014). Folivorous NHPs, such as doucs and howlers, are rarely housed in captivity, due, in part, to the complexities associated with replicating their wild diets, and thus maintaining ideal health. In addition, red-shanked doucs have been historically very difficult to manage in captive settings due to an inadequate understanding of their dietary requirements and predisposition for developing health-related issues. Digestive-associated morbidity and mortality, including conditions such as gastric distress (vomiting & diarrhea) and gastric amebiasis, continue to occur commonly among captive individuals (Janssen 1994; Ruempler 1998b; Loomis et al. 1983; Edwards & Killmar 2004). To highlight the level of difficulty involved with housing doucs in captivity, consider the fact that less than 50% of the captive doucs sampled in this study survived more than one year after sample collection was performed. This suggests the important role of maintaining keystone microbial genetic and functional diversity in conservation of NHPs.

Recent studies have shown that modern humans have lost a substantial portion of their natural microbial diversity (Moeller et al. 2014; Martínez et al. 2015; Clemente et al. 2015). Clemente et al. (2015) found that the microbiome of Yanomami Amerindians, an uncontacted human tribe who live seminomadic hunter-gatherer lifestyles in the Amazon, was more diverse than semitransculturated Guahibo Amerindians and Malawians, which in turn had more diverse microbiomes than Americans living a westernized lifestyle. Our findings suggest the existence of a strong link between lifestyle and gut bacterial diversity for NHPs, similar to what has been shown for humans. Given recent studies suggesting that modern humans have lost a substantial portion of their natural microbial diversity, and the substantial data presented in this study showing massive losses in gut microbiome diversity in captive primates, mapping the link between primate phylogeny, gut microbial genetics, and the functional adaptability of the host species may provide useful insight into the evolution, health, and conservation of primates, as well as allow for NHPs to serve as models to better understand the development of human diseases linked to diet, such as obesity.

Determination of keystone bacterial taxa in wild and captive NHP

In this study, we investigated how NHPs co-evolved with bacteria. We examined the microbial communities of two NHP species, the red-shanked douc (*Pygathrix nemaeus*) and the mantled howling monkey (*Alouatta palliata*), two species with highly divergent natural diets and gut physiologies. Samples were collected from both species living under wild and captive conditions, and were compared to one another. The foregut-fermenting

douc and hindgut-fermenting howler utilize different digestive strategies to meet their energetic demands. Despite their anatomical differences, doucs and howlers are both folivorous NHPs, meaning the majority of their diet is composed of leaves, and although their diets in the wild are compositionally quite different, both doucs and howlers consume a diet which is both nutritionally poor and difficult to process compared to diets consumed by non-folivores. By comparing subjects with two distinctly different anatomical conformations, representative of two evolutionary products, we were able to use doucs and howlers as examples of coevolution with bacteria, and specifically to investigate the potential role microbial communities played in the evolutionary changes in primate digestive physiology related to diet.

Initially, we compared the microbiomes of wild doucs and howlers and saw major differences in gut microbiome composition between the two NHP species. This was expected, as other studies examining multiple NHP populations have also shown gut microbiomes of wild primates to be species-specific both among NHP species living in the same general geographic area (McCord et al. 2014; Yildirim et al. 2010), as well as those separated by large geographic differences (McCord et al. 2014; Ochman et al. 2010).

Following our comparison of wild douc and howler microbiomes, we were able to examine whether or not changes in lifestyle result in a reduction in a species host microbiome signature, as our study included captive and wild individuals of two NHP

species, doucs and howlers. Although the gut microbiome genetic and functional diversity of howlers and doucs were highly divergent in the wild, we found that captivity causes their gut microbiomes to converge. The observed convergence of captive NHP gut microbiomes suggests that lifestyle changes have a dramatic impact on gut microbiome composition. This analysis demonstrates a substantial loss in interspecies gut microbiome variation in captivity, as well as a dramatic decrease in within-individual bacterial genetic and functional biodiversity and a domination of the gut by non-native taxa.

Parallel effects between captivity and westernization on gut microbiome composition of NHPs and humans

In a meta-analysis including wild, captive, and semi-captive NHPs and previously published modern and ancient human microbiomes, we have found that captivity causes the NHP microbiome to converge along an axis of dysbiosis leading toward the modern westernized human microbiome, implying that our study of loss of NHP microbiome diversity may shed light on processes that drive loss of important microbial genetic diversity in humans as well. When we plotted our samples by dietary preference, clear clustering patterns were visible. The split between captive herbivores and omnivores shows that despite the massive convergence of NHP microbiomes in captivity, diet still drives part of the interindividual diversity. However, some interindividual variability remains within captive NHPs, suggesting that factors other than diet are contributing to the loss of key genetic and functional biodiversity in the gut microbiome.

In order to understand the similarities in gut microbiome composition between captive primates and westernized humans, we focused on bacterial taxa with known associations with specific dietary factors, such as protein and fiber. We see that one of the marked effects of captivity on the gut microbiome of NHPs, as well as westernization on the gut microbiome of humans, is an increase in relative abundance of *Bacteroides*. Using both wild ape and human microbiomes, Moeller et al. (2014) determined that *Bacteroides* has increased in frequency in humans living in the USA greater than fivefold since their divergence from other human populations. The bacterial genus *Bacteroides* has a known positive association with the consumption of a diet rich in animal fat and protein (Moeller et al. 2014; Wu et al. 2011), which are major components of a Western diet. A Western diet is considered to be a diet high in fat and animal protein (e.g., red meat), high in sugar, and low in plant-based fiber (Hold 2013; Myers 2004; Martinez-Medina et al. 2014). Previous studies examining the relationship between dietary patterns and dysbiosis suggest a strong association between Western lifestyle, notably diet, and a dysbiotic gut microbiome (Hold 2013; Myers 2004; Martinez-Medina et al. 2014), as the Western diet is evolutionarily discordant from the diet of ancestral humans (Hold 2013; Cordain et al. 2005). Taken together, relative abundance of *Bacteroides* in the gut appears to be strongly regulated by dietary intake, and when found in high abundance, suggests the presence of dysbiosis. From our analysis, it appears that captivity moves captive NHPs in the same direction along the bacteroides gradient as westernization does to humans. One possible explanation is that the same factors, including diet, that are causing the shift in humans are causing the shift in NHPs.

The fact that captivity appears to cause captive NHPs, notably doucs, to shift to severe gut dysbiosis, means captive NHPs could potentially serve as models to study what is happening to the human gut microbiome in highly westernized countries like the USA, and also likely in humans over time. Additionally, given that substantially more human microbiome data is currently available, including established diet-microbiome links based on many studies with large sample sizes, we can use human studies to aid in our interpretation of the diet-microbiome relationship evident in NHPs, as well as to develop mitigation strategies to address health issues in captive primates, which arise as a result of being dysbiotic due to the consumption of inappropriate diets. It appears that the future of microbiome research holds great promise, as the enteric microbial adaptations that facilitate efficient extraction of key nutrients can guide the creation of appropriate diets, both for humans and NHPs alike, which are conducive to maintaining optimal health.

Conclusion

In this study, our goal was to provide novel information regarding the link between lifestyle, primate gut microbiome diversity, and adaptation to diet. Existing research studying the links between lifestyle, primate gut microbiome diversity, and adaptation to diet is severely limited in its ability to identify mechanisms for the loss of keystone microbial genes and functions due to captivity or loss of habitat. A unique component of this study was the examination of multiple primate populations, including populations of two NHP species living under various environmental conditions, as well as eight NHP species all living under similar environmental conditions. Overall, our results showed that

gut microbiome composition of wild NHPs differ greatly, while captive NHP gut microbiomes converge to yield a similar composition, and captive NHPs trends toward the modern human gut microbiome, implying that parallel processes may be driving recent loss of core microbial biodiversity in humans.

Table 3.1 Primate species and associated lifestyles included in this study.

NHP species	**Lifestyle**	**Provenance**
Red-shanked douc (*Pygathrix nemaeus*)	Wild	Son Tra Nature Reserve (Da Nang, Vietnam)
	Semi-captive	Endangered Primate Rescue Center (Ninh Binh, Vietnam)
	Captive	Singapore Zoo (Singapore, Singapore)
	Captive	Philadelphia Zoo (Philadelphia, PA, USA)
Mantled howling monkey (*Alouatta palliata*)	Wild	Hacienda La Pacifica (Guanacaste, Costa Rica)
	Captive	Las Pumas Rescue Center (Guanacaste, Costa Rica)
Western lowland gorilla (*Gorilla gorilla gorilla*)	Captive	Como Zoo (Saint Paul, MN, USA)
Sumatran orangutan (*Pongo abelii*)	Captive	Como Zoo (Saint Paul, MN, USA)

De Brazza's monkey (*Cercopithecus neglectus*)	Captive	Como Zoo (Saint Paul, MN, USA)
Black-handed spider monkey (*Ateles geoffroyi*)	Captive	Como Zoo (Saint Paul, MN, USA)
White-faced saki (*Pithecia pithecia*)	Captive	Como Zoo (Saint Paul, MN, USA)
Blue-eyed black lemur (*Eulemur macaco flavifrons*)	Captive	Como Zoo (Saint Paul, MN, USA)
Emperor tamarin (*Saguinus imperator subgrisescens*)	Captive	Como Zoo (Saint Paul, MN, USA)
Geoffroy's tamarin (*Saguinus geoffroyi*)	Captive	Como Zoo (Saint Paul, MN, USA)

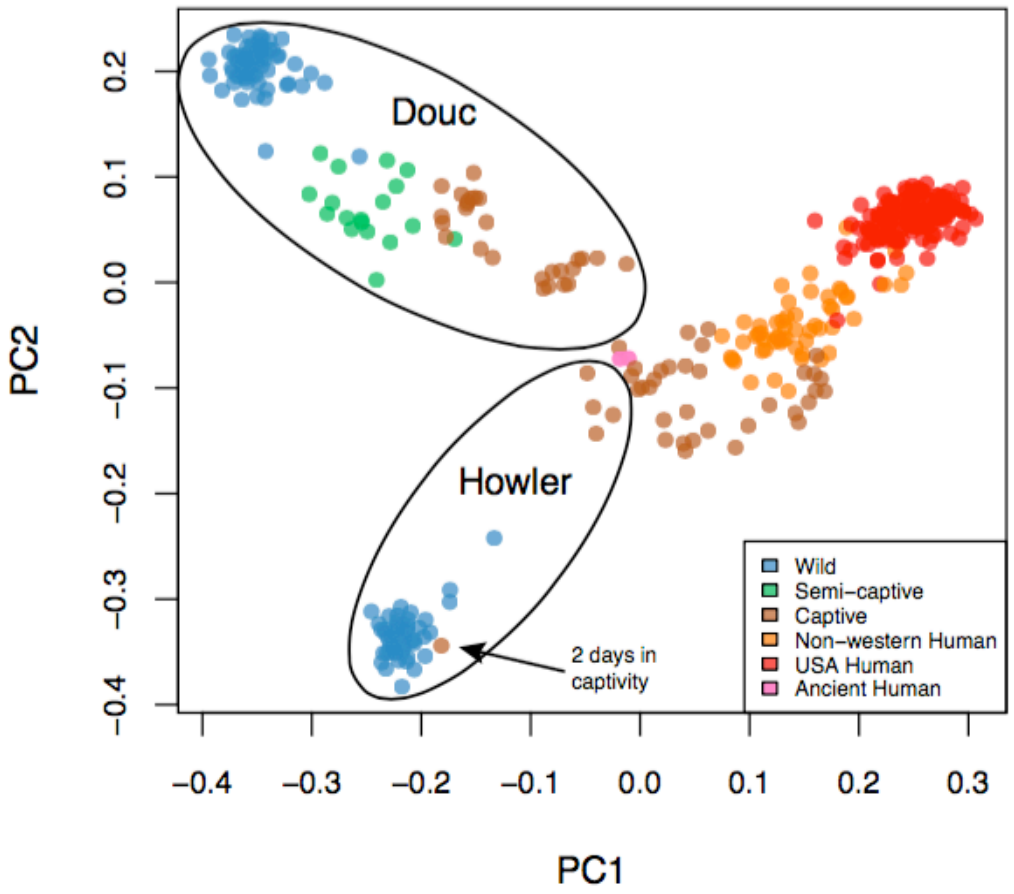

Figure 3.1 Captive primate dysbiosis converges toward the modern western human microbiome. Principal coordinates plot of unweighted UniFrac distances showing ecological distance between gut microbial communities in wild, semi-captive (from a sanctuary), and captive non-human primates, as well as non-westernized humans and humans living in the USA. Although in wild populations the douc and howler microbiomes are highly distinctive, captivity causes them to converge toward the same composition. Semi-captive doucs (green) fall in between wild and captive doucs along the same axis of convergence. The axis of convergence is associated with dysbiosis due to dramatic loss of native gut species and high rates of gastrointestinal disease in the

captive individuals. The axis of convergence continues on to non-westernized human populations (Malawi and Venezuela) and finally to the modern USA human microbiome.

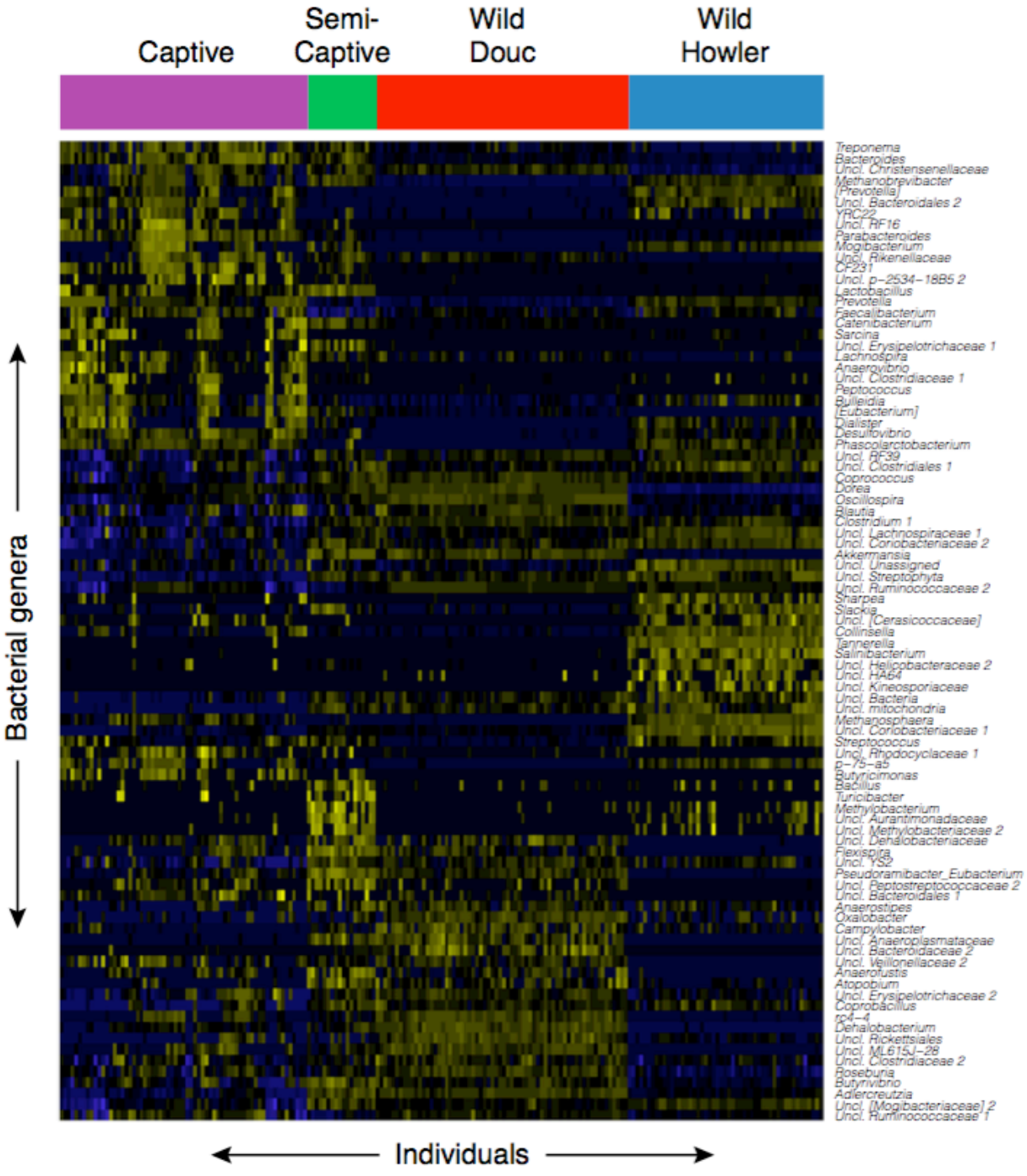

Figure 3.2 Heatmap of discriminative taxa by lifestyle. Highly predictive taxa for discriminating the Captive, Semi-captive, and Wild Douc, and Wild Howler groups. Predictive taxa included are those with a 0.001 mean decrease in error when removed as determined by the random forests classifier. The classifier was able to correctly predict the group label for unseen samples for all but one of 251 samples (99.6% accuracy). Semi-captive individuals show an overlap between some of the wild douc genera and some of the captive animal genera. Captive NHPs contain large blocks of bacterial genera that are absent from wild NHPs, and vice versa, indicating a massive shift in taxonomic composition between the populations. Wild doucs and howlers lose most of their native signature gut bacteria in captivity.

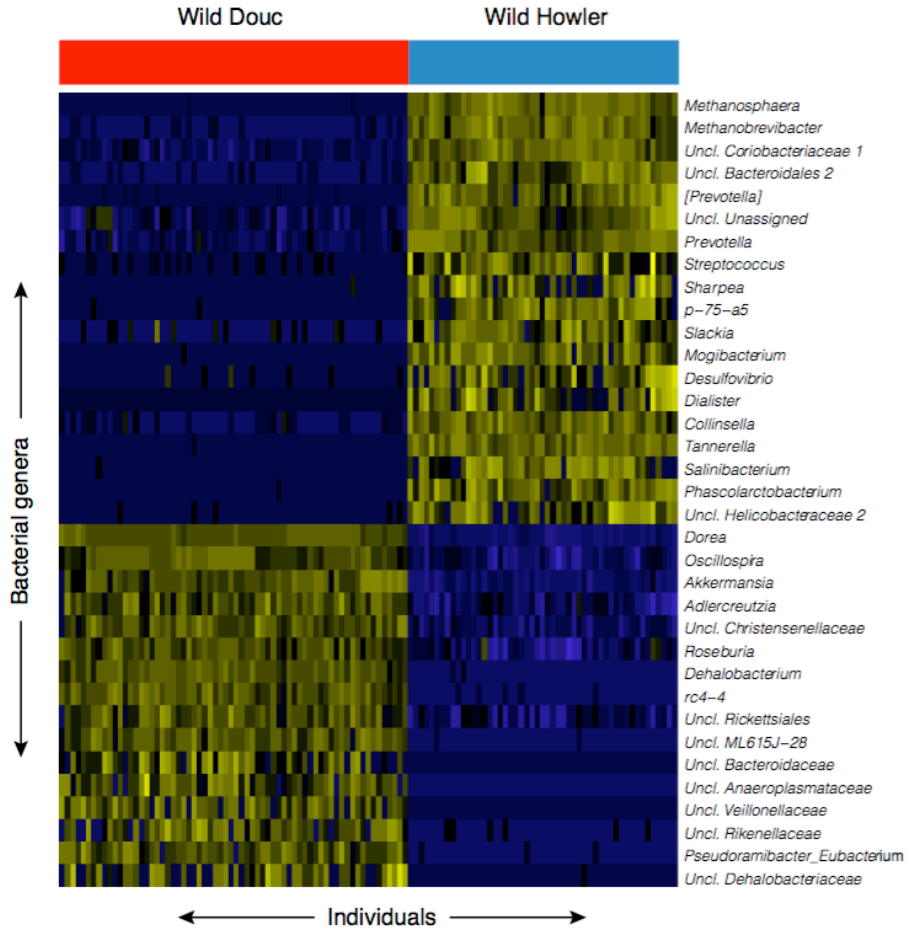

Figure 3.3 Heatmap of most predictive taxa discriminating gut microbiomes of two primate species. The transformed relative abundance of the 20 most strongly predictive bacterial genera for discriminating between two species of primates, the red-shanked douc and the mantled howling monkey, as determined by the random forests classifier. This heatmap shows very strong signature species for each of these groups.

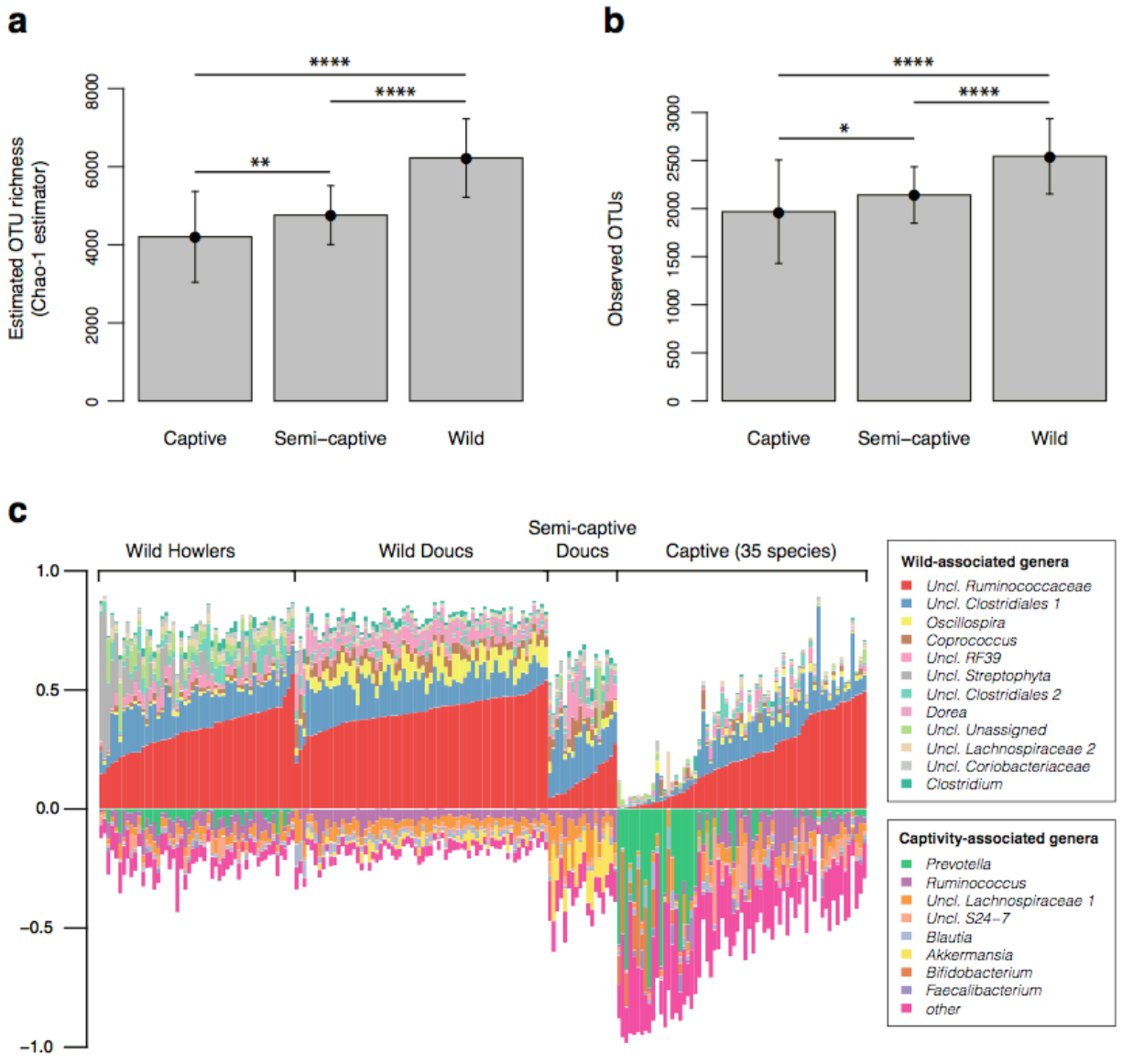

Figure 3.4 Captivity is associated with dramatic reductions in native primate microbiota. Bar plots of mean and spread of gut microbial biodiversity, as measured by the number of species-like operational taxonomic units (OTUs) in the gut microbiome, of wild, semi-captive, and captive populations of NHPs using the Chao-1 estimator of total OTU richness (**a**), and the total number of observed OTUs (**b**). These indicate a significant loss of biodiversity from wild populations to semi-captive populations, and

again from semi-captive to captive. Error bars correspond to standard deviation, and asterisks denoted significant differences at $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). (**c**) Stacked bar plot of relative abundance of the 20 most abundant genera across all wild and captive individuals. Bars above zero correspond to genera more prevalent in wild primates; bars below zero correspond to genera more common in captive primates.

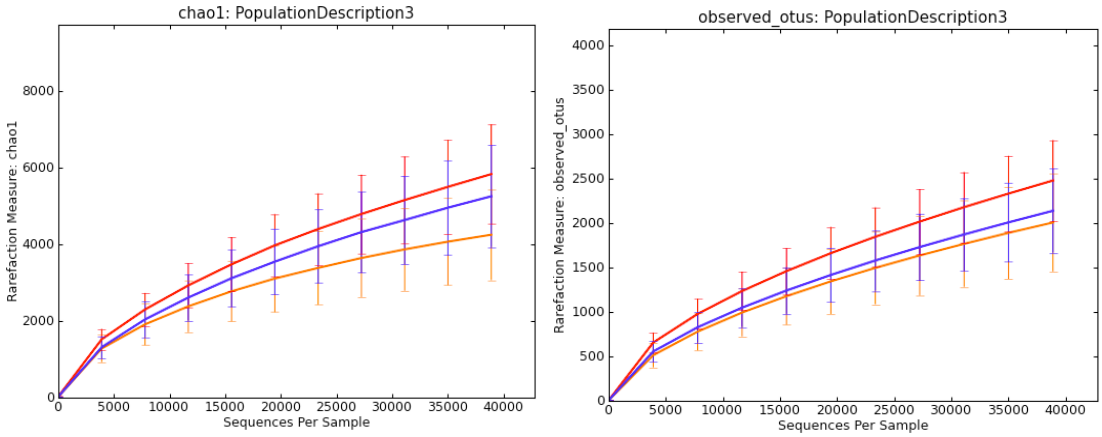

Figure 3.5 Rarefaction curves for different primate groups, including Chao-1 index and observed OTUs. Red (top): wild; blue (middle): semi-captive; orange (bottom): captive.

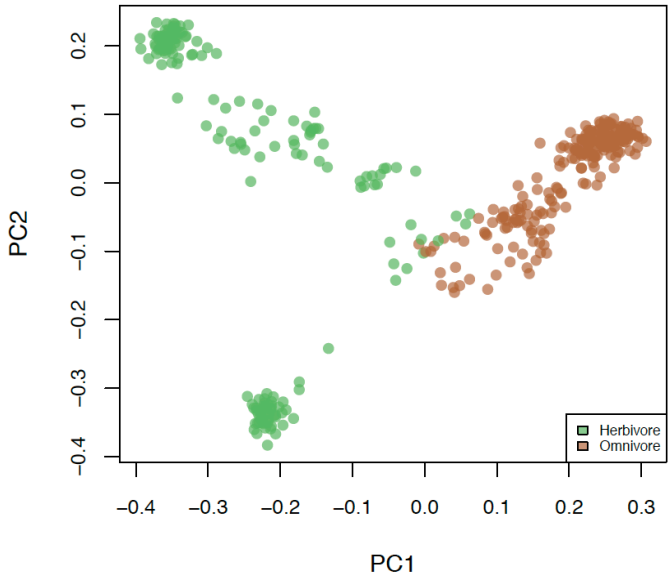

Figure 3.6 Diet is a major driver of gut microbial community structure in captive and wild nonhuman primates, as well as humans. The same samples plotted in Figure 1, but colored by dietary preference (herbivore vs. omnivore). The split between captive herbivores and omnivores shows that despite the massive convergence of primate microbiomes in captivity, diet still drives part of the interindividual diversity.

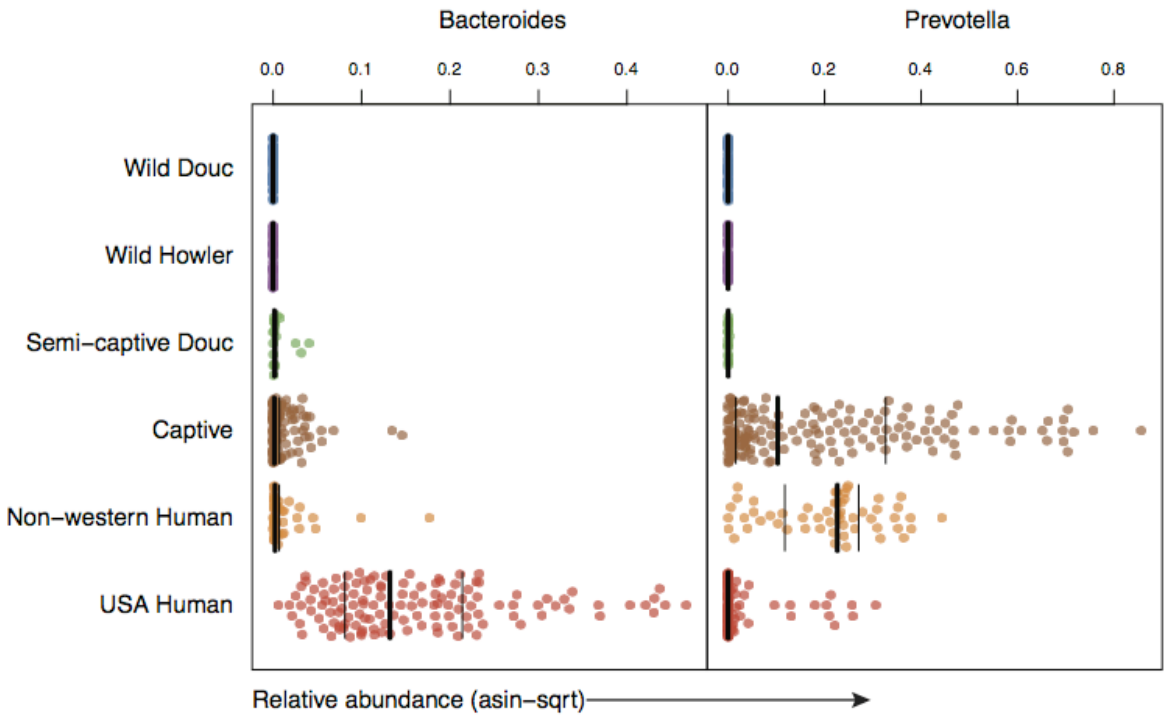

Figure 3.7 Captive primates acquire Bacteroides and Prevotella, the dominant genera in the modern human gut microbiome. (a) A beeswarm plot of the arc-sin square-root relative abundance of bacterial genera *Bacteroides* and *Prevotella*, the two dominant modern human gut microbiome genera, shown in wild, semi-captive, and captive NHPs, as well as in non-westernized and westernized humans. As populations move away from the wild state toward the captive state their microbiomes acquire these dominant human gut bacterial genera.

Chapter 4. General Conclusion

One mechanism for the preservation of threatened species is the creation of sustainable captive populations that may act as a genetic repository that buffers against extinction. According to the IUCN red book, the internationally accepted standard for the listing of endangered and threatened species, nonhuman primates, such as red-shanked doucs, are among the most endangered species in the world. Maintenance of these animals as captive populations that could provide a breeding reservoir for public awareness and education, and for re-populating restored habitats, has not been achieved due to an inadequate understanding of their nutritional requirements. The same has been true for many other colobine species. Red-shanked doucs are folivorous primates and are especially susceptible to gastric disorders when maintained on artificial (i.e., commercially prepared) diets in captivity. Improving the artificial diet is limited due to critical gaps in our understanding of local fibrous vegetation, including chemical composition and structural components, and the enteric microbial adaptations of doucs that facilitate efficient extraction of key nutrients. The overall focus of my dissertation was to better understand the symbiotic relationships between host and microbe relative to diet within the gut. Through the studies detailed in this dissertation, my collaborators and I sought to build essential baseline data correlating wild versus captive diets with microbial community structure using the red-shanked douc model. In the studies detailed in this dissertation, the douc was used as a model system to study the relationships between dietary composition and microbial community activity within the gastrointestinal tract. A direct outcome of this work is the identification of microbial and

dietary biomarkers associated with optimal douc digestive capability. A broader outcome is the establishment of a scientific research model to study the correlations between microbiome, diet, and primate evolution. This model can be applied to any scenario involving difficulties rearing primates in captivity, and thus paves the way for the development of novel approaches to effectively do so.

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