COMPARATIVE GASTROINTESTINAL MICROBIOMES OF FEEDING SPECIALIST SCIURUS ABERTI AND GENERALIST S. NIGER

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A thesis submitted to the Graduate Faculty of the University of Colorado Colorado Springs in partial fulfillment of the requirements for the degree of Master of Sciences Department of Biology 2017
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.......May 4\textsuperscript{th}, 2017
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ABSTRACT

The nutritional value of an organism’s diet plays an integral part in the evolution of their feeding strategy. In return, the morphology of the gastrointestinal (GI) tract evolves to facilitate either an intake, or retention-maximizing feeding strategy. The microbial diversity harbored within separate compartments of the GI tract shows variation on temporal, spatial, sex-specific, host phylogenetic, interspecific, and intraspecific levels. Variation in microbial community diversity seen in dietary generalist and specialist species offers an opportunity to explore the relationship seen between host diet, phylogeny, GI morphology, and symbiotic microbial composition. I compared the GI microbiomes of Abert’s squirrels (*Sciurus aberti*) and Eastern fox squirrels (*S. niger*) to evaluate interspecific and intraspecific microbial community composition. I hypothesized that the dietary and GI morphological differences between the two species of tree squirrels would result in uniquely different communities and abundance of gut bacteria. Abert’s squirrels exhibit a specialized diet consisting predominately of ponderosa pine (*Pinus ponderosa*) structures. Fox squirrels exhibit a generalist diet. Abert’s squirrels have a GI tract that is longer and composed of more surface area than fox squirrels. Four females of each species were collected (n=8) from a ponderosa pine-Gambel oak (*Quercus gambelii*) forest. I collected tissue samples from the stomach, small intestine, cecum, and large intestine, in addition to a fecal sample. Bacterial DNA samples were isolated and the V4 region of the 16s rRNA was used for sequence alignment. Operational taxonomic units (OTUs) were assigned using the workflow provided by QIIME. Results show that microbial communities form distinct clusters in both species, but also within the upper and lower regions of the gastrointestinal tract. At the GI
region and species level, *Prevotella copri* and *Prevotella* spp. were the most important features for the machine learning analysis. This analysis used an algorithm to determine which OTUs were the best predictors of sample origin. Abert’s squirrels had less diversity in microbial communities between individuals than fox squirrels, which may be explained by their specialist diet. There were also functional differences identified from intraspecific comparisons of GI tract region using a PICRUSt analysis.
DEDICATION

I would like to dedicate this thesis to my parents Mike and Kathy Reed, whose abounding support and love have sustained me throughout this arduous, yet rewarding journey.
ACKNOWLEDGEMENTS

I would be remiss to believe that I could have accomplished this goal without my advisor, Dr. Jon Pigage, who took a chance on me when no one else would, and gave me the opportunity to prove my worth as a graduate student. Dr. Helen Pigage has been invaluable in her assistance in the field and during the long process of reviews and edits to this body of work. Dr. Jeremy Bono filled the role as my unofficial second advisor. I could not have gotten this far without his guidance and encouragement in the lab and during the process of analyzing the data. Dr. Meghan Lybecker provided her insight and knowledge of microbial systems that helped formulate our experimental design. I appreciate Dr. David Hale for allowing us to utilize the United States Air Force Academy grounds to conduct the field work for this project. Cody Glickman’s expertise in the bioinformatic software used to analyze these data was critical to my completion of this research. In conclusion, I am truly grateful to the network of scientists that have mentored me throughout these last two years.
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CHAPTER 1

LITERATURE REVIEW

Overview

This review begins with the introduction of the topic of the symbiosis between hosts and their gut microbial community. The physiological roles that are associated with gut flora are discussed, with importance being placed on the metabolizing of indigestible plant polysaccharides and plant secondary compounds (PSCs) by bacteria associated with herbivorous mammals. Diversity in the composition of microbial communities is summarized into phylogenetic, GI spatial, temporal, habitat spatial, and sex-hormone induced variation. The evolution of dietary feeding strategies and the associated GI tract morphology is discussed as it pertains to herbivorous mammals. I highlight the significant differences in Abert’s and fox squirrel GI tract morphology and diet, and their sympatric distribution along the Front Range of Colorado. The reason for my choice of the 16s rRNA gene for bacteria taxonomic analysis is addressed. The content of this review highlights the background research conducted to design the study to investigate the microbiomes of Abert’s and fox squirrels.

Microbiome

Multicellular organisms have evolved for roughly 1.2 billion years, and their co-evolutionary relationship with microbial communities has likely shaped the natural history of vertebrates (Ley et al. 2008b). Within the human body, there are an estimated 1.3 trillion bacterial cells living on or in our bodies (Sender et al. 2016). Bacterial symbiosis is responsible for many physiological processes including immunity, metabolism, digestion, and nutrient absorption. The bacteria within the GI tract are responsible for the breakdown of complex
carbohydrates including dietary fibers, production of short chain fatty acids (SCFAs), metabolism of toxins and PSCs, and synthesis of vitamins (Cummings & Macfarlane 1997; Ley et al. 2008b; Kohl & Dearing 2016).

The complete array of microbial lineages that live in a particular environment is known as the microbiota, while the microbiome refers to the full complement of genes expressed by the microbiota (Ley et al. 2008b). Studies on microbiomes have focused on soil as well as vertebrate and invertebrate GI tract, skin, and vaginal ecosystems. These studies largely explored the relationships between host microbial communities and host gut morphology, diet, phylogeny, habitat, season, and sex. Diet, host phylogeny, and GI tract morphology have shown to be the most influential variables in clustering of microbiota communities between organisms (Ley et al. 2008a).

For placental mammals, the inoculation of the GI tract microbiota is derived from both maternal and environmental microbes. The process begins during birth, and is relatively unstable until approximately two to four years of age for humans. Human microbial ecology can also be influenced by many epigenetic factors such as mode of infant delivery, antibiotic exposure, neonatal nutrition, adult nutrition, stress, age, and degree of hygiene (Mackie et al. 1999; Penders et al. 2006; Palmer et al. 2007; Ley et al. 2008b; Tanaka et al. 2009; Koenig et al. 2011).

The changes in gut microbial communities may affect host reproductive fitness by altering digestive efficiency, host nutrition, immune response, and stress response. (Amato et al. 2013; 2015; 2016). These effects allow studies of mammalian microbiomes to draw inferences about the role that gut microbiota composition plays on the natural selection and evolution of host species. The stability of gut microbial communities has direct effects on the physiological homeostasis of the host. A shift in the composition of the microbiota, a process known as
dysbiosis, has ramifications for host immunity and thus increases risk of disease (Brown et al. 2012). Alterations in the microbial composition of the human gut has been linked to obesity, Crohn’s disease, irritable bowel disease, and type-2 diabetes (Larsen et al. 2010; Joossens et al. 2011; Chassard et al. 2012). Recent evidence has shown that through interactions with the gut-brain axis, the bidirectional communication system between the central nervous system and the GI tract, the gut microbiome can also influence neural development, cognition, and behavior. Through this gut-brain communication pathway, the state of gut microbial dysbiosis can induce mental illness, such as depressive-like behaviors (Rogers et al. 2016).

**Short Chain Fatty Acid Production**

Short chain fatty acids are organic fatty acids which arise from bacterial fermentation of polysaccharide, oligosaccharide, protein, peptide, and glycoprotein precursors in the colon. The fermentation of organic matter by anaerobic bacteria housed in the gut produces usable energy for microbial growth and maintenance, as well as other metabolic end products for use by the host (Cummings, 1983). The abundance and community structure of the microbiome, in addition to the substrate source, or diet, within the host will determine the production of SCFAs (Cummings, 1983).

Most hindgut fermenting herbivores have a large cecum, and parts of the proximal colon have developed in length and diameter to accommodate large volumes of plant material. This increased fermentation chamber is the source of SCFA production and can account for up to 80% of maintenance energy in herbivores (Rechkemmer et al. 1988). Although fox squirrels are hindgut-fermenting herbivores, their diet is richer in nutrients, requiring less time to digest and convert into energy.
The morphology of the GI tract is not static, but adapts to the changes in physical and chemical characteristics of the diet and the level of food intake (Barboza et al. 2010). Abert’s squirrels have evolved a hindgut that is particularly enlarged when compared to the fox squirrel. The largest differences are seen in the length, weight, and surface area measurements of the cecum and colon. The cecum is on average 125% larger than the cecum of a fox squirrel, and is consistent with the enlarged cecum of other specialized herbivores such as koalas (Phascolarctos cinereus). This adaptation may be the result of selection pressures generated by the need to maximize the food value of its low-quality diet by allowing symbiotic microbes to digest fiber that cannot be broken down until it reaches the cecum, at which point the anaerobic bacteria produce SCFAs and detoxify terpenes, resin acids, and tannins associated with ponderosa pine (Snyder 1992).

**Microbial Degradation of Plant Secondary Compounds**

Herbivorous mammals have been engaged in an evolutionary arms race with the plants they target. Herbivores must rely on their co-evolutionary history with bacteria, predominately in the phyla Firmicutes and Bacteroidetes, to produce enzymes that metabolize harmful or deterrent plant-secondary compounds (Barboza et al. 2010). The GI tract microbial structure is a dynamic community that responds to exposure to PSCs and other xenobiotics. Mammals and their gut microbes must adapt to find new pathways to breakdown plant secondary metabolism byproducts that are toxic digestive inhibitors, and diuretics that deter herbivores from consuming large quantities of the plant (Dearing et al. 2005). Plants are nature’s alchemists, and it is the production of novel chemicals that allows them to maximize their fitness by deterring herbivores from consuming them. For herbivores that are specialists with their host plants, a strategy of dealing with the volumes of PSCs must be efficient at degradation, but also excretion of the PSCs
so that the absorption of these toxins is kept at non-lethal concentrations (Torregrossa et al. 2012).

Studies conducted by Shipley et al. (2012) that investigated the difference in the toxicity levels of specialist versus generalist herbivores used pygmy rabbits (Brachylagus idahoensis) which subsist almost entirely on sagebrush (Artemisia spp.) and mountain cottontail rabbits (Sylvilagus nuttali) as their generalist species. Sagebrush accounts for nearly 99% of the pygmy rabbit’s diet in winter, and contains high levels of monoterpenes, terpenes, sesquiterpene lactones, and phenolics. In this study, they examined the mechanisms used by the specialist pygmy rabbit to metabolize 1,8-cineole that was laced into their food. They found that the pygmy rabbit consumed greater amounts of food with higher concentrations of PSCs than generalists, and that their feces and urine contained more PSC metabolites, suggesting that specialist herbivores have evolved mechanisms that control the absorption of toxins related to their host plant (Shipley et al. 2012).

A different strategy has been observed in Abert’s squirrels in studies conducted by Snyder (1992). In feeding trials, he found that food laced with ecologically realistic concentrations of either beta-pinene or beta-phellandrene were eaten significantly less than control food (Snyder 1992). Snyder then designed a way to test this hypothesis that Abert’s squirrels are selecting ponderosa pine (Pinus ponderosa) trees based on the chemical composition of their resin. He found that the phloem of the ponderosa pine trees targeted by Abert’s squirrels for a source of food contain lower concentrations of tannins than do non-target trees. Abert’s squirrels may avoid the dangerous concentrations of PSCs by a combination of behavioral and physiological adaptations that rely on selective herbivory and microbial communities to control their absorption of toxins (Snyder 1992).
Kohl & Dearing (2016) designed a study using several species of woodrat to evaluate the hypothesis that ingestion of toxic diets by herbivores is facilitated by the gut microbiota. They have proposed that the functional role of the foregut in rodents may be to house microbes that are capable of detoxification. Foregut detoxification would allow for the metabolism and inactivation of PSCs before absorption in the small intestine (Kohl & Dearing 2016). Metagenomic studies conducted on the microbial functionality in the foregut of desert woodrats (Neotoma lepida) showed that there were higher abundances of genes associated with the metabolism of aromatic compounds in animals that were feeding predominantly on creosote bush, a plant with many known harmful secondary compounds (Kohl et al. 2014). The animal would seem to benefit from metabolizing PSCs in the foregut to render the digested food benign as quickly as possible.

**Differences in Microbiomes Between Taxa**

Studies by Ley et al. (2008a) have investigated the clustering of microbiota across host taxonomy and have shown that diet, more than host phylogeny, drives the composition of microbial communities. Host-associated microbiota allows specialized diets among the vertebrates. There is a clear distinction between the microbiota represented in herbivores, carnivores, and omnivores. Within herbivorous mammals, there is further specialization to accommodate hind-gut versus fore-gut fermentation. Results have suggested that as mammals underwent convergent evolution in the morphological adaptations of their guts to herbivory; their microbiota arrived as similar compositional configurations in unrelated hosts with similar gut structures (Ley et al. 2008a). Exceptions include folivores like the red panda (Ailurus fulgens) and giant panda (Ailuropoda melanoleuca), whose guts still resemble that of their carnivorous ancestors, and have microbiota that cluster with other carnivores (Ley et al. 2008a).
A study conducted by Delsuc et al. (2014) on myrmecophagous mammals (ant/termite eating specialists) used the highly specialized diet shared among these organisms as a model system to examine the effects of convergent evolution on microbiota composition. They hypothesized that because the mammals feed on social insects whose protein and caloric value is locked within a chitin exoskeleton, they rely on symbiotic chitinolytic microbes to optimize their protein nutritional intake. This study adds support to the theory that host diet is a strong evolutionary force shaping the composition of host microbiota. Notable results indicate that the termite-eating aardwolf is an outlier within the Carnivora, and that its microbiome clusters more closely with other specialized myrmecophagous vertebrates, such as the armadillo and aardvark, than it does with its closest living relative, the spotted-hyena (Delsuc et al. 2014).

Gastrointestinal Tract Morphology

Small mammalian herbivores need much more energy and protein per unit body mass than larger herbivores. To achieve an efficient system in which nutrients are converted to energy, many small herbivores have an enlarged cecum that serves as a fermentation reservoir for their fibrous diet (Sakaguchi, 2003). The Optimal Digestion Theory predicts that the digestive strategy that endows an animal with the greatest fitness payoff is that which maximizes the net rate of energy released from ingested food. This model predicts that optimal digestion time varies among foods, being longer for poor quality foods than the time for higher quality foods. Thus, animals eating poorer-quality foods should have larger digestive tracts (Hume, 1989).

Abert’s squirrels are specialists, depending on ponderosa pine stands for nesting sites and food (Snyder 1992). They feed on inner bark, seeds, twigs, buds, and young cones from the ponderosa pine and on hypogeous fungi growing both above and below ground. The fungal spores remaining in the squirrel’s feces contribute to the spread of the mycorrhizal community
associated with ponderosa pine. Because these squirrels do not cache food for winter, from late
fall to early spring they remain active at clipping terminal twigs to consume the inner bark as the
main source of forage. Only about 10% of the weight of twigs cut is consumed (Snyder 1992).
Abert’s squirrels have evolved a distinctively large GI tract to make use of a retention-
maximizing strategy to process this high volume, low quality diet. Host trees appear to be
selected by their low levels of certain monoterpenes and differ both chemically and
physiologically from non-host trees (Capretta et al. 1980; Snyder 1992).

Fox squirrels have a generalist diet and eat a variety of food, including nuts, such as
acorns and walnuts; twigs, buds, and tender leaves of many tree species, including cottonwood
and elm; various berries, apples, Russian-olives, and other fleshy fruits; small grains; and corn
(Yeager, 1959). Their cecum, small intestine, large intestine, and overall GI length are
significantly shorter than Abert’s squirrels. In addition, fox squirrels have significantly smaller
overall GI tract surface area (Murphy et al. 1999). These differences in morphology result in an
intake-maximizing strategy, rather than retention-maximizing. The comparative GI tract
morphology of fox and Abert’s squirrels is an excellent example of the adaptations to a nutrient
poor versus nutrient rich diet.

Changes in Microbial Diversity in Gastrointestinal Tract

Research conducted on the human microbiome has shown that our microbial habitats are
not isolated from one another, but are interconnected throughout the human body. Although
these habitats are not isolated, they do vary in environmental conditions present for bacterial
growth, and thus can harbor unique communities (Costello et al. 2012). The GI tract thus
provides a heterogenous ecosystem to study the changes in microbial diversity that are affected
by the changes in oxygen levels, pH, and substrate abundance (Savage 1977). A study conducted
by Dubos et al. (1967) on the indigenous flora of the GI tract found that aerotolerant bacteria, such as enterococci and lactobacilli, are predominant in the stomach and small intestine, and obligate anaerobic species, such as Clostridia and Bacteroides, are predominant in the large intestine.

An analysis of the variation in microbial composition throughout the human GI tract compared to fecal composition showed that lineages present in mucosal libraries were distinct from stool libraries. Eckburg et al. (2005) found inconsistencies in the subpopulations of mucosal communities, and suggested that the pattern of distribution of mucosal bacteria did not follow a homogeneous gradient along the longitudinal axis of the colon. Furthermore, the patchiness of microbial communities did not display an obvious pattern along the course of the colon, but may reflect microanatomic niches. A high level of bacterial diversity and novel lineages was found in both the stool and mucosal libraries, and Eckburg et al. (2005) postulated that the fecal microbiota represents a combination of shed mucosal bacteria and a separate nonadherent luminal populations.

Research conducted by van der Wielen et al. (2002) on the microbiota of broiler chickens (Gallus gallus domesticus) has investigated the unique microbial ecosystems within separate structures along the GI tract. Their results indicated that although crop, duodenum, and ilium were more similar to each other than either was to the cecum, every part of the intestinal tract has its own specific bacterial community and thus can be seen as separate ecosystems.

Another example of the spatial variation of microbiota within the GI tract is provided by a study conducted by de Oliveira et al. (2013). In this study, they dissected the GI tract of a Brazilian Nelore steer (Bos taurus). This herbivorous, foregut fermenter was the single sample for this study. The researchers collected luminal samples from three distinct GI tract regions:
forestomach, small and large intestine. All segments from these three regions were sampled as follows: forestomach: rumen, reticulum, omasum, and abomasum; small intestine: duodenum, jejunum, and ileum; and large intestine: cecum, colon and feces. Their principal coordinate analysis (PCOA) plot revealed that each of the three distinct GI regions clustered separately. The large intestine had more diversity of bacteria than the small intestine or forestomach.

A study conducted on the spatial variation of microbial diversity along the GI tract of mice was conducted by Turnbaugh et al. (2009). They sacrificed adult mice and collected samples from the luminal contents of the stomach, small intestine, cecum, colon, and a fecal sample. Significant increases in the percent representation of Bacteroidetes was observed moving from the small intestine to the cecum, colon, and feces. Lowest levels of overall diversity were observed at the proximal intestine, while the highest levels corresponded to samples taken from the colon and feces (Turnbaugh et al. 2009).

The inoculation of germ-free mice (Mus musculus) with a standard set of 8 aerotolerant and anaerobic bacteria species known as the altered Schaedler flora (ASF) species has been used to characterize the spatial heterogeneity of bacterial communities within the GI tract. A study conducted by Sarma-Rupavtarm et al. (2004) showed that the diversity of strains and relative distribution changed substantially over the GI tract. They found that the aerotolerant anaerobe Lactobacillus murinus comprised nearly 100% of community composition in the esophagus, and declined rapidly along the length of the GI tract. Anaerobes were generally absent in the esophagus, occurred in low numbers in the stomach, and increased sharply over the length of the small intestine, comprising 99.99% community composition of the cecum and colon. Clostridium spp., Flexistipes spp., Eubacterium plexicaudatum, and Bacteroides spp. followed similar distribution patterns along the length of the GI tract, increasing in numbers from the small intestine through the colon.
In addition to comparing community composition along the length of the GI tract in mice, Sarma-Rupavtarm et al. (2004) collected fecal samples to analyze the distinction between colonic bacterial species and those found in the feces. They found considerable differences in the distribution of ASF species in the colon and feces, with bacterial species occurring in orders of magnitude higher in the colonic environment than in the feces. A later study conducted by Michelland et al. (2009) also evaluated the differences in microbial community composition of the feces and the forestomach of bovines. They found significant variation between samples from the forestomach (ventral rumen, dorsal rumen, and reticulum) and the feces.

Temporal Variation

A study conducted over a ten-month period on Mexican black howler monkeys (Alouatta pigra) by Amato et al. (2013; 2015; 2016) has shown that the relative abundances of individual bacterial taxa change over time in correlation with changes in host diet. The authors propose that energy and nutrient production by the gut microbiota acts as a buffer against seasonal variation in food availability.

Over a two-year period Maurice et al. (2015) monitored the changes in microbial composition of wood mice (Apodemus sylvaticus). They observed a seasonal shift in composition that coincided with the expected timing of an annual transition to a seed-based diet from a more insect-based diet. Specifically, Lactobacillus species abundance was significantly higher in the spring, whereas Alistipes spp. and Helicobacter spp. were consistently enriched in the fall.

Hibernating animals undergo prolonged fasting in addition to physiological and morphological changes of the GI tract, which make them an intriguing model to investigate interrelationships between host physiology and the gut microbial community (Stevenson et al. 2014). Microbiome studies involving hibernating host species monitor the response of microbial
communities to a cyclically dynamic environment. Changes in food intake during hibernation periods affect the substrates available for microbial communities, allowing competition over resources to alter community composition. Winter fasting limits the availability of degradable substrates to support growth of gut microbes. Decreased metabolic rates associated with core body temperature drops during a state of torpor could potentially alter microbial communities. The intestinal immune system is also remodeled during hibernation, which can alter the environment in which microbial communities reside. The gut microbiota composition of thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) undergoes seasonal restructuring over an annual hibernation cycle due to the variation of diet composition and intake related to the cycle of fasting and reemergence from hibernation. Phylogenetic diversity and number of observed operational taxonomic units (OTUs) were observed to be lowest in late winter and highest in the spring after a two-week period of refeeding (Carey *et al.* 2013).

**Sex-Dependent Variation**

Host sex hormones can modulate microbiota composition in mammals (Markle *et al.* 2013; Sankaran-Walters *et al.* 2013). In an experiment in which male and female mouse microbiota were transferred to the opposite sex to observe the changes in metabolic outcomes, transfer of male, but not female, microbiota lowered serum concentrations of glycerophospholipid and sphingolipid long-chain fatty acids. In a subsequent test to determine whether testosterone transferred from the male donor was the cause of the metabotype changes, they performed another transfer in which the female recipients received an androgen receptor antagonist. The blockage of the sex hormone receptor successfully negated the effects seen in previous transfers, supporting that testosterone elevation caused by male microbiome transfer was responsible for the changes in host metabolomic phenotypes (Markle *et al.* 2013).
The effect of host sex hormones on the functional genetic profile of the human gut microbiome was examined by Sankaran-Walters et al. (2013). The gut mucosa is a site of interaction between the gut microbiome and the host immune system. By evaluating the upregulation of gene expression related to immune function in the gut microenvironment, they measured significant differences in the immune response between males and females. This study demonstrated that host-microbe interactions differ at the level of immune activation, and specifically that females had a higher baseline level of immune activation compared to males (Sankaran-Walters et al. 2013).

Spatial Variation in Microbial Diversity

A study on the microbiome of howler monkeys revealed distinct differences in microbial species richness and diversity among groups occupying distinct habitats. The samples were collected from monkeys in southeastern Mexico that inhabited either a continuous, evergreen rainforest (CR), an evergreen rainforest fragment 4.5 km from the CR (FR), a continuous, semi-deciduous forest (SD), and a wildlife center (captive). Diet differed among the habitats, showing that both FR and captive howler monkeys consumed a diet with severely reduced diversity. Microbial species richness clustered by habitat, revealing that howler monkeys inhabiting the CR contained as many as six times the number of microbial OTUs as captives, and up to twice as many OTUs as SD and FR forests (Amato et al. 2013).

A lack of microbial species diversity may reduce microbial functional groups, making the microbiome less efficient, less resistant to disturbance, more susceptible to pathogenic invasion, and elevated stress levels (Costello et al. 2012). Further research on the effects of habitat degradation on the mammalian microbiome could become another avenue towards understanding how to construct conservation management plans.
*Sciurus aberti*

*Sciurus aberti* is a large tree squirrel known commonly as the Abert’s squirrel, or tassel-eared squirrel. Abert’s squirrels are found in ponderosa pine communities in the Southwest, usually between 1800 and 3000 m, in portions of Wyoming, Colorado, New Mexico, Arizona, and Utah in the United States and in the Sierra Madre Occidental from Northern Sonora and Chihuahua to Southern Durango in Mexico (Nash & Seaman, 1977). Six subspecies are currently recognized, and populations are relatively isolated due to discontinuous distribution of stands of ponderosa pine. Subspecies include: *Sciurus aberti kaibabensis*, *S.a. aberti*, *S.a. barberi-durangi*, *S.a. ferreusE*, *S.a. ferreusW*, and *S.a. chuscensis* (Wettstein et al. 1995).

Abert’s squirrel is a specialized mammalian herbivore with its host, the ponderosa pine. The squirrel’s diet varies seasonally, but is predominately composed of tissues from the ponderosa pine and other species that occur in close association with it. They obtain phloem primarily from terminal twigs in the upper crown of target trees. The process involves gnawing completely through the base of the twig, discarding the terminal needle cluster, stripping away the outer bark, revealing the edible layer of inner bark which is consumed and the remainder of the twig is dropped to the ground (Snyder 1992).

Abert’s squirrels will subsist up to six months out of the year on a food source that is known to contain high concentrations of compounds, such as phenolics and terpenoids, which are deterrent or even toxic to other herbivores (Keeling & Bohlmann 2006; Li *et al.* 2012). The ponderosa pine resin contains monoterpenes, which are a major class of naturally occurring chemical compounds found in the pine tissue. The phloem of the ponderosa pine trees that are targeted by Abert’s squirrels for a source of food contain lower concentrations of tannins than do non-target trees (Snyder 1992). Target trees can be identified by an arrangement of needle
clusters and stripped twigs surrounding the base of the tree. In feeding trials conducted by Snyder, he found that food laced with ecologically realistic concentrations of either beta-pinene or beta-phellandrene were eaten significantly less than control food. Snyder proposed that Abert’s squirrels probably select individual trees in which chemical and physiological defenses are insufficient to deter feeding, and the nutritive value of the phloem makes continued twig clipping worthwhile (Snyder 1992).

*Sciurus niger*

*Sciurus niger* is a large tree squirrel commonly known as the fox squirrel. Native to the eastern and central United States and southern Canada, the fox squirrel has been introduced into the western United States. Fox squirrels are a relatively new species to Colorado. A combination of deliberate introductions in the early 1900’s and natural invasion has resulted in the fox squirrel becoming common along the South Platte, Republican, and Arkansas rivers (Geluso et al. 2004). In this western edge of their range, fox squirrels invade habitats characterized with low-land shelterbelts, urban areas, and riparian woodlands. In the Black Forest of Colorado, Abert’s squirrels have experienced a population decline attributed to competition over habitat selection (Armstrong et al. 2010) by the fox squirrel.

**Using 16S rRNA Gene to Separate Operational Taxonomic Units**

When conducting research aimed at classifying lineages of bacteria within the microbiota, it is necessary to identify a gene of interest that will be used in a species alignment. Ribosomal RNA is a component of all self-replicating systems, is readily isolated, and its sequence changes slowly over time, permitting the detection of relatedness among very distant species. The 16s rRNA gene is highly conserved due to its important function in coding for the assembly of the 16s small ribosomal subunit. The primary structure of the 16s rRNA is highly
conserved, and species having 70% or greater DNA similarity usually have more than 97% sequence identity. These 3% or 45-nucleotide differences are not evenly scattered along the primary structure of the molecule but are concentrated mainly into nine hypervariable (V) regions (Stackebrandt & Goebel 1994). A study conducted by Kim et al. (2011) compared the efficacy of these hypervariable regions at estimating operational taxonomic units (OTUs). Their results showed that for analysis of bacteria, the V1-V3 and the V1-V4 regions should be targeted. These regions are 484 and 652 base pairs long, respectively. Enough variation exists within the 16s rRNA gene across bacterial lineages to make this gene an ideal candidate for performing metagenomic analysis of host microbiota.
CHAPTER 2

INTRODUCTION

The bacterial communities within the gastrointestinal (GI) tract have likely shaped the evolutionary history of vertebrates (Ley et al. 2008a). Bacterial symbiosis is responsible for many physiological processes including immunity, metabolism, digestion, and nutrient absorption. The bacteria within the GI tract are responsible for the breakdown of complex carbohydrates including dietary fibers, production of short chain fatty acids (SCFAs), metabolism of toxins and plant secondary compounds (PSCs), and synthesis of vitamins (Cummings & Macfarlane 1997; Ley et al. 2008b; Kohl & Dearing 2016). Studies on gut microbiota have largely explored the relationships between host microbial communities and host gut morphology, diet, phylogeny, habitat, season, and sex.

Diet, host phylogeny, and GI tract morphology have been shown to be the most influential variables in clustering of microbiota communities among organisms (Ley et al. 2008a). Studies by Ley et al. (2008a) have investigated the clustering of microbiota across host taxonomy and shown that diet and GI tract morphology, more so than host phylogeny, drives the composition of microbial communities. For instance, in a study to examine the relatedness of myrmecophagous mammals and their microbiomes, Delsuc et al. (2014) showed that the termite-eating aardwolf (Proteles cristata) is an outlier within the class Carnivora, as its microbiome clusters more closely with other specialized myrmecophagous mammals, such as the armadillo (Dasypus novemcinctus) and aardvark (Orycteropus afer), than it does with its closest living relative, the spotted-hyena (Crocuta crocuta). The effect of GI tract morphology on bacterial community composition was described by Ley et al. (2008a), who found that herbivores clustered into two groups that corresponded generally to foregut fermenters and hindgut fermenters. In
addition to foregut and hindgut groups, omnivores separated into hindgut fermenters and those with simple guts (Ley et al. 2008a).

Variation within the microbial community has been shown to occur spatially along the lumen of the GI tract. The GI tract is an excellent example of various microenvironments due to the spatial heterogeneity of pH, oxygen content, and substrate availability (Savage 1977). Spatial variation of microbial diversity along the GI tract of mice (*Mus musculus*) was observed by Turnbaugh et al. (2009) at the stomach, small intestine, cecum, colon, and in a fecal sample. Significant increases in the percent representation of Bacteroidetes were observed moving from the small intestine to the cecum, colon, and feces. Lowest levels of overall diversity were observed at the proximal intestine, while the highest levels corresponded to samples taken from the colon and feces (Turnbaugh et al. 2009).

Herbivores rely on the symbiotic microbial community in their GI tract to breakdown indigestible carbohydrates in their diet. Four components of a herbivorous diet are cell wall, cell contents, plant exudates, and anti-herbivore, deterrent PSCs (Hume 1989). The cell wall of plants contains fiber composed of polysaccharides and lignin that are not degradable by mammalian enzymes, and must be metabolized through the action of symbiotic gut microorganisms that utilize these carbohydrates (Van Soest 1996). Because herbivorous mammals depend on gut microorganisms to produce the cellulases, hemicellulases, or pectinases to digest the fibrous components of their diets, their fermentation chambers that house the microorganisms have become an important evolutionary adaptation. (Van Soest 1996).

Herbivorous mammals can develop a generalist or specialist diet, and the nutritional quality of the diet has strong influence on the morphology of the GI tract and consequently, how rapidly the animal moves food through the digestive system. For example, the GI tract of a
retention-maximizing herbivore tends to be extremely elongated, with greater tissue surface area and weight. High fiber, low quality diets need to be retained within the GI tract for longer than for an organism with a higher quality diet, and thus the morphology of the GI tract has adapted for this increased digestion time. The GI tract of an intake-maximizing herbivore is typically shorter in length to rapidly move digesting food from the stomach through the small and large intestines for excretion. This strategy allows organisms to move food through the digestive tract as quickly as possible to maximize the quantity of food that can be processed (Murphy et al. 1999).

In addition to the indigestible carbohydrates contained in the cell wall of plant material, herbivores are exposed to PSCs that have diverse physiological effects, ranging from direct toxicity to digestion impairment (Dearing et al. 2005). Herbivores that adopt a specialist diet in association with a host plant have different strategies to regulate PSCs than generalist species (Torregrossa et al. 2012). Studies that investigate the microbial community composition of feeding specialists versus generalists have focused on desert woodrat species (Neotoma spp.), pygmy rabbits (Brachylagus idahoensis) and cottontails (Sylvilagus spp.) (Sorensen et al. 2004; Ley et al. 2008a; Shipley et al. 2012). A study conducted by Sorensen et al. (2004) investigated the differences in the absorption of alpha-pinene, the predominant monoterpene in one seeded juniper (Juniperus monosperma), by two species of wood rat. The levels of alpha-pinene in the feces of the dietary specialist, Neotoma stephensi, and the generalist, N. albigula, were used to evaluate whether specialists absorbed fewer toxins derived from their diet. Their results showed that specialists had mechanisms in place to reduce the levels of toxins absorbed into the blood. They proposed that microbial degradation of monoterpenes could be one of the mechanisms, and a subsequent study conducted by Kohl et al. (2016) confirmed that symbiotic microbes do indeed degrade dietary toxins in the foregut of wood rats.
Variation in bacterial community composition has also been documented among habitat types. This habitat-specific diversity was shown in a study conducted on black howler monkeys (*Alouatta pigra*) in southeastern Mexico that inhabited either a continuous, evergreen rainforest (CR), an evergreen rainforest fragment 4.5 km from the CR (FR), a continuous, semi-deciduous forest (SD), and a wildlife center (captive). Diet differed among the habitats, showing that both FR and captive howlers consumed a diet with severely reduced diversity. Microbial species richness clustered by habitat, revealing that howlers inhabiting the CR contained as many as six times the number of microbial OTUs as captives, and up to twice as many OTUs as SD and FR forests (Amato *et al.* 2013).

Seasonal variation in the microbiome has been documented to occur in response to resource availability throughout the changing seasons. A study conducted over a ten-month period on Mexican black howler monkeys by Amato *et al.* (2013; 2015; 2016) has shown that the relative abundances of individual bacterial taxa change over time in correlation with changes in host diet. The authors propose that energy and nutrient production by the gut microbiota acts as a buffer against seasonal variation in food availability. This seasonal response to food availability was also observed by Maurice *et al.* (2015), who monitored the changes in microbial composition of wood mice (*Apodemus sylvaticus*) over a two-year period. They observed a seasonal shift in composition that coincided with the expected timing of an annual transition to a seed-based diet from a more insect-based diet.

This study took advantage of the fox and Abert’s squirrel’s sympatric habitat along the Front Range of Colorado to investigate the interrelationship between diet and GI morphology on microbial community composition of a dietary specialist and generalist. Fox squirrels (*Sciurus niger*) have a generalist diet and eat a variety of food, including nuts, such as acorns (*Quercus* spp.) and walnuts (*Juglans* spp.), twigs, buds, and tender leaves of many tree species, including
cottonwood (*Populus* spp.) and elm (*Ulmus* spp.), various berries, apples (*Malus* spp.), Russian-olives (*Elaeagnus* spp.), and other fleshy fruits; small grains; and corn (*Zea mays*) (Yeager 1959). Abert’s squirrels (*Sciurus aberti*) are a specialized mammalian herbivore associated with ponderosa pine (*Pinus ponderosa*). The squirrel’s diet varies seasonally, predominately composed of cones, seeds, and phloem from the ponderosa pine, but also heavily relies on mycorrhizal fungi during the months of June through November, which during some seasons approaches 100% of their stomach volume (States & Wettstein 1998). While some species of tree squirrels occasionally strip the bark of trees to supplement their diet, the Abert’s squirrel relies on the ponderosa’s phloem year-round, primarily from terminal twigs in the upper crown of target trees. From January through April, the Abert’s squirrel will feed almost exclusively on phloem, constituting a high fiber, low nutrient diet (States & Wettstein 1998). This dietary specialization is unique amongst mammalian herbivores. Abert’s squirrels subsist up to six months of the year on a food source highly concentrated with chemical compounds, such as phenolics and terpenoids, which are deterrent or even toxic to other herbivores (Keeling & Bohlmann 2006; Li *et al.* 2012). Snyder investigated the strategies that Abert’s squirrels utilize to deal with the terpenoids and phenolics in their diet by characterizing the chemical composition of resin in target versus non-target ponderosa pine trees (Snyder 1992). Abert’s squirrels have evolved a distinctively large GI tract to make use of a retention-maximizing strategy to process this high volume, low quality diet (Murphy *et al.* 1999), and do not cache food like their relative, the fox squirrel (Armstrong *et al.* 2010). The fox squirrel cecum, small intestine, large intestine, and overall GI length are significantly shorter than Abert’s squirrels. In addition, fox squirrels have significantly smaller overall GI tract surface area (Murphy *et al.* 1999). These differences in morphology result in an intake-maximizing strategy.
Due to the differences in GI morphology and diet between the two species of tree squirrels, I hypothesized that there would be significant differences in the microbial diversity seen at the interspecific, intraspecific, and GI tract level. Because of the high fiber diet of the Abert’s squirrel, I predicted that a unique microbial community is responsible for the production of cellulase and other cell wall metabolizing enzymes. In addition, I hypothesized a functional difference in the microbial metagenome of both species that would add support to previous evidence that herbivorous specialists have evolved a suite of genes responsible for the metabolism of PSCs.
CHAPTER 3

MATERIALS AND METHODS

Trapping and Sample Collection

All animals were trapped using the Havahart live traps (24” x 7” x 7”) (Havahart, Lititz, Pennsylvania) in a mixed ponderosa pine and Gamble oak (*Quercus gambelii*) forest from March 15th, 2016 through April 22nd, 2016. This time window was selected to control for seasonal variation in the gut microbiome and to sample Abert’s squirrels while they were still subsisting predominately on cortical tissue of ponderosa pine. A variety of bait was used, including peanut butter, soy butter, and roasted shelled peanuts. The best trapping results were seen using the roasted shelled peanuts. Male squirrels were released at the site of capture. To control for sex-dependent variation in gut microbiome, female squirrels were used exclusively in this study. Female squirrels were taken to the University of Colorado Colorado Springs (UCCS) and euthanized via inhalation of Isoflurane. All animals were trapped, handled, and euthanized following protocol approved by the Institutional Animal Care and Use Committee (IACUC) (Protocol # UCCS-16-001) and Colorado Parks and Wildlife Scientific Collection License # 16TR2104. Dissections were conducted in an aseptic environment at UCCS immediately following euthanasia.

Once the GI tract was removed from the animal, incisions were made into the stomach, small intestine, cecum, and large intestine and each sampling site was swabbed three times with sterile swabs. This included one stomach site, three small intestine sites, one cecum site, two large intestine sites, and two fecal samples collected at the most distal end of the large intestine. The total number of swabs per animal equaled 27. The small intestine was measured (cm) from the pyloric valve to the ileo-cecal junction. Total length was divided by three, then the mid-point
of each section of the small intestine was removed and the inner lining of the intestinal wall swabbed. Likewise, the large intestine was measured (cm) and divided by two, then the midpoint of each section was removed for swabbing. The stomach and cecum were swabbed by making a single incision to insert the swabs to collect material from the inner walls of the structure. Each swab was then inserted into its own labeled Eppendorf tube, and all samples from each animal were kept in separate containers stored at -80° C.

**DNA Extraction and 16s rRNA Gene Sequencing**

DNA extractions were performed using the MoBio PowerSoil DNA Isolation Kits (QIAGEN, Carlsbad, California) following manufacture’s procedure. Sample concentration (ng/µl) and 260/280 ratios were measured using a Thermo Scientific NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts). An additional DNA extraction from a replicate sample was performed if the first sample did not meet a minimum threshold of 30 ng/µl. All samples met Beijing Genomics Institute (BGI) requirements of DNA mass of 1µg < m < 2µg, concentration > 20 ng/µl, with a total volume range between 20-100 µl. DNA isolates were stored at -80° C until shipment. Samples were sequenced using Illumina MiSeq platform at BGI Americas (Cambridge, Massachusetts). Primers were selected that would sequence the entirety of the V4 region of the 16s rRNA. Amplicons were sequenced using 125 base pair paired end reads to allow complete coverage of the V4 region.

**Data Analysis**

Data returned in FASTQ formatted files were then imported into the software program Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso *et al.* 2010). QIIME is a pipeline for performing microbial community analysis that integrates many third party tools which has become one of the standards in the field. Operational taxonomic units were selected
using the pick_open_reference_otus.py command using the default threshold of 97% similarity and Greengenes database (Lawrence Berkeley Laboratory). Cyanobacteria (Hanshew et al. 2013) and Unassigned phyla were filtered from the OTU table using the filter_taxa_from_otu_table.py. Additional quality filtering removed OTUs with a low count using filter_otus_from_otu_table.py with a threshold of --min_count_fraction .005 (Bokulich et al., 2013). Sequences per sample ranged from a minimum of 55 to a maximum of 140,373 with a mean of 49,984. Core diversity analysis was conducted using the core_diversity_analysis.py command with a sequencing depth of 10,000 sequences per sample.

A beta diversity OTU table was created using the default beta_diversity.py command and the unweighted unifrac OTU table produced from the taxa filtering and minimum count threshold. The make_distance_boxplots.py command was used to produce the distance matrices that would be used to make pairwise comparisons of the following categories taken from our metadata table (-f "Species,GI,SampleID,Individual,Description"). The categories of interest were species and region of GI tract (upper or lower) in which the samples were taken (labeled GI in metadata table). Samples taken from the stomach and three regions within the small intestine were grouped into the upper GI, and the cecum, two regions of the large intestine, and the fecal samples were grouped into the lower GI.

Data were then imported into RStudio (Allaire, Boston, Massachusetts) to make distance boxplots from the beta diversity distance matrices. An Ensemble random forest algorithm was used to extract OTU feature importance. For our machine learning stage, we used the QIIME commands supervised_learning.py -c GI -e cv10 and supervised_learning.py -c Species -e cv10 with our finalized OTU table as our input, metadata table, and the -e cv10 argument which produces a 10-fold cross validation, and provides mean and standard deviation of error for the
predictions. Pairwise comparisons of the beta diversity values were made between species and between GI regions.

Core microbiome analysis was used to identify OTUs shared between the two species of squirrels. Our core microbiome species Venn diagram was built in RStudio using the VennDiagram package and the 75% core OTU text file built in QIIME.

To gain more insight to the functionality of the microbial communities observed in both species, a PICRUSSt (phylogenetic investigation of communities by reconstruction of unobserved states) analysis was conducted. PICRUSSt is a computational approach to predict the functional composition of a metagenome using 16s rRNA data and a database of reference genomes. PICRUSSt uses an extended ancestral-state reconstruction algorithm to predict which gene families are present and then combines gene families to estimate the composite metagenome (Langille et al. 2013). Metagenomic sequencing of microbiomes aims to sample all genes from a microbial community to elucidate the metabolic and functional profiles associated with host microbiota. Data produced from PICRUSSt were then analyzed in STAMP (statistical analysis of metagenomic profiles) (Parks et al. 2014).
CHAPTER 4

RESULTS

*Alpha* rarefaction indices of both species show that our sampling effort was sufficient to detect the majority of bacterial diversity represented. *Alpha* diversity measurements show that Abert’s squirrels were trending higher in species richness than fox squirrels (Fig 1). A similar trend was also observed in the lower GI region of both species, suggesting the cecum, large intestine, and fecal samples were higher in bacterial species richness. This finding is consistent with a study conducted by Turnbaugh *et al.* (2009) where the highest levels of bacterial species richness were found in the colon and feces, while lower levels were present in samples taken from the stomach and small intestine.

![Fig 1](image)

**Fig 1.** *Alpha* rarefaction indexes for species and GI tract region (A-Lower/F-Lower = Abert’s/fox cecum, large intestine, fecal samples; A-Upper/F Upper = Abert’s/fox stomach and small intestine samples). Rarefaction curves show sampling effort was sufficient to capture species richness in all samples. Results show that for both species, the lower GI tract regions trended higher in species richness than upper GI tract regions. Abert’s squirrels trended to have higher species richness than fox squirrels.
Dominant phyla for both species were Bacteroidetes, Firmicutes, Proteobacteria, and Verrucomicrobia (Appendix A1) which accounted for 28.8%, 34.6%, 29.3%, and 7.2%, respectively, for the total combined composition. Bacteroidetes are Gram negative, non-spore forming, aerobic or anaerobic microorganisms that include two main genera, Bacteroides, and Prevotella (Appendix A1). Bacteroides spp. are well known as they contain many genes encoding glycoside hydrolase activity. Prevotella is associated with high fiber diets and includes many genes functionally responsible for the metabolism of complex polysaccharides (Rampelli et al. 2015). Taxa within Bacteroides and Firmicutes are both known to metabolize fructans, complex plant polysaccharides, which are generally insoluble and indigestible by host-derived enzymes (Sonnenburg et al. 2010). The primary fermentation products from Bacteroides spp. include: acetate, propionate, and succinate from carbohydrates (Appendix B1). Firmicutes are Gram positive microorganisms that may form spores, are anaerobic or aerobic, and produce similar products from carbohydrates, but can also metabolize proteins and amino acids to produce branched chain fatty acids (BCFAs), indoles, sulfides, phenols, amines, NH₃, H₂, CO₂, CH₄ (Appendix B1) (Jacobs et al. 2009).

Proteobacteria comprise the largest phylum of Gram-negative bacteria and have diverse ecological and pathogenic importance. Proteobacteria, such as Escherichia coli, Campylobacter concisus and enterohpatic Helicobacter (Appendix A1), have all been associated with the pathogenesis of inflammatory bowel disease in humans (Mukhopadhya et al. 2012). The two dominant classes of Proteobacteria in fox and Abert’s squirrels were Gammaproteobacteria and Epsilonproteobacteria, the latter of which has been linked to animal models of colitis (Chalifoux et al. 1981). Main fermentation products of Proteobacteria include: lactate, acetate, succinate, formate from carbohydrates; sulfide from sulfate, H₂S, and mercaptans (Appendix B1) (Jacobs et al. 2009).
The dominant orders present in the upper GI region for Abert’s and fox squirrels were Firmicutes in the order Lactobacillales and Proteobacteria in the order Enterobacteriales (Appendix A1). While both species of squirrel harbored nearly equal percentages of Lactobacillales, the fox squirrel upper GI community composition had close to 10% more Enterobacteriales. In Abert’s squirrels, Pasteurellales, another member of the Proteobacteria, accounted for 14.24% of the upper GI community, compared to only 0.92% in fox squirrels. The family Pasteurellaceae are obligate parasites or commensals of vertebrates, colonizing mainly the mucosal surfaces of respiratory tract, reproductive tracts, and the intestinal tract. Most taxa represent potential pathogens (Kuhnert et al. 2008). The lower GI regions of both fox and Abert’s squirrels contained nearly equal percentages of Bacteroidales. Abert’s squirrel lower GI tract contained higher percentages of Clostridiales, and Campylobacterales, whereas fox squirrel lower GI tract had a higher percentage of Verrucomicrobiales (16.21%) than Abert’s squirrel lower GI community composition (3.3%). Akkermansia mucinifila, a species in Verrucomicrobiales, has been shown to produce sulfate, acetate, succinate, propionate and ethanol from fermentation of mucin in the GI tract (Appendix B1) (Derrien et al. 2004, Louis & Flint 2017). One individual Abert’s squirrel contained an average Ruminococcus community composition of 23.58% of the lower GI. This genus includes two species, Ruminococcus albus and Ruminococcus flavenfaciens, which are known cellulolytic bacterial species (Krause et al. 2003). By contrast, no fox squirrel samples had any representation of this genus.
Results from the pairwise comparisons for *beta* diversity between species *S. aberti* and *S. niger* produced significant differences (Fig 2).

**Fig 2.** *Beta* diversity boxplot of Unifrac measurements for microbial community composition of Abert’s and fox squirrels (0 = more similar, 1 = less similar). Mean Unifrac measurements for both species were significantly different from one another, suggesting that microbial community composition is heavily influenced by diet and GI tract morphology. Pairwise comparison of *beta* values (t stat = 7.38, p-value = 3.36E-13)
Pairwise comparisons for beta diversity between GI regions were broken down into four comparisons. Results show significant differences between the upper and lower GI regions at both interspecific and intraspecific levels (Table 1, Fig 3).

**Table 1.** Beta diversity pairwise comparisons for gastrointestinal regions show significant differences occurring at interspecific and intraspecific levels.

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>t statistic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-Upper</td>
<td>F-Lower</td>
<td>15.57</td>
<td>0*</td>
</tr>
<tr>
<td>F-Upper</td>
<td>A-Upper</td>
<td>3.37</td>
<td>8.64E-04</td>
</tr>
<tr>
<td>F-Lower</td>
<td>A-Lower</td>
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<td>8.77E-14</td>
</tr>
<tr>
<td>A-Lower</td>
<td>A-Upper</td>
<td>-14.15</td>
<td>2.22E-33</td>
</tr>
</tbody>
</table>

* p-value was below the numeric threshold of the QIIME statistical analysis

**Fig 3.** Beta diversity boxplot of Unifrac Measurements for upper and lower GI tract regions in both Abert’s and fox squirrels (0 = more similar, 1 = less similar). (A-Upper/F Upper = Abert’s/fox stomach and small intestine samples; A-Lower/F-Lower = Abert’s/fox cecum, large intestine, fecal samples). Results show significant differences occurring between upper and lower GI tract regions both at the interspecific and intraspecific levels. Smaller mean beta diversity measurements for the lower GI tract region in both species shows that microbial diversity is more similar between individuals of the same species for lower GI tract comparisons than for the upper GI tract.
The supervised_learning.py analysis produced a confusion matrix for each category specified to be evaluated (GI and Species) that showed our True/Predicted ratio for *S. aberti* and *S. niger* to be 81.25% and 96.875%, respectively. This matrix shows the percentage of success that the machine learning process had in classifying a sample as belonging to its appropriate category. The feature_importance_scores.txt file contain the statistical analysis of the most successful OTUs to predict the category of origin. For both the GI and Species analysis it was found that *Prevotella copri* and *Prevotella* spp. were the most important features for the machine learning analysis. The confusion matrix showed that machine learning was more successful at predicting fox squirrels and the lower GI region for both species (Table 2).

**Table 2.** Machine learning found that using *Prevotella* spp. as a classifier was the most successful OTU in predicting sample origin. *Prevotella* spp. is associated with high fiber, low quality diets. The presence/absence of *Prevotella* spp. is shown here to be the best predictor of sample origin, and may have implications of the influences that diet has on the microbial community composition of Abert’s and fox squirrels.

<table>
<thead>
<tr>
<th>True\Predicted</th>
<th>Abert's</th>
<th>Fox</th>
<th>Class Success</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abert's</td>
<td>26</td>
<td>6</td>
<td>0.81</td>
</tr>
<tr>
<td>Fox</td>
<td>1</td>
<td>31</td>
<td>0.97</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>True\Predicted</th>
<th>A-Lower</th>
<th>A-Upper</th>
<th>F-Lower</th>
<th>F-Upper</th>
<th>Class Success</th>
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<tbody>
<tr>
<td>A-Lower</td>
<td>15</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0.94</td>
</tr>
<tr>
<td>A-Upper</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td>5</td>
<td>0.69</td>
</tr>
<tr>
<td>F-Lower</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>1</td>
<td>0.94</td>
</tr>
<tr>
<td>F-Upper</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>14</td>
<td>0.88</td>
</tr>
</tbody>
</table>
Using the threshold of 75% overlap in all samples from fox and all samples from Abert’s squirrels we found that the fox squirrel core microbiome was composed of nine OTUs, and Abert’s squirrel core microbiome had eighteen OTUs. There were seven OTUs shared in the core microbiome of the Abert's and fox squirrels. The families in the core microbiome shared between the two species were Prevotellaceae, Lactobacillaceae, Enterobacteriaceae, Helicobacteraceae, Streptococccaceae, Bacteroidaceae, and Veillonellaceae. Operational taxonomic units found in the Abert’s squirrel core microbiome that were not shared by the fox squirrel were *Prevotella copri*, *Prevotella spp.*, *Lactobacillus reuteri*, *Cerasicoccaceae*, *Lachnospiraceae*, several species of *Ruminococccaceae*, *Bacteroides spp.*, and *Faecalibacterium prausnitzii*. Operational taxonomic units found in the fox squirrel core microbiome that were not shared by the Abert’s squirrel were *Sutterella spp.* and *Akkermansia spp.* (Fig 4).

**Fig 4.** Shared OTUs in core microbiome of Abert’s and fox squirrel: Prevotellaceae, Lactobacillaceae, Enterobacteriaceae, Helicobacteraceae, Streptococccaceae, Bacteroidaceae, and Veillonellaceae. Not shared by fox: *Prevotella copri*, *Prevotella spp.*, *Lactobacillus reuteri*, *Cerasicoccaceae*, *Lacnospiraceae*, several species of *Ruminococccaceae*, *Bacteroides spp.*, and *Faecalibacterium prausnitzii*. Not shared by Abert: *Sutterella spp.* and *Akkermansia spp.*
PICRUSt (Langille et al. 2013) results showed no significant differences in functional metagenomic composition between species, interspecific GI regions, or interspecific along the GI tract. However, significant differences were observed between upper and lower GI tract regions within species. Both fox and Abert’s squirrels contained a higher proportion of sequences associated with metabolism of terpenoids and polyketides, xenobiotics, lipids, and other amino acids in the upper GI tract (stomach and small intestine) than in the lower GI tract (cecum, large intestine, and fecal sample) (Fig 5, Fig 6).

**Fig 5.** Functional metagenome of fox squirrel upper and lower GI regions. Significant differences in amino acid, xenobiotic, lipid, terpenoids and polyketide metabolism all could play integral roles in the digestion and detoxification of plant material.
Fig 6. Functional metagenome of Abert’s squirrel upper and lower GI regions. Significant differences in amino acid, xenobiotic, lipid, terpenoids and polyketide metabolism all could play integral roles in the digestion and detoxification of plant material.
CHAPTER 5

DISCUSSION

My project was designed to further the understanding of the relationship between microbial communities and their host diet, feeding strategy, and GI tract morphology. I examined the microbiota of Abert’s and fox squirrels, which provided an excellent comparison of a closely related feeding generalist and specialist. The retention-maximizing strategy of the Abert’s squirrel has resulted in a morphologically distinct GI tract, which shows increased length and surface area when compared to the intake-maximizing, fox squirrel. The diet of the Abert’s squirrel is higher in PSCs (Snyder 1992), which led me to explore through metagenomic analysis whether functional differences exist between the microbial communities of the squirrel species. Abert’s squirrel’s diet is also heavily reliant on high fiber phloem from December-May, and fungi from June-November (States & Wettstein 1998), which I predicted would necessitate a different microbial community to produce cellulase and other cell wall degrading enzymes than the fox squirrel. I examined how these evolutionary changes have resulted in distinct communities of microbes responsible for the physiological and metabolic processes associated with their distinct diets.

Due to the variety of environmental factors that dynamically shift from the stomach, small intestine, cecum, large intestine, and fecal samples, we expected to find variation in the microbial communities along the length of the GI tract. The diversity between GI regions was significant on both intraspecific and interspecific levels. Beta diversity scores show that the lower GI tracts for both species have less variation between individuals than do the upper GI tracts (Fig 3). This may be explained by the specificity of bacteria responsible for producing SCFAs in the cecum, which closely resemble the clusters of bacteria identified in fecal samples.
Species richness (alpha diversity) was also trending lower for the stomach and small intestine (Fig 1), which is consistent with findings from studies done by Turnbaugh et al. (2009) conducted on the spatial heterogeneity of GI tract flora.

Variation in the microbial communities seen in feeding specialists and generalists was evaluated through the diversity seen between individuals of the same species, a metric known as beta diversity. Beta diversity scores for fox squirrels were higher than for Abert’s squirrels (Fig 2). I interpreted that for a feeding generalist, I could expect more variation in microbial communities between individuals resulting from the wider breadth in digestible food items. For the Abert’s squirrel, I observed lower beta diversity which could be explained by the narrow range in diet, and therefore, less variation between individual’s microbial communities that metabolize the high fiber, low nutrient diet. The study was limited by the ability to investigate only one example of sympatric feeding generalist and specialist species whose ancestral link is separated only at the species level. An in-depth knowledge of the differences in microbial diversity associated with feeding generalists and specialists will require the research of microbiomes harbored in many more organisms. Further research on host microbial degradation of PSCs seen in generalist and specialist species may provide more insight. Promising work has been done on specialist and generalist species of wood rat (Neotoma stephensi, N. albigula) and pygmy rabbit (Brachylagus idahoensis) and mountain cottontail (Sylvilagus nuttalira) systems (Kohl & Dearing 2016; Shipley et al. 2012). More exploration into the microbiota of koala (Phascolarctos cinereus) and wombat (Vombatus ursinus), as well as bushy tail possum (Trichosurus vulpecula) and ringtail possum (Pseudocheirus peregrinus) may provide additional support to the diversity differences we observed in our system.

The machine learning analysis successfully identified the most important features (OTUs) that were associated with each sample that distinguished species and GI tract region from one
another. The machine learning analysis identified *Prevotella* spp. as being the most important feature in classifying samples (Table 2). I believe this is attributed to the differences in the fiber content and nutritional value in the diet of Abert’s and fox squirrels. The increased abundance of *Prevotella* spp. is usually associated with long-term intake of diets rich in fiber, as it is known to have mucin oligosaccharide degradation importance (Wright *et al.* 2000). A study by Schnorr *et al.* (2014) compared the microbiomes of Hadza hunter-gatherer tribes living in the Rift Valley of Africa to the microbiomes of Italian families. They found that the Hadza microbial communities had a significantly greater abundance of *Prevotella* spp., which may be explained by their high fiber, low-quality diet. Kovatcheva-Datchary *et al.* (2015) showed that humans with improved glucose metabolism after the introduction of an indigestible carbohydrate have increased *Prevotella* spp. in their gut microbiota. Human subjects who responded positively to the introduction of barley kernel-based bread (BKB) were shown to have a higher habitual fiber intake in their diet prior to the experiment, which has been associated with increased abundance of *Prevotella* spp. These researchers believe that metagenomic analysis of the functional repertoire of *Prevotella* spp. suggest that dietary fibers present in BKB can be more efficiently metabolized by humans with higher levels of *Prevotella* spp. due the bacteria’s ability to ferment complex polysaccharides. I propose that the increased abundance of *Prevotella* spp. in the Abert’s squirrel microbial community is the result of the high intake of fibrous ponderosa pine phloem.

Core microbiome analysis was conducted to show that there could exist unique communities of integral bacteria responsible for the diets associated with feedings specialists and generalists. The results of the core microbiome analysis show that the core group of OTUs is larger in Abert’s squirrels than in fox squirrels (Fig 4). I propose that this is further evidence that the low degree of variation between all Abert’s squirrel samples, as shown by the low beta
diversity value and high core microbiome OTU count, is the result of their specialist diet. It could be that herbivorous specialists contain a larger core microbiome because their diet varies little between individuals, and the OTUs represented are necessary for the metabolism of their diet, thus would be highly represented in all samples. Eleven OTUs are found only in the core microbiome of Abert’s, and not fox squirrels. By contrast, only two OTUs are found in the fox squirrel core microbiome that are not shared by the Abert’s squirrel core microbiome. Four of these OTUs that are unique to the Abert’s squirrel core microbiome contain species from the families Prevotellaceae or Ruminococcaceae, which are associated with high fiber, low quality diets and cellulolytic functionality. This supports the hypothesis that the specialist diet of ponderosa pine phloem necessitates a different suite of bacteria to detoxify and metabolize their food to prevent accumulation and absorption of PSCs. Future metagenomic studies on the Abert’s squirrel microbiome will be necessary to support this hypothesis.

By examining the functionality of the metagenome associated with the microbial communities in both species of squirrels, we hoped to uncover significant interspecific differences that would explain the ability to metabolize the phloem of ponderosa pine tissue, and their respective PSCs. The results of the PICRUSt analysis show that the stomach and small intestine of both species had a higher abundance of bacterial genes that are functionally related to the metabolism of terpenoid and polyketides, lipids, and other xenobiotics (Fig 5, Fig 6). Kohl & Dearing (2016) designed a system using several species of woodrat to evaluate the hypothesis that ingestion of toxic diets by herbivores is facilitated by the gut microbiota. They proposed that the functional role of the foregut in rodents may be to house microbes that are capable of detoxification. Foregut detoxification would allow for the metabolism and inactivation of PSCs before absorption in the small intestine (Kohl & Dearing 2016). Metagenomic studies conducted on the microbial functionality in the foregut of desert woodrats (Neotoma lepida) showed that
there were higher abundances of genes associated with the metabolism of aromatic compounds in animals feeding predominantly on creosote bush (*Larrea tridentate*), a plant with many known harmful secondary compounds (Kohl et al. 2014). The animal would seem to benefit from metabolizing plant-secondary compounds in the foregut to render the food benign as quickly as possible. This may explain why both fox and Abert’s squirrels contained higher abundances of genes associated with deterrent chemical compounds in the stomach and small intestine than in the hindgut. We did not however, find any significant differences occurring between the two species of squirrels in regards to their ability to metabolize high fiber diets that contain PSCs.

The metagenomic analysis conducted by PICRUSt uses 16s rRNA data to extrapolate the gut microbial metagenome. While this is a useful tool, I believe that a full metagenomic analysis of the remaining samples would perhaps uncover additional functional differences at the species level that were missed in my analysis. It seems unlikely that, when comparing the gut microbial metagenome of fox and Abert’s squirrels, the latter’s diet would not require a significantly different proportion of bacterial genes that function to metabolize PSCs given the high levels of terpenoids, tannins, beta-pinene and beta-phellandrene in the phloem of ponderosa pine.

A future direction of this study could involve collecting microbiome data for the closest living species to the Abert’s and fox squirrel. Having data on these generalist species, the Eastern gray (*Sciurus carolinensis*) and Western gray (*S. griseus*) squirrel, may offer additional support that the overall differences in bacterial community diversity seen in Abert’s and fox squirrels is primarily influenced by their diet, and not phylogeny.

These studies on the microbial composition of generalist and specialist species are important to our understanding of evolutionary ecology. Specialist herbivores have evolved host-microbe functional adaptations that have allowed them to fill unique ecological niches. The role
that their gut microbes play in the digestion and detoxification of their host plants can be examined through these metagenomic approaches, and the evidence we gather can help us to understand the microbial influence on the evolution of herbivorous specialization.


APPENDICES

Appendix A

Appendix A1  Bacterial classification pertinent to microbiota of *Sciurus aberti* and *S. niger*.

**Domain Bacteria**

**Phylum – Bacteroidetes (Gram negative)**

Class – Bacteroidia

Order – Bacteroidales

Family – *Bacteroidaceae* (6 genera)

*Bacteroides* *

Family – *Prevotellaceae* (5 genera)

*Prevotella*

**Phylum – Proteobacteria**

Class – Betaproteobacteria

Order – Burkholdariales

Family – *Alcaligenaceae* (21 genera)

*Alcaligenes*

Class – Epsilonproteobacteria

Order – Campylobacterales

Family – *Campylobacteriaceae* (6 genera)

*Campylobacter*

Family – *Helicobacterales* (6 genera)

*Helicobacter*

Order - Pasteurellales

Family – *Pasteurellaceae* (22 genera)

*Pasteurella*

**Phylum – “Verrucomicrobia”**

Class – Verrucomicrobiiae

Order – Verrumicrobiales

Family – *Verrucomicrobiaceae* (8 genera)

*Verrucomicrobium*
Phylum – Firmicutes (Gram positive)
   Class – Bacilli/Firmibacteria
      Order – Latobacillales
         Family – Enterococcaceae (7 genera)
            Enterococcus
         Family – Lactobacillaceae (4 genera)
            Lactobacillus
         Family Streptococcaceae (3 genera)
            Streptococcus
   Class – Clostridia
      Order – Clostridiales
         Family – Clostridaceae (37 genera)
            Clostridium
         Family – Lachnospiraceae (28 genera)
            Lachnospira
   Class - Negativicutes
      Order – Veillonellales
         Family – Veillonellaceae (8 genera)
            Veillonella
      Order - Ruminococcales
         Family – Ruminococcaceae (17 genera)
            Ruminococcus

*One representative genus listed for each family of bacteria
http://www.bacterio.net/-classifphyla.html
The majority have not been isolated and researched. The fermentation end-products are only indicative of some cultured representatives of a family as:

<table>
<thead>
<tr>
<th>Family</th>
<th>Important Families</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycetales</td>
<td>Phylum Ptilinae</td>
</tr>
<tr>
<td>Bacillaceae</td>
<td>Prosthecobacteriales</td>
</tr>
<tr>
<td>Alcaligenaceae</td>
<td>Pseudomonadaceae</td>
</tr>
<tr>
<td>Acetobacteraceae</td>
<td>Vibrionaceae</td>
</tr>
</tbody>
</table>

Some fermentation products include:

- SUlphate, sucluate, propionate, and ethanol from muchin
- SUlphate, sulphite, H2S, mercaptans
- Iacetate, acetate, succinate, formate from carbohydrates,
- Phenols, amine, NH3, H2, CO2 from proteins and amino-acids
- Iacetate, propionate from carbohydrates; B. C. A. A. indirect, sulphones, acetate, formate, L- and D-iacetate, pyruvate, acetate, propionate, succinate from carbohydrates.