ABSTRACT

MCKENNEY, ERIN ALISON. The Effects of Phylogeny and Ecology on Microbiota in Captive Primate and Carnivore Species. (Under the direction of Vivek Fellner and Melissa Ashwell).

Nutrition, as a form of preventative medicine and a foundation for good health, has great importance for animals in captive settings. Microbial populations play a key role in the process of digestion for nutrient use by the animal. Differences in the diversity and fermentation activity of microbial populations associated with specific animal hosts arise from dissimilar diets and divergent phylogenies. This project aims to compare the community composition and fermentation activity of gastrointestinal bacteria found in gorillas (*Gorilla gorilla,* n=3), chimpanzees (*Pan troglodytes,* n=12), baboons (*Papio hamadryas,* n=17), and binturongs (*Arctictis binturong,* n=*10*). Host species were chosen which exemplified feeding strategies across the omnivorous spectrum. All animals within each species were fed a standardized diet for two weeks prior to sample collection. Diets were different and fixed for each species. Fecal specimens were collected within two hours of defecation and placed into pre-warmed vacuum Thermos® flasks and immediately transported to the lab. Fresh pooled fecal inoculums were added to pre-weighed diet substrate and incubated at 37°C for 24h. Fermentation products were measured and analyzed using the Proc Mixed procedure in SAS. DNA was extracted from frozen fecal samples and pooled within species. A region of the *cpn60* gene from the bacterial species was sequenced for species identification. Phylogenetic trees were compiled using the PHYlogeny Inference Package (PHYLIP), and biodiversity indices were calculated to quantitatively compare the microbial population compositions. Significant differences were observed across species for short chain fatty acid ($p<0.0001$) and methane ($p<0.001$) production, as well as neutral detergent fiber ($p<0.05$) and dry matter ($p=0.0001$) digestibility. Microbial composition analysis shows that dissimilar microbial communities are associated with each host species. While the composition of diets offered may contribute to differences in fermentation, the trends seen in our microbial diversity data suggest inherent microbial patterns are associated with energy requirements of the host. This link between patterns of energy usage and host-specific endosymbionts is consistent with previous findings in humans and non-human primate species.

The Effects of Phylogeny and Ecology on Microbiota in Captive Primate and Carnivore Species

by
Erin Alison McKenney

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Animal Science

Raleigh, North Carolina

2011

APPROVED BY:

Vivek Fellner Committee Co-Chair	Melissa Ashwell Committee Co-Chair
Jennifer Campbell Committee Member	Joanna Lambert External Committee Member

DEDICATION

The more you know, the more you can create. ~Julia Child

Only she who attempts the absurd can achieve the impossible. –Robin Morgan

To the teachers who shared everything they learned, and then encouraged me to keep asking questions.

BIOGRAPHY

Erin was born on August 23, 1985, in Jacksonville, Florida, to Craig and Linnea McKenney. The family settled in Indianapolis where Erin grew up with her younger siblings, Lesley and Ian, and a succession of pets. In 1997 the McKenneys relocated to New Bern, North Carolina, where Erin completed high school before earning a Bachelors of Science at the University of North Carolina in Chapel Hill. While in college, Erin spent a semester abroad in Coleraine, Northern Ireland and played piccolo in the marching band. After graduating from Carolina with a Biology major and Chemistry minor, Erin moved to Orlando, Florida, to work for Disney's Animal Kingdom as a Nutrition & Research Professional Intern. In August, 2009, Erin began the MS program in Animal Science at North Carolina State University. After defending her Master's thesis, she will travel to Namibia to perform conservational field research and study wildlife management. Other summer plans include a Natural Science teaching position at the NC Governor's School. In August, 2011 Erin will begin her PhD in Biology, studying lemurs at Duke University.

ACKNOWLEDGMENTS

I could not have made this academic journey without help. For that, I would like to give thanks:

To my family, for telling me they were proud and for sharing my excitement, and somehow keeping their eyes from glazing over during long-winded scientific rants;

To Katie Sullivan, for nurturing my initial interests in animal nutrition and bolstering me through the grad school experience;

To Vivek Fellner, for his good-humored advice, encouragement and support—the embodiment of what it is to be a mentor;

To Melissa Ashwell, for challenging me beyond my preconceptions;

To Jennifer Campbell, for her valuable insight and enthusiastic participation;

To Joanna Lambert, for her collaborative efforts from afar—her long-distance nurturing and feedback have been instrumental in honing my knowledge of primatology;

To Kimberly Ange-van Heugten, for guiding my involvement at conferences and for introducing me to researchers with similar interests;

To Sarah Jo McLeod, for her smile and insurmountable, bright shiny joy in the face of fecal inoculations;

To Audrey O'Nan, for her determination and for pushing me to take ownership;

To Rich Bergl at the NC Zoo and Kathryn Bertok at the Carolina Tiger Rescue, for jumping on board to make this project possible—and to Terry Webb, Jennifer Ireland, Chris Goldston, and the animal keepers, commissary staff, and volunteers at both institutions, for negotiating the logistics of science and animal husbandry;

To fellow students, for company, commiseration, and countless office/dance/coffee parties;

And to Kevin, for putting up with my (extra)curricular stress, and for making study sessions fun…for his unwaning optimism, and for being my best motivation and favorite part of coming home.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADF – Acid detergent fiber

ATP – Adenosine triphosphate

CoT – Correction time

DGGE – Denaturing gradient gel electrophoresis

DM – Dry matter

DNA – Deoxyribonucleic acid

FASTA – FAST-All

GIT – Gastrointestinal tract

HMW – High molecular weight

MRT – Mean retention time

NDF – Neutral detergent fiber

PCR – Polymerase chain reaction

SCFA – Short chain fatty acids

TCA – Tricarboxylic acid

TGGE – Temperature gradient gel electrophoresis

UBC – Uncultured bacterium clone

UPFBC – Uncultured pig fecal bacterium clone

USBC – Uncultured soil bacterium clone

CHAPTER 1: LITERATURE REVIEW

Adaptation to Dietary Niches

The importance of understanding the complex array of adaptations that make an animal suited to its environment cannot be overestimated. Nutritional ecology comprises a wide range of fields which includes evolutionary biology, ecology, life history, behavior, nutrition, morphology, and physiology (Raubenheimer et al., 2009).

Cellulose is the most abundant form of carbohydrate in the world, yet no vertebrate has evolved the ability to synthesize endogenous cellulase (Stevens and Hume, 1998; Lambert, 2007). Thus, in order to exploit vegetative food sources, it has been necessary for mammals to adopt fungi, protozoans, and bacteria as endosymbiotic producers of cellulolytic enzymes (Hofmann, 1989; Lambert, 2007).

Most current knowledge of mammalian gastrointestinal microflora is based on studies of domestic ruminants. However, as Hofmann (1989) pointed out, a single domestic model cannot adequately represent the remarkable ruminant diversity born of variances in climate, habitat pressure, behavior, and ecological opportunity. In fact, many exotic species become ill and can die if they are offered only diets formulated to meet the needs of domestic animals (Hofmann, 1989). Primate biologists are challenged to link observations of feeding and foraging in the wild with knowledge of nutrition in captive primates (Lambert, 2010). For example, while fruit exhibits wide interspecific variation, it generally provides nonstructural carbohydrates (like fructose) which are easy to digest. Leaves, on the other hand, contain high amounts of structural carbohydrates (like fiber) and must be digested over a longer period of time to yield energy (Lambert, 2007). Studies suggest that primates, like herbivores, strive to achieve nutritional balance in the wild by consuming a variety of food items (Felton et al., 2009). While little is known of the nutrient intake of healthy primates in wild populations (Rothman et al., 2008), apes are generally called dietary specialists and rely on high-quality foods (Lambert and Fellner, in press). Primates' multifaceted ecology *in situ*, coupled with their complex nutrient requirements, makes their management in captivity an ongoing challenge to conservationists.

Feeding Strategies

While Hofmann's observations only directly apply to certain ungulates, his concepts of evolutionary adaptation are pertinent across mammalian taxa. For example, most primates are omnivorous, consuming a combination of plant and animal matter (Lambert, 2007). The three basic ruminant feeding strategies Hofmann (1989) depicts can be used to describe primate feeding ecology along the omnivorous spectrum as well. Roughage eaters, adapted to consume and digest forages high in fiber, could be grazing water buffalo (*Bubalus bubalis)* or lowland gorillas (*Gorilla gorilla*) (Watkins et al., 2010). Goats (*Capra aegagrus*) and olive baboons (*Papio anubis*) exemplify intermediate feeding, marked by opportunistic selectivity from a variety of potential food items. Concentrate selectors are known to distinctly prefer easily digestible forages high in soluble carbohydrates. This last category comprises the tiny dik-dik antelope (genus *Madoqua*) as well as frugivores like the chimpanzee (*Pan troglodytes*) (Watkins et al., 2010) and, interestingly, the binturong (*Arctictis binturong*)—an divergent carnivore. Four of the carnivores studied in Mediterranean Europe by Rosalino and Santos-Reis (2009) ate more than 30 fruit species, and overall consumption favored fleshy fruits (63%) to their dry counterparts. However, while carnivores in southern latitudes generally consume more diverse diets which include vegetable material, Carnivore species consuming a diet of mainly fruits—like *A. binturong*—are rare (Rosalino and Santos-Reis, 2009).

Vegetation availability changes with elevation and the nutritional composition may vary for a single plant species across space and time (Rothman et al., 2008). An animal's gross intake is a product of its environment and physiological state, as well as social dynamics either within its own group hierarchy or between competing populations (Lambert, 2010). While myriad food items may be available, animals mix and match specific food items non-randomly to achieve a complete nutritional combination of macronutrients, vitamins, and minerals. This self-imposed selection technique is ubiquitous among all living creatures (Raubenheimer et al., 2009). Macro- and micro-nutrient requirements drive all nutrition ecology interactions, and vary with body size, metabolism, lifestyle, and digestive system. Felton et al. (2009) list five models for achieving these nutritional goals, through (1)

energy maximization, (2) nitrogen or protein maximization, (3) avoidance or regulation of secondary plant compounds, (4) limiting intake of dietary fiber, (5) nutrient balancing, or through a combination of strategies.

Nutritional goals drive food selection based on the properties of each item, which dictate their inclusion and ranking in daily intake. Determining the nutritional goal governing diet selection for a given primate species increases understanding of primate behavior, ecology, and human evolution. That knowledge can be further applied to zoo husbandry, comparative physiology studies, and maintenance of primate populations in managed ecosystems (Felton et al., 2009). However, pinpointing an animal's primary motivation in selecting a particular diet can be extremely difficult. For example, a diet apparently selected to maximize energy may, in fact, result from a need to ingest high-protein items.

Nitrogen is necessary for metabolic processes, cell growth, and gene expression. While Primate species have low protein requirements, especially compared to carnivorous mammals (Lambert, 2010), nitrogen deficiency in any animal may limit health, reproduction, and survival. Primates are sometimes observed selecting plant parts that are rich in protein, such as younger leaves instead of older growth, and supplement otherwise herbivorous diets with insects and small prey, in order to meet their needs. However, items ingested may be misleading as to the animal's actual nutrient gain.

Foods are complex mixtures, and their components interact to exert a variety of influences on their consumer (Raubenheimer et al., 2009). Some plant secondary metabolites (PSM) may restrict the uptake of certain nutrients, but others protect against internal parasites—or may counteract the detrimental effects of other ingested compounds. PSM levels vary among plant species as well as among individual plants within a species, and increased nutrient intake may ameliorate harmful effects (Felton et al., 2009). African apes avoid exposure to tannins, toxins, and other secondary plant compounds, and manage to maintain consumption of higher-quality components year-round, regardless of scarcity—which often means that they must increase their range (Lambert, 2007).

It is difficult to assess the nutritional value of a single food item—or, subsequently, an animal's criterion for selecting it—due to the associative effects of other items consumed

(that is, interactions between food items that affect nutrient availability), as well as confounding compounds within the food item itself (Felton et al., 2009). For example, carbon derived from different sources may have varied post-ingestive consequences: insoluble fibers in plant cell walls can almost be considered a type of secondary plant compound due to their antifeedant and antinutritional effects on the animals that consume them (Raubenheimer et al., 2009; Lambert, 2007). To avoid or compensate for these adverse effects, *Gorilla* and *Papio* species select leaves with a higher protein:fiber ratio; but tannins present in the leaves may bind crude protein, rendering it inaccessible. When assessing nutrient intake, the importance of considering availability cannot be overemphasized (Rothman et al., 2008a).

The source of dietary fiber, in turn, may also significantly affect microbial metabolism. Fecal inoculums from chimpanzees fed high-psyllium diets exhibit lower pH and higher short chain fatty acids (SCFA) and β-glucosidase levels, indicating an increase in fermentation. Similar results have been seen in previous studies of the colonic microbiota isolated from humans (*Homo sapiens*) and vervet primates (Genus *Chlorocebus*) (Costa et al., 1989).

In general, primates are extremely selective. They eat only a small percentage of the plant species accessible in their natural habitat, and may consume a given species only at certain times of the day—and even then, select only a specific portion of the plant such as the stem or leaf (Lambert, 2007). Preferred foods are those which are high in nutrient density and easy to find and access, but a single food item often exhibits only one of the optimal traits. Lower-density foods, which yield lower energy, are more ubiquitous, while highly nutritious foods are often rare or protected in a hard husk, shell, or other covering (Lambert, 2010). Fruit, as an ephemeral resource, is scattered both spatially and temporally. Therefore, frugivorous animals must develop fall-back strategies for those times when preferred foods are unavailable. In these cases, animals may resort to eating bark, leaves, and other alternative vegetative food sources (Lambert, 2007). The replacements may be predictable, staple items; or they may be 'fillers,' obtained opportunistically (Lambert, 2010).

Often, different animal species may evolve morphological adaptations to eat different parts of a single type of fruit. For example, the black colobus (*Colobus satanus*) consumes

the young seeds of hard fruits, while the red-faced spider monkey (*Ateles paniscus*) swallows the fruit whole because it lacks the dentition needed to chew (Lambert, 2010). Competitive pressures may also affect food selection—for example, in a social group the alpha-male gets first choice and the subordinate males must pick through the leftovers. In the wild, the constant variability of food availability across habitats induces varying degrees of feeding competition between species and populations of primates (Lambert, 2007). But these dynamics may also have severe ramifications in captive settings: broadcast feeding a balanced diet on exhibit does not necessarily provide a complete diet to each individual housed therein. In this case, availability is affected by social hierarchy as well as food properties.

Anatomical Morphologies

The chemical, physical, and nutritional properties of the diet for a given species' ecology may dictate that animal's adaptations—specifically features of the GI anatomy and other characteristics of the digestive system. For example, all omnivores are hindgut fermenters—meaning they harbor microbes in the cecum and colon, where digestion of fiber occurs after the ingesta has been initially processed in the acidic stomach (Bergman, 1990; Watkins et al., 2010). Primates utilize a plethora of morphological adaptations to procure food in myriad ways; there is virtually no food item that is not eaten by some species of primate (Lambert, 1998). This dietary and digestive flexibility has allowed the Primate order to exploit more potential food items, minimizing intra- and inter-specific competition, and allowing increased evolutionary capacity over wide ranges of habitat (Lambert, 2007).

Regardless of food selection or items consumed, gut limitations and intake of indigestible cell wall material may impact the availability of nutrients (Felton et al., 2009). The volume of a digestive cavity is generally proportional to the production of SCFAs within it. However, the quantity of absorbed organic acids is limited by the available surface area (Lambert, 1998). The intestine varies both in total length compared to the length of the body, and in the relative lengths of the midgut and hindgut sections. The ratio of intestine: body length is a useful value for comparison between animals with different body sizes or feeding

strategies. Table 1 gives a list of animals and their intestinal length to body length ratios, as well as their phylogenetic and physiological classifications.

A longer digestive tract allows digesta to be retained for a greater period of time before excretion, which in turn maximizes digestive efficiency of microbial and host processes as well as the absorption of nutrient products. A change in retention times dependent on dietary quality has been observed across several taxa. The relationship between digestion and diet includes anatomical, physiological, and feeding ecology interactions (Lambert, 1998). In general, there is an association between the anatomy of an animal's digestive tract and what that animal eats. A simple stomach, relatively short small intestine, and unspecialized colon will likely belong to a species whose diet contains easily digested, nonchitinous animal matter—namely, a carnivore such as the domestic cat (*Felis catus*) or (see Table 1). Animals eating plant parts such as leaves, exudates, and seeds, which are high in polysaccharides, rely on a complex stomach, large colon, and/or cecum for nutrient extraction; examples of these animals include the domestic cow (*Bos taurus*) and horse (*Equus ferus*) (see Table 1). Animals exhibiting high frugivory—such as the binturong (see Table 1)—are often characterized by a highly unspecialized gut, and any morphological differences generally arise from dietary supplementation with insects or leaves (Lambert, 1998; Lambert, 2007).

Similar to gut specialization, intestinal length tends to increase with dietary fiber. Retention of digesta in hindgut fermenters is often aided by the presence of an enlarged cecum or by an extended or haustrated colon. While the human appendix is a present-day shadow of its former glory—a mere vestigial organ—nonhuman primates have retained a cecum, which in some lemurs and monkeys is quite well-developed (Stevens and Hume, 1998). The passage rate of digesta links digestive physiology and feeding ecology (Lambert, 1998). As the efficiency of energy extraction is critical for understanding of an animal's feeding strategy, the digestive morphology is of obvious interest (Lambert and Fellner, in press).

Modularity is the capacity of an animal or species to adjust or regulate its digestion when diet is changed. Many vertebrate taxa show a considerable GI flexibility in response to

diet. Adaptations include enzyme activity, intestinal transport and absorption rates, morphological capacity, and digestive retention times (Lambert, 1998). The Primate order exhibits extraordinary diversity in feeding and foraging-related adaptations. Some of these modifications are shared across taxa, while others are unique to primates (Lambert, 2007). One must take into account the physiological and anatomical components making up the GI tract, as well as factors such as retention times, gut motility and modularity, before any generalizations can be made with regards to body and gut size, diet, or digestion (Lambert, 1998).

Microbial Fermentation

There is more to digestion than gut size and anatomy alone; foods differ widely in their capacity to yield energy, and some require additional digestive processing by endogenous enzymes or microbial fermentation before their energy can be utilized (Lambert, 1998). Mutualistic bacteria play a key role in digesting the cellulose and hemicellulose that make up plant cell walls, as well as the chitin in insect exoskeletons, and converting the structural carbohydrates into short chain fatty acid end products—mainly the short chain fatty acids (SCFA) acetate (A), propionate (P), and butyrate (B)—that would otherwise be nutritionally inaccessible to the host (Bergman, 1990; Lambert, 1998; Stevens and Hume, 1998). Equation 1 summarizes this process below.

$$CHO \xrightarrow{microbial\ \ fermentation} SCFA + CO_2 + H_2 \xrightarrow{methanogenesis} CH_4 \qquad \textbf{Equation 1.}$$

In the great apes, fermentation occurs in the hind gut, where the enlarged colon lends an increased capacity to digest fiber and detoxify secondary plant compounds, yielding substantial contributions to ATP production (Kisidayova et al., 2009; Lambert, 1998; Lambert and Fellner, in press; Stevens and Hume, 1998). This colonic system of fermentation is best-suited for mammals which typically ingest foods of moderate to high digestibility.

The colon in great apes is an important site of fermentation and production of SCFA. In fact, the short chain fatty acids (SCFA) produced during fermentation processes have been detected in the lower GI tract or feces of all mammalian species studied to date, including humans (Lambert, 1998; Bergman, 1990). High levels of dietary cellulose affect fermentation

activity, specifically by decreasing in vitro dry matter digestibility (IVDMD) gas. The production of hydrogen gas promotes methane formation, which corresponds to inefficient utilization of energy. High-fiber diets, therefore, are more efficiently utilized by microbes and their hosts.

Short chain fatty acids, however, are absorbed and used as a source of energy (Stevens and Hume, 1998). Up to 44% of energy intake may be converted to short chain fatty acids in the large intestine and cecum of hindgut fermenters, and the products provide 6-9% of total energy requirements in humans (Leser and Mølbak, 2009). Proteolytic enzymes are not present in the colon wall of hindgut fermenters, and they are believed to be unable to transport the amino acids and vitamins produced by microbes. This disadvantage has led to cecotrophy in many species, and increases the value of SCFA contribution to the host energy budget.

Total SCFA concentrations in the cecum, large colon, rectum, and feces range from 30-240 mM and average 70-120 mM (Bergman, 1990). In ruminants like sheep (*Ovis aries*) and cattle, SCFA concentrations between 60-120 mM have been measured, compared with 65-235 mM in the primate hindgut (Stevens and Hume, 1998). Considerable amounts of acetate are formed in humans by CO_2-reducing bacteria, via an indirect pathway, as opposed to direct formation from glucose (Wolin et al., 1999). In humans, fat formed from acetate provides energy to hepatic and peripheral tissues. Lambert and Fellner (in press) reported data for primate fermentation which suggest alternate routes of hydrogen removal, and provide indirect evidence of acetogenic bacteria. Recent evidence also indicates considerable levels of fermentation in the cecum and colon of humans and non-ruminants (Bergman, 1990). While humans typically consume high-quality diets, free-ranging great apes derive a nontrivial amount of their daily metabolic energy requirements from the products of hind-gut fermentation (Watkins et al., 2010).

While acetate accounts for the vast majority of SCFA produced, propionate and butyrate vary with diet. For example, colonic microbes such as *Roseburia, Eubacterium rectale,* and *Faecalibacterium prausnitzii*-like organisms are known to produce large amounts of butyrate from starch in humans and animals, and their populations are vulnerable

to changes in dietary carbohydrates (Bergman, 1990, Leser and Mølbak, 2009). Along similar lines, Wolin et al. (1999) found that increased colonic starch favors the growth of starch-using bacteria. This corroborates the Costa et al. study (1989), which deduced that consuming fermentable dietary fiber over long periods of time may affect microecology as well as microbial activity. This induced dietary effect on intestinal populations suggests that seasonal availability of produce and forages may have profound ramifications on the ability of the microbiota to meet host energy requirements, by introducing carbohydrate substrates which bacteria are not well-adapted to digest. The effect of dietary change on microbial efficiency may have subsequent nutritional impact not only on free-ranging animals in changing habitats *in situ*, but also on animals managed in captivity.

Lambert and Fellner observed high A:P ratios after 24 hours in fermentation chambers containing primate fecal inoculum (in press). While *G. gorilla, P. troglodytes, and P. hamadryas* all exhibited intermediate acetate concentrations, *P. hamadryas* had the highest propionate concentrations compared to intermediate levels in *G. gorilla and P. troglodytes*. *G. gorilla* and *P. troglodytes* microbes, however, produced the highest methane levels—suggesting that they assimilated feed stuffs least efficiently. Overall ratios of A:P:B range from 75:15:10 to 40:40:20 and are similar across species between hindgut fermenters and ruminants, but individual animal species are home to distinct and relatively stable populations of indigenous bacteria (Bergman, 1990; Stevens and Hume, 1998). Low pH and rapid transit times decrease or inhibit microbial growth, while more neutral pH and prolonged retention promote population density and diversity (Stevens and Hume, 1998).

The SCFA concentrations at various sites along the GIT are a result of the types of bacteria on-site as well as the retention time. Even in forestomach fermenters a considerable amount of fiber is digested in the large intestine (Bergman, 1990). Significant differences in fermentation activity may correlate to a shift in microbiota (Kisidayova et al., 2009).

Microbial effects on animal health

The gut microbiota plays an important role in health and production (Torok et al., 2008). The normal relationship between a healthy host and its microbiota can be described as a homeostatic symbiosis. Conversely, the presence of novel pathogenic species or an

imbalance in autochthonous constituents can be detrimental to performance and wellbeing (Paliy et al., 2009). Gastrointestinal microorganisms are shown to have significant impact on energy and nutrient uptake and utilization, immunology, and nonspecific resistance to infection. The bacteria within a population interact with their co-constituents as well as with the animal host (Torok et al., 2008). Analysis of intestinal transcription using microarrays established that colonization has decided influence on diverse and fundamental physiological functions, the range and kind of which suggest that the microflora can have considerable effects on host metabolism (Leser and Mølbak, 2009).

Colonic microbial activity has a variety of effects on host physiology and nutrient metabolism, including the provision of additional energy to the host and prevention of osmotic diarrhea (Costa et al., 1989). Short chain fatty acids, in themselves, promote GI cell growth and decrease the luminal pH to increase further absorption of nutrients (Stevens and Hume, 1998; Lambert and Fellner, in press). Acetate is a substrate for lipogenesis in cattle, but there are also other beneficial effects of SCFA such as increased DNA synthesis and vasodilation (Bergman, 1990). Butyrate is associated with improved epithelial health and cell growth. It also promotes differentiation and helps to regulate cell proliferation and apoptosis, thereby inhibiting the growth of cancer cells (Bergman, 1990; Wolin et al., 1999; Leser and Mølbak, 2009). Propionate helps lower blood cholesterol in humans and animals by down-regulating cholesterol synthesis, and fuels ATP production in the tricarboxylic acid (TCA) cycle (Bergman, 1990). Thus, the endosymbiotic population has significant influence on animal health and nutrition (Wei et al., 2007).

Just as feeding concentrates to domestic ruminants can promote *Lactobacillus* growth and causes acidosis, feeding a diet that does not suit the morphology or evolutionary track of a monogastric animal can have dire consequences. The specific dynamics and characteristics of microbial communities, their metabolism, and their impact on primate biology all remain poorly known (Lambert and Fellner, in press).

The population within a particular host may affect other macroorganisms as well as itself. Take, for instance, toxoplasmosis—an infection caused by *Toxoplasma gondii* protozoa. The disease is notorious for its transmission from domestic cats to pregnant women

and any other warm-blooded animal with a weakened immune system. The feline intestinal microbiota is important to companion animal health and also as a potential reservoir for zoonotic pathogens and strains resistant to antibiotics, both of which pose threats to human health (Desai et al., 2009). Coccidiosis, a leading cause of death in the poultry industry and a common illness among domestic dogs (*Canis familiaris*) and cats, is caused by a bacterial infection of the GIT and can spread to humans or other animals. Animal production plants, zoos, and other captive exotic facilities can also be potential biosecurity threats, as they provide homes for susceptible hosts—and thus may function as sources of zoonotic disease.

Target Host Species

Apes invariably rely on high-quality foods which are relatively low in fiber—especially ripe fruit (Lambert, 2007). From an ecological standpoint, apes represent a much lower biomass and exhibit more specialized dietary feeding strategies than cercopithecoids, which are more diverse and have a higher carrying capacity (also known as environmental maximum load) within their respective habitats (Lambert, 2007). These characteristics make the apes less adaptive and more susceptible to changes in food availability and habitat destruction, and higher-priority candidates for conservation efforts.

In this study, *Gorilla gorilla* (a partial folivore that eats ripe fruits when they're seasonally available)*, Papio hamadryas* (an omnivore that opportunistically feeds on a diverse array of food items)*, Pan troglodytes,* and *Arctictis binturong* (two frugivores) were selected to represent points along the omnivorous continuum. The GI anatomy and physiology represent a trade-off between retention of digesta to access nutrients and the total mass of food that can be ingested and processed. Fermentation of plants must occur to extract protein and energy from high-fiber diets, and the primary approach is to extract nutrients slowly. On the opposite end of the spectrum, fruit must be eaten in large quantities and processed in the digestive tract quickly (Lambert, 1998). Intermediate feeders employ a blend of strategies, often foraging opportunistically. The binturong exhibits chimp-like frugivory, but as a carnivore the animal faces unique challenges to nutrient extraction.

Gorilla gorilla

While small primates need high quality foods to access readily available energy quickly, large primates need less energy relative to their bodyweight to live, but have the capacity to consume higher quantities of food—meaning they can afford to eat lower quality diets to meet their absolute caloric requirements. Gorillas consume fruit when it is seasonably available, but this availability varies geographically; in the case of the mountain gorilla (*Gorilla beringei beringei*), leaves and the flexible green peel on plant stems are staple foods and comprise the majority of the diet even when fruits are consumed (Tutin et al., 1991; Rothman et al., 2008). Wild mountain gorillas in Bwindi National Park were seen to eat 84 different foods during focal observations. Dry weight and energy intake remained constant across seasons, so the change in food items during periods of increased frugivory resulted in higher concentrations of water-soluble carbohydrates and lower concentrations of protein and fiber. Overall, age and sex were determined to have an effect on nutrient intake (Rothman et al., 2008).

Wild gorilla diets generally contain about 40% neutral detergent fiber and have a total digestibility of about 60 percent (Kisidayova et al., 2009; Rothman et al., 2008). Western lowland gorillas (*Gorilla gorilla gorilla*), however, are able to consume fruit to varying degrees year-round, and are highly selective for plant parts low in fiber and tannins but high in protein, sugar and other soluble carbohydrates (Lambert, 2007).*Gorilla gorilla* individuals averaging 103 kg body weight had a digesta transit time of 37 hours, much longer than *Homo sapiens* (26 hours for 67 kg) but expected due to lower-quality diet. The extended retention of digesta is critical, as fermentation products may provide wild Western gorillas up to 30-60% of their daily metabolic energy (Watkins et al., 2010).

The gorilla gastrointestinal tract is characterized by a large colon which houses the microbes needed to digest a high-fiber diet and process the condensed tannins present in many food items. The mean retention times (MRT) measured for a gorilla weighing 51 kg were 45 hours for fluid and 57 hours for particles; overall, MRT increased proportionally to the fiber content of the diet (Stevens and Hume, 1998). The extent of bacterial influence on digestive function, food choice, and detoxification of secondary plant compounds in gorillas

remains undetermined (Frey et al., 2006), but up to 63% of the day is spent feeding and passage rate of ingesta is slow to allow more time for fermentation to occur (Lambert, 2007). These adaptations enable the gorilla to fall back on lower-quality, higher fiber foods when staple diet items are scarce (Rothman et al., 2008), though *Gorilla gorilla* are known to maintain fruit in their diet throughout the year by increasing their foraging efforts (Lambert, 2007).

In wild *Gorilla beringei* individuals, juveniles and adult females ate diets of a similar composition to that consumed by the (adult male) silverbacks—but to a higher total amount, in order to achieve the higher protein intake per metabolic body unit ($kg^{0.75}$) needed to support growth and reproduction, respectively. Juveniles also consumed higher mineral levels compared to adult gorillas (Rothman et al., 2008). However, a gorilla's actual nutrient requirements at any physiological stage are unknown, and must be estimated based on the accepted requirements of humans, other non-human primates, and swine—all of which have a wide range of body weights, feeding habits, and gastrointestinal morphologies—among other characteristics which factor heavily. Thus, it is quite difficult to ascertain what a "good" diet might comprise.

Papio hamadryas

Baboons (Genus *Papio*) are flexible, eclectic omnivores (Clemens and Philips, 1980), and require intermediate retention times to process a variety of food items which are gathered opportunistically (Swedell et al., 2008). By one year of age, Kenyan yellow baboons (*Papio cynocephalus*) are reported to have ingested more than 200 different food items—a far greater selection than is generally foraged by apes (Lambert, 2007). Such dietary flexibility is likely related to the baboon's digestive strategy (Lambert, 2010). As such, their gastrointestinal tracts are less specialized than those of their folivorous counterparts.

Clemens and Philips (1980) measured shorter mean retention times for *Papio cynocephalus* than for the herbivorous Sykes monkey (*Cercopithecus mitis*) for all markers, despite similar pH values along the digestive tract for both species. Nakamura et al. (2009) used denaturing gradient gel electrophoresis (DGGE) analysis to identify the dominant microbial organisms in primates based on the differential denaturing characteristics of DNA

sequences isolated from different bacteria. Their results indicated that sooty mangabeys (*Cercocebus atys*)—another omnivorous primate whose diet comprises primarily fruits and seeds—harbor more uniform sulfate reducing bacteria communities than Hamadryas baboons (*P. hamadryas*). The discrepancy makes sense, as baboons consume a greater variety of the available food items in their environment (Nakamura et al., 2009).

Several *Papio* species have been reported to vary their daily protein intake in relation to diet composition and season (Felton et al., 2009). SCFA concentrations measured in the sacculated *P. cynocephalus* caeco-colic regions were comparable to ruminant forestomach production values, as well as those for the hindgut of the pony (*Equus ferus caballus*), rabbit (Family *Leporidae*), and pig (*Sus scrofa*). Furthermore, the concentration peaked in the cecum then decreased as digesta moved distally into the rectum, suggesting luminal absorption by the host (Clemens and Philips, 1980).

Pan troglogytes

Chimpanzees in the wild are consistently observed to derive 87-98% of their daily intake from plant sources, while the remaining 4-6% is supplemented by insects and small prey (Tutin et al., 1991; Lambert, 2007). In Kibale National Park in Uganda, chimps eat ripe fruit almost exclusively; individual items may vary, but the majority of the diet is always comprised of succulent fruit (Lambert, 2007; Kisidayova et al., 2009; Watkins et al., 2010). In most tropical forests fruit-bearing trees either have staggered seasons, or produce fruit either in small quantities or in abundance but by individual trees which are widely scattered. Because fruit is a relatively limited resource, it is vulnerable to monopoly by dominant individuals within the chimps' social hierarchy. Therefore, *P. Troglodytes* and other frugivorous primates are more limited in troop and foraging group size than their less specialized counterparts (Lambert, 2010). When fruit was absolutely unavailable, Lambert (2007) observed chimps eating the pith (central tissues for nutrient storage) of terrestrial herbaceous vegetation.

A wild-based diet fed to captive chimpanzees by Kisidayova et al. (2009) contained 34% neutral detergent fiber (NDF) and had a total digestibility of 54.3%. In an experiment comparing the short chain fatty acids produced from high fiber versus low fiber diets, the low

methane levels and acetogenesis observed in microbes adapted to high-fiber diets was similar to fermentation profiles seen in wild chimpanzees (Kisidayova et al., 2009). The fresh produce items fed in captivity, however, are generally high in soluble sugars and low in fiber compared to the diets of wild, free-ranging *P. troglodytes.* This dietary discrepancy may be to blame for the rising incidence of diabetes and obesity in captive individuals worldwide.

The intestinal fermentation observed in chimpanzees is more similar to that in humans than either to gorillas or orangutans (Genus *Pongo*) (Kisidayova et al., 2009), and the products of fermentation provide wild chimpanzees with 21-33% of their daily metabolic energy (Watkins et al., 2010). Transit times are also similar: 23.2 hours in *P. troglogytes* (40-50 kg), versus 26 hours in *Homo sapiens* (67 kg) (Lambert, 1998). These shared characteristics make *P. troglodytes* a potentially useful model for humans in nutritional studies, in addition to existing genetic research.

Arctictis binturong

Though it is taxonomically classified as a Carnivore, the binturong is one of several palm civet species that eat mostly fruit supplemented with a variety of insects and small vertebrate prey (Carpo et al, 2002; Grassman et al, 2005; Mudappa et al., 2010). High activity levels during the wet season correspond to opportunistic feeding on figs located outside of the typical dry season range. Range size is affected by seasonal changes in resource availability (Grassman et al, 2005), and population density is significantly lower in logging sites compared to primary forests.

Binturongs comprise an intriguing collection of adaptations to frugivorous living. Convergent evolutionary traits are extensive among Carnivore species, but only the bear-cat and kinkajou (*Potus flavus*) use the shared morphologies to eat fruit-based (rather than meat-based) diets (Van Valkenburgh, 2007). The skull houses dentition similar to that of a dog or cat (Carpo et al, 2002). Prominent canine teeth, originally intended to pierce and grip prey, now serve to puncture ripened fruits. The finely honed carnassial molars, once used as scissors to shear meat from bone, are equally well-adapted for cutting soft fruit flesh away from a pit (personal observation).

A unique digestive morphology adds to *A. binturong*'s specialized alterations. In 2002, necropsies were performed on two unrelated specimens, both of which died of natural causes at the Carolina Tiger Rescue facility. Average lengths of 130cm (4 feet, 3.2 inches) and 58cm (1 foot, 10.8 inches) were recorded for the small and large intestines, respectively, with the total length of the gastrointestinal tract averaging 188 centimeters (6 feet, 2 inches) from pyloric sphincter to anus (Carpo et al, 2002). At just over twice the body length, the approximately 2:1 ratio is noticeably smaller than those recorded for other Carnivores (see Table 1).

Further, the Carpo et al. (2002) necropsies revealed the complete absence of a cecum. The lack of a cecum, coupled with the comparatively short tract to body length ratio, suggest that the binturong may be less efficient in digesting and utilizing the nutrients in fruit than most other monogastric animals that have a cecum (Carpo et al, 2001). Dierenfeld (2003) investigated the digestive efficiency of carnivores after consuming a diet of 66% canned and dry primate diet, 33% fruit and vegetables, and 7% green produce. She found the apparent digestibility in binturongs to be lower than that of the dwarf mongoose (*Helogale parvula*), another carnivore which has retained ancestral feeding habits. Discrepancies may be due to the novelty of protein and fat in the binturong's experimental diet, when one considers that the animal consumes mostly fruits in the wild—potentially as a source of water (Carpo et al, 2002; Mudappa et al., 2010). Nevertheless, Dierenfeld's data uphold the suspicion that *A. binturong* anatomy is ill-suited for complete digestion, and help to explain the expulsion of undigested fruit and vegetable material in feces (Carpo et al, 2002;). Indeed, it is common to find intact peels and citrus pulp in the excrement (personal observation)—most likely because these components contain higher proportions of structural and insoluble fibers.

Binturongs ingest considerably large volumes of figs (*Ficus*), among other food items, to meet their nutrient requirements (Carpo et al, 2002); but the animals' binging tendencies are a poor indication of fig nutritional content. Though the *Ficus'* varying levels of protein, carbohydrates, and lipids are lower than other native fruits, they are characterized by high levels of fiber, pulp water content, and minerals. Calcium is especially abundant, occurring at up to three times the concentration measured in non-fig fruits. Extra boosts of

protein are provided to the binturongs in the form of hatched larvae laid in the fruit by pollinator wasps, parasites, and other insects, though binturongs may require a mixture of *Ficus* species to provide adequate (and balanced) nutrition (Shanahan et al, 2001).

Occupying such a specialized niche presents nutritional challenges to *A. binturong*. Even if an individual consumes a variety of figs of diverse species, most fig-wasps will already have departed from ripe figs, and remnants of larvae corpses are unlikely to retain significant nutritional value. The likelihood of consumption occurring in time to access maximum potential protein is small. On the other hand, to eat figs earlier than the optimal stage of ripeness is to gain protein at the cost of simple sugars. An unripened fig is much higher in starch and structural carbohydrates, and thus less available to the binturong digestive system.

Even if *A. binturong* managed to adjust its foraging strategy to include a wider array of food items, the species would still be at a physiological disadvantage from a nutritional standpoint. Binturongs' fast passage rates and low digestive efficiencies serve to prevent complete nutrient extraction. *Ficus annulata* seeds in the feces of captive binturongs germinate readily after a retention time of over three hours (Shanahan et al, 2001). The seeds' retained viability speaks to the ineffectiveness of the digestive process, and agrees with the observed inability of binturongs to digest neutral detergent fiber (Dierenfeld, 2003).

Binturongs in captivity occasionally require digestive enzyme supplements to promote full utilization of maintenance diets (personal observation). This inhibited ability to break down and assimilate ingested produce raises questions regarding binturong physiology. Do binturongs utterly lack endogenous enzymes, or are the enzymes expressed simply not suited to extracting nutrients from the domestic food items offered in captivity? In either case, the absence of catalytic activity certainly has ramifications for nutrient extraction within the animal.

Species exhibiting foregut or hindgut fermentation are able to extract up to 50% more of the metabolizable energy available from figs than binturongs (Shanahan et al, 2001). Throughout several trial runs, binturong fecal samples have exhibited a complete lack of fermentation—that is, neither methane nor short chain fatty acids were produced when

inoculums were exposed to appropriate substrates (Lambert and Fellner, in press). It is unsurprising that experimental fermentation chambers were devoid of activity, considering the minimal retention times observed *in vivo*. However, when coupled with the comparatively low-quality foods *A. binturong* consumes in the wild, its success as a frugivore can only be described as perplexing.

Characterizing the Gastrointestinal Microflora

Fermentation Profiles

Fecal samples are commonly utilized in veterinary diagnosis, and can be obtained through use of non-invasive methods (Desai et al., 2009). Excrement has been used for some time to diagnose intestinal disease and assess diets and feed additives; and in 1989 Costa et al. pronounced feces a valid substitute for colonic inoculums in fermentation studies. After 48 hours, no significant differences (p>0.05) were found in viscosity, pH, SCFA production, or ammonia nitrogen concentrations between chambers inoculated with feces or colonic content procured from adult male African green monkeys (Genus *chlorocebus*). A short-term dietary adaptation prior to sampling is practical, after which a viable microbial system, similar in function to primate microflora found in the colon, can be recreated using fecal samples (Costa et al., 1989).

Samples are collected immediately after excretion and transported in sealed bags and coolers (Wei et al., 2007). Warm feces and buffer are combined and filtered to create an inoculum which is then added to substrate in an anaerobic chamber. After a predetermined time, the volume of gas which has accumulated is measured using gas chromatography (Kisidayova et al., 2009). Residues can then be dried and weighed for analysis. NDF and acid detergent fiber (ADF) are measured using the Van Soest (1991) detergent analysis method. These measurements do not account for pectin, but they do quantify the insoluble fibers. In vitro dry matter digestibility quantifies how much of the substrate is used by the microbes (Kisidayova et al., 2009). In pigs, 8-26% of the total dietary carbohydrates are fermented per day. In humans, 50-60g of ingested carbohydrates yield 0.5-0.6 moles of SCFA, which can then be converted to 140-180 kcal of energy (Bergman, 1990).

Mammals, reptiles, and birds all produce methane (Nakamura et al., 2009), though hindgut fermenters vary in their capacity to harbor methanogens in the digestive tract. The actual methanogenic status of a mammal seems to result from its evolutionary track, rather than dietary substrate, though Kisidayova et al. (2009) did report that gas production decreased as cellulose increased in chimpanzee and orangutan diets. Passage rate, colonic pH, and host genetics all have a major influence on the dominant hydrogenotrophic pathways in the colon. Most Old World monkeys produce methane, and hydrogen-utilizing microbiota have been shown to directly impact colonic health. Although measurements of fecal methane emissions have been used to detect methanogens in non-human primates, the diversity of methane-producing microbes in primates has not been quantified (Nakamura et al., 2009).

Population Dynamics

The colonic microbiome exhibits a surprising degree of homeostasis: despite a constant flow of digested content, the bacterial populations within the colon maintain high species richness at stable densities (Leser and Mølbak, 2009). However, there is complex interplay between microbes, the host, and the diet. In a study of 116 individual captive and exotic animals belonging to 54 mammalian species, Ley et al. (2008) demonstrated that the microbiota clustered by host order, gut morphology, diet, and fiber index, regardless of habitat or geographic location. Therefore, a change in fermentation activity could feasibly arise from an alteration in the community makeup, a variation in the proportions of pre-existing species, or a modification in the phenotypic expression of a constant population in response to a novel substrate (Torok et al., 2008).

Characterization of the GI microflora was originally accomplished by plating fecal or intestinal samples on agar, and identifying and quantifying resultant colonies. The technique did not support growth of anaerobic bacteria—which are estimated to reach 10^4-10^6/g of digesta in humans, and 10^{10}-10^{11}/g of rumen fluid—meaning only 0.001% to 15% of the microorganisms from a population may actually have been cultured from samples (Stevens and Hume, 1998; Hill et al., 2002). Even species able to grow in aerobic environments may be sensitive to antibiotics or other selection agents added to the media (Chaban et al., 2009). Additionally, results were biased toward *E. coli* and other bacterial species that grow

exceptionally well in nutrient agar—and against the gram-negative species that do not (Stevens and Hume, 1998; Chaban et al., 2000).

The identification of many bacteria remains restricted by the lack of knowledge of appropriate conditions needed to culture anaerobic and other species (Torok et al., 2008). Metagenomic studies target microbial communities in the environment, using molecular methods to overcome the problems presented by anaerobic culturing (Desai et al., 2009). Recent molecular studies of gastrointestinal populations in poultry, for example, have targeted bacterial DNA to yield valuable insight to microbial community composition (Torok et al., 2008). Such knowledge can be applied to increase production in industry settings, or to improve the health and nutrition of conserved species kept in captive facilities. Direct extraction methods, cloning and analysis provide phylogenetic and functional information on cultural microbes as well as those that, as yet, remain unculturable (Leser and Mølbak, 2009).

Molecular technologies have been used extensively to characterize bacterial community compositions in the GI tracts of several species (Wei et al., 2007). Distinctive microbial populations have been associated with the gender, diet, and species of their host, and with specific locations along the GI tract. For example, the bacteria associated with the mucus layer differ from those of the lumen across host species, and microflora appear to be host specific and subject to host-related factors of control—though, due to the difficulties involved in sampling their locations, the populations are not well characterized (Leser and Mølbak, 2009). The ratio of anaerobic:aerobic bacterial species increased in chimpanzees fed high-fiber diets, and, in general, the ratios are higher for individuals in the wild (Benno et al., 1987). For all animals studied, the endosymbiotic classes detected and their relative abundances have been found to vary over time—and many taxa identified in the macaque intestine were not previously known to exist there (McKenna et al., 2008).

The composition of fecal microbiota is known to vary widely through life, as well as with diet and in various disease states (Hill et al., 2002). Although there is evidence of changing microbial populations among individuals within a species, limited research has been done to investigate the effects of feeding strategies on microbial populations across species. While the diets consumed by different animals can vary widely, Watkins et al.

(2010) showed that even when (vegan) humans and great apes consume a similar diet, the levels of phytanic acid and other microbial products of fermentation differ significantly. Their study suggests that, while diet does play a role in influencing microbial populations and digestive activity, humans and animal species also have inherent microbial communities which provide specific types and levels of fermentation products to meet the hosts' energetic needs (Watkins et al., 2010).

A critical limitation in assessing microbial diversity is the accurate isolation and characterization of microbial DNA which represent internal populations. Animal models are often sacrificed to provide access to samples from GI-tract regions which fecal samples cannot accurately represent, but this technique is not feasible for exotic animals in captivity (Leser and Mølbak, 2009). Fortunately, while the gut section of origin has been shown to significantly impact microbial population dynamics, no significant diet-associated differences were found within the upper small intestine by Torok et al. (2008). Assuming the same holds true among monogastric mammals, the same principles should apply: that the hindgut will experience the highest degree of dietary effect, making fecal samples a more accurate representation of the gut flora of interest. While fecal microbes are not a perfect model for gastrointestinal populations, the non-invasive technique is invaluable when studying protected animals (Kisidayova et al., 2009).

Several analytical approaches have been used to characterize microbial populations. DGGE and temperature gradient gel electrophoresis (TGGE) separate DNA fragments based on their structural properties—denaturing characteristics and annealing temperatures, respectively—and restriction fragment length polymorphism analysis separates homologous DNA samples based on differences in the locations of restriction enzyme sites (Kisidayova et al., 2009; Nakamura et al., 2009). Bacteria can be classified rapidly by their unique banding patterns in the gel, which provide a visual fingerprint for each of the microbial types found in complex populations. Nakamura et al. (2009) used the technique to study and compare the microflora associated with sooty mangabeys and Hamadryas baboons (Figure 1), and other researchers have employed the technique for similar applications.

While the prior molecular approaches can be performed quickly, these methods for comparing population dynamics cannot identify individual species as is done in DNA sequencing (Hill et al., 2002). To date, identification strategies have focused on detecting a single species or subspecies, detecting an entire genus, or detecting a subset of species while excluding others; but cloning allows the analysis of complex communities (Chaban et al., 2009), making this the optimal technique for sampling and characterizing the microbiomes in several mammalian species.

Prior to the extraction of microbial DNA, storing samples at -80°C ensures that DNA degradation will be kept to a minimum (Chaban et al., 2009; Claesson et al., 2009; Desai et al., 2009; Nakamura et al., 2009). The Qiagen QIAamp DNA stool kit has been used to extract microbial DNA from feces of several species including humans, domestic pigs (*Sus scrofa domesticus)*, chickens (*Gallus gallus domesticus),* cows (*Bos taurus)*, dogs (*Canis familiaris)*, mountain gorillas (*Gorilla gorilla gorilla),* giant pandas *(Ailuropoda melanoleuca)*, rhesus macaques (*Macaca mulatta*), baboons (*Papio hamadryas)*, and sooty mangabeys (*Cercocebus atys)* (Chaban et al, 2009; Claesson et al, 2009; Li et al, 2003; McKenna et al, 2008; Nakamura et al, 2009; Nsubuga et al, 2004; Yu and Morrison, 2004; Wei et al, 2007).

The two most commonly used gene regions for bacterial species identification are the *16S r*RNA- and *cpn60*-encoding sequences. While the 1.6kb *16S* gene is widely accepted as the conventional region of choice, *cpn60*'s shorter length (549-567bp), variability and high resolution have gained scientific interest in recent years (Hill et al., 2002; Hill et al., 2006; Chaban et al., 2009).

Also known as *HSP60* or *GroEL*, the *cpn60* gene—so named because it encodes a 60-kDa chaperonin protein—is ubiquitous among Eubacteria. The essential and highly conserved sequence displays moderate diversity, making the gene useful in taxonomic studies of bacteria (Maynard et al., 2005). Universal primers are available for amplification, and its use has been well-established for analyzing the phylogenies of complex samples: the cpnDB is the second largest database available to the public (Schellenberg et al., 2009; Masson et al., 2006; Hill et al., 2006; Chaban et al., 2009). While the *cpn60* collection is not

as extensive as GenBank®, limiting its comparative reference capability, the pitfall is a small tradeoff compared to the high discriminatory power of the gene. Furthermore, the cpnDB is updated regularly and often, and its content is included in GenBank®, and thus accessible through all GenBank® searches.

*16S r*RNA-encoding DNA sequences have a high chance of generating multiple sequence matches in the *r*RNA database. Conversely, the shorter *cpn60* sequence is more likely to have fewer and more accurate matches (Schellenberg et al., 2009). The overall variability of the chaperonin-60 target gene is similar across bacterial species and distributed uniformly along the entire length of the sequence. The *16S r*RNA gene, however, is more highly conserved: even the full-length of a *16S r*RNA gene sequence does not always have a sufficient resolving power to confidently identify the bacterial species of origin (Claesson et al., 2009). Complete segments from different genera may be over 97% similar, while sequences from within the same species—or genome—may show less similarity to one another. The inability to distinguish highly similar sequences can limit the resolution power of *16S r*RNA to identification at the family level, as in the McKenna et al. (2008) study of the macaque GI microbiome (Figure 2). By contrast, the increased diversity of *cpn60* makes it more phylogenetically informative and thus more useful in differential analysis of complex samples (Hill et al., 2002; Maynard et al., 2005; Masson et al., 2006; Desai et al., 2009; Olson et al., 2010).

Schellenberg et al. (2009) studied the vaginal microbiome using pyrosequencing, a method that detects the pyrophosphate released during nucleotide incorporation and thus uses synthesis to determine the order of nucleotides within the sequence of interest. For each individual sample analyzed, the *cpn60* universal target (UT) resulted in nearly complete sampling of the taxonomic richness, while *16S* resolution was limited for closely related species (Schellenberg et al., 2009). Use of the latter may give an incomplete assessment of complex microbiota composition (Claesson et al., 2009). Phylogenetically, similar trends have been observed when *cpn60*-based identification was used, compared to the *16S r*RNA target. However, an increased intraspecies diversity was seen for chaperonin 60: for example, 11 *cpn60* alleles were detected for *Streptococcus intermedius* in the Olson et al. (2010) cystic

fibrosis study of human sputum samples, while *16S* had only a single allele, making it less discriminating. Currently, most exotic animals' GIT microflora remain uncharacterized, much less compared across taxa. The present lack of knowledge makes a gene region of increased identification capability, such as *cpn60*, highly desirable to glean maximum insight to the gut populations.

The high sensitivity and specificity make polymerase chain reaction (PCR) the most commonly employed tool used for molecular studies (Maynard et al., 2005); however, detection of the gastrointestinal constituents using PCR techniques may be limited. Standardized pooling of DNA samples is both cost- and time- effective, but detection is biased toward the target of highest abundance observed in any individual included in the pool (Desai et al., 2009). While biochemical identification—based on structural and functional characteristics—is more definite than phenotyping—rooted in physical traits (Chaban et al., 2000)—Wei et al. (2007) found that the bacterial composition differed between fecal samples and locations within the digestive tract. This discrepancy may, in part, be due to the concentration-time (CoT) effect, whereby rare bacteria are overrepresented while the abundant species are under-represented, yielding a distortion in the actual relative abundances (Hill et al., 2002; Desai et al., 2009).

Studies of the porcine fecal microflora have shown that the dominant bacterial species is present in numbers at least an order of magnitude greater than the next most prevalent species, and that culturable organisms are retrieved in abundances that vary over several orders of magnitude (Hill et al., 2002). For example, when 1,125 clones were analyzed in the Hill et al. experiment (2002), a total of 398 unique sequences were identified. Therefore, if small proportions of an original sample are tested, less abundant species may be missed (Chaban et al, 2000). As our project aims to identify the major players within endangered, threatened, or vulnerable host species, amplicons from pooled non-invasive samples were deemed the most appropriate substrate for measurement and a total of 384 colonies per host species were collected to represent those dominant constituents in spite of normalization. PCR artifacts could also be a potential source of error; but since most of the sequence variation within observed clusters of related clones resulted from a change in the third codon

position, the Hill et al. (2002) study concluded that the dissimilarities reflected real biological diversity.

The pGEM-T Easy Vector Kit is widely used to transform a PCR product into competent *E. coli* for mass production by the cells, which are plated on LB agar with X-galactose (Wei et al, 2007; Chaban et al., 2009; Desai et al., 2009). Successful ligation of an insert interrupts the *lacZ* gene, resulting in white clones which are easily distinguished from their blue counterparts (Wei et al., 2007; Nakamura et al., 2009). Ligation efficiency can be increased by an ethanol precipitation reaction, prior to transformation, to increase the number of white clones per reaction (Zhu and Dean, 1999). Cloning PCR products may introduce biases to the data set, since colonies containing inserts of different sizes may not have equal growth success on selection plates (Schellenberg et al, 2009). To avoid this issue, PCR products can be gel-purified using the QIAquick purification kit (Qiagen), and only products of the desired length—in our case, between 500-700bp—are used in the cloning reaction (Maynard et al., 2005; Masson et al., 2006; McKenna et al., 2008; Desai et al., 2009; Kisidayova et al., 2009).

Dye-terminator sequencing reactions are commonly used to fluorescently tag nucleotide bases on PCR fragments (Maruyama et al., 2009). The labeled extension products are then injected into a denaturing flowable polymer and separated using capillary electrophoresis based on size and total charge. The fragments pass across a laser beam, causing the dyes on the terminal nucleotides to fluoresce, and the bases are detected in the same order as the template to determine the original sequence (Applied Biostystems). Resulting gene sequences are trimmed for quality and to delete vector overhangs in processing software, yielding an output file in FAST-All (.FASTA) format (Hill et al., 2002; Hill et al., 2006). The FASTA file is used to generate a succession of alignments between each pair of sequences within the dataset. The pairwise alignments are achieved with dynamic programming, which calculates the mathematically optimal alignment for a set of sequences using a given table of scores for nucleotide matches and mismatches and insertion/deletion penalties and compiles a distance matrix from the numeric results (Thompson et al., 1994; Chenna et al., 2003). The Neighbor-Joining method calculates

branch-lengths from the distance matrix which are proportional to the estimated divergence along every branch, and an unrooted guide tree is produced to cluster the sequences in a way that minimizes the sum of the branch lengths (Thompson et al., 1994; Chenna et al., 2003). The pairwise alignments are then progressively incorporated into a multiple alignment which follows the guide tree branching order and comprises all sequences in the original dataset (Thompson et al., 1994; Chenna et al., 2003).

Aligned sequences are compared to identify duplicate PCR products. One representative sequence can be used to represent each set of redundant amplicons to avoid false inflation of sequence abundance (Hill et al., 2002). Each unique sequence (or representative of a unique PCR product) is compared to an online nucleotide database using the Basic Local Alignment Search Tool (BLAST) in order to identify its nearest neighbor among previously isolated sequences (Hill et al., 2002; Wei et al., 2007; Desai et al., 2009; Kisidayova et al., 2009; Olson et al., 2010) found in the GenBank nucleotide database at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=Nucleotides). Sequences whose closest match is an as yet uncultured bacterium clone are then grouped with the next nearest neighbor result that is known at the genus and species level.

Sequence identities are grouped into operational taxonomic units (OTUs) of unique species, and the sequence with the highest BLAST score from each OTU is chosen as a representative of that OTU, to build a phylogenetic tree in a software package such as the PHYlogeny Inference Package (PHYLIP) (Figure 3) (Hill et al., 2002; Hill et al., 2006; Desai et al., 2009; Olson et al., 2010). For lack of complete taxonomic characterization of uncultured bacteria, phylogenetic trees allow those clones' relatedness to be visualized until all bacterial species can be classified. Known identities retrieved from BLAST can also be used to check PHYLIP's accuracy of sequence placement within trees.

While PHYLIP provides a method for visually comparing the evolutionary relatedness between isolated bacteria, the software does not provide a method for quantitative comparison of the microflora across mammalian species. Diversity indices, however, are widely used to numerically describe population dynamics in ecological studies of any scale—

and are especially useful in describing complex microbial populations (Martin, 2002; Johnson et al., 2009; Tap et al., 2009). Species richness (S) is the total number of species observed in a sample, and describes the spread of organisms represented. Simpson's Diversity Index (D) measures the probability that two individuals randomly sampled from the population of interest will belong to the same species, based on the abundance of each species (n) and the total number of organisms present in the sample (N) (Johnson et al., 2009; Tap et al., 2009). A high value of D equates to low diversity; however, the complement of Simpson's Index (1-D) is often reported to avoid confusion. Simpson's Complement reports the probability that two randomly selected individuals will belong to different species, and the value increases with increasing diversity (Equation 2) (Johnson et al., 2009).

$$1 - D = \frac{\Sigma\left(n\left(n-1\right)\right)}{N\left(N-1\right)} \qquad \textbf{Equation 2.}$$

The Shannon-Weaver Diversity Index (H') measures the total biodiversity as a result of both species richness (S) and evenness, using the abundance of each species (p) (Equation 3) (Martin, 2002; Johnson et al., 2009).

$$H' = -\sum_{i=1}^{S}\left(p_i \ln p_i\right) \qquad \textbf{Equation 3.}$$

Evenness (J') compares the relative abundance of each species—that is, it shows how evenly each organism is represented within the sample (see Equation 4) (Johnson et al., 2009).

$$J' = \frac{H'}{H_{\max}} = \frac{H'}{\ln(S)} \qquad \textbf{Equation 4.}$$

As one of the most complex microbial populations known to man, the gastrointestinal microbiome has yet to be fully characterized or understood. The microbiota also has a profound impact on host health and nutrition, making it a subject of interest for human and animal researchers alike. By identifying the bacterial constituents and quantifying their relationships with one another and the host, we can further our comprehension of a vital enterosymbiosis. Resulting knowledge will have huge ramifications for medical and dietary management, for humans and in animal husbandry.

Recent studies indicate that GI microbial diversity is influenced by both the diet and phylogeny of the host, and that there are is higher similarity of bacterial community structures found in mammals within the same species than in mammals of different species (Leser and Mølbak, 2009). McKenna et al. (2008) also found that microbiota clustered by species among humans, macaques, rhesus monkeys, and mice, despite the diversity within each host species; and Watkins et al. (2010) suggest that to some extent these unique microbial populations are inherent to their hosts, regardless of dietary effects.

This project aims to determine microbial fermentation and genetic profiles for comparison between primate and carnivore species which occupy nutritional niches across the spectrum of omnivorous feeding strategies. *Gorilla gorilla, Papio hamadryas, Pan troglodytes* were selected to represent three distinct strategies employed by primates; and *Arctictis binturong* serves as a valuable comparison with *P. troglodytes*, with respect to the impact that GIT morphology may have when two species occupy a similar niche. Based on previous findings, we expect to find distinct endosymbiotic communities associated with phylogenies, feeding strategies, and fermentation activities.

LITERATURE CITED

Benno, Y., K. Itoh, Y. Miyao, T. Mitsuoka. 1987. Comparison of fecal microflora between wild Japanese monkeys in a snowy area and laboratory-reared Japanese monkeys. *Japanese Journal of Veterinary Science* 49(6): 1059-1064.

Bergman, E. N. 1990. Energy contributions of short chain fatty acids from the gastrointestinal tract in various species. *Physiological Reviews* 70(2): 567-590.

Chaban, B., K. M. Musil, C. G. Himsworth, J. E. Hill. 2009. Development of *cpn60*-based real-time quantitative PCR assays for the detection of 14 *Campylobacter* species and application to screening of canine fecal samples. *Applied and Environmental Microbiology* 75(10): 3055-3061.

Chenna, R., H. Sugawara, T. Koike, R. Lopez, T. J. Gibson, D. G. Higgins, J. D. Thompson. 2003. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Research* 31(13):3497-3500.

Claesson, M. J., O. O'Sullivan, Q. Wang, J. Nikkila, J. R. Marchesi, H. Smidt, W. M. de Vos, R. Paul Ross, P. W. O'Toole. 2009. *PLoS ONE* 4(8): e6669.

Clemens, E., B. Phillips. 1980. Organic acid production and digesta movement in the gastrointestinal tract of the baboon and Sykes monkey. *Comparative Biochemistry and Physiology* 66A: 529-532.

Costa, M. A., T. Mehta, J. R. Males. 1989. Effects of dietary cellulose and psyllium husk on monkey colonic microbial metabolism in continuous culture. *Journal of Nutrition* 119: 979-985.

Desai, A. R., K. M. Musil, A. P. Carr, J. E. Hill. 2009. Characterization and quantification of feline fecal microbiota using *cpn60* sequence-based methods and investigation of animal-to-animal variation in microbial population structure. *Veterinary Microbiology* 137: 120-128.

Dierenfeld, E. S. 2003. Viverrid digestive physiology: comparison of binturongs (*Arctictis binturong*) and dwarf mongoose (*Helgale parvula*). *Proceeings of the Conference of the Nutrition Advidsory Group (NAG) of the American Zoo and Aquarium Association (AZA) on Zoo and Wildlife Nutrition:* 52.

Felton, A. M., A. Felton, D. B. Lindenmayer, W. J. Foley. 2009. Nutritional goals of wild primates. *Functional ecology* 23: 70-78.

Frey, J. C., J. M. Rothman, A. N. Pell, J. B. Nizeyi, M. R. Cranfield, E. R. Angert. 2006. Fecal bacterial diversity in a wild gorilla. *Applied and Environmental Microbiology* 72(5): 3788-3792.

Grassman, Jr., L. I., M. E. Tewes, N. J. Silvy. 2005. Ranging, habitat use and activity patterns of binturong *Arctictis binturong* and yellow-throated marten *Martes flavigula* in north-central Thailand. *Wildlife Biology* 11(1): 49-57.

Henneberg, M., V. Sarafis, K. Mathers. 1998. Human adaptations to meat eating. *Human Evolution* 13(3-4): 229-234.

Hill, J. E., R. P. Seipp, M. Betts, L. Hawkins, A. G. Van Kessel, W. L. Crosby, S. M. Hemmingsen. 2002. Extensive profiling of a complex microbial community by high-throughput sequencing. *Applied and Environmental Microbiology* 68(6): 3055-3066.

Hill, J. E., A. Paccagnella, K. Law, P. L. Melito, D. L. Woodward, L. Price, A. H. Leung, Lai-King Ng, S. M. Hemmingsen, S. H. Goh. 2006. Identification of *Campylobacter* spp. And discrimination from *Heliobacter* and *Arcobacter* spp. By direct sequencing of PCR-amplified *cpn60* sequences and comparison to cpnDB, a chaperonin reference sequence database. *Journal of Medical Microbiology* 55: 393-399.

Hofmann, R. R. 1989. Evolutionary steps of ecophysiological adaptation and diversification of ruminants: a comparative view of their digestive system. *Oecologia* 78: 443-457.

Johnson, M. C., A. A. Devine, J. C. Ellis, A. M. Grunden, V. Fellner. 2009. Effects of antibiotics and oil on microbial profiles and fermentation in mixed cultures of ruminal microorganisms. *Journal of Dairy Science* 92(9):4467-4480.

Kisidayova, S., Z. Varadyova, P. Pristas, M. Piknova, K. Nigutova, K. J. Petrzelkova, I. Profousova, K. Schovancova, J. Kamler, D. Modry. 2009. Effects of high- and low-fiber diets on fecal fermentation and fecal microbial populations of captive chimpanzees. *American Journal of Primatology* 71: 548-557.

Lambert, J. E. 1998. Primate digestion: interactions among anatomy, physiology, and feeding ecology. *Evolutionary Anthropology* 7(1): 8-20.

Lambert, J. E. (2007). The biology and evolution of ape and monkey feeding. In Henke W., Rothe H., Tattersall I. (eds.), *Handbook of Anthropology Vol II*

(pp. 1207-1234). New York NY: Springer-Verlag.

Lambert, J. E. (2010). Primate Nutritional Ecology: feeding biology and diet at ecological and evolutionary scales. In Campbell C., Fuentes A., MacKinnon K., Bearder S., Stumpf R. (eds.), *Primates in Perspective, 2nd Edition*. Oxford University Press, Oxford, UK.

Lambert, J.E., V. Fellner. In press. *In vitro* fermentation of dietary carbohydrates consumed by African apes and monkeys: preliminary results for interpreting microbial and digestive strategy. *International Journal of Primatology.*

Leser, T. D., L. Molbak. 2009. Better living through microbial action: the benefits of the mammalian gastrointestinal microbiota on the host. *Environmental Microbiology* 11(9): 2194-2206.

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

Li, M., J. Gong, M. Cottrill, H. Yu, Cl de Lange, J. Burton, E. Topp. 2003. Evaluation of QIAamp® DNA Stool Mini Kit for ecological studies of gut microbiota. *Journal of Microbiological Methods* 54(1): 13-20.

Masson, L., C. Maynard, R. Brousseau, S. Goh, S. M. Hemmingsen, J. E. Hill, A. Paccagnella, R. Oda, N. Kimura. 2006. Identification of pathogenic *Heliobacter* species by chaperonin-60 differentiation on plastic DNA arrays. *Genomics* 87: 104-112.

Maynard, C. F. Bethiaume, K. Lemarchand, J. Harel, P. Payment, P. Bayardelle, L. Masson, R. Brousseau. 2005. *Applied and Environmental Microbiology* 71(12): 8548-8557.

Martin, A. P. 2002. Phylogenetic approaches for describing and comparing the diversity of microbial communities. *Applied and Environmental Microbiology* 68(8):3674-2682.

Maruyama, F., T. Nozawa, C. Aikawa, A. Sakurai, I. Nakagawa. 2009. Cost effective DNA sequencing and template preparation from bacterial colonies and plasmids. *Journal of Bioscience and Bioengineering* 107(4):471-473.

McKenna, P., C. Hoffmann, N. Minkah, P. P. Aye, A. Lackner, Z. Liu, C. A. Lozupone, M. Hamady, R. Knight, F. D. Bushman. 2008. The macaque

gut microbiome in health, lentiviral infection, and chronic enterocolitis. *PLoS Pathogens* 4(2): e20.

Mudappa, D., Kumar, A. and Chellam, R. 2010. Diet and fruit choice of the brown palm civet *Paradoxurus jerdoni*, a viverrid endemic to the Western Ghats rainforest, India. *Tropical Conservation Science* Vol. 3 (3):282-300.

Nakamura, N., S. R. Leigh, R. I. Mackie, H. R. Gaskins. 2009. Microbial community analysis of rectal methanogens and sulfate reducing bacteria in two non-human primate species. *Journal of Medical Primatology* 38: 360-370.

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

Olson, A. B., C. D. Sibley, L. Schmidt, M. A. Wilcox, M. G. Surette, C. R. Corbett. 2010. Development of real-time PCR assays for detection of the *Streptococcus milleri* group from Cystic fibrosis clinical specimens using targets for *cpn60* and 16S rRNA genes. *Journal of Clinical Microbiology* 48(4): 1150-1160.

Paliy, Ol., H. Kenche, F. Abernathy, S. Michail. 2009. High-throughput quantitative analysis of the human intestinal microbiota with a phylogenetic microarray. *Applied and Environmental Microbiology* 75(11):3572-3579.

Raubenheimer, D., S. J. Simpson, D. Mayntz. 2009. Nutrition, ecology and nutritional ecology: toward an integrated framework. *Functional Ecology* 23: 4-16.

Rosalina L. M., M. Santos-Reis. 2009. Fruit consumption by carnivores in Mediterranean Europe. *Mammal Review* 39: 67-78.

Rothman J. M., E. S. Dierenfeld, H. F. Hintz, A. N. Pell. 2008. Nutritional quality of gorilla diets: consequences of age, sex, and season. *Oecologia* 115: 111-122.

Schellenberg, J., M. G. Links, J. E. Hill, T. J. Dumonceaux, G. A. Peters, S. Tyler, T. B. Ball, A. Severini, F. A. Plummer. 2009. Pyrosequencing of the chaperonin-60 (*cpn60*) universal target as a tool for determining the

composition of microbial communities. *Applied and Environmental Microbiology* 75(9): 2889-2898.

Shanahan, M., S. So, S. G. Compton, R. Corlett. 2001. Fig-eating by vertebrate frugivores: a global review. *Biological Reviews of the Cambridge Philosophical Society* 76(4): 529-572.

Stevens, C. E., and I. D. Hume. 1998. Contributions of microbes in vertebrate gastrointestinal tract to production and conservation of nutrients. *Physiological Reviews* 78(2): 393-427.

Swedell, L., G. Hailemeskel, A. Schreier. 2008. Composition and seasonality of diet in wild Hamadryas baboons: preliminary findings from Filoha. *Folia Primatol* 79:476-490. doi:10.1159/000164431.

Tap, J., S. Mondot, F. Levenez, E. Pelletier, C. Caron, J. Furet, E. Ugarte, R. Munoz-Tamayo, D. Paslier, R. Nalin, J. Dore, M. Leclerc. 2009. Towards the human intestinal microbiota phylogenetic core. *Environmental Microbiology*. doi:10.1111/j.1462-2920.2009.01982.x

Thompson, J. D., D. G. Higgins, T. J. Gibson. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22(22):4673-4680.

Torok, V. A., K. Ophel-Keller, M. Loo, R. J. Hughes. 2008. Application of methods for identifying broiler chicken gut bacterial species linked with increased energy metabolism. *Applied and Environmental Microbiology* 74(3): 783-291.

Tutin, C. E. G., M. Fernandez, M. E. Rogers, E. A. Williamson, W. C. McGrew, S. A. Altmann, D. A. T. Southgate, I. Crowe, C. E. G. Tutin, A. Whiten, N. L. Conklin, L. Barrett. 1991. Foraging profiles of sympatric lowland gorillas and chimpanzees in the Lope Reserve, Gabon [and Discussion]. *Philosophical Transactions: Biological Sciences* 334(1270):179-186.

Van Soest, P. J., J. B. Robertson, B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science* 74: 3583.

Van Valkenburgh, B. 2007. *Déjà vu:* the evolution of feeding morphologies in the Carnivora. *Integrative and Comparative Biology* 47(1): 147-163.

Watkins, P. A., A. B. Moser, C. B. Toomer, S. J. Steinberg, H. W. Moser, M. W. Karaman, K. Ramaswamy, K. D. Siegmund, D. R. Lee, J. J. Ely, O. A. Ryder, J. G. Hacia. 2010. Identification of differences in human and great ape phytanic acid metabolism that could influence gene expression profiles and physiological functions. *BMC Physiology* 10:19. Doi:10.1186/1472-6793-10-19.

Wei, G., H. Lu, Z. Zhou, H. Xie, A. Wang, K. Nelson, L. Zhao. 2007. The microbial community in the feces of the Giant Panda (*Ailuropoda melanoleuca*) as determined by PCR-TGGE profiling and clone library analysis. *Microbial Ecology* 54: 194-202.

Wireman, J., M. Lowe, A. Spiro, Y. Zhang, A. Sornborger, A. O. Summers. 2006. Quantitative, longitudinal profiling of the primate fecal microbiota reveals idiosyncratic, dynamic communities. *Environmental Microbiology* 8(3): 490-503.

Wolin, J. J., T. J. Miller, S. Yerry, Y. C. Zhang, S. Bank, G. A. Weaver. 1999. Changes in fermentation pattern of fecal microbial communities associated with a drug treatment that increases dietary starch in the human colon. *Applied and Environmental Microbiology* 65: 2807-2812.

Yu, Z., M. Morrison. 2004. Improved extraction of PCR-quality community DNA from digesta and fecal samples. *BioTechniques* 36:808-812.

Zhu, H., and R. A. Dean. 1999. A novel method for increasing the transformation efficiency of *Escherichia coli*—application for bacterial artificial chromosome library construction. *Nucleic Acids Research* 27(3):910-911.

Table 1. Ratio of intestine to body length for mammalian species (Henneberg et al., 1998; Carpo et al., 2002; Wei et al, 2007).

Genus species	**Common name**	**Feeding strategy**	**Intestine:Body**
Bos Taurus	domestic cow	Herbivore	20
Equus ferus caballus	domestic horse	Herbivore	12
Papio hamadryas	baboon	Omnivore	8
Canis familiaris	domestic dog	Omnivore	6
Homo sapiens	human	Omnivore	5
Felis catus	domestic cat	Carnivore	4
Ailuropoda melanoleuca	giant panda	Herbivore	4
Arctictis binturong	binturong	Frugivore	2

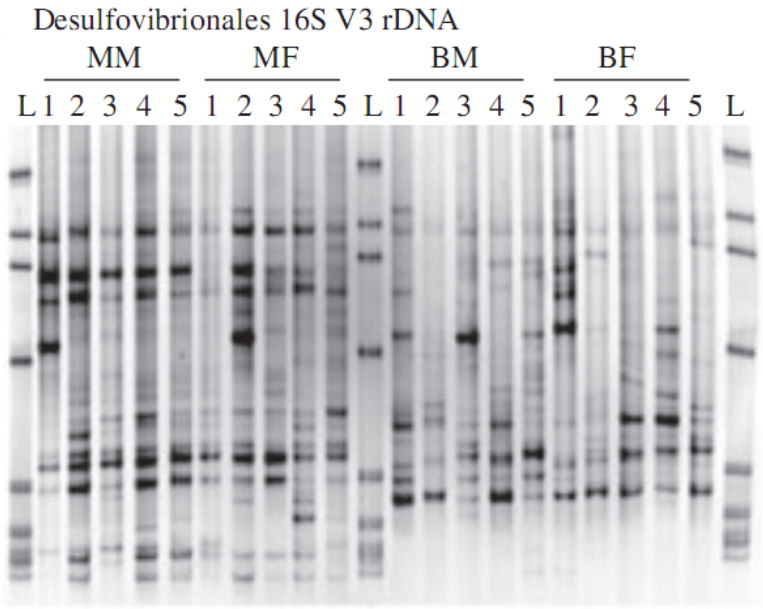


Figure 1. Denaturing gradient gel electrophoresis (DGGE) profiles of Desulfovibrionales 16S V3 rDNA obtained from mangabeys (MM: male, MF: female) and baboons (BM: male, BF: female). Numbers in each lane indicate different animals. L represents ladder (Nakamura et al, 2009).

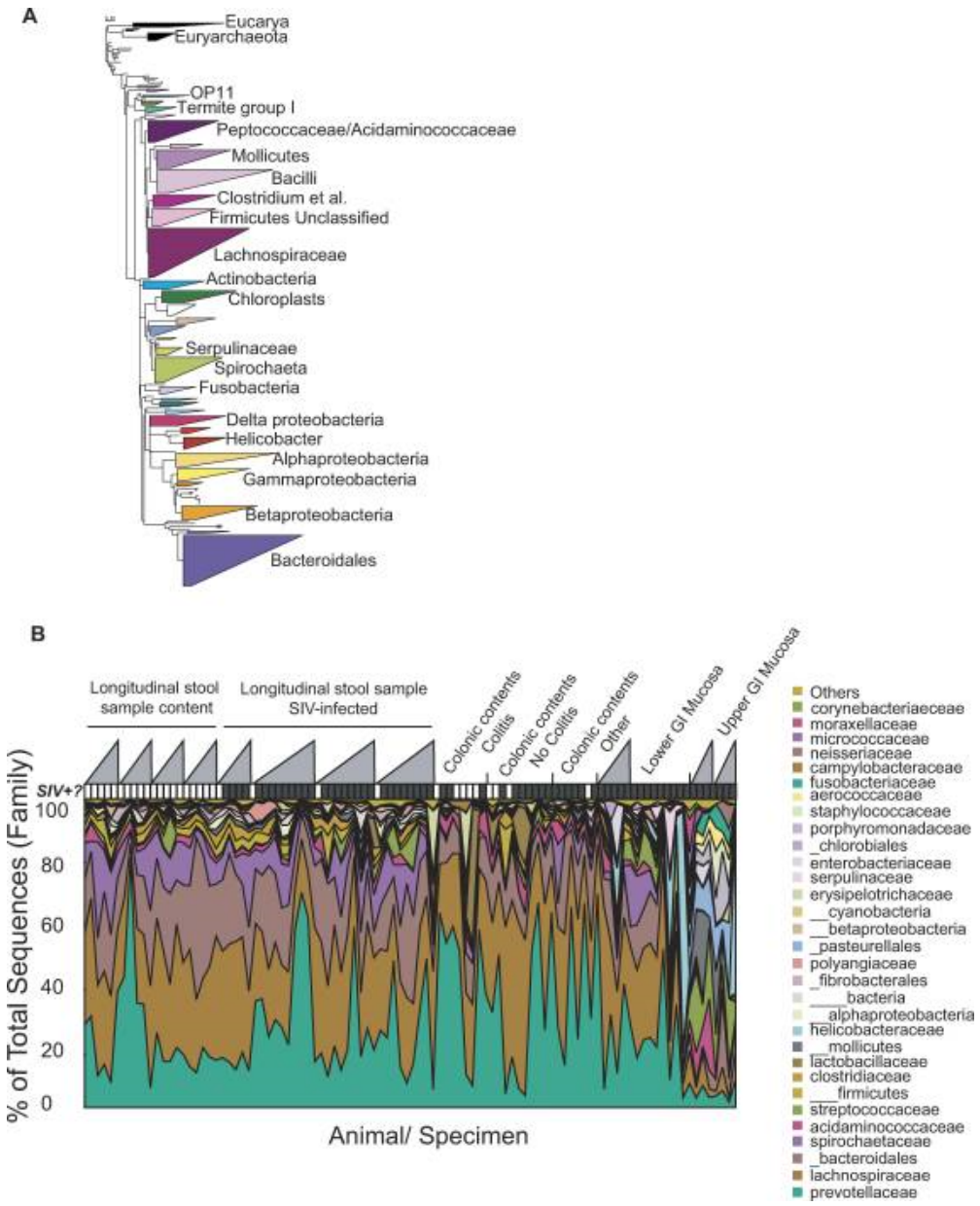


Figure 2. Bacteria composing the macaque GI microbiome: (A) Bacterial taxa identified from pyrosequencing data after alignment with the ARB 16S rRNA gene database. The size of each triangle indicates the relative number of OTUs within each taxa (100% identity threshold). (B) Summery of the bacterial taxa present in each gut community sampled, indicating the individual and temporal variation in the macaque GI microbiota. Taxa corresponding to bacterial phyla are indicated with the triple underscore before the name, classes by a double underscore, orders by single underscores, and families by no underscore.

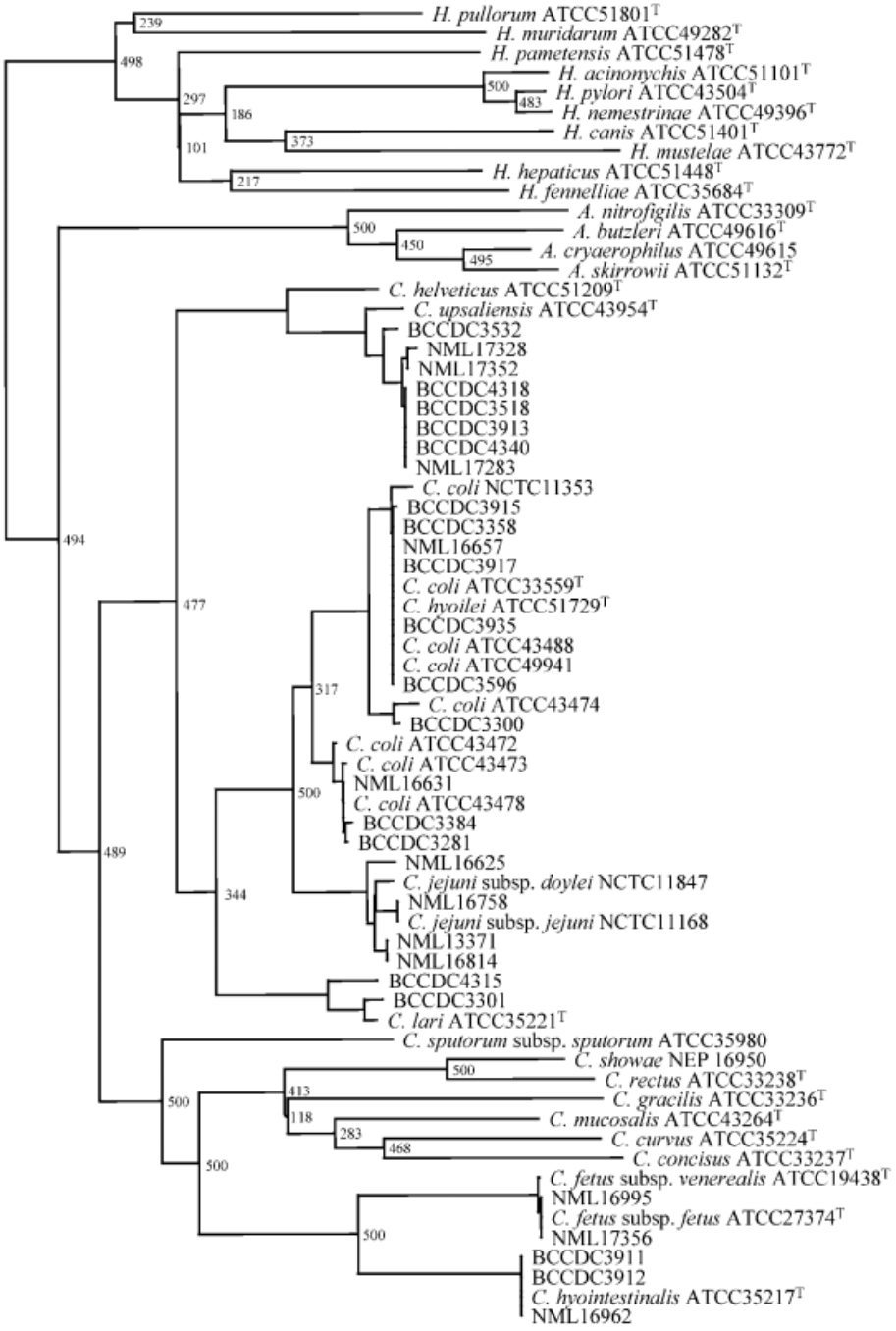


Figure 3. Inferred phylogenetic relationships of *Campylobacter, Heliobacter* and *Arcobacter* strains based on partial *cpn60* sequences. The tree is a consensus of neighbor-joined trees, with bootstrap values indicated at branch-points (Hill et al., 2006).

CHAPTER 2:
THE EFFECTS OF PHYLOGENY ON THE CHARACTERISTICS OF THE MICROBIOME ASSOCIATED WITH PRIMATE AND CARNIVORE SPECIES

ABSTRACT

Fecal samples were collected from 3 gorillas (*Gorilla gorilla*), 12 chimpanzees (*Pan troglodytes*), 17 Hamadryas baboons (*Papio hamadryas*), and 10 binturongs (*Arctictis binturong*) to characterize and compare the GIT microflora associated with each host species. Each species was fed a different, fixed, standardized diet for two weeks prior to collection. Fresh fecal samples were pooled within species and blended with buffer solution; the inoculums were added to 1g of the appropriate ground dietary substrate, sealed and incubated at 37°C. Methane concentration and SCFA were measured after 24 hours, and all fermentation products were analyzed using the Proc Mixed procedure in SAS. Microbial DNA was extracted from individual fecal samples frozen at -70°C, and combined into standardized pools for each host species. A region of the bacterial *cpn60* gene was sequenced to identify microbial species by comparison with the GenBank database, and biodiversity indices were calculated to compare the composition of microbial populations across host species. Gene libraries were aligned in Clustal X, and phylogenetic trees were compiled using the PHYlogeny Inference Package (PHYLIP). Significant differences were observed across species for short chain fatty acid ($p<0.0001$) and methane ($p<0.001$) production, as well as neutral detergent fiber ($p<0.05$) and dry matter ($p=0.0001$) digestibility. Molecular analysis suggests that microbial biodiversity increases with dietary fiber and the complexity of nutritional niches *in situ*. This link between patterns of energy usage and host-specific endosymbionts is consistent with previous findings in humans and non-human primate species.

INTRODUCTION

The vertebrate gut is home to a vast community of bacteria. The host immune system modulates the composition of the population, cultivating mutualistic endosymbionts that help meet host energy requirements (McKenna et al., 2008; Watkins et al., 2010). At densities of 10^{11}-10^{12}/mL, the digestive tract is among the most highly concentrated microbial habitats known to man (Stevens and Hume, 1998; Leser and Molbak, 2009). Imbalance of the microbiota has been linked to host disease states in humans and non-human primates (McKenna et al., 2008; Torok et al., 2008; Claesson et al., 2009; Desai et al., 2009), and microbes living in the gastrointestinal tract (GIT) directly impact host health and nutrition across mammalian taxa (Stevens and Hume, 1998; Leser and Mølbak, 2009). Population dynamics of the microflora vary among animals, over time and between hindgut segments within individuals, by gender, and according to feeding strategy (Stevens and Hume, 1998; Hill et al., 2002; McKenna et al., 2008; Torok et al., 2008; Claesson et al, 2009; Desai et al., 2009; Leser and Mølbak, 2009). Ingested food acts as a source of nutrients as well as a vector of disease, making the digestive tract a key location for immune defense as well as normal homeostasis (McKenna et al., 2008).

Carbohydrate metabolism pathways, derived from studies of rumen digestion, have helped to define the bacterial role in digestive processes. Monosaccharides are absorbed directly by the host, and disaccharides may be utilized after hydrolysis; but fibrous polysaccharides must first undergo microbial fermentation. This process provides a source of short chain fatty acids (SCFA)—mainly acetate, propionate, and butyrate—which serve as substrates for ATP-production and gluconeogenesis, and improve the health of forestomach and hindgut epithelia (Lambert, 2010; Bergman, 1990; Stevens and Hume, 1998). Microbes also provide an additional source of amino acids and vitamin B to ruminants and non-ruminants. However, the degree of hindgut absorptive efficiency in monogastrics is unknown, and without coprophagy nutritional contributions to this latter group of animals may be limited (Stevens and Hume, 1998).

The gastrointestinal tract is home to an interactive population of coevolved microflora. All bacterial species ferment a portion of the digesta to produce cellular

components and SCFA which can be used by the host to meet energy requirements (Bergman, 1990). Therefore, quantifying microbial products cannot by itself lend insight to the bacterial species present or proportions thereof. Furthermore, the dietary substrate itself not only promotes the growth of specific populations, but it changes the metabolic activity of microbes and impacts the quantities and nature of fermentation products (Bergman, 1990). This suggests that an animal's diet can also have decided impact on its microflora (Watkins et al., 2010).

In a study of 166 mammalian species, Ley et al. (2008) found that gut microbial populations clustered by animal taxonomy, GI morphology, and diet composition, for captive as well as wild subjects. Digestive physiology encompasses an animal's evolutionary past and its environmental present (Hofmann, 1989). Conservation efforts must take both phylogeny and ecology into account when managing exotic species in captivity to ensure their future. The characterization of microbial populations, therefore, is of utmost importance in understanding and evaluating animal health. The distinct microflora residing within a host result from that animal's characteristics and living situation, and in turn the bacterial population specifically affects host digestion, nutrition, and well-being.

Recent studies indicate that GI microbial diversity is influenced by both the diet and phylogeny of the host, and that there is higher similarity of bacterial community structures found in mammals within the same species than in mammals of different species (Leser and Mølbak, 2009). McKenna et al. (2008) compared the relatedness of microbial populations isolated from humans, macaques, rhesus monkeys, and mice fed different diets. The microbiota were found to cluster by host species, despite the diversity among and between individuals, and within each host species. Watkins et al. (2010) suggest that to some extent these unique core microbial populations are inherent to their hosts, regardless of dietary effects. This project aims to determine microbial profiles between primate and carnivore species that occupy distinct nutritional niches within the spectrum of omnivorous feeding strategies. We expect to find distinct endosymbiotic communities associated with *Gorilla gorilla* (a folivore that consumes ripe fruits whenever possible)*, Papio hamadryads* (an opportunistic omnivore that eats a wide variety of food items)*, Pan troglodytes* (a

frugivorous primate), and *Arctictis binturong* (a frugivorous carnivore) based on their phylogenies.

MATERIALS AND METHODS

Animals and Experimental Diets

Animals used in this study were either housed at the North Carolina Zoo in Asheboro, NC (baboons, chimpanzees, and gorillas) or at the Carolina Tiger Rescue in Pittsboro, NC (binturongs). The animals' species, genders, and ages are listed in Table 2. All primate species were housed in troops, and samples were collected from indoor holding. Binturongs were housed individually or in pairs, in outdoor enclosures. The enclosure dimensions, temperature were recorded (Table 3). Each species was fed a different diet, formulated to mimic the composition of wild intake with commercially available food items while still delivering complete nutrition. All animals were fed their respective predetermined diets for two weeks prior to sample collection. A representative diet for each species was dried for 48 hours at 55°C, and the components were weighed and recorded (Tables 4 and 5) before being ground to a uniform powder with a Waring blender, to be used as dietary substrate for *in vitro* fermentation.

A total of 42 fecal specimens (one from each individual) were collected within two hours of defecation and sealed in Ziplock® bags labeled with the animal species, donor ID (where possible), and the date. Samples were collected during the summer and fall to minimize the effects of ambient temperature on microbial viability. Gorillas (n=3) and binturongs (n=10) were housed individually, so their samples were labeled by name. Baboons (n=17) and chimpanzees (n=12) were housed in troops, so each fresh sample was numbered for tracking purposes although the individual donor was unknown.

In Vitro Fermentation

Bags containing the fresh feces were placed into pre-warmed vacuum Thermos® flasks and immediately transported to the lab. Upon arrival, equal weights of fresh feces were mixed and 20g of the composited samples from each species were quantitatively mixed in a Waring blender under a constant flow of CO_2 to restore anaerobic conditions. Inoculums

were prepared by diluting 1 part fresh feces with 5 parts of a previously warmed (37°C) media and buffer. Buffer composition matched the formula developed by Sunvold et al (1995). Inoculums were blended for 15s, filtered through four layers of cheesecloth, and added to culture bottles containing 1.0 grams of pre-weighed dietary substrate. Appropriate blank controls contained only the inoculums and no substrate or only substrate and 30 mL of medium (without feces). After being purged with CO_2, bottles were sealed with crimp tops—with a rubber center lining for gas measurements—and placed either on ice (t=0 hours) or in 37°C water baths for 24h. All controls and experimental diets were run in triplicate (k=3).

At the end of 24h, bottles were removed from the water bath and placed on ice to stop microbial activity. Gas samples were withdrawn from the bottle headspace immediately prior to removing the caps, and methane concentration was determined using a CP-3800 gas chromatograph with a Porapak mol sieve column (Varian) according to the methods described by Johnson et al. (2009). Following methane analysis, culture bottles were opened and a 5-mL culture sample was processed for analysis of short chain fatty acids (SCFA) using a Nukol fused silica capillary column on a CP-3380 gas chromatograph (Varian) following the Johnson et al. (2009) methodology. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) in the remaining residue was determined sequentially using an Ankom fiber extractor (Ankom Technologies, Fairport, NY) according to the method of Van Soest et al. (1991) without amylase and sodium sulfite (Table 6).

The Proc Mixed procedure (SAS®, Cary, NC) was used to analyze the SCFA, methane, and digestibility of NDF, ADF, and DM. The model (Equation 5) includes the dependent variables and accounts for fixed phylogenetic and random effects.

$$Y_{ijk} = \mu + A_i + \beta_j + E_{ijk} \qquad \textbf{Equation 5.}$$

In this model, Y_{ijk} represents the variable of interest, μ is the mean of the respective input values, A_i represents fixed host species, β_j is random replicate effect, and E_{ijk} is the random error component.

Genetic Analysis

An aliquot of each fresh fecal specimen was stored at -80°C to prevent DNA degradation until subsequent determination of microbial profiles. Microbial DNA was

extracted individually from each sample using the QIAamp Stool Mini Kit (QIAGEN). The procedure followed the manufacturer's protocol, except for the following modification. Four 2.0-mm zirconia beads were added to 250 mg of stool in a 2-mL beadbeater tube, and shaken on settings time=6 and speed=48 in a Mini-BeadBeater-1 to mechanically lyse samples as described by Zoetendal (2006). DNA concentration and quality was determined using a Nanodrop-1000 and visualized on a 1% agarose gel.

Standardized pools were compiled using equal weights of DNA from the individual samples within each species which yielded high molecular weight (HMW) DNA (all binturongs, chimpanzees, and gorillas; and 10 of 17 baboons). Fifty nanograms of pooled template DNA were added to a PCR reaction with a total volume of 50 µL in a 1.5-mL reaction tube (Table 7). A region of the *cpn60* gene was amplified using the universal PCR primers H279 and H280 designed by Goh et al. (1996).

The gel-purified *cpn60* insert was cloned into the pGEM®-T Easy vector per the manufacturer's protocol (Promega) (Table 8) and transformed into TOP10 OneShot® Electrocomp™ Cells (Invitrogen). Three hundred eighty-four colonies were picked from each library (Figure 4). Plasmid DNA was isolated using the Gentra Puregene Cell Kit (QIAGEN) (Table 9) and sequenced using the M13F (-41) primer and the ABI Big Dye® Terminator v3.1 Cycle Sequencing Kit (Table 10).

Sequences were trimmed using Codon Code Analysis v3.7.1 software to eliminate vector overhangs and measure the quality of bases. Sequences under 50 base pairs were discarded; all other sequences were exported in FASTA format. Sequences were aligned using the ClustalX 2.0 software and compared to identify duplicate PCR products. For each group of redundant sequences, a single sequence was chosen to represent the duplicates to avoid false inflation of diversity counts. The unique sequences remaining from each library were compared to the GenBank® database using the Blastn function (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=Nucleotides). The top hit for each sequence was recorded; for sequences whose closest match was an as yet unidentified bacterium clone (UBC), the *Genus* and *species* of the nearest known neighbor were also recorded.

Representative sequences for every unique clone identity were aligned by species in ClustalX (Chenna et al., 2003). Using the PHYLIP software package (Felsenstein, 2005), the alignments were sampled by Seqboot for random bootstrap analysis to calculate the relatedness of samples based on maximum probability, and the distances between branches were calculated with Dnadist. The Neighbor program constructed a dendogram from the nucleotide distance matrix using the neighbor-joining method for multiple data sets, which minimizes the sum of the branch lengths. The Consense program incorporated all neighbor trees into a single consensus tree, and completed trees for each library were viewed in TreeView 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

All unique sequences were grouped by known *Genus* and *species* into operational taxonomic units (OTUs). The species richness and abundance of each OTU were used to calculate Simpson's Diversity Index (D) (Johnson et al, 2009; Tap et al., 2009), the Shannon-Weaver Index (H') (Johnson et al, 2009; Martin, 2002), and evenness (J') (Johnson et al, 2009) to numerically describe and compare populations between species.

RESULTS

In vitro fermentation

Analysis of SCFA and methane concentration after 24 hours showed distinct fermentation profiles associated with species (Figure 5). No fermentation was observed in fecal samples from *A. binturong* in preliminary studies, while total millimolar SCFA concentration was significant at $p<0.0001$ for all primate species in this experiment (Figure 5A). In baboons the acetate and valerate levels were significant at $p=0.0017$ and $p=0.0256$, respectively. Valerate in gorillas was insignificant ($p=0.0825$). All other SCFA levels were significant at $p<0.0001$. Acetate:propionate ratios were all significant ($p<0.0001$), and were all significantly different from one another ($p=0.0001$) (Figure 5D). Methane concentration in chimpanzees and gorillas was significantly greater than that in baboons ($p<0.0001$) (Figure 6), indicating that the microbes in baboons utilize substrate more efficiently.

Molecular analysis

At least 85% of the 384 white colonies originally isolated from each host species yielded unique sequences longer than 300bp for genetic analysis (Table 11). While this project provides preliminary sequencing data, the clones assigned a known *Genus* and *species* using the Blastn search comprise a diverse taxonomic spread (Table 12) which exhibits strong a strong positive correlation to host dietary ecology.

The phylogenetic trees in Figure 7 comprise all OTUs identified within the *A. binturong*, *P. troglodytes*, *P. hamadryas,* and *G. gorilla* libraries. The majority of bootstrap values (seen at each node) were 50 or higher (out of 100), lending confidence to the calculated tree. Primate collections contained considerably more OTUs due to the greater proportion of uncultured bacteria identified compared with the binturong. The nearest known relative of uncultured clones in the GenBank® database was corroborated by the branch placement next to known species in the Consense tree (PHYLIP).

The taxonomic breakdown of all bacterial clones, based on identification using the Blastn function, and abundance at each level, is shown in Table 13. The shared and unique genera were plotted to show overlap between libraries (Figure 8). The relative abundance of bacterial classes detected in each clone library is illustrated in Figure 9, and the OTU

abundances used to calculate diversity indices for each clone library are found in Table 14. As seen in Figure 8 at the generic level, the populations associated with each host species are unique but exhibit some overlap. Furthermore, the binturong microflora was dominated by the Bacilli class, which belongs to the phylum Firmicutes (Table 15)—a trend observed across mammalian taxa (Leser and Mølbak, 2009; Desai et al., 2009).

Richness, evenness (J'), Simpson's Diversity Index (D), and the Shannon-Weaver Index (H') were calculated for each library so that the microflora could be numerically quantified and compared across host species (Table 16). High richness and evenness contribute to high overall diversity, evidenced by increased Simpson's Complement (1-D) and Shannon-Weaver Index (H') values. In general, an increase in diversity was observed with increasing GIT length and specialization, as dietary fiber increaseds, and as feeding strategies moved from frugivory → folivory → omnivory. Binturongs exhibited the lowest overall diversity, and peaked with the baboons (Figure 10).

DISCUSSION

The baboon fecal samples in this experiment produced a SCFA concentration of 56.69 mmol at t=24hours. SCFA concentrations ranging from 65-235 mM have been reported in the hindgut of humans and baboons (Stevens and Hume, 1998). Clemens and Phillips (1980) measured 170 mmol/L and 90-160 mmol/L SCFA produced in the cecum and colon, respectively, when baboons were fed primate biscuit. Fecal inoculums are a less invasive alternative to intestinal sampling and provide comparable fermentation results to colonic inoculums (Costa et al., 2005). The SCFA concentrations measured for chimpanzee samples in this experiment were within the range of the values recorded for fecal microbes isolated from chimpanzee feces by Kisidayova et al. (2009).

It has been calculated that SCFA in the lower gut may contribute between 5 and 30% of the energy requirements for the entire body; and in the rumen the SCFA can provide up to 70% of the body's requirements. In humans, 6-10% of whole body requirement was met when fed 50-60 grams of carbohydrates comprised of 15 g fiber and 35-50 g sugar and/or starch. (Bergman, 1990; Stevens and Hume, 1998). The figure increased in developing countries, due to increased SCFA production from a higher proportion of fiber intake (Bergman, 1990). Increased fiber also increases the SCFA content in the hindgut of several mammalian species, including the vervet monkey (Stevens and Hume, 1998), and similar trends were seen in the primate species studied in this experiment. This suggests that non-human primates may also derive increased energetic benefit from high-fiber items foraged *in situ* or fed in captive settings. Milton and McBee (1983) found that 31% of the daily energy requirements for free-ranging mantled howler monkey (*Alouatta palliata*) was derived from products of microbial fermentation, and according to Watkins et al. (2010) this value is 30-60% for wild gorillas. While the degree of energy requirement garnered by the species studied in this project has not been quantified, the symbiotic benefit could play an instrumental role in primate success. This leaves the binturong—which exhibited no fermentation activity in preliminary studies by Lambert and Fellner (in press)—at an apparent disadvantage, unless the carnivore has an appreciably lower energetic requirement than the primate species we studied. In recent studies by Pontzer et al. (2010), free-ranging

orangutans were found to have lower daily energetic expenditure than most known mammalian species. The orangutan (Genus *Pongo*) is a frugivorous primate native to rainforests in southeast Indonesia which has evolved a decreased metabolism in response to food scarcity. *Pongo*'s habitat and nutritional niche overlap with those of *A. binturong*, and these similarities may allow it to serve as a model for the binturong's adaptations.

Individual SCFA are produced by bacteria from different diet components, and metabolized differently by the host. Acetate and butyrate are known to contribute to ruminant energy requirements through conversion to ketone bodies (Stevens and Hume, 1998). Acetate is fermented from structural carbohydrates, and in all species studied to date acetate accounts for over 90% of arterial and peripheral blood SCFA levels and is the primary energy source used by muscle and adipose tissue (Bergman, 1990). In this project, acetate concentrations increased significantly with dietary fiber content and as host species feeding strategy moved from frugivorous (chimpanzee) to omnivorous (baboon) to frugivorous (gorilla) (Figure 5). Butyrate is also converted to ketone bodies; but their high concentrations in portal blood samples suggest hepatic processing (Bergman, 1990). As chimpanzee fecal samples produced significantly lower butyrate concentrations than baboon or gorilla inoculums (Figure 5), it may be inferred that hepatic tissues in *P. troglodytes* depend less heavily on butyrate as an energy source than those of the other primate species. The glucogeneogenic potential of propionate in nonruminants is unclear, as the animals are less dependent on endogenous sources of glucose (Bergman, 1990). However, increased levels of propionate in the portal blood compared with hepatic, arterial, and peripheral venous blood samples suggest that the liver removes much of the propionate for metabolism (Bergman, 1990). Thus, it is likely that propionate retains its role (as a product of starch and pectin, and a metabolic precursor of glucose) in primates—though propionate concentrations were significantly lower in chimpanzee and gorilla samples than in baboon samples (Figure 5).

While absolute SCFA concentration values (like those in Figure 5B) tell us how substrates are utilized by bacteria, the molar percentages (Figure 5C) shed light on which microbes contribute the most to fermentation. For example, the diets consumed by *Gorilla gorilla* contained higher levels of structural fiber components than those eaten by *Pan*

troglodytes or *Papio hamadryas* individuals (Table 5). The microflora associated with *G. gorilla* fermented the cellulose and hemicellulose to a greater extent than those isolated from *P. troglodytes* and *P. hamadryas,* producing significantly higher concentrations of acetate and methane, whereas in the latter host species starches and pectins were utilized to a greater extent to produce high concentrations of propionate (Figure 5B). From the molar percentages (Figure 5B) it is evident that *G. gorilla* bacterial populations are dominated by acetogenic species—that is, cellulolytic bacteria which are highly adapted to digesting cell wall structures. Propiogenic (44.88%) and acetogenic (38.14%) bacteria, then, comprise the majority of *P. troglodytes* microflora, while *P. hamadryas* population dynamics are dominated by propiogenic (52.41%) and butyrate-forming (31.43%) bacterial species. This is interesting because, based on the dietary composition analysis the chimpanzee and baboon diets comprised similar percentages of NDF, ADF, and non-fiber carbohydrates (Table 5). Meanwhile, the binturong diet contained a comparable proportion of total carbohydrates to the primate diets (77.84% in binturong versus 81.12% in baboon, 82.4% in chimpanzee, and 86.86% in gorilla)—yet its microflora did not produce any SCFA in preliminary studies (Lambert and Fellner, in press).

All SCFA are absorbed readily over the entire lower digestive tract (Bergman, 1990). Transport is mostly passive (Stevens and Hume, 1998), and absorption of SCFA involves sodium bicarbonate (Bergman, 1990). While total SCFA absorption from the lumen is comparable between pigs (8-10 $uMcm^{-2}h^{-1}$), dogs (9 $uMcm^{-2}h^{-1}$), and humans (6-12 $uMcm^{-2}h^{-1}$), molar proportions of individual SCFA in blood vary and are not similar either to rumen or gut contents (Bergman, 1990). This is a clear indication that each individual SCFA is metabolized to a different extent by the gut epithelium, and furthermore may play a significant role in osmotic homeostasis by conserving sodium and water (Bergman, 1990). Water retention may be important for baboons and other species living in arid climates, while salt sequestering would have higher priority in the binturong or other animals in tropical forests, where constant rainfall leaches minerals from the soil and the food web.

The major phyla detected in the libraries constructed for *A. binturong, P. hamadrays, P. troglodytes,* and *G. gorilla* were *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*. While

Proteobacteria was the second most prevalent phylum recorded in the primate species, results may have been skewed by the high proportions of (as yet) unidentified bacteria isolated. Interestingly, while the binturong microflora contained a level of *Firmicutes* similar to proportions detected in human studies, all 214 clones belonged to a single species (*Weisella N65)*. All mammals studied to date—especially humans—have been characterized by a predominance of the *Firmicutes* and *Bacteroidetes* phyla, although the diversity of constituents within these broad taxonomic groups is huge (Leser and Mølbak, 2009; Desai et al., 2009). In contrast to the more even and diverse populations in human GI tracts, binturong microbiota appear to contain fewer constituents, and are skewed by a single highly dominant species.

It is especially interesting to note that, while primates and binturongs were housed in facilities 24.7km (15.3 mi) apart and were fed different diets, there were still several genera shared between the binturong and one or more of the primate species (Figure 8). Our findings suggest that, while bacteria are generally ubiquitous and diet can impact GIT populations, microflora are refined based on morphology, retention time, and phylogeny—a point made previously by Ley et al. (2008).Typical genera present in the human GIT include *Bacteroides, Eubacterium, Peptostreptococcus, Bifidobacterium,* and *Fusobacterium* (Bergman, 1990). Only the gorilla library contained any clones identified as belonging to the above genera (2 *Bacteroides* and 1 *Eubacterium)*. However, the high incidence of unknown isolates and the comparatively small libraries compiled may have, as yet, prevented the discovery of similar trends in the primate microflora.

Several of the clones isolated in this project were identified as having been previously detected in rumen samples, suggesting that colonic microbes may perform similar digestive functions to those endemic to cattle (Stevens and Hume, 1998). OTUs identified in this project that were shared with cattle include *Ruminococcus albus, R. flavefaciens, Butyrivibrio fibrisolvens, Succinivibrio dextrinosolvens,* and *Lactobacillus* species—an unsurprising list, when their fiber digesting abilities are taken into account.

While overall bootstrap values at or above 50 (out of 100) lend credibility to sequence-based phylogenetic analysis, there was a trend of decreasing confidence as the

number of uncultured clones isolated from each host species increased. For example, binturong fecal samples yielded 24 unique OTUs, of which only three were unclassified; and the average bootstrap value for that tree was 78. Baboon samples yielded 59 unique OTUs, of which 46 are as yet unidentified, and an average bootstrap value of 69. Chimpanzee samples yielded 88 total unique OTUs with 97 unidentified clones, and exhibited a further decrease in the average bootstrap value, to 46. Gorilla results bore closer numerical resemplance to baboons, with 51 unidentified clones out of 78 total unique OTUs, and an average bootstrap value of 67.

The dominant bacterial families isolated by Wireman et al. (2006) from rhesus and cynomolgus monkeys (*Macaca fascicularis*) were identified as *Clostridium-Eubacterium* and *Lactobacillus*, respectively. While both families were detected in this project (1 *Clostridiaceae* and 1 *Lactobacilliaceae* in binturong; 2 *Lactobacilliaceae* in baboon; 1 *Lactobacilliaceae* in chimpanzee; 1 *Eubacteraceae* and 2 *Lactobacilliaceae* in gorilla), their prevalence was far from dominant in any of the host species of interest. Populations of *E. coli, Bifidobacteria,* and *Enterococci* were positively correlated with one another within the animals throughout the course of the Wireman et al. experiment (2006), but the bacteria were only detected in the binturong library (1 each of *E. coli* and *Enterococcus)*.

Hill et al. (2002) isolated a pig fecal clone with 82% nucleotide ID similarity to *Burkholder vietnamiensis,* a *β-Proteobacteria*. This was interesting because no prior studies reported this bacterial family's presence in fecal flora. However, *Burkholderia cenacepacia* was detected in the gorilla and chimpanzee libraries (1 clone in each), and several species have been found in the soil's rhizosphere. Since all primate samples were collected from indoor enclosures, it is unlikely that *B. cenacepacia* was present due to ground contamination—though captive animals sometimes exhibit pica, and geophagy would certainly provide a means of transmitting the species. As the genus is known to have pathogenic effects against plants and humans, perhaps it is not surprising to find this group of organisms present in feces (Hill et al., 2002).

An increase in fecal microbial diversity has been found associated with herbivores, compared to samples from carnivorous animals (Desai et al., 2009). Our libraries showed a similar increase in diversity as dietary fiber increased (Figure 10). Nakamura et al. (2009) also found that baboons hosted less uniform microbial populations than mangabeys. The difference in community diversity may be explained by a difference in wild diet, as mangabeys feed largely on plant parts (such as leaves, fruits, flowers, and seeds) supplemented with insects, while baboons generally select from a wider array of plant items, insects, and small prey. Similar trends were observed when diversity indices were calculated and compared for the libraries in this project (see Figure 10).

Host diet and GIT morphology clearly play a key role in shaping the core microbiome. While the binturong and Giant Panda (*Ailuropoda melanoleuca*) have both diverged from their ancestral carnivory to consume fruit- and foliage-based diets, respectively, the Giant Panda houses a much more uniform microbial population compared with the binturong (see Figure 11). The less diverse bacterial community detected in the Giant Panda may develop in response to the animal's specialized diet (comprised mainly of bamboo)—and the core microbiome stability described by Wei et al. (2007) may be possible due to the increased length of the intestinal tract, compared with the binturong's scanty measurements.

Species found residing in the GIT of the Giant Panda were unexpected when only the animal's intake was considered: the microflora were more typical of a Carnivore, and ill-suited to a high-cellulose diet (Wei et al., 2007). This discrepancy between substrate and microbial ability may contribute to overall digestive inefficiency. In the case of *A. binturong,* a population of low diversity and uneven representation was observed, compared to the chimpanzee—despite the similarly frugivorous diets both animals have evolved to consume in the wild.

Dietary adequacy is determined based on knowledge of nutrient supply, digestibility, and a given animal's requirements (Rothman et al., 2008). Community shifts in primate microflora have been induced by varying fiber intake (Kisidayova et al., 2009), which in turn was associated with geographic and seasonal changes. In the Kisidayova et al. (2009) study,

microbial populations that were acclimated to high fiber diets produced increased SCFA and decreased methane concentrations compared to populations adapted to low-fiber diets, suggesting that bacteria utilize substrate more efficiently when their normal diet contains high levels of fiber. The relative efficiency of fecal microbiota did not appear to correlate with the levels of dietary fiber measured in the substrate fed to each host species, but fermentation by the baboon microbiota produced significantly less methane than chimpanzee or gorilla samples, despite similar total SCFA concentration. However, Ley et al. (2008) and Tap et al. (2009) also demonstrated that there is a distinct core microbiota associated with host species, which is conserved despite the transient effects of location or day-to-day diet.

A challenge inherent to the management of exotic species in captive settings is the translation of diets observed *in situ* to appropriate alternative regimens in which cultivated items are substituted for native fare. Domestic produce, however, is altered in its composition from that found in nature. When observed *in situ*, frugivorous diets are considered less digestible than folivorous diets if the proportion of indigestible seeds (in the fruit) offsets the amount of indigestible fiber (in the leaves) (Rothman et al., 2008). By contrast, digestibility is hardly a concern for commercially-available fruits. The cultivated crops have been selected to contain minimal seeds and high levels of water and sugar to achieve a desirable palatability for human consumers. As such, domestic fruits are no longer nutritionally equal—or even analogous—to their wild-growing counterparts.

Without composition analysis of all food items, an offered diet may be nutritionally imbalanced and inappropriate for its recipient. The inflated levels of simple carbohydrates resulting from incorrectly formulated diets are known to increase incidence of obesity and diabetes in non-human primates. Furthermore, in species like *A. binturong* which have considerably high passage rates, even a diet high in soluble carbohydrates will not easily satisfy energetic or nutritional requirements without a rapid transport mechanism to aid in nutrient absorption. Dietary and nutritional research may help gain insight into a particular species' needs in captivity—a factor especially important in preserving threatened and endangered species (Lambert, 2010).

From a conservational perspective, a population of endosymbionts which is not complementary to host intake will not provide much benefit to the animal. On the contrary, as seen with the Giant Panda, specialists will be rendered more vulnerable to minor changes in habitat and food availability (Wei et al., 2007). Such may be the case for the gorilla, chimpanzee, and binturong, if their habitats continue to be fractured by human activity. As three of the four animals studied in this project are considered threatened or endangered in the wild, a more complete understanding of the microflora—which play such a key role in providing nutrients, and which are implicated in overall health—must be achieved to maintain the remaining populations. Characterizing the fermentation activity and population demographics associated with each host of interest is the first step toward gaining that understanding, and may provide future methods for population monitoring and management.

CONCLUSIONS

Unique microflora were observed in each primate species, even though zoo diets were prepared in the same commissary using stock produce. Several bacterial genera were shared among primate and carnivore hosts, despite differences in diet and geographical location. Each bacterial population utilized their substrates differently, to produce different levels of SCFA. Many isolated clones were previously identified in pig feces (Hill et al. 2002) or rumen samples (Stevens and Hume, 1998). Animals appear to host microbial populations that are adapted to their specific diet and morphology.

LITERATURE CITED

Bergman, E. N. 1990. Energy contributions of short chain fatty acids from the gastrointestinal tract in various species. *Physiological Reviews* 70(2): 567-590.

Clemens, E., B. Phillips. 1980. Organic acid production and digesta movement in the gastrointestinal tract of the baboon and Sykes monkey. *Comparative Biochemistry and Physiology* 66A: 529-532.

Costa, M. A., T. Mehta, J. R. Males. 1989. Effects of dietary cellulose and psyllium hisk on monkey colonic microbial metabolism in continuous culture. *Journal of Nutrition* 119: 979-985.

Claesson, M. J., O. O'Sullivan, Q. Wang, J. Nikkila, J. R. Marchesi, H. Smidt, W. M. de Vos, R. Paul Ross, P. W. O'Toole. 2009. *PLoS ONE* 4(8): e6669.

Desai, A. R., K. M. Musil, A. P. Carr, J. E. Hill. 2009. Characterization and quantification of feline fecal microbiota using *cpn60* sequence-based methods and investigation of animal-to-animal variation in microbial population structure. *Veterinary Microbiology* 137: 120-128.

Felsenstein, J. 2005. PHIP (Phylogeny Inference Package) version 3.6. *Distributed by author. Department of Genome Sciences, University of Washington, Seattle.*

Goh, S. H., Potter, S., Wood, J. O., Hemmingsen, S. M., Reynolds, R. P., Chow, A. W. 1996. HSP60 gene sequences as universal targets for microbial species identification: studies with coagulase-negative staphylococci. *Journal of Clinical Microbiology* 34: 818-823.

Hill, J. E., R. P. Seipp, M. Betts, L. Hawkins, A. G. Van Kessel, W. L. Crosby, S. M. Hemmingsen. 2002. Extensive profiling of a complex microbial community by high-throughput sequencing. *Applied and Environmental Microbiology* 68(6): 3055-3066.

Hofmann, R. R. 1989. Evolutionary steps of ecophysiological adaptation and diversification of ruminants: a comparative view of their digestive system. *Oecologia* 78: 443-457.

Johnson, M. C., A. A. Devine, J. C. Ellis, A. M. Grunden, V. Fellner. 2009. Effects of antibiotics and oil on microbial profiles and fermentation in mixed cultures of ruminal microorganisms. *Journal of Dairy Science* 92(9):4467-4480.

Kisidayova, S., Z. Varadyova, P. Pristas, M. Piknova, K. Nigutova, K. J. Petrzelkova, I. Profousova, K. Schovancova, J. Kamler, D. Modry. 2009. Effects of high- and low-

fiber diets on fecal fermentation and fecal microbial populations of captive chimpanzees. *American Journal of Primatology* 71: 548-557.

Lambert, J. E. (2010). Primate Nutritional Ecology: feeding biology and diet at ecological and evolutionary scales. In Campbell C., Fuentes A., MacKinnon K., Bearder S., Stumpf R. (eds.), *Primates in Perspective, 2nd Edition*. Oxford University Press, Oxford, UK.

Lambert, J.E., V. Fellner. In press. *In vitro* fermentation of dietary carbohydrates consumed by African apes and monkeys: preliminary results for interpreting microbial and digestive strategy. *International Journal of Primatology.*

Leser, T. D., L. Molbak. 2009. Better living through microbial action: the benefits of the mammalian gastrointestinal microbiota on the host. *Environmental Microbiology* 11(9): 2194-2206.

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

Martin, A. P. 2002. Phylogenetic approaches for describing and comparing the diversity of microbial communities. *Applied and Environmental Microbiology* 68(8):3674-2682.

McKenna, P., C. Hoffmann, N. Minkah, P. P. Aye, A. Lackner, Z. Liu, C. A. Lozupone, M. Hamady, R. Knight, F. D. Bushman. 2008. The macaque gut microbiome in health, lentiviral infection, and chronic enterocolitis. *PLoS Pathogens* 4(2): e20.

Milton, K., R. H. McBee. 1983. Rates of fermentative digestion in the howler monkey, *Alouatta palliate* (primates: Ceboidea). *Comparative Biochemistry and Physiology Part A: Physiology* 74(1): 29-31.

Nakamura, N., S. R. Leigh, R. I. Mackie, H. R. Gaskins. 2009. Microbial community analysis of rectal methanogens and sulfate reducing bacteria in two non-human primate species. *Journal of Medical Primatology* 38: 360-370.

Pontzer, H., D. A. Raichlen, R. W. Shumaker, C. Ocobock, S. A. Wich. 2010. Metabolic adaptation for low energy throughput in orangutans. *PNAS* 107(32): 14048-14052.

Rothman J. M., E. S. Dierenfeld, H. F. Hintz, A. N. Pell. 2008. Nutritional quality of gorilla diets: consequences of age, sex, and season. *Oecologia* 115: 111-122.

Stevens, C. E., and I. D. Hume. 1998. Contributions of microbes in vertebrate gastrointestinal tract to production and conservation of nutrients. *Physiological Reviews* 78(2): 393-

427.

Sunvold, G. D., H. S. Hussein, G. C. Fahey, Jr., N. R. Merchen, G. A. Reinhart. 1995. In vitro fermentation of cellulose, beet pulp, citrus pulp, and citrus pectin using fecal inoculums from cats, dogs, horses, humans, and pigs and ruminal fluid from cattle. *Journal of Animal Science* 73: 3639-3648.

Tap, J., S. Mondot, F. Levenez, E. Pelletier, C. Caron, J. Furet, E. Ugarte, R. Munoz-Tamayo, D. Paslier, R. Nalin, J. Dore, M. Leclerc. 2009. Towards the human intestinal microbiota phylogenetic core. *Environmental Microbiology*. doi:10.1111/j.1462-2920.2009.01982.x

Torok, V. A., K. Ophel-Keller, M. Loo, R. J. Hughes. 2008. Application of methods for identifying broiler chicken gut bacterial species linked with increased energy metabolism. *Applied and Environmental Microbiology* 74(3): 783-291.

Van Soest, P. J., J. B. Robertson, B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science* 74: 3583.

Watkins, P. A., A. B. Moser, C. B. Toomer, S. J. Steinberg, H. W. Moser, M. W. Karaman, K. Ramaswamy, K. D. Siegmund, D. R. Lee, J. J. Ely, O. A. Ryder, J. G. Hacia. 2010. Identification of differences in human and great ape phytanic acid metabolism that could influence gene expression profiles and physiological functions. *BMC Physiology* 10:19. Doi:10.1186/1472-6793-10-19.

Wei, G., H. Lu, Z. Zhou, H. Xie, A. Wang, K. Nelson, L. Zhao. 2007. The microbial community in the feces of the Giant Panda (*Ailuropoda melanoleuca*) as determined by PCR-TGGE profiling and clone library analysis. *Microbial Ecology* 54: 194-202.

Wireman, J., M. Lowe, A. Spiro, Y. Zhang, A. Sornborger, A. O. Summers. 2006. Quantitative, longitudinal profiling of the primate fecal microbiota reveals idiosyncratic, dynamic communities. *Environmental Microbiology* 8(3): 490-503.

Zhu, H., and R. A. Dean. 1999. A novel method for increasing the transformation efficiency of *Escherichia coli*—application for bacterial artificial chromosome library construction. *Nucleic Acids Research* 27(3):910-911.

Zoetendal, E. G., H. G. Heilig, E. S. Klaassens, C. C. Booijink, M. Kleerebezem, H. Smidt, W. M. de Vos. 2006. Isolation of DNA from bacterial samples of the human gastrointestinal tract. *Nature Protocol* 1: 870-873.

Table 2. Animal demographics

Species	Location	Gender	Name	Age
Binturong	Pittsboro, NC	Male	Cholo	20
		Male	BJ	18
		Male	Bok	20
		Male	Keanu	17
		Male	Rotten	19
		Male	Shroom	17
		Male	Tristan	15
		Male	Coda	14
		Male	Jonas	14
		Female	Becky	14
Baboon	Asheboro, NC	Male	Addis	13
		Male	Gondar	13
		Male	Geb	7
		Male	Tukio	6
		Female	Sally	27
		Female	Marta	23
		Female	Marjie	21
		Female	Matilda	21
		Female	Mendi	13
		Female	Ras Mitat	8
		Female	Sudi	7
		Female	Candy	6
		Female	Rhea	7
Chimpanzee	Asheboro, NC	Male	Hondo	36
		Male	Jonathon	15
		Male	Sokoto	10
		Male	Lance	10
		Male	Tendall	12
		Female	Terry	42
		Female	Maggie	38
		Female	Ruthi	40
		Female	Amy	28
		Female	Tammy	40
		Female	Maki	17
		Female	Ruby	15
Gorilla	Asheboro	Male	N'Kosi	19
		Female	Acacia	16
		Female	Jamani	12

Table 3. Enclosure dimensions and termperatures on sample collection dates

Species	Binturong	Baboon	Chimpanzee	Gorilla	
Dimensions (ft^2)	400	700	1160	1132	
Temperature (°F)	84	76	68	80	66
Collection date	June 6	October 22	July 12	July 26	October 22

Table 4. Composition of standardized diets in grams, measured on a dry matter basis

Item	Binturong	Baboon	Chimpanzee	Gorilla
apple	11.15	8.04	28.52	
avocado				43.75
banana	51.8	15.63	24.32	
cabbage		5.89		68.21
canteloupe	6.29			
carrot		11.83	19.71	62.18
cauliflower				
celery		3.46	17.18	36.81
collard greens		2.7		
cucumber				2.49
dog food "mush"	141.53			
grapefruit		2.28	4.96	
green beans		6.81	12.03	18.25
green leaf lettuce				26.65
green pepper				18.88
kale		2.17	6.65	47.93
kiwi				42.9
Marion Leafeater		131.51	265.82	
orange	14.24	10.56	23.76	
pears	8.52			
PMI fiber plus		133.26	254.81	
romaine		3.34	24.81	49.27
sweet potato		3.18	18.94	
swiss chard		2.08		
tomato	2.71			
Total	**233.53**	**342.74**	**701.51**	**417.32**

Table 5. Dry matter composition of diet, categorized by (A) food type and (B) analyte

A) Food Type	Unit	Binturong	Baboon	Chimpanzee	Gorilla
Fruit	g	94.71	36.51	81.56	86.65
Leafy Greens	g	--	16.18	31.46	191.06
Pelleted Feed	g	141.53	264.77	520.63	--
Vegetables	g	--	25.28	67.86	138.61
Total	g	236.24	342.74	701.51	416.32

B) Analyte	Unit	Binturong	Baboon	Chimpanzee	Gorilla
Crude protein	%	14.97	21.73	21.25	12.81
Neutral detergent fiber	%	10.89	17.49	18.41	21.04
Acid detergent fiber	%	3.63	13.86	14.33	20.72
Non-fiber carbohydrate	%	63.32	49.77	49.66	45.10
Fat	%	4.53	4.00	3.85	12.47
Calcium	%	1.19	1.31	1.40	0.76
Phosphorus	%	0.80	0.62	0.63	0.31
Sulfur	%	0.18	0.34	0.34	0.51
Magnesium	%	0.12	0.20	0.21	0.21
Sodium	%	0.28	0.21	0.22	0.27
Potassium	%	1.05	1.14	1.37	2.19
Copper	ppm	12.00	23.00	61.00	7.00
Iron	ppm	70.00	407.00	445.00	222.00
Manganese	ppm	12.00	92.00	101.00	34.00
Zinc	ppm	141.00	105.00	113.00	22.00
Ash	%	62.9	7.01	6.83	8.58

Table 6. Fiber analysis of dietary residue (post-fermentation) on a dry matter basis

Fiber composition	Binturong	Baboon	Chimpanzee	Gorilla
% NDF	18.14	25.51	22.89	20.12
% ADF	3.43	11.77	11.42	13.16

Table 7. PCR reaction used to amplify *cpn60* gene region

Reagent	Volume (µL)
5X Go Taq Buffer with 1.5mM $MgCl_2$	10.00
10mM dntps	1.00
Go Taq polymerase	0.50
PCR Enhancer	5.00
20uM H279	1.25
20uM H280	1.25
Water	25.50
10mg/mL BSA	0.50
Total Reaction Volume	**50.00**

Table 8. TA cloning reaction using pGEM®-T Easy vector

Reagent	Volume (µL)
Purified PCR product	1
2x Rapid Ligation Buffer	5
pGEM® Easy Vector	2
T4 DNA Ligase	1
H_20	1
Total Reaction Volume	10

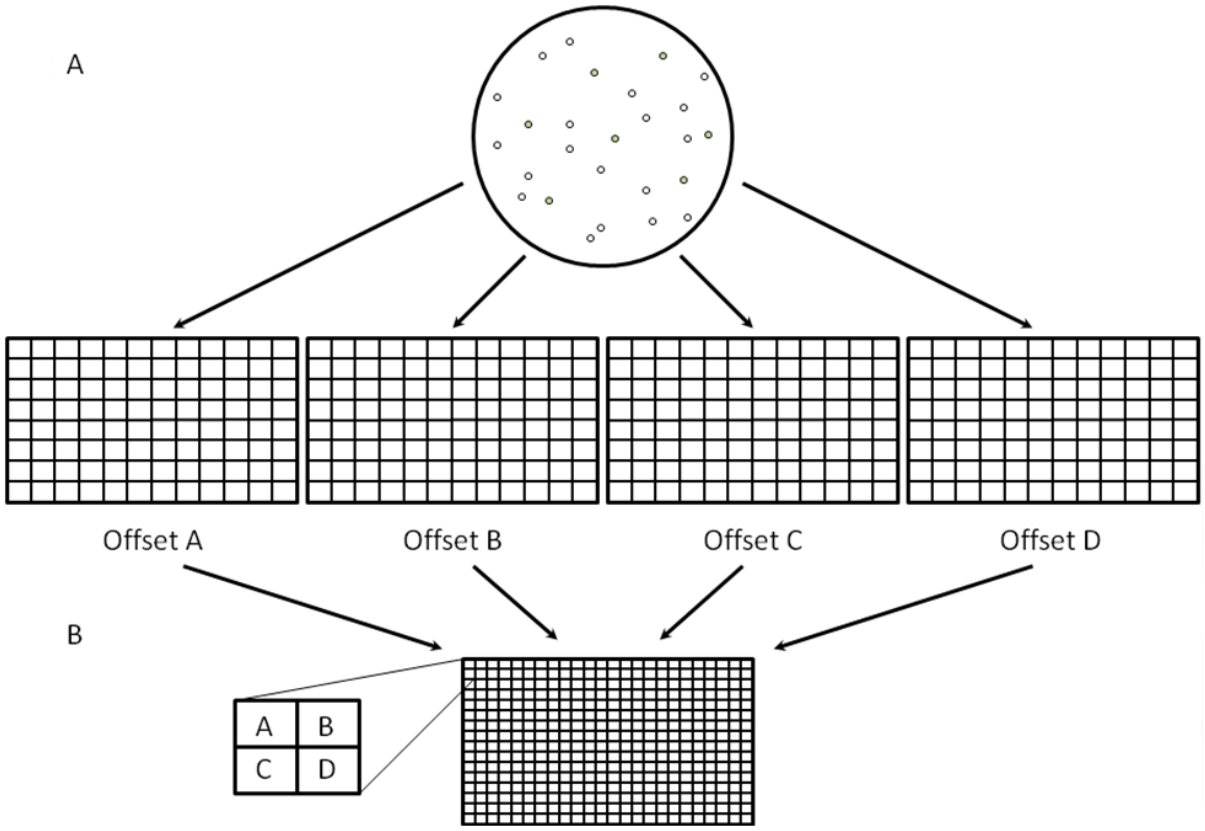


Figure 4. Diagram of experimental setup: A) White transformant colonies are picked to four 96 deep well plates for incubation. B) Cell cultures are combined with glycerol in a 384 well plate to make stock solution.

Table 9. Composition of Plasmid Prep Solutions I, II, and III.

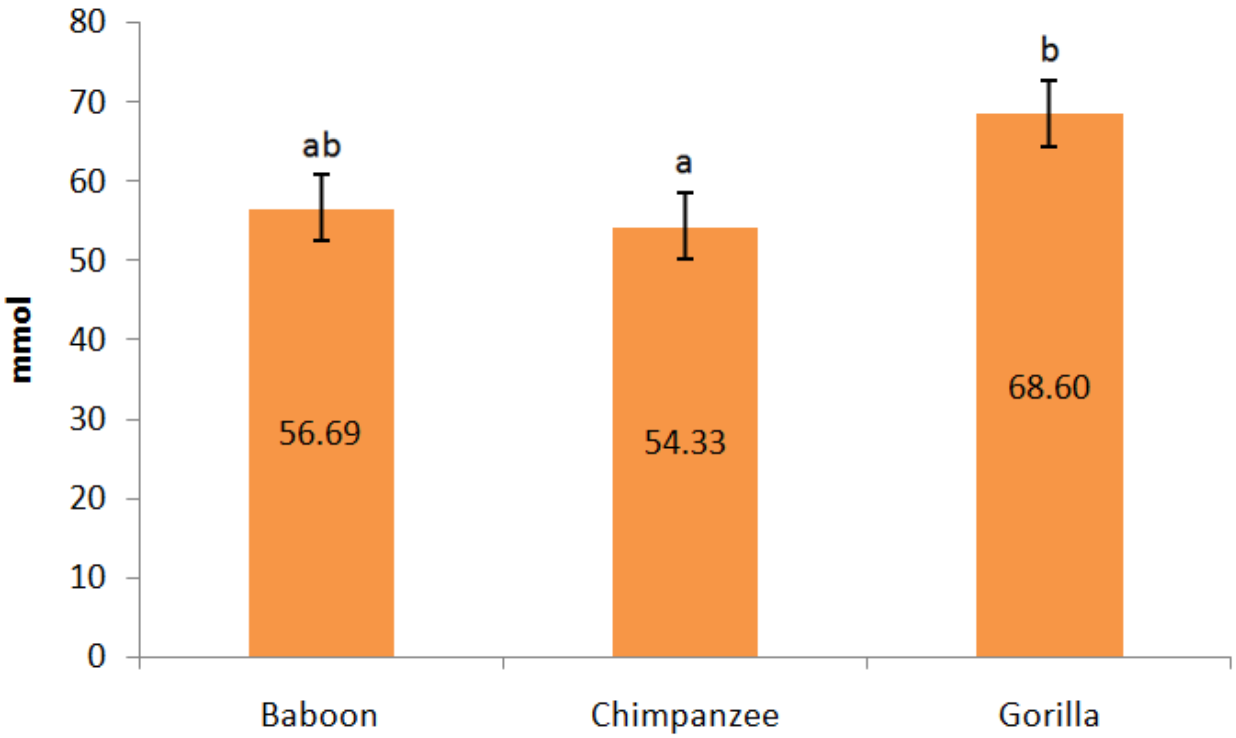
Plasmid Prep Solution	Reagent	Amount
I	50mM glucose	9g
	25mM Tris HCl, pH 8	25mL of 1M stock
	10mM EDTA, pH 8	20mL of 500mM stock
	dH2O	Bring to 1L
II	10M NaOH	20mL
	10% SDS	100mL
	dH2O	Bring to 1L
III	Potassium acetate (KOAC)	294g
	dH2O	Bring to 1L

Table 10. Big Dye reactions, standardized by species

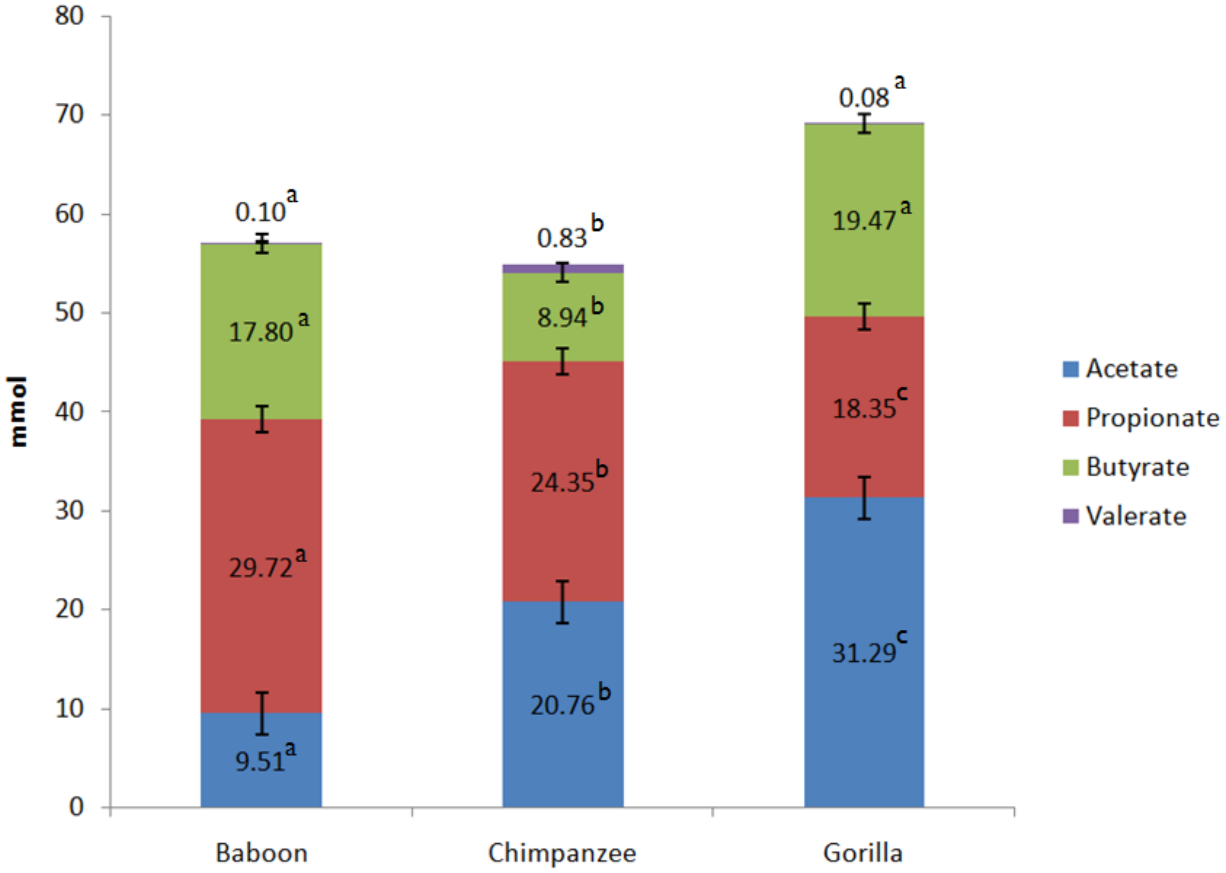
Reagent	Binturong	Chimpanzee	Baboon	Gorilla
H20	3.6		--	
Plasmid DNA	3		6.6	
BigDye® buffer	2			
3.2pm forward primer	1			
BigDye® terminator v3.1	0.4			
Total volume	10			

Figure 5. Fermentation activity observed at t=24 hours, compared across primate species: A) total millimoles of SCFA produced; B) millimoless of acetate, propionate, butyrate, and valerate; C) molar percentages of acetate, propionate, butyrate, and valerate; and D) A:P ratios

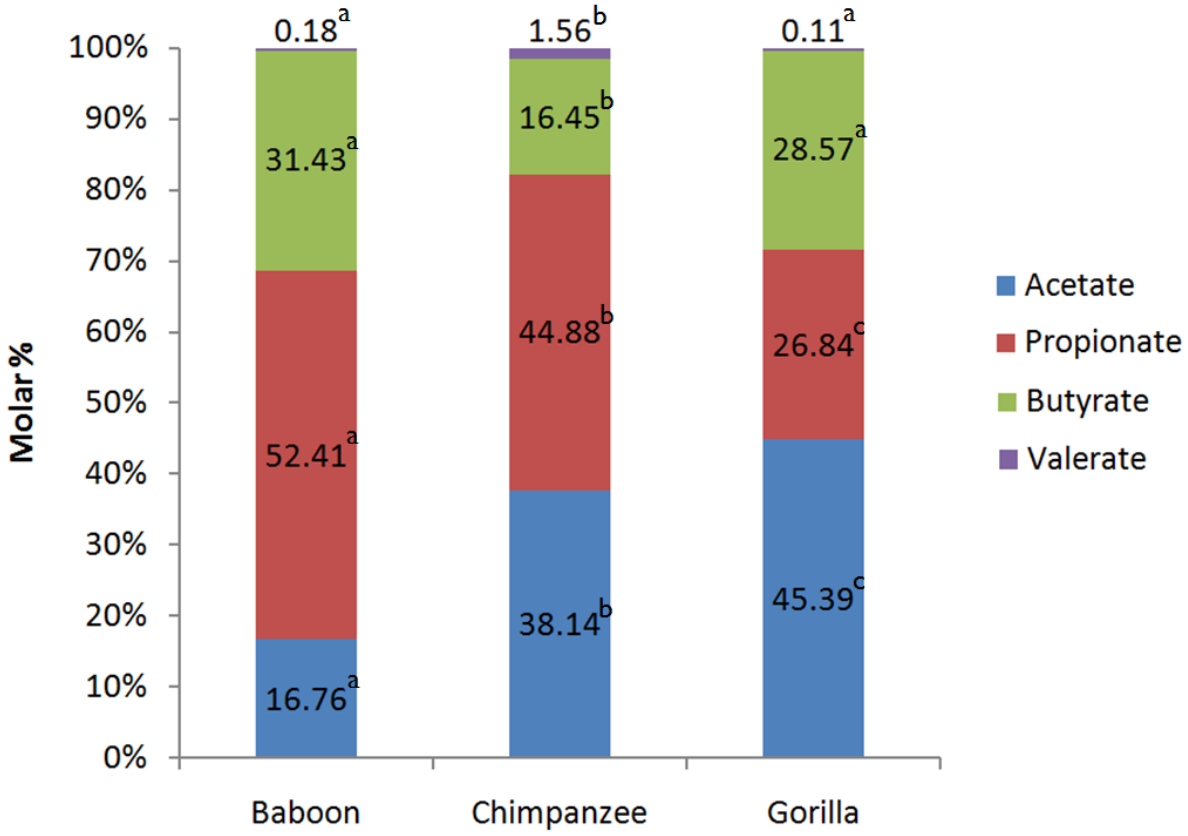
A


80
70
60
50
40
30
20
10
0
mmol
ab
a
b
56.69
54.33
68.60
Baboon
Chimpanzee
Gorilla

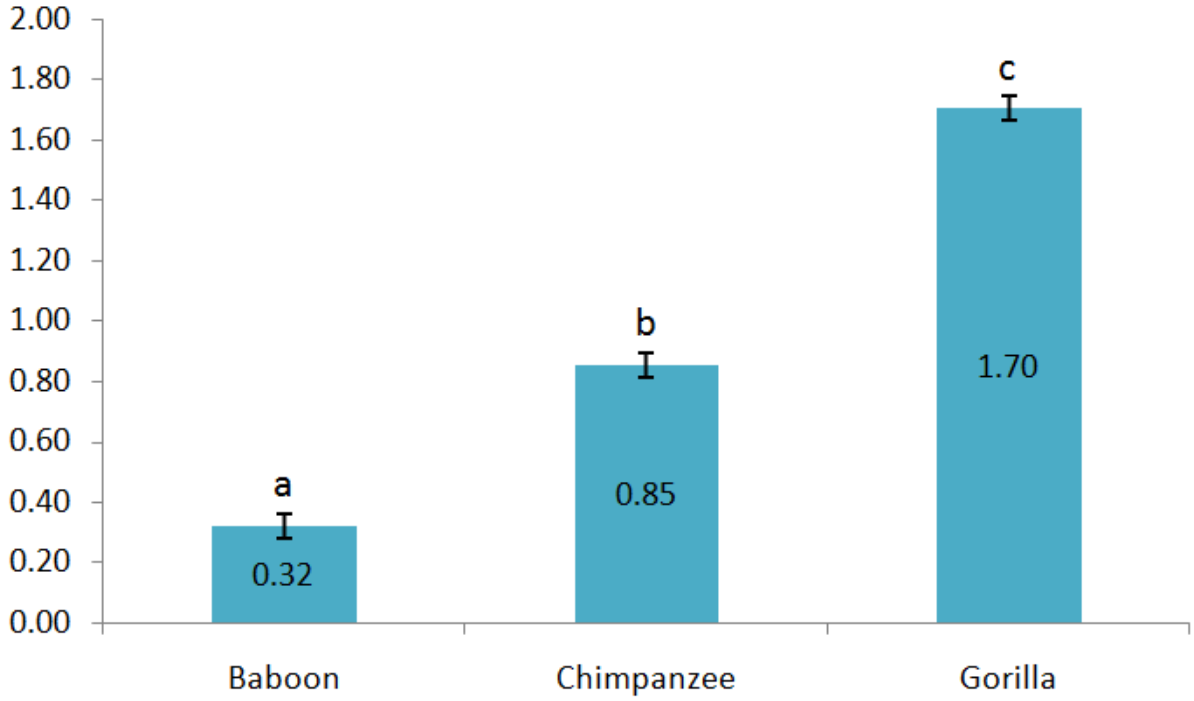
B


80
70
60
50
40
30
20
10
0
mmol
0.10a
17.80a
29.72a
9.51a
0.83b
8.94b
24.35b
20.76b
0.08a
19.47a
18.35c
31.29c
Baboon
Chimpanzee
Gorilla
Acetate
Propionate
Butyrate
Valerate

C


Molar%
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0.18a
1.56b
0.11a
31.43a
16.45b
28.57a
52.41a
44.88b
26.84c
16.76a
38.14b
45.39c
Baboon
Chimpanzee
Gorilla
Acetate
Propionate
Butyrate
Valerate

D


2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
a
0.32
b
0.85
c
1.70
Baboon
Chimpanzee
Gorilla

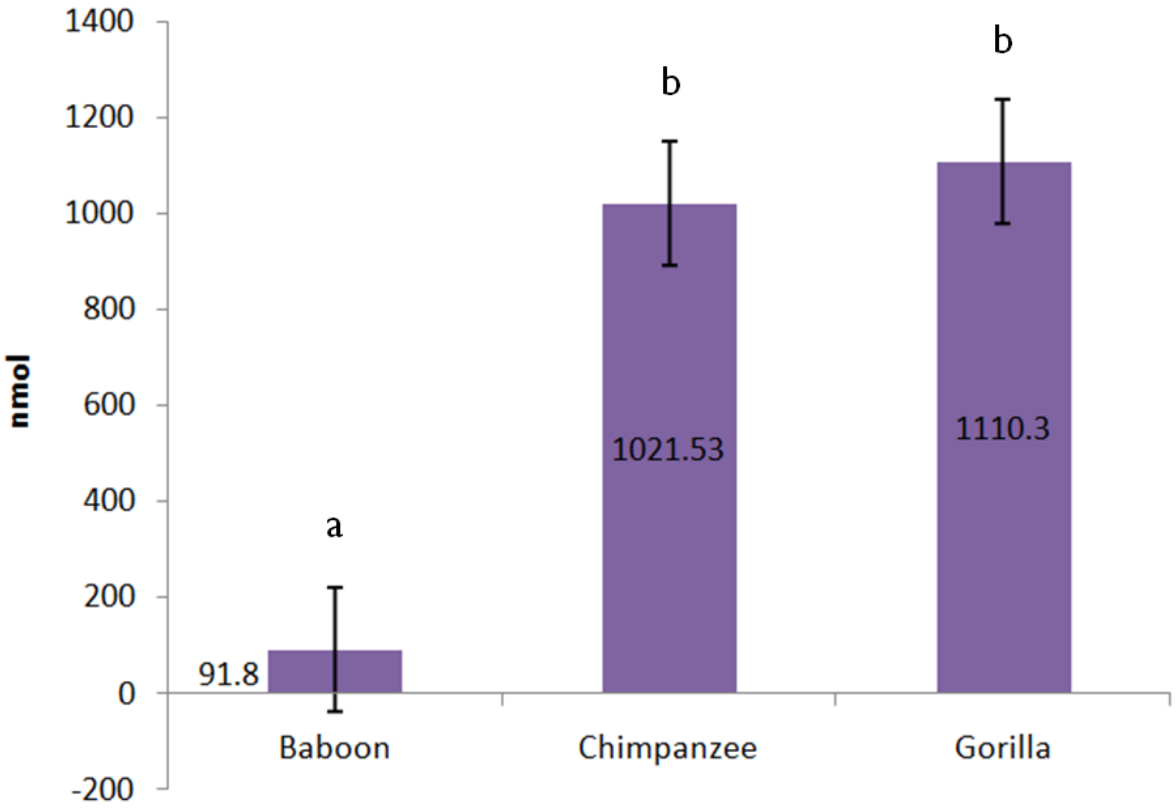


Figure 6. Methane concentration observed in primate samples at t=24 hours

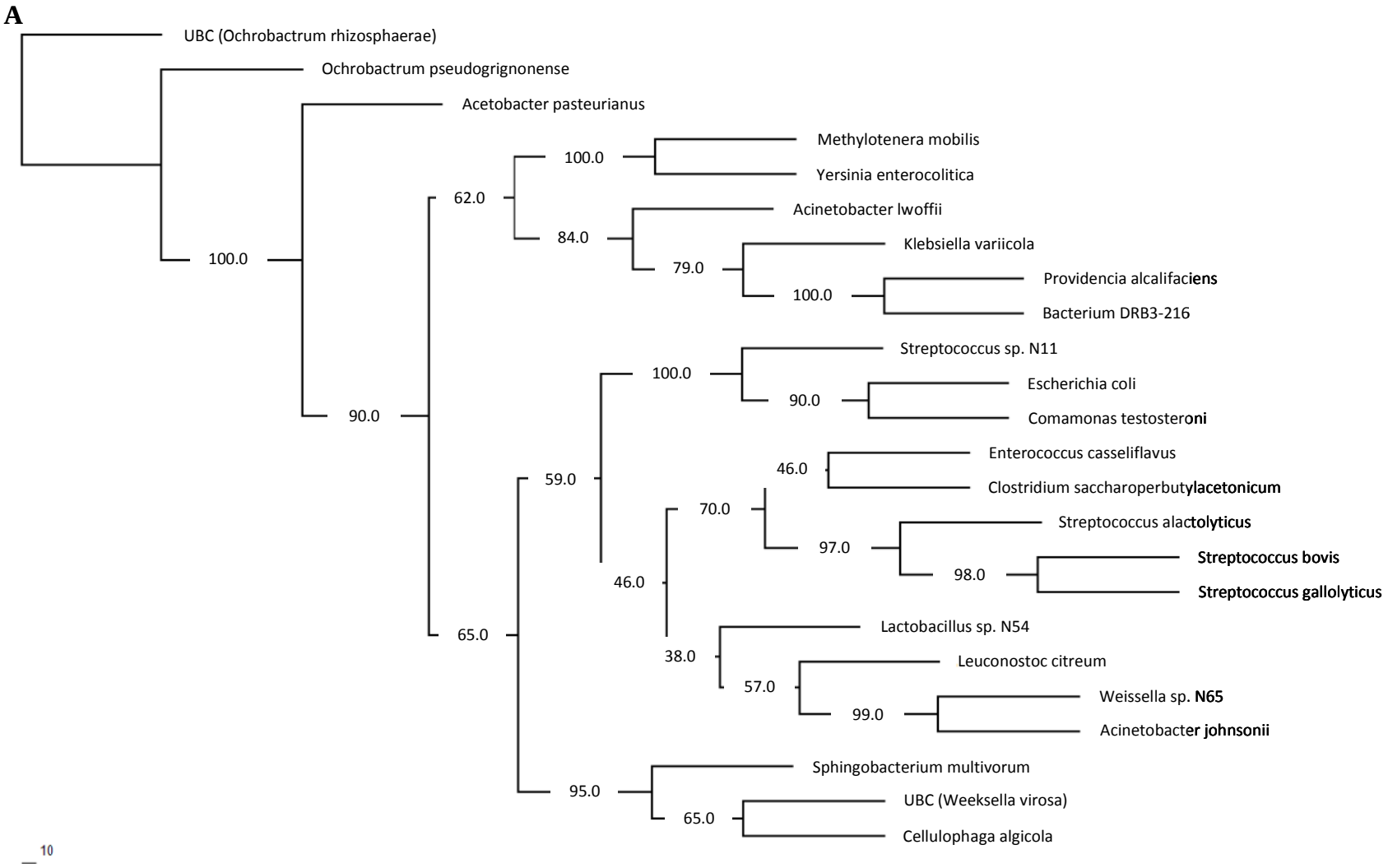
Table 11. Isolate count

	Binturong	Baboon	Chimpanzee	Gorilla
Total unique clones	339	367	353	327
***Genus, species* known**	336	50	65	123
Uncultured bacterial clones	3	317	288	204

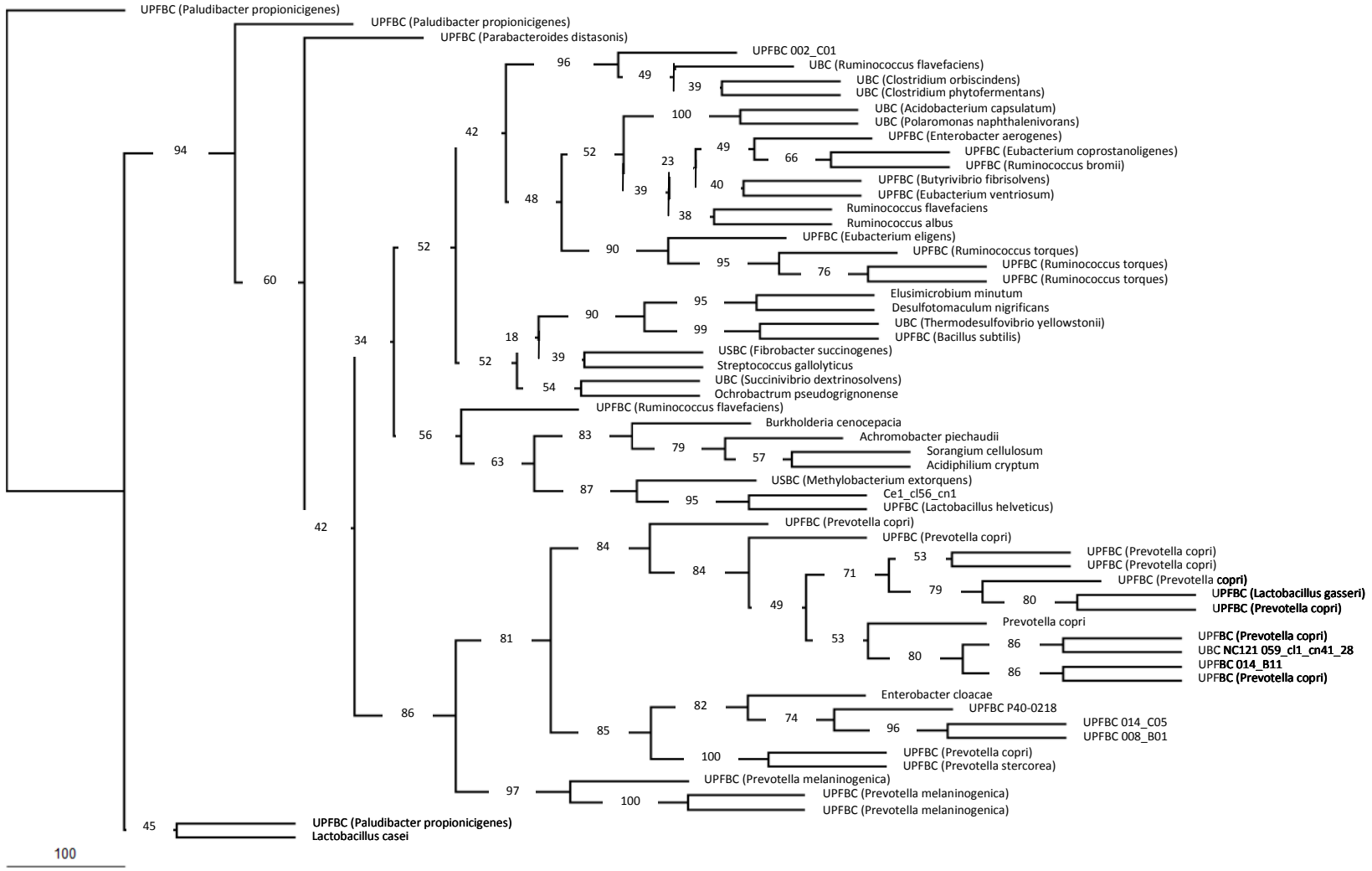
Table 12. Taxonomic composition of gene libraries

Host species	Genera	Families	Orders	Classes	Phyla
Binturong	18	12	10	7	3
Chimpanzee	12	12	9	8	4
Baboon	17	17	15	11	6
Gorilla	22	20	15	12	6

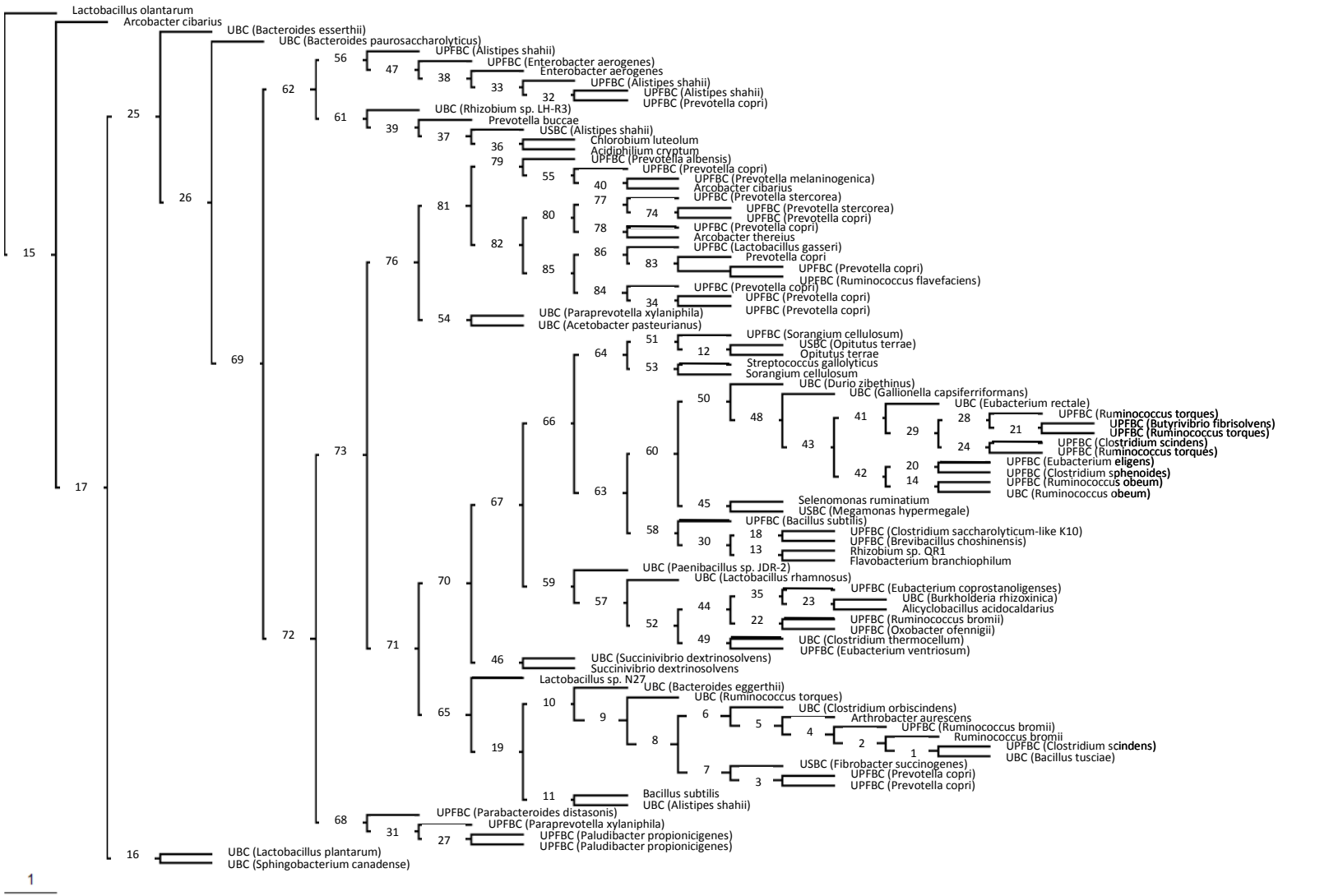
Figure 7. Phylogenetic tree showing microbial relatedness for unique sequences isolated from A) *Arctictis binturong*, B) *Papio hamadryas*, C) *Pan troglodytes*, and D) *Gorilla gorilla* fecal samples. UBC = Uncultured Bacterium Clone; UPFBC = Uncultured Pig Fecal Bacterium Clone; USBC = Uncultured Soil Bacterium Clone


A
UBC (Ochrobactrum rhizosphaerae)
Ochrobactrum pseudogrignonense
Acetobacter pasteurianus
Methylotenera mobilis
Yersinia enterocolitica
Acinetobacter lwoffii
Klebsiella variicola
Providencia alcalifaciens
Bacterium DRB3-216
Streptococcus sp. N11
Escherichia coli
Comamonas testosteroni
Enterococcus casseliflavus
Clostridium saccharoperbutylacetonicum
Streptococcus alactolyticus
Streptococcus bovis
Streptococcus gallolyticus
Lactobacillus sp. N54
Leuconostoc citreum
Weissella sp. N65
Acinetobacter johnsonii
Sphingobacterium multivorum
UBC (Weeksella virosa)
Cellulophaga algicola
100.0
62.0
84.0
79.0
100.0
100.0
90.0
90.0
59.0
46.0
46.0
70.0
97.0
98.0
65.0
38.0
57.0
99.0
95.0
65.0
10

B


UPFBC (Paludibacter propionicigenes)
UPFBC (Paludibacter propionicigenes)
UPFBC (Parabacteroides distasonis)
UPFBC 002_C01
UBC (Ruminococcus flavefaciens)
UBC (Clostridium orbiscindens)
UBC (Clostridium phytofermentans)
UBC (Acidobacterium capsulatum)
UBC (Polaromonas naphthalenivorans)
UPFBC (Enterobacter aerogenes)
UPFBC (Eubacterium coprostanoligenes)
UPFBC (Ruminococcus bromii)
UPFBC (Butyrivibrio fibrisolvens)
UPFBC (Eubacterium ventriosum)
Ruminococcus flavefaciens
Ruminococcus albus
UPFBC (Eubacterium eligens)
UPFBC (Ruminococcus torques)
UPFBC (Ruminococcus torques)
UPFBC (Ruminococcus torques)
Elusimicrobium minutum
Desulfotomaculum nigrificans
UBC (Thermodesulfovibrio yellowstonii)
UPFBC (Bacillus subtilis)
USBC (Fibrobacter succinogenes)
Streptococcus gallolyticus
UBC (Succinivibrio dextrinosolvens)
Ochrobactrum pseudogrignonense
UPFBC (Ruminococcus flavefaciens)
Burkholderia cenocepacia
Achromobacter piechaudii
Sorangium cellulosum
Acidiphilium cryptum
USBC (Methylobacterium extorquens)
Ce1_cl56_cn1
UPFBC (Lactobacillus helveticus)
UPFBC (Prevotella copri)
UPFBC (Prevotella copri)
UPFBC (Prevotella copri)
UPFBC (Prevotella copri)
UPFBC (Prevotella copri)
UPFBC (Lactobacillus gasseri)
UPFBC (Prevotella copri)
Prevotella copri
UPFBC (Prevotella copri)
UBC NC121 059_cl1_cn41_28
UPFBC 014_B11
UPFBC (Prevotella copri)
Enterobacter cloacae
UPFBC P40-0218
UPFBC 014_C05
UPFBC 008_B01
UPFBC (Prevotella copri)
UPFBC (Prevotella stercorea)
UPFBC (Prevotella melaninogenica)
UPFBC (Prevotella melaninogenica)
UPFBC (Prevotella melaninogenica)
UPFBC (Paludibacter propionicigenes)
Lactobacillus casei
100

C


Lactobacillus olantarum
Arcobacter cibarius
UBC (Bacteroides esserthii)
UBC (Bacteroides paurosaccharolyticus)
UPFBC (Alistipes shahii)
UPFBC (Enterobacter aerogenes)
Enterobacter aerogenes
UPFBC (Alistipes shahii)
UPFBC (Alistipes shahii)
UPFBC (Prevotella copri)
UBC (Rhizobium sp. LH-R3)
Prevotella buccae
USBC (Alistipes shahii)
Chlorobium luteolum
Acidiphilium cryptum
UPFBC (Prevotella albensis)
UPFBC (Prevotella copri)
UPFBC (Prevotella melaninogenica)
Arcobacter cibarius
UPFBC (Prevotella stercorea)
UPFBC (Prevotella stercorea)
UPFBC (Prevotella copri)
UPFBC (Prevotella copri)
Arcobacter thereius
UPFBC (Lactobacillus gasseri)
Prevotella copri
UPFBC (Prevotella copri)
UPFBC (Ruminococcus flavefaciens)
UPFBC (Prevotella copri)
UPFBC (Prevotella copri)
UPFBC (Prevotella copri)
UBC (Paraprevotella xylaniphila)
UBC (Acetobacter pasteurianus)
UPFBC (Sorangium cellulosum)
USBC (Opitutus terrae)
Opitutus terrae
Streptococcus gallolyticus
Sorangium cellulosum
UBC (Durio zibethinus)
UBC (Gallionella capsiferriformans)
UBC (Eubacterium rectale)
UPFBC (Ruminococcus torques)
UPFBC (Butyrivibrio fibrisolvens)
UPFBC (Ruminococcus torques)
UPFBC (Clostridium scindens)
UPFBC (Ruminococcus torques)
UPFBC (Eubacterium eligens)
UPFBC (Clostridium sphenoides)
UPFBC (Ruminococcus obeum)
UBC (Ruminococcus obeum)
Selenomonas ruminatium
USBC (Megamonas hypermegale)
UPFBC (Bacillus subtilis)
UPFBC (Clostridium saccharolyticum-like K10)
UPFBC (Brevibacillus choshinensis)
Rhizobium sp. QR1
Flavobacterium branchiophilum
UBC (Paenibacillus sp. JDR-2)
UBC (Lactobacillus rhamnosus)
UPFBC (Eubacterium coprostanoligenses)
UBC (Burkholderia rhizoxinica)
Alicyclobacillus acidocaldarius
UPFBC (Ruminococcus bromii)
UPFBC (Oxobacter ofennigii)
UBC (Clostridium thermocellum)
UPFBC (Eubacterium ventriosum)
UBC (Succinivibrio dextrinosolvens)
Succinivibrio dextrinosolvens
Lactobacillus sp. N27
UBC (Bacteroides eggerthii)
UBC (Ruminococcus torques)
UBC (Clostridium orbiscindens)
Arthrobacter aurescens
UPFBC (Ruminococcus bromii)
Ruminococcus bromii
UPFBC (Clostridium scindens)
UBC (Bacillus tusciae)
USBC (Fibrobacter succinogenes)
UPFBC (Prevotella copri)
UPFBC (Prevotella copri)
Bacillus subtilis
UBC (Alistipes shahii)
UPFBC (Parabacteroides distasonis)
UPFBC (Paraprevotella xylaniphila)
UPFBC (Paludibacter propionicigenes)
UPFBC (Paludibacter propionicigenes)
UBC (Lactobacillus plantarum)
UBC (Sphingobacterium canadense)
1

D

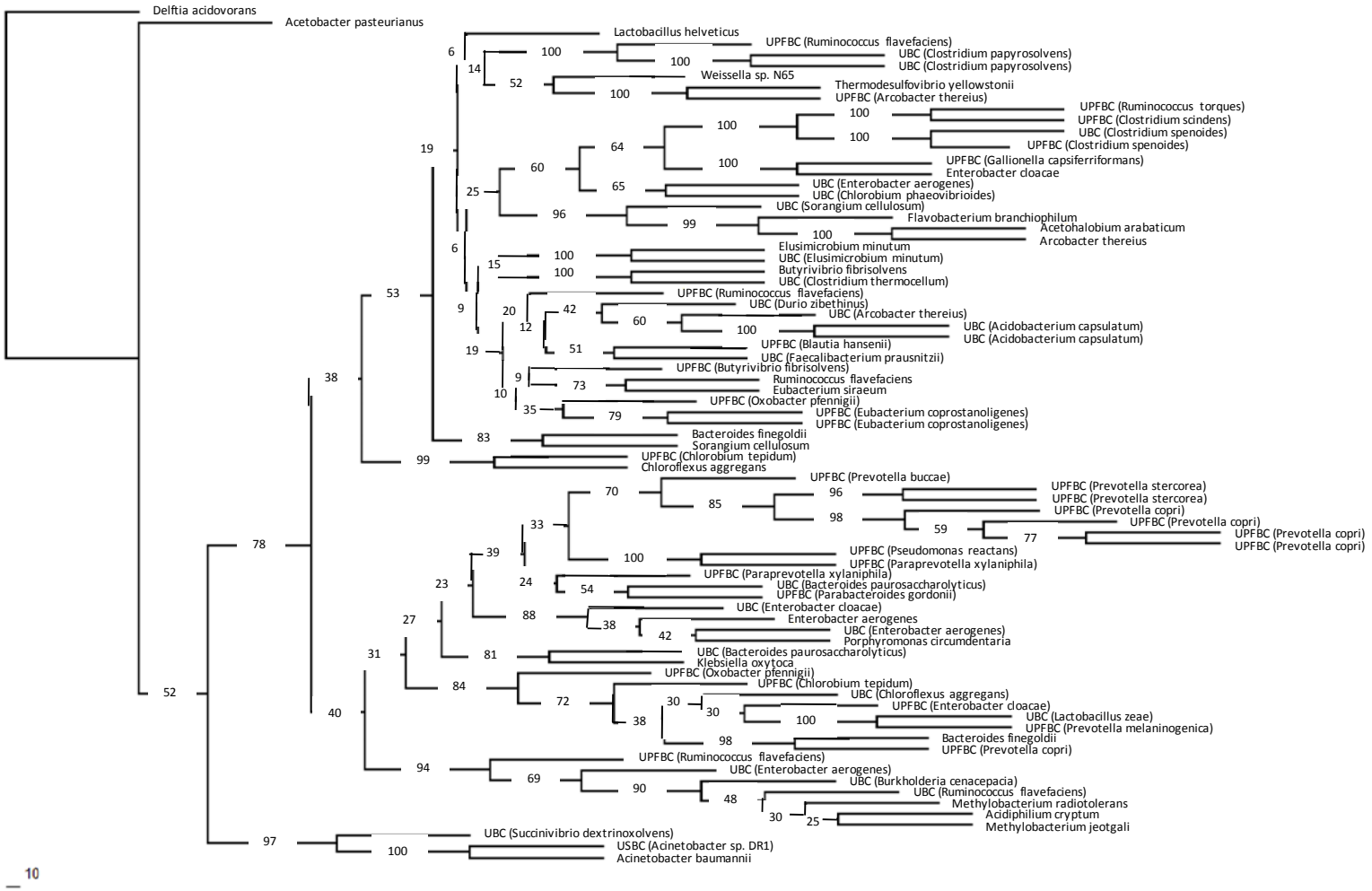

Delftia acidovorans
Acetobacter pasteurianus
Lactobacillus helveticus
UPFBC (Ruminococcus flavefaciens)
UBC (Clostridium papyrosolvens)
UBC (Clostridium papyrosolvens)
Weissella sp. N65
Thermodesulfovibrio yellowstonii
UPFBC (Arcobacter thereius)
UPFBC (Ruminococcus torques)
UPFBC (Clostridium scindens)
UBC (Clostridium spenoides)
UPFBC (Clostridium spenoides)
UPFBC (Gallionella capsiferriformans)
Enterobacter cloacae
UBC (Enterobacter aerogenes)
UBC (Chlorobium phaeovibrioides)
UBC (Sorangium cellulosum)
Flavobacterium branchiophilum
Acetohalobium arabaticum
Arcobacter thereius
Elusimicrobium minutum
UBC (Elusimicrobium minutum)
Butyrivibrio fibrisolvens
UBC (Clostridium thermocellum)
UPFBC (Ruminococcus flavefaciens)
UBC (Durio zibethinus)
UBC (Arcobacter thereius)
UBC (Acidobacterium capsulatum)
UBC (Acidobacterium capsulatum)
UPFBC (Blautia hansenii)
UBC (Faecalibacterium prausnitzii)
UPFBC (Butyrivibrio fibrisolvens)
Ruminococcus flavefaciens
Eubacterium siraeum
UPFBC (Oxobacter pfennigii)
UPFBC (Eubacterium coprostanoligenes)
UPFBC (Eubacterium coprostanoligenes)
Bacteroides finegoldii
Sorangium cellulosum
UPFBC (Chlorobium tepidum)
Chloroflexus aggregans
UPFBC (Prevotella buccae)
UPFBC (Prevotella stercorea)
UPFBC (Prevotella stercorea)
UPFBC (Prevotella copri)
UPFBC (Prevotella copri)
UPFBC (Prevotella copri)
UPFBC (Prevotella copri)
UPFBC (Pseudomonas reactans)
UPFBC (Paraprevotella xylaniphila)
UPFBC (Paraprevotella xylaniphila)
UBC (Bacteroides paurosaccharolyticus)
UPFBC (Parabacteroides gordonii)
UBC (Enterobacter cloacae)
Enterobacter aerogenes
UBC (Enterobacter aerogenes)
Porphyromonas circumdentaria
UBC (Bacteroides paurosaccharolyticus)
Klebsiella oxytoca
UPFBC (Oxobacter pfennigii)
UPFBC (Chlorobium tepidum)
UBC (Chloroflexus aggregans)
UPFBC (Enterobacter cloacae)
UBC (Lactobacillus zeae)
UPFBC (Prevotella melaninogenica)
Bacteroides finegoldii
UPFBC (Prevotella copri)
UPFBC (Ruminococcus flavefaciens)
UBC (Enterobacter aerogenes)
UBC (Burkholderia cenacepacia)
UBC (Ruminococcus flavefaciens)
Methylobacterium radiotolerans
Acidiphilium cryptum
Methylobacterium jeotgali
UBC (Succinivibrio dextrinoxolvens)
USBC (Acinetobacter sp. DR1)
Acinetobacter baumannii
10

Table 13. Taxonomic breakdown of Microbial populations by (A)species, (B) genus, (C) family, (D) order, (E) class, and (F) phylum

(A) Species	Binturong	Baboon	Chimpanzee	Gorilla
Acetobacter pasteurianus	7			1
Acetohalobium arabaticum				2
Achromobacter piechaudii			1	
Acidiphilium cryptum		9	29	13
Acinetobacter baumannii				4
Acinetobacter johnsonii	1			
Acinetobacter lwoffii	1			
Alicyclobacillus acidocaldarius		1		
Arcobacter cibarius		4		1
Arcobacter thereius		1		4
Arthrobacter aurescens TC1		1		
Bacillus subtilis BSn5		1		
Bacterium DRB3-216	2			
Bacteroides finegoldii				2
Burkholderia cenocepacia			1	1
Butyrivibrio fibrisolvens				1
Cellulophaga algicola	2			
Chlorobium luteolum DSM 273		1		
Chloroflexus aggregans				1
Clostridium saccharoperbutylacetonicum	1			
Comamonas testosteroni	18			
Desulfotomaculum nigrificans			1	
Durio zibethinus				3
Elusimicrobium minutum			2	1
Enterobacter aerogenes		1		6
Enterobacter cloacae			1	5
Enterococcus casseliflavus	1			
Esherichia coli	1			
Eubacterium siraeum 70/3				1
Flavobacterium branchiophilum		5		1
Klebsiella oxytoca				1
Klebsiella variicola	1			
Lactobacillus casei			1	
Lactobacillus helveticus				3
Lactobacillus plantarum		1		

Table 13 (continued)

Lactobacillus sp. N27		1		
Lactobacillus sp. N54	1			
Leuconostoc citreum	1			
Methylobacterium jeotgali				3
Methylobacterium radiotolerans				1
Methylotenera mobilis	4			
Ochrobactrum pseudogrignonense	2		2	
Opitutus terrae		1		
Porphyromonas circumdentaria				1
Prevotella buccae		5		
Prevotella copri		1	5	
Providencia alcalifaciens	2			
Rhizobium sp. QR1		3		
Ruminococcus albus			17	
Ruminococcus bromii L2-63		7		
Ruminococcus flavefaciens			1	61
Selenomonas ruminantium		1		
Sorangium cellulosum		4	1	6
Sphingobacterium multivorum	80			
Streptococcus alactolyticus	6			
Streptococcus bovis	1			
Streptococcus gallolyticus	3	1	2	
Streptococcus sp. N11	4			
Succinivibrio dextrinosolvens		1		
Thermodesulfovibrio yellowstonii				1
Uncultured bacterium clone	3	90	20	76
Uncultured pig fecal bacterium clone		219	270	126
Uncultured soil bacterium clone		8	2	2
Weissella sp. N65	196			1
Yersinia enterocolitica	1			

(B) Genus	**Binturong**	**Baboon**	**Chimpanzee**	**Gorilla**
Acetobacter	7			1
Acetohalobium				2
Achromobacter			1	
Acidiphilium		9	29	13
Acinetobacter	2			4
Alicyclobacillus		1		

Table 13 (continued)

Arcobacter		5		5
Arthrobacter		1		
Bacillus		1		
Bacterium	2			
Bacteroides				2
Burkholderia			1	1
Butyrivibrio				1
Cellulophaga	2			
Chlorobium		1		
Chloroflexus				1
Clostridium	1			
Comamonas	18			
Desulfotomaculum			1	
Durio				3
Elusimicrobium			2	1
Enterobacter		1	1	11
Enterococcus	1			
Esherichia	1			
Eubacterium				1
Flavobacterium		5		1
Klebsiella	1			1
Lactobacillus	1	2	1	3
Leuconostoc	1			
Methylobacterium				4
Methylotenera	4			
Ochrobactrum	2		2	
Opitutus		1		
Porphyromonas				1
Prevotella		6	5	
Providencia	2			
Rhizobium		3		
Ruminococcus		7	18	61
Selenomonas		1		
Sorangium		4	1	6
Sphingobacterium	80			
Streptococcus	14	1	2	
Succinivibrio		1		
Thermodesulfovibrio				1

Table 13 (continued)

Uncultured bacterium clone	3	90	20	76
Uncultured pig fecal bacterium clone		219	270	126
Uncultured soil bacterium clone		8	2	2
Weissella	196			1
Yersinia	1			

(C) Family	**Binturong**	**Baboon**	**Chimpanzee**	**Gorilla**
Acetobacteraceae	7	9	29	14
Alcaligenaceae	0	0	1	0
Alicyclobacillaceae	0	1	0	0
Bacillaceae	0	1	0	0
Bacteroidaceae	0	0	0	2
Brucellaceae	2	0	2	0
Burkholderiaceae	0	0	1	1
Campylobacteraceae	0	5	0	5
Chlorobiaceae	0	1	0	0
Chloroflexaceae	0	0	0	1
Clostridiaceae	1	0	0	0
Comamonadaceae	18	0	0	1
Elusimicrobiaceae	0	0	2	1
Enterobacteriaceae	7	1	1	12
Eubacteriaceae	0	0	0	1
Flavobacteriaceae	2	5	0	1
Halobacteroidaceae	0	0	0	2
Lachnospiraceae	0	0	0	1
Lactobacillaceae	1	2	1	3
Leuconostocaceae	198	0	0	1
Methylobacteriaceae	0	0	0	4
Methylophilaceae	4	0	0	0
Micrococcaceae	0	1	0	0
Moraxellaceae	2	0	0	4
Nitrospiraceae	0	0	0	1
Opitutaceae	0	1	0	0
Peptococcaceae	0	0	1	0
Polyangiaceae	0	4	1	6

Table 13 (continued)

Porphyromonadaceae	0	0	0	1
Prevotellaceae	0	6	5	0
Rhizobiaceae	0	3	0	0
Ruminococcaceae	0	7	18	61
Sphingobacteriaceae	80	0	0	0
Streptococcaceae	14	1	2	0
Succinivibrionaceae	0	1	0	0
Veillonellaceae	0	1	0	0
Uncultured	3	317	289	204

(D)Order	**Binturong**	**Baboon**	**Chimpanzee**	**Gorilla**
Actinomycetales	0	1	0	0
Aeromonadales	0	1	0	0
Bacillales	0	2	0	0
Bacteroidales	0	6	5	3
Burkholderiales	18	0	2	2
Campylobacterales	0	5	0	5
Chlorobiales	0	1	0	0
Chloroflexales	0	0	0	1
Clostridiales	1	7	19	63
Elusimicrobiales	0	0	2	1
Enterobacteriales	7	1	1	12
Flavobacteriales	2	5	0	1
Halanaerobiales	0	0	0	2
Lactobacillales	213	3	3	4
Methylophilales	4	0	0	0
Myxococcales	0	4	1	6
Nitrospirales	0	0	0	1
Opitutales	0	1	0	0
Pseudomonadales	2	0	0	4
Rhizobiales	2	3	2	4
Rhodospirillales	7	9	29	14
Selenomonadales	0	1	0	0
Sphingobacteriales	80	0	0	0
Unknown	3	317	289	204

Table 13 (continued)

(E) Class	Binturong	Baboon	Chimpanzee	Gorilla
Actinobacteria	0	1	0	0
Alphaproteobacteria	9	12	31	18
Bacilli	213	5	3	4
Bacteroidia	0	6	5	3
Betaproteobacteria	22	0	2	2
Chloroflexi	0	0	0	1
Clostridia	1	8	19	65
Deltaproteobacteria	0	4	1	6
Elusimicrobia	0	0	2	1
Epsilonproteobacteria	0	5	0	5
Flavobacteria	2	5	0	1
Gammaproteobacteria	9	2	1	16
Negativicutes	0	1	0	0
Nitrospira	0	0	0	1
Opitutae	0	1	0	0
Sphingobacteria	80	0	0	0
Unknown	3	317	289	204

(F) Phylum	Binturong	Baboon	Chimpanzee	Gorilla
Actinobacteria	0	1	0	0
Bacteroidetes	82	11	5	4
Chlorobi	0	1	0	0
Chloroflexi	0	0	0	1
Elusimicrobia	0	0	2	1
Firmicutes	214	13	22	69
Nitrospirae	0	0	0	1
Proteobacteria	40	23	35	47
Verrucomicrobia	0	1	0	0
Unknown	3	317	289	204

Table 14. Operational taxonomic units and abundances used to calculate diversity indices

Operational Taxonomic Unit	Binturong	Baboon	Chimpanzee	Gorilla
Acetobacter pasteurianus	7	1		1
Acetohalobium arabaticum				2
Achromobacter piechaudii			1	
Acidiphilium cryptum		9	29	13
Acidobacterium capsulatum			5	3
Acinetobacter baumannii				4
Acinetobacter johnsonii	1			
Acinetobacter lwoffii	1			
Acinetobacter sp. DR1				2
Alicyclobacillus acidocaldarius		1		
Alistipes shahii		10		
Arcobacter cibarius		4		1
Arcobacter thereius		1		7
Arthrobacter aurescens TC1		1		
Bacillus subtilis		4	13	
Bacillus tusciae		1		1
Bacterium DRB3-216	2			
Bacteroides eggerthii		2		
Bacteroides finegoldii				2
Bacteroides paurosaccharolyticus		2		17
Bifidobacterium dentium				1
Blautia hansenii				1
Brevibacillus choshinensis		1		
Burkholderia cenocepacia			1	1
Burkholderia rhizoxinica		1		
Butyrivibrio fibrisolvens		1	11	5
ce1_cl56_cn1			1	
Cellulophaga algicola	2			
Chlorobium luteolum DSM 273		1		
Chlorobium phaeovibrioides				4
Chlorobium tepidum				3
Chloroflexus aggregans				2
Clostridium orbiscindens		1	1	
Clostridium papyrosolvens				6
Clostridium phytofermentans			3	
Clostridium saccharolyticum-like K10		2		
Clostridium saccharoperbutylacetonicum	1			
Clostridium scindens		3		1
Clostridium sphenoides		1		7

Table 14 (continued)

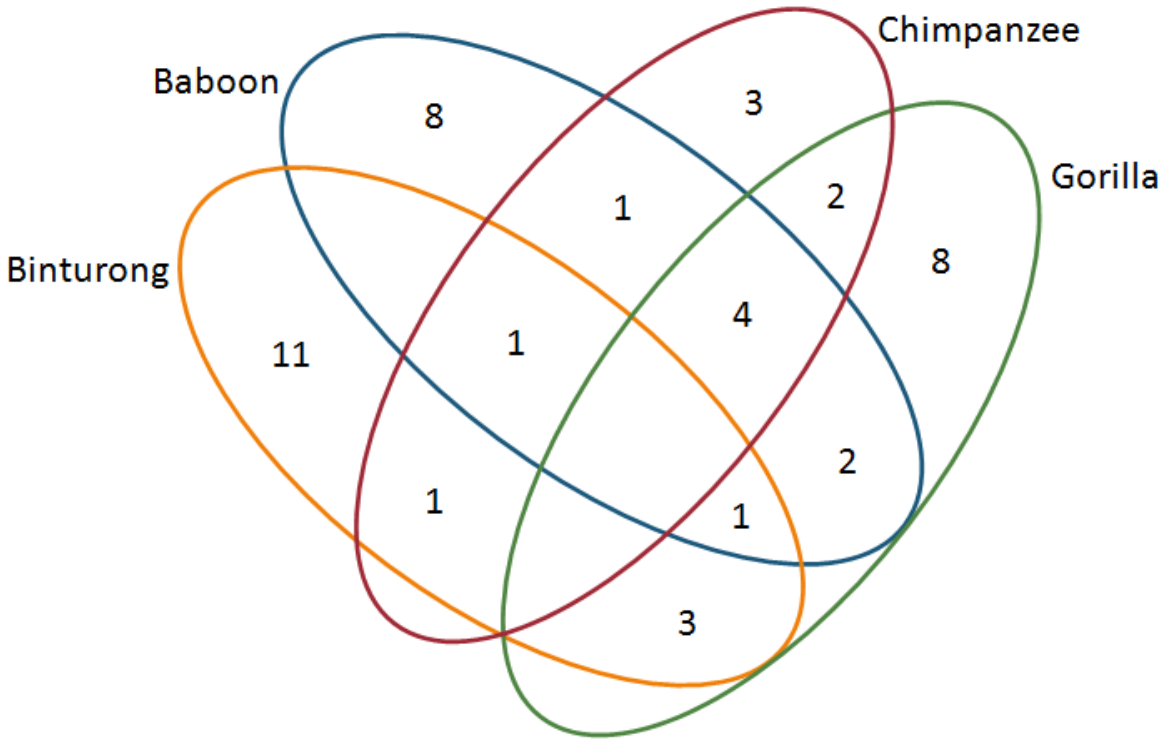
Clostridium thermocellum		1		2
Comamonas testosteroni	20			
Desulfotomaculum nigrificans			1	
Durio zibethinus		2		
Elusimicrobium minutum			2	6
Enterobacter aerogenes		28	2	11
Enterobacter cloacae			1	53
Enterococcus casseliflavus	1			
Esherichia coli	1			
Eubacterium coprostanoligenes		2	12	2
Eubacterium eligens		1	2	
Eubacterium rectale		2		
Eubacterium siraeum 70/3				1
Eubacterium ventriosum		2	2	
Faecalibacterium prausnitzii				1
Fibrobacter succinogenes		3	1	
Flavobacterium branchiophilum		5		1
Gallionella capsiferriformans		7		1
Klebsiella oxytoca				1
Klebsiella variicola	1			
Lactobacillus casei			1	
Lactobacillus gasseri		2	1	
Lactobacillus helveticus			4	3
Lactobacillus plantarum		43		
Lactobacillus rhamnosus		2		
Lactobacillus sp. N27		1		
Lactobacillus sp. N54	1			
Lactobacillus zeae				1
Leuconostoc citreum	1			
Megamonas hypermegale		2		
Methylobacterium extorquens			1	
Methylobacterium jeotgali				3
Methylobacterium radiotolerans				1
Methylotenera mobilis	4			
Ochrobactrum pseudogrignonense	2		2	
Ochrobactrum rhizosphaerae	1			
Opitutus terrae		3		
Oxobacter pfennigii		3		4
Paenibacillus sp. JDR-2		1		
Paludibacter propionicigenes		6	29	
Parabacteroides distasonis		5	2	
Parabacteroides gordonii				2

Table 14 (continued)

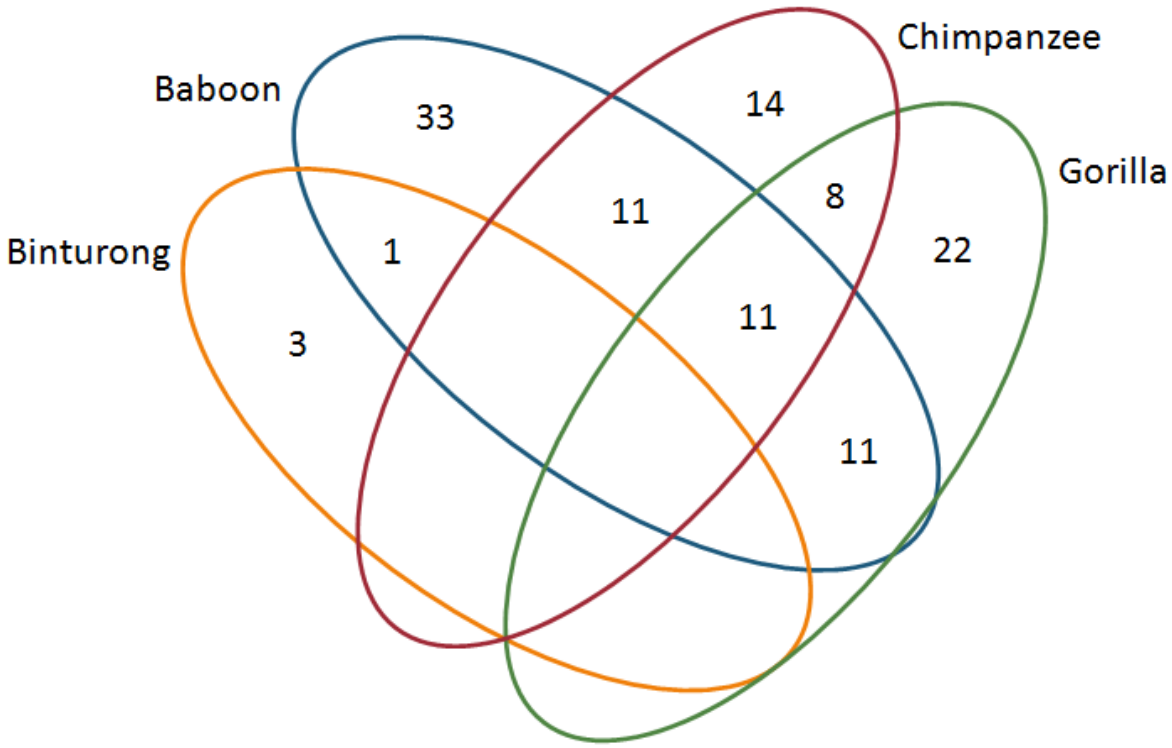
Paraprevotella xylaniphila		18		11
Polaromonas napthalenivorans			1	
Porphyromonas circumdentaria				1
Prevotella albensis		1		
Prevotella buccae		5		1
Prevotella buccalis			1	
Prevotella copri		61	75	21
Prevotella melaninogenica		5	7	1
Prevotella stercorea		29	70	9
Providencia alcalifaciens	2			
Pseudomonas reactans				1
Rhizobium sp. LH-R3		1		
Rhizobium sp. QR1		3		
Ruminococcus albus			17	
Ruminococcus bromii		9	1	
Ruminococcus flavefaciens		20	25	87
Ruminococcus obeum		32		
Ruminococcus torques		5	15	1
Selenomonas ruminantium		1		
Sorangium cellulosum		5	1	8
Sphingobacterium canadense		1		
Sphingobacterium multivorum	81			
Streptococcus alactolyticus	6			
Streptococcus bovis	1			
Streptococcus gallolyticus	3	1	2	
Streptococcus sp. N11	4			
Succinivibrio dextrinosolvens		2	4	4
Thermodesulfovibria yellowstonii			1	1
UPFBC 002_C01			1	
UPFBC 008_B01			1	
UPFBC 014_C05			1	
UPFBC P40-0218			1	
Weeksella virosa	1			
Weissella sp. N65	203			1
Yersinia enterocolitica	1			

Figure 8. Overlap between gene libraries. A) Sequences with known hits were compared at the Genus level, while B) sequences identified as uncultured clones were compared using the accession numbers retrieved from the Blastn search in GenBank®.

A



B



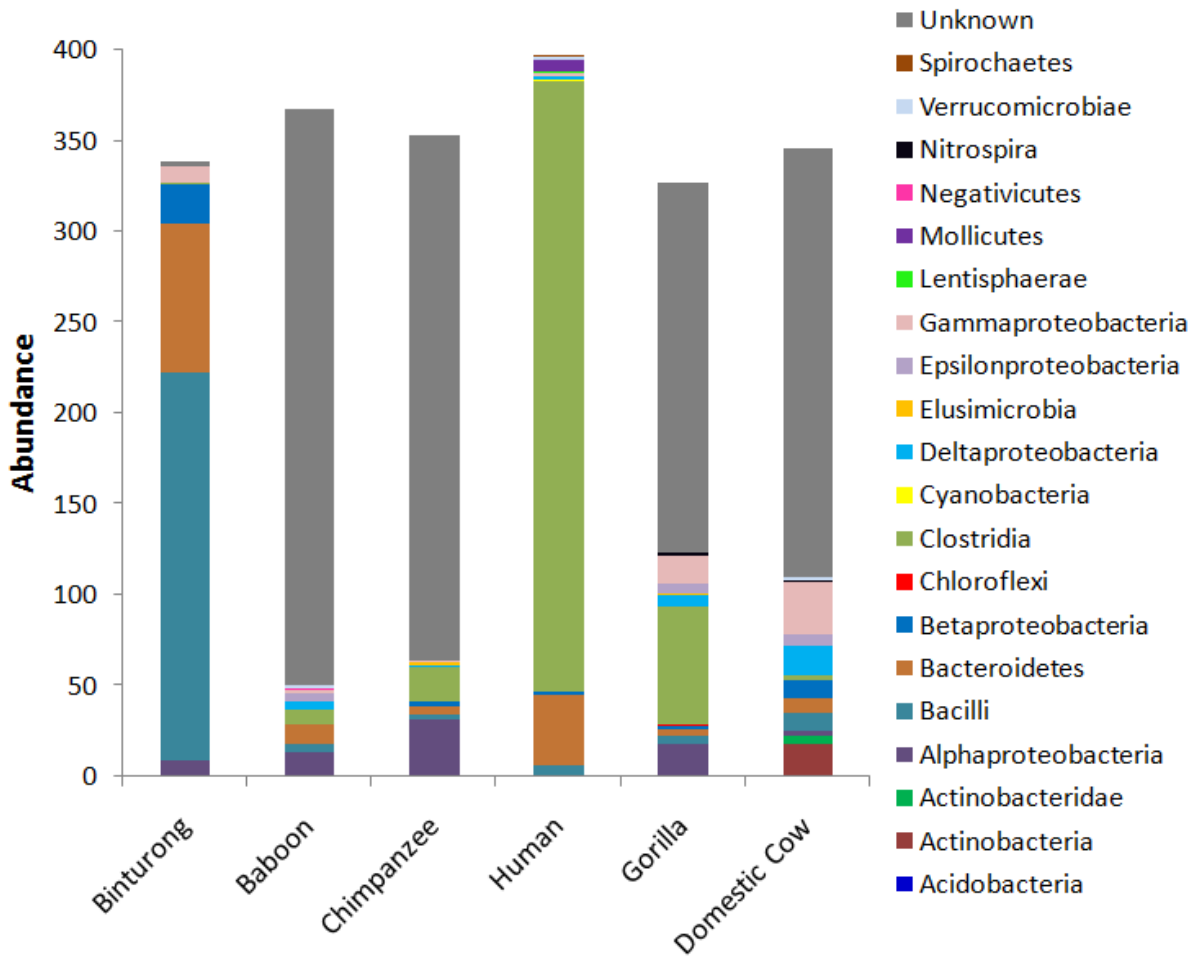


Figure 9. Composition of microbial populations at the class level, compared with results from studies of *Homo sapiens* (Paliy et al., 2009) and *Bos taurus* (Johnson et al., 2009).

Table 15. Abundance (%) of major phyla detected in gene libraries

Phylum	Binturong	Baboon	Chimpanzee	Gorilla
Bacteroidetes	82	11	5	4
Firmicutes	214	13	22	69
Proteobacteria	40	23	35	47
Unknown	3	86	82	62

Table 16. Diversity indices calculated for microbial populations

Host	Binturong	Baboon	Chimpanzee	Gorilla
Richness	24	56	42	51
Evenness (J')	0.24	0.54	0.45	0.50
Simpson's Diversity Index (D)	0.397	0.069	0.109	0.111
Simpson's Complement (1-D)	0.603	0.931	0.891	0.889
Shannon-Weaver Index (H')	1.43	3.17	2.63	2.89

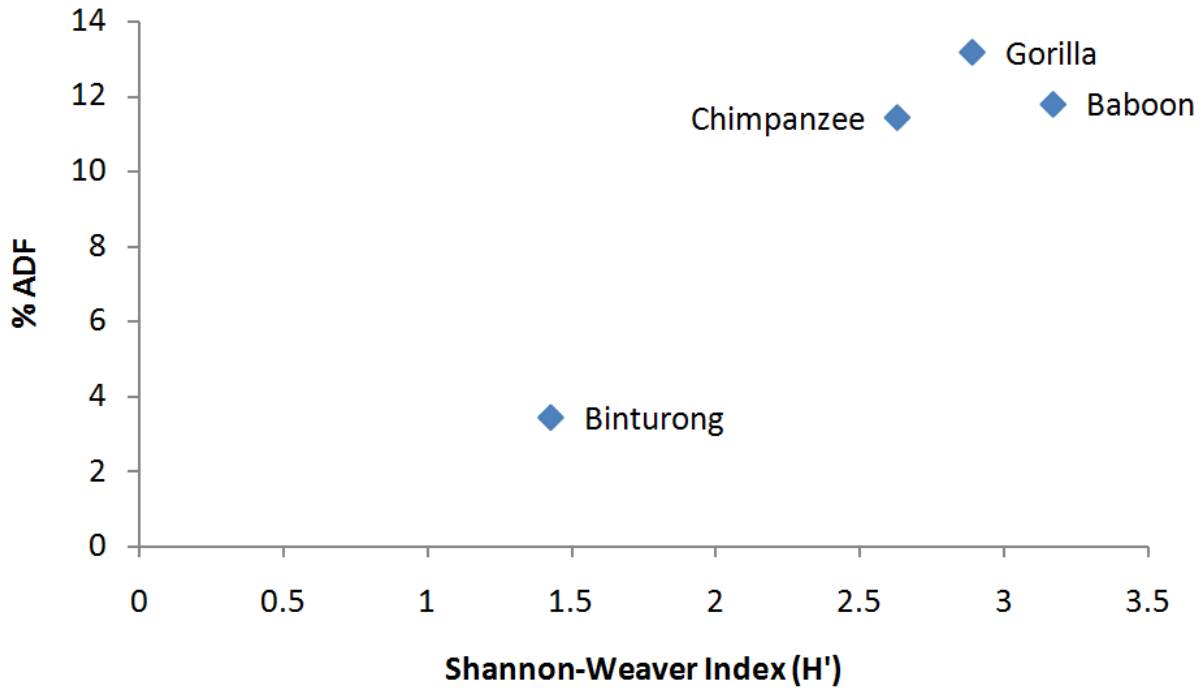


Figure 10. Percent dietary acid detergent fiber on a dry matter basis, versus Shannon-Weaver Diversity Index (H') value for microbial populations, associated with *A. binturong, P. troglodytes, G. gorilla,* and *P. hamadryas*

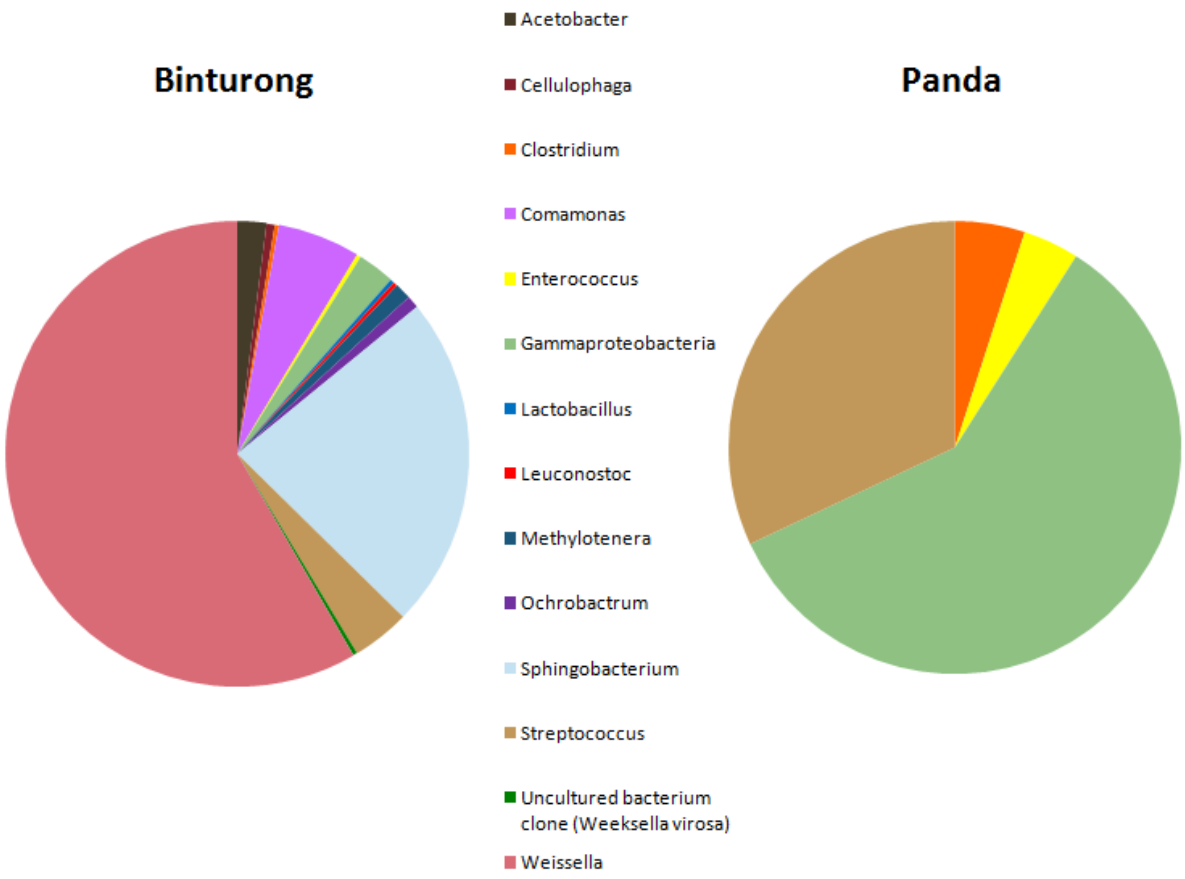


Figure 11. Comparison of binturong microbial population demographics with Giant Panda (*Ailuropoda melanoleuca*) data from Wei et al. (2007)

SUMMARY

The microbial fermentation activity and population profiles differed across mammalian hosts with changes in diet and phylogeny. Specifically, SCFA and methane production differed significantly ($p<0.0001$ and $p<0.001$, respectively), as did the amount of neutral detergent fiber ($p<0.05$) and dry matter ($p=0.0001$) digested. Total millimolar concentrations and molar percentages of SCFA produced also indicated that the microflora associated with each host species utilized substrates uniquely, and was dominated by bacteria with different fermentative functions. The gene libraries isolated exhibit a variety of endosymbiotic populations linked with different mammalian hosts. Diversity values calculated for each host species' microflora increased with the level of acid detergent fiber measured in the animals' diet. Our findings suggest that, while daily intake certainly affects the gut microbiota, so too do the host's ecological feeding strategy and evolutionary origins.

IMPLICATIONS FOR FUTURE STUDIES

Diet and environment, whether *in situ* or captive living conditions, do affect bacterial populations. While representative microbial populations remained stable within individual Giant Pandas over the course of the Wei et al. study (2007), the proportions of predominant *Streptococcus, Enterobacteria, Clostridium,* and *Enterococcus* changed over time, as well as among hosts. Not only can the gut microbial profile change over relatively short periods of time in response to diet or health status (McKenna et al., 2008; Kisidayova et al., 2009), but the endosymbiotic constituents are highly sensitive to antibiotics and toxins as well as changes in ingested substrate (Johnson et al., 2009). Wireman et al. (2006) and Torok et al. (2008) also observed that, even among animals which were isolated and uniformly fed and housed, the gastrointestinal populations appeared to be in rapid, continual change.

Further study is needed to improve our understanding. Even knowing the constituents and dynamics within a bacterial population cannot tell us which species perform specific fermentation activities. *Ruminococcus* and *Fibrobacter* species play a key role in digesting cellulose and other fiber components in typical ruminants, for example, but little is known of the actions of most other endosymbionts (Wei et al., 2007). The *Bacteriodetes* comprise a majority of the mammalian distal intestinal microflora, but their role within that complex community is not well understood beyond the digestion of dietary nutrients to produce vitamins and capsular glycans, which they use to interact with the host immune system (Desai et al., 2009). An increased ratio of *Bacteroidetes:Firmicutes,* moreover, is associated with obesity in humans. Similar knowledge of population dynamics in exotic animals could provide a useful tool in non-invasive diagnostics or intervention for health purposes—especially in primate species prone to obesity in captivity—and form a basis of information for application in free-ranging populations.

Desai et al. (2009) measured an inter-individual variation in sequence target abundances of two orders of magnitude or greater among *Felis catus* fecal samples. Caesson et al. (2009) also found that the fecal microbiota isolated from adult human subjects are highly individual-specific. In this project, we made standardized pools within species to detect the major microbial constituents associated with each host—and to stay within budget

and time constraints. However, analyses of the original samples, taken from known individuals, would be useful in comparing population dynamics within the host species as they will likely differ with the age, sex, health, and life stage of each animal. Frey et al. (2006) further pointed out that clone libraries, like any PCR-based method, are subject to bias. Future comparison to T-RFLP analyses may be a useful tool in further validating our findings, and providing a more accurate look at the composition and taxonomic richness than is provided by the current approach.

A healthy GI population sets the stage for good overall host health, making microbial balance a solid foundation for captive animal management. The emerging 'omics' technologies will make it possible to understand the microbial activities, functions and interactions within the gut, from the perspective of systems biology (Leser and Mølbak, 2009). Metagenomic sequencing will allow the alignment of target microbial species, their genes, and their digestive function within the host. Studies must be conducted to compare the community composition in captive versus wild individuals, and the role those populations play in the digestive process, to optimize management strategies and maximize understanding of populations *in situ*. Also, it is currently unknown how accurate fecal inoculums are in representing regions down the length of the intestine. While fermentation profiles are not significantly different between fecal and colonic inoculums, microbial populations on the epithelial surface are different from those found in the lumen contents, between the hindgut segment, and within fecal samples (Stevens and Hume, 1998). Future projects will be instrumental in furthering our comprehension of the internal locations and functions of micro communities.