The gastrointestinal tract microbiota of the obligate bamboo foragers, the giant panda 

(*Ailuropoda melanoleuca*) and the red panda (*Ailurus fulgens*)

By

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Previously, the exact phylogenetic relationship between the giant panda (Ailuropoda melanoleuca) and the red panda (Ailurus fulgens) has been unclear, but with the advent of molecular sequencing technologies, these two animals are now known to be distantly related. Although taxonomically distinct, these two panda species have several things in common, primarily their almost exclusively bamboo diet, but also their similar physical adaptations to their diet and their sympatry in some locations. What was unknown was if their similarities also extended to their gastrointestinal tract (GIT) microbiota. Using next-generation 16S rRNA parallel sequencing technologies, we uncovered several factors that impact bacterial communities of the pandas and also found that the two panda species harbor distinct microbial communities. In general, the Firmicutes and the Proteobacteria dominate both pandas’ gut microbiomes, with lesser contributions by the Actinobacteria and the Bacteroidetes. However, for the red panda, sex, age, and season significantly alter GIT microbiota. For giant pandas, we found that bacterial communities differed significantly between their normal fecal samples and
mucus excretions. Together, these data display the plasticity of their bacterial communities, with several factors shaping GIT microbiota. As both species are highly threatened, understanding the relationship between the panda and their gut microbiome has given insight into the overall health of these uniquely herbivorous carnivores.
DEDICATION

This work is dedicated to my family. They have given me endless support and encouragement throughout this sometimes arduous process. Thank you.
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LIST OF ABBREVIATIONS

16S rRNA: 16S ribosomal ribonucleic acid
AA: amino acid
CAZG: Chinese Association Of Zoo and Gardens
CBSG: Conservation Breeding Specialist Group
CD: Crohn’s disease
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid
dNTPs: deoxynucleoside triphosphate
DOB: date of birth
DXL: Daxiangling Mountains
EHA: effective habitat area
FA: fatty acid
GALT: gut associated lymphoid tissue
GIT: gastrointestinal tract
GPCRN: Giant Panda Conservation Reserve Network
HCC: habitat carrying capacity
HCL: hydrochloric acid
IACUC: Institutional Animal Care and Use Committee
IBD: inflammatory bowel syndrome
IEC: intestinal epithelial cells
IgA: Immunglobulin A
LAB: lactic acid bacteria
LS: Liangshan Mountain
MHA: minimum habitat area
MS: Minshan Mountains
NFPP: Natural Forest Protection Program
NGS: next-generation sequencing
NMDS: nonmetric multidimensional scaling
NPN: non-protein nitrogen
ORF: open reading frame
OTU: operational taxonomic unit
PCoA: principal coordinate analysis
PCR: polymerase chain reaction
QIN: Qingling Mountains
QIO: Qionglai Mountains
SE: standard error
SFA: Stage Forestry Administration
SLCP: Sloping Land Conversion Program
TAG: triacylglycerol
TGGE: temperature gradient gel electrophoresis
TLR: toll-like receptor
UC: ulcerative colitis
VFA: volative fatty acid
XXL: Xiaoxiangling Mountains
DEFINITIONS

Anthropogenic effects include impacts on biophysical environments, biodiversity and other resources resulting from human activity.

Digestible energy is the total amount of energy that can be absorbed.

Ex situ translates to “out of place.” In conservation, ex situ is the process of protecting a species outside of its natural habitat, i.e. removing part of the population from a threatened habitat and placing them in a new location which may be a wild area or within the care of humans.

Fecal energy is the total amount of energy that is not available for digestible energy (undigested food, metabolic byproducts, etc).

A flagship species is a popular, charismatic species that serve as symbols and rallying points to stimulate conservation awareness and action. At the larger scale these include animals such as condors, pandas, rhinos, large cats and large primates, while at the smaller scale they include orchids, cacti, and invertebrates such as large butterflies and stick insects. Flagship species may serve as both indicators and/or umbrella species and also provide a highly visible reminder of the progress of a particular conservation management plan.

Gaseous energy is a byproduct of metabolism, and used as an electron acceptor in anaerobic rumen (methane, CO2).

Gross energy is the total amount of energy in the diet.
Heat increment is the heat released from the inefficient metabolism of digestive fermentation and nutrient metabolism.

*In situ* translates to “in its original place.” In conservation, *in situ* is the on-site conservation or conservation of genetic resources in natural populations.

**Metabolizable energy** is the total amount of energy that can be available to tissues for metabolism.

**Microbiome** describes the collective genomes of the microorganisms that reside in an environmental niche or the microorganisms themselves.

**Microbiota** comprises the ecological community of commensal, symbiotic and pathogenic microorganisms that share the human body.

**Net energy** is the total amount of energy that can be used for work.

**Operational taxonomic unit** is an operational definition of a species or group of species often used when only DNA sequence data is available.

**Sympaty** indicates the use of the same or overlapping geographical areas by populations of related species without interbreeding.

**Urinary energy** is the residue (nitrogen) of nutrient metabolism.
CHAPTER I
LITERATURE REVIEW

The giant panda (*Ailuropoda melanoleuca*) is one of the most well-known endangered species globally (Lu, Wang, Garshelis, 2014). Although not quite as widely known, there is another panda, the red panda (*Ailurus fulgens*); often referred to as the lesser panda, it is considered a vulnerable species (Wang et al., 2012). Together the pandas serve as a flagship species for all endangered species (Johnson et al., 1996). These species face considerable threats, but they are also of great interest for study due to their uniqueness. Both pandas display a diet paradox, that is, they are classified as carnivores, but exhibit an almost exclusively bamboo diet (Schaller et al., 1985; Roberts and Gittleman, 1984). As diet greatly affects gastrointestinal tract microbial communities (Maslowski and Mackay, 2011), the relationship between a physiological carnivore and a dietary herbivore is of extreme interest. The focus of the following dissertation aims to examine the interplay in this relationship and gain insight into these unique species.

**The Giant Panda**

**Overall Status**

Historically, giant pandas’ habitat covered southern, middle and north-west China, northern Myanmar, northern Vietnam, Laos, and Thailand, but currently their habitat is confined to six mountain ranges on the eastern edge of the Tibetan Plateau in
China: Qinling, Minshan, Qionglai, Liangshan, Daxiangling, and Xiaoxiangling (Figure 1.1) (Wei et al., 2012). Molecular and traditional censusing methods estimate the giant panda population to be 3,000 and 1,600 individuals, respectively (Zhan et al., 2006). With such a small population, there is great need to understand factors that impact the giant panda. As neither a prey or predator species, they are not outcompeted by other species. In fact, most of their threats are considered anthropogenic effects, primarily through habitat loss and fragmentation (Zhan et al., 2007).

Figure 1.1 The a) historical, and b) current range of the giant panda

Notes: adapted by Wei et al., (2012).
Qin, Qingling mountains; MS, Minshan Mountains; QIO, Qionglai Mountains; DXL, Daxiangling Mountains; XXL Xiaoxiangling Mountains; LS, Liangshan Mountains.

**Habitat Loss and Fragmentation**

As the primary threat to pandas, habitat loss and fragmentation comes in many forms. The majority of lost panda habitat has occurred from human-related activities
such as logging, agriculture, and an increase in infrastructure (i.e. building of roads) as they have lost approximately half of their habitat from the mid-1970s-1980s (Fan et al., 2011; Kang et al., 2014). An increase in human settlements also attributed to the drastic decrease in suitable habitat for giant pandas (Fan et al., 2011; Kang et al., 2014; Ellis et al., 2006; Hull et al., 2014; Liu et al., 1999). As human populations increase, so do their demand for resources, and wood in particular. Rural communities rely heavily on the use of fuelwood to heat their homes, and their harvesting of wood for fuel has encroached on giant panda habitat (Fan et al., 2014; Bearer et al., 2008; He et al., 2009).

Greater human populations lead to more habitat fragmentation (Zhu et al., 2010; Liu et al., 1999). There are approximately thirty-three, isolated populations that make up the fragmented giant panda habitat with few corridors to allow for genetic exchange (SFA, 2015). Dispersal is very important for gene flow, and with few corridors this is greatly limited. Dispersal is also very important for females selecting den sites for birthing (Zhang et al., 2007), and for bamboo selection, especially in times of bamboo flowering (Hull et al., 2010; Zhang et al., 2007; Hunter, Jinhe, and Dierenfeld, 2003; Gong et al., 2010). In the 1970s and 1980s, bamboo flowering events and subsequent bamboo die-off resulted in the deaths of 141 giant pandas. In studies by Wu et al. (1996) and Carter et al. (1999), bamboo die-off post-flowering was not the cause of decreasing panda population; their inability to disperse to other areas with viable bamboo stand due to anthropogenic effects was the cause. Prior to human influence, this problem was nonexistent as giant pandas could travel to another bamboo stand when needed. However, with such fragmented habitat without proper corridors, this is not possible.
China: The Giant Pandas’ Last Habitat

Over-exploitation of lands and resources deteriorated forest ecosystems, leading to the erosion of approximately 38% of China’s land area (Wenhua, 2004). To combat these issues, China has implemented several programs to protect and restore forests. Two State Forestry Administration programs that have had success are the Natural Forest Protection Program (NFPP) and the Sloping Land Conversion Program (SLCP) (Wenhua, 2004; Yin et al., 2005). Combined, forest cover has rebounded from 5.2% in 1950 to 20.4% in 2009 (Yin et al., 2005; Wang et al., 2011; Song et al., 2014; Dornelas et al., 2013; Zhao and Shao, 2002). China also established over 2,000 reserves to protect biodiversity (Wenhua, 2004; Wu et al., 2011).

Unfortunately, many reserves implemented for giant panda protection did not take into account what habitat is suitable for giant pandas. According to several studies, the giant panda conservation reserve network (GPCRN) was designed around administrative or judicial lines, not actually considering habitat suitability for giant pandas (Gong et al., 2010a; Yin et al., 2005; Gong et al., 2011). Depending on the mountain region, these studies also determined that only 20-60% of the land that lies within these reserves are suitable for giant pandas (Gong et al., 2010; Feng et al., 2009; Hu et al., 2007; Li et al., 2013; Xu et al., 2009). Several of these studies also observed approximately 35% of suitable panda habitat fell outside the boundaries of the reserves (Feng et al., 2009; Gong et al., 2010; Xu et al., 2009).

To be considered suitable habitat, the area must fit certain abiotic and biotic criteria (Table 1.1). Giant pandas prefer mixed conifer and deciduous broadleaf forests with arrow and umbrella bamboo (Liu et al., 1999; Hu et al., 2007; Xu et al., 2009;
Songer et al., 2012). The elevation of these areas must range from 1500-2750 m with a gentle slope of less than 30 degrees (Liu et al., 1999b; Hu et al., 2007; Qi et al., 2012). These areas must also not lie near human settlements or roadways (Fan et al., 2011; Kang et al., 2014; Liu et al., 1999a; Bearer et al., 2008; Liu et al., 1999b; Feng et al., 2009; Hu et al., 2007; Gong et al., 2010). However, climate change may affect what habitat is suitable for giant pandas. Predicted climate change is expected to reduce suitable habitat 60% from what is currently suitable (Fan et al., 2014; Songer et al., 2012). Also, only 37% of this area is currently protected under the GPCRN (Fan et al., 2014; Songer et al., 2012). New areas may become available, but they are far from current habitat, and only 15% of lands will fall within the GPCRN (Songer et al., 2012).

Table 1.1 Abiotic and biotic factors used to determine suitability of habitat for giant pandas.

<table>
<thead>
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<th>Factor</th>
<th>Highly suitable</th>
<th>Suitable</th>
<th>Marginally suitable</th>
<th>Unsuitable</th>
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<td>2250&lt;x≤2750</td>
<td>1500&lt;x≤2250</td>
<td>≤1500</td>
<td>&gt;3750</td>
</tr>
<tr>
<td>Slope (degree)</td>
<td>≤15</td>
<td>15&lt;x≤30</td>
<td>30&lt;x≤45</td>
<td>&gt;45</td>
</tr>
<tr>
<td>Vegetation</td>
<td>Mixed conifer and deciduous broadleaf forest</td>
<td>Deciduous broadleaf forest, conifer forest</td>
<td>Evergreen broadleaf forest, missed evergreen and deciduous broadleaf forest</td>
<td>Brush, meadow, no vegetation</td>
</tr>
<tr>
<td>Bamboo</td>
<td>Arrow and umbrella bamboo</td>
<td>Arrow and umbrella bamboo</td>
<td>Other bamboo species</td>
<td>No bamboo</td>
</tr>
</tbody>
</table>

Adapted from Liu et al., (1999).

*Adaptive management of giant panda habitat areas*

There is a great need for adaptive management strategies in areas containing giant panda habitat. Swaisgood et al. (2011) suggested several forms of adaptive management including: the annexation of suitable habitat, the continuation of the logging ban, the
reforestation in areas that fit certain criteria, the transition from fuelwood as an energy source, and the reduction in human expansion.

To increase the giant panda population there must be an increase in effective habitat area. As mentioned above, there are several areas of suitable habitat that do not fall within the GPCRN (Feng et al., 2009; Hu et al., 2007; Gong et al., 2010; Xu et al., 2009). Therefore, these areas are in need of being annexed to increase the protected area for wildlife. This annexation includes the addition of corridors to connect these highly fragmented populations. As of 2012, approximately 28,272 km$^2$ remained as suitable habitat (effective habitat area, EHA) for giant pandas across all six mountain ranges, and this value contains all suitable habitat regardless of whether it lies within the GPCRN. If a new reserves system was drawn up to comprise all suitable habitat for giant pandas, the habitat carrying capacity (HCC) of this area would be either 2872 individuals (minimal habitat area: 10.1 km$^2$) (Fan et al., 2011) or 5985 individuals (minimal habitat area: 4.8 km$^2$) (Hu et al., 2007) according to which minimal habitat area (MHA) selected. The habitat carrying capacity was calculated using the following formula (Fan et al., 2011):

$$HCC = \frac{EHA}{MHA}$$

1.1

The continuation of the logging ban would help reduce the amount of forest cover lost. Forest cover loss degrades panda habitat and/or makes it inaccessible (Liu et al., 1999a; Liu et al., 1999b; Wenhua, 2004; Zhao and Shao, 2002). This ban should cover all areas of current and potential giant panda habitat, and increased monitoring in the area would help reduce illegal logging in areas.
Reforestation of areas that could be deemed suitable for giant panda habitat would also allow for the increase in giant panda population. These areas would consist of lands that lie in elevations between 1500-2750 km with gentle slopes (Liu et al., 1999b; Hu et al., 2007; Qi et al., 2012). This action could restore areas that lie within the GPCRN and areas adjacent to the reserves, and create corridors. These forests should be planted with mixed conifer and deciduous broadleaf trees to reestablish a functional forest ecosystem (Liu et al., 1999b; Hu et al., 2007; Xu et al., 2009).

In order to reduce the need for fuelwood in areas within or adjacent to giant panda reserves, an ecologically sustainable alternative must be provided. The harvesting of fuelwood degrades panda habitat, and reducing the need for fuelwood would reduce habitat degradation in and around the reserve systems (Bearer et al., 2008; He et al., 2009; Liu et al., 1999b; Wenhua, 2004; Li et al., 2013). Some alternative fuel sources could consist of transitioning these communities to electricity or methane (biogas stoves) (Li et al., 2013; He et al., 2009; Bearer et al., 2008). This transition would also increase the livelihood of the local community by providing them a more stable fuel source.

Probably one of the most important adaptive management strategies to help increase giant panda populations would be the reduction in human expansion in areas suitable for panda habitat. Anthropogenic effects majorly contribute to the loss of forest cover, forest resources, and corridors, thus reducing the effective habitat area for giant pandas. Liu et al. (1999b) determined that the factor that influenced panda population the most in the Wolong reserve was the human population. To increase the suitable panda habitat and the giant panda population, there must be a decrease in human populations in and adjacent to these areas.
Programs such as the Natural Forest Protection Program and the Grain to Green Program have been successful in achieving their goal of restoring forest cover and conserving the natural forest (Li et al., 2013). Li et al. observed that 94% of villages in the Qinling and Sichuan mountain regions had either no change or an increase in forest cover, and 90% of townships exhibited either forest regeneration or had effectively protected remaining forests (Li et al., 2013). However, not all villages had the same results; some regions that rely heavily on land resources such as fuelwood and mining exhibited a loss of forest cover (Li et al., 2013).

**Gastrointestinal Disease**

Giant pandas also face non-anthropogenic effects, such as gastrointestinal disease. As an aim of the biomedical survey carried out by the Conservation Breeding Specialist Group (CBSG) requested by the Chinese Association of Zoo and Gardens (CAZG), the group investigated issues related to mortality, veterinary issues, and nutrition (Wildt et al., 2006). The relationship between the giant pandas' obligate bamboo diet and nutrition was of supreme interest, as nutrition and nutritional status has a major impact on the overall health of animals. During growth and development, reproduction, and disease resistance, the impacts of nutritional status can be seen (Wildt et al., 2006).

Nutritional impacts on giant pandas are important, as the leading cause of death both wild and captive neonates and adult pandas is gastrointestinal disease (Loeffler et al., 2006, Qiu and Mainka, 1993). Qiu and Mainka’s study (years 1938-1992) observed 48% and 30% of captive and wild bears, respectively, succumbed to GIT disease. Also, 33% of wild bears suffered from malnutrition (Qiu and Mainka, 1993). Chronic gastrointestinal disease occurs in some adult bears, ongoing abdominal discomfort and
loss of appetite, typically followed by excretion of mucus. These mucous excretions have been weakly linked to pathogenic organisms and nutritional value of bamboo, but the cause remains unknown (Loeffler et al., 2006).

Bacterial infections are also prevalent in both adult bears and neonates. Adults can experience frequent hemorrhagic enteritis and diarrhea, and often these episodes implicated *E. coli*, hemolytic/non-hemolytic *Streptococcus* and *Salmonella*, and *Clostridium* spp. Bacterial infections are also common in neonates of less than 30 days old, with *Escherichia coli*, *Staphylococcus* spp., and *Psuedomonas* spp. identified as the pathogenic organisms. The weaning process also incites gastrointestinal distress in cubs, with some individuals displaying symptoms such as diarrhea, abnormal stool, abdominal pain, mucous passage, and vomiting (Loeffler et al., 2006).

Pandas are also plagued with parasitic infections. Approximately 50 % of *in situ* giant panda mortality (years 2001-2005) is associated with visceral larval migran infections, increasing in probability since 1971 (Zhang J. et al., 2007). *Ex situ* deaths due to heavy parasite loads has also been observed, and *Baylisascaris schroderi* has been found consistently in giant pandas (Zhang J. et al., 2007, Loeffler et al., 2006). Along with numerous negative health issues, the loss of nutrient absorption can lead to a decrease in the panda’s health status (Loeffler et al., 2006).

### The Red Panda

#### Overall Status

The red panda (*Ailurus fulgens*) is native to the Himalayas and China (Figure 2) (Choudhury, 2001; Wei et al., 1999a). As the only extant member of the family Ailuroidea and a member of the superfamily Musteloidea, they are most closely related to
members of Mustelidae (weasels, otters, martens, and badgers), Procyonidae (raccoons, coatis, kinkajous, olingos, ringtails and cacomistles), and Mephitidae (skunks) (Flynn et al., 2000). The \textit{ex situ} red panda population consists of 10,000 total individuals (Wang et al., 2012) and is declining; this decline in wild populations has been primarily attributed to habitat loss and fragmentation from deforestation, as well as poaching (Choudhury, 2001; Wei et al., 1999a).

\textbf{Red Panda Habitat}

Red pandas prefer temperate forests as their habitat, as they typically have bamboo-thicket understories (Roberts and Gittleman, 1984). In some areas, the red panda and the giant panda are sympatric species, as they both are found in the Minshan, Qionglai, Liangshan and Xiangling mountain areas in western Sichuan Province, China (Figure 1.2) (Qi et al., 2009; Zhang et al., 2004, Wei et al., 2000, Wei et al., 1999a, Johnson et al., 1988). Red pandas also inhabit the temperate forests of India, but they can also be found tropical and subtropical forests of India’s Meghalaya Plateau (Choudhury, 2001).
Figure 1.2  The historical and current range of the red panda
Adapted from Wang et al. (2008).

*Habitat Loss and Fragmentation*

Like the giant panda, the red panda also is threatened by habitat loss and fragmentation, as it is considered the species greatest threat. Habitat loss and fragmentation has occurred primarily as an anthropogenic effect, with high human population growth in and adjacent to their range (Choudhury, 2001). This great increase in human population has increased the need for farming and deforestation in these areas, and the red panda has felt the impacts (Choudhury, 2001). In some areas, the suitable forest habitat for red pandas has been decimated, and the red pandas are near extinction in the western part of its range (Roberts and Gittleman, 1984; Choudhury, 2001). Deforestation from commercial logging, firewood, farming, and grazing of domestic stock is the largest threat to red panda survival (Choudhury, 2001; Wei et al., 1999a). In addition, the development of infrastructure such as dams, roads, and industries has had detrimental effects on red panda habitat.
Not only does the loss of habitat and its fragmentation directly affect red pandas, but it also indirectly affects habitat by isolating red panda populations and making corridors non-existent, which can lead to serious ecological consequences, especially populations with low densities (Wei and Zhang, 2011; Chettri et al., 2007). This isolation can lead to inbreeding depression and genetic stochasticity, eventually leading to population reduction (Wei et al., 1998, Wei and Zhang, 2011).

Other Threats

Red pandas also suffer from other threats, such as poaching. This is prevalent in regions that red panda habitat are not under control of government enforcement, where hunting of red pandas is for sport and their pelts, in addition to locals capturing cubs as pets (Ghose and Dutta, 2011; Wei and Zhang, 2011). In areas where red pandas interact with domestic livestock and/or feral dogs, there is also a high occurrence of mortality for red pandas (Ghose and Dutta, 2011).

Conservation Efforts

The red panda is protected under CITES Appendix I, Schedule I of the Indian Wild Life Protection Act (1972), category II species under the Wild Animal Protection Law in China and Nepal, a Red Listed Vulnerable A2ace species in China, and the Wildlife Act (1994) of Myanmar. In India, there are 20 protected areas that cover approximately 33 % of suitable habitat (Choudhury, 2001), and 35 protected areas in China, covering approximately 42.4 % of suitable habitat (Wei et al., 1998). In Nepal and Bhutan, there are eight and five protected areas, respectively.
Although there are some conservation efforts for red pandas, when compared to the giant pandas, these efforts are much less, and in some cases, the red pandas appear to be “forgotten” (Wei and Zhang, 2011). Luckily for the red panda, they are sympatric in certain areas, and the protection of the giant panda will extend to protect them. However, there is a great need to establish new reserve systems for the red panda, and new corridors need to be developed to connect isolated species (Wei and Zhang, 2011).

**Captive Populations**

In captivity, there are approximately 500 red pandas, with 200 individuals living in North American institutions (ZIMS, 2014). Although captive populations are managed by breeding programs, breeding is not as successful as might be expected. Only one-third of the global *A. fulgens* population is breeding during their peak reproductive age (Loeffler, 2011), and cubs born in North American institutions have high first year mortality (approximately 50%). This high mortality may be attributed to many factors, but of great interest are factors related to their captive diet (Loeffler, 2011).

**Nutritional and Gastrointestinal Disorders**

Diet-related problems were very prevalent in captive pandas, as observed in the years 1982-1995, but currently, young cubs suffer more from insufficient milk production or inadequate maternal care, which are indirectly nutritionally related (Nijiboer and Dierenfeld, 2011). Red pandas also suffer from gastric ulcers, but altering their diet to include a higher-roughage component appears to improve the condition of red pandas. Like giant pandas, red pandas also have episodes of mucous stools, but the cause remains unclear (Phillipa and Ramsay, 2011).
In juveniles, pathology reports indicate that red pandas are prone to GIT disturbances, such as gastritis, erosion and ulceration of the stomach, and enteritis and ulceration of the intestines, indicated as the primary cause of death. In some cases pathogenic microorganisms, such as *Campylobacter jejuni* and *Clostridium perfringens* were isolated in juvenile red pandas presenting symptoms of enteritis. In zoo-wide assessments Al-Soud et al. (2003) and Stirling et al. (2008) found other pathogens, *Helicobacter spp.*, and *Campylobacter jejuni* and *Yersinia enterocolitica*, respectively, in captive red pandas.

**Similarities And Differences In The Panda Species**

As both obligate bamboo foragers sharing the name “panda,” similarities between these two species are apparent. The giant and red panda are sympatric in some locations, share a comparable gastrointestinal tract, both consume bamboo as their diet staple, and have adapted to this diet in similar ways. However, they are very different as well. They are not closely related, and some of their physiological adaptations to their bamboo diet are different.

**Gastrointestinal Tract**

The pandas’ bamboo diet leads to an interesting diet paradox; although their diet is herbivorous, their gastrointestinal tract (GIT) is structurally similar to other carnivores (Davis, 1964; Roberts and Gittleman, 1984; Flower, 1870; Schaller et al., 1985). Specifically, pandas have simple stomachs (Figure 1.3) and lack a cecum (Davis, 1964). However, they do have slight differences when compared to other carnivores; pandas have a reduced length of small intestine, an increased relative surface area of the large
intestine, and a smaller liver (Davis, 1964). These anatomical features are distinct from other herbivores, which have highly developed rumens and ceca that favor plant biomass degradation. In particular, the GIT of herbivores is thought to promote the retention of microbes specialized for the breakdown and fermentation of plant biomass.

Figure 1.3  Stomach of the a) giant panda and b) red panda
As described by Davis (1964) and Flower (1870).

The Pandas Bamboo Diet

Both giant and red pandas are taxonomically classified as carnivores and exhibit a carnivorous gut physiology (Schaller et al., 1985; Roberts and Gittleman, 1984). It is unclear why these taxonomic carnivores have selected an herbivorous diet. Some attribute this shift to the loss of function of the umami taste receptor (Zhao et al., 2010), as the open reading frame of the T1R1 was found no longer intact (Li et al., 2010; Zhao et al., 2010). Estimating that pseudogenization of the gene occurred about 4.2 million years ago (Ma), this is approximately the same time giant pandas switched to a bamboo diet (Zhao et al., 2010). However, this open reading frame (ORF) is found intact in other herbivorous species, so that alone cannot be the reason for the switch (Zhao et al., 2010).
A study by Jin et al. (2011) found that giant panda’s dopamine metabolic pathway is incompetent, which suggests that unusual metabolic processes dictate their unusual bamboo diet. However, this has not been examined in the red panda, therefore, there is no conclusive evidence to determine why they also shifted to their bamboo diet.

As specialized herbivores, they consume large quantities of bamboo. Although nutrient values differ according to species and plant part, bamboo is considered a woody fibrous plant than contains high levels of cellulose, hemicellulose and lignin in both its leaf and culm parts (Christian et al., 2015). Giant pandas primarily consume both the leaf and culm plant parts, and red pandas consume the leaf part; they both consume shoots when available. In particular, giant pandas consume several bamboo species, and their diet consists of approximately 99% bamboo (Schaller et al., 1985), whereas red pandas almost exclusively eat *Sinarundinaria fangiana* in China, but have a more variable diet, as only 90% of their diet is comprised of bamboo leaves (Table 1.2) (Roberts and Gittleman, 1984, Nijiboer and Dierenfeld, 2011). Red pandas have been observed eating small mammals, birds, eggs, blossoms and berries (Nijiboer and Dierenfeld, 2011).

Both pandas have been observed undergoing a seasonal shift in feeding behavior (Hansen et al. 2010, Tarou et al., 2006; Williams et al., Wei et al., 1999; Loeffler, 2011, Nijiboer and Dierenfeld, 2011; Yonzon and Hunter, 1989; Wei et al., 1999b; Reid, Hu, and Huang, 1991; Johnson, Scaller, and Hu, 1988; Zhang et al., 2009), and it is believed that this change may be due to shifting nutritive levels in the bamboo. Both wild and captive giant pandas have been observed preferring the leaf portion of bamboo throughout most of the year, but switching their preference to the culm portion in late spring (Schaller et al., 1985; Hansen et al., 2010; Williams et al., 2012). Red pandas do
not consume the culm portion, but they have been observed changing preference in bamboo leaves throughout the year (Wei et al., 2000). During the spring, pandas supplement their diet with bamboo shoots, and in the summer, they consume fruits (Schaller et al., 1985, Wei et al., 1999a; Wei et al., 1999b; Reid, Hu, and Huang, 1991; Johnson, Schaller, and Hu, 1988; Zhang et al., 2009). In captivity, pandas are fed a diet that mimics the wild diet in bamboo and fruit composition with the addition of a commercially prepared high-fiber biscuit that ensures a stable nutrient supply (Nijiboer and Dierenfeld, 2011).

Nutritive Values Differ According To Season

Bamboo’s nutritive composition varies by season and stage of growth (Christian et al., 2015; Okahisa et al., 2006), and this could be driving the seasonal shift observed in pandas. In particular, the portion only consumed by giant pandas, the culm section, is more fibrous, as it gives structure to the plant; therefore, culm parts usually contain higher levels of lignin. However, in the spring and summer months, percent of total lignin decreases in the culm, and there is an observable increase in free glucose in the culm (Okahisa et al., 2006). At this time, lignification increases in the leaf portion (Van Soest, 1996). Not only does lignification increase in the leaf portion during the spring, but there is also an increase in silica (Schaller et al., 1985). Both lignin and silica are almost completely indigestible by higher order mammals, and silica, in particular, can inhibit cell wall digestion in herbivores (Schaller et al., 1985).
Table 1.2  Bamboo consumed by *in situ* pandas.

<table>
<thead>
<tr>
<th>Species name</th>
<th>Region</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Giant panda</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bashania fargesii</em></td>
<td>Min, Qinling, Qionglai</td>
<td>Pan, 1988; Wang, 1989; Li, 1997</td>
</tr>
<tr>
<td><em>Chimonobambusa</em></td>
<td>Min, Qionglai, Xiangling</td>
<td>Wang, 1989; Li 1997</td>
</tr>
<tr>
<td><em>Fargesia denudate</em></td>
<td>Min</td>
<td>Wang, 1989; Li 1997</td>
</tr>
<tr>
<td><em>F. ferax</em></td>
<td>Xiangling, Liang</td>
<td>Wang, 1989; Li 1997</td>
</tr>
<tr>
<td><em>F. rufa</em></td>
<td>Min</td>
<td>Wang, 1989; Li 1997</td>
</tr>
<tr>
<td><em>F. scrabrida</em></td>
<td>Min, Qionglai</td>
<td>Wang, 1989; Li 1997</td>
</tr>
<tr>
<td><em>F. robusta</em></td>
<td>Min, Qinling, Qionglai</td>
<td>Schaller et al., 1985; Pan, 1988;</td>
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<td></td>
<td></td>
<td>Wang, 1989; Li 1997</td>
</tr>
<tr>
<td><em>Qiongzhuea opienensis</em></td>
<td>Xiangling, Liang</td>
<td>Wang, 1989; Li 1997</td>
</tr>
<tr>
<td><em>Pyllostachys nigra</em></td>
<td>Qinling</td>
<td>Pan, 1988</td>
</tr>
<tr>
<td><em>Yushania chungii</em></td>
<td>Min, Qinling, Qionglai</td>
<td>Wang, 1989</td>
</tr>
<tr>
<td><em>Bashania fangiana</em></td>
<td>Min, Xiangling, Liang</td>
<td>Schaller et al., 1985; Wang, 1989;</td>
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<td></td>
<td></td>
<td>Li 1997</td>
</tr>
<tr>
<td><em>F. nitida</em></td>
<td>Min, Qinling, Qionglai</td>
<td>Schaller et al., 1985; Wang, 1989;</td>
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<td></td>
<td></td>
<td>Li 1997</td>
</tr>
<tr>
<td><em>Y. confuse</em></td>
<td>Xiangling, Liang</td>
<td>Wang, 1989</td>
</tr>
<tr>
<td><strong>Red panda</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Phyllostachys spp.</em></td>
<td></td>
<td>Nijiboer and Dierenfeld, 2011</td>
</tr>
<tr>
<td><em>Sinarundinaria spp.</em></td>
<td></td>
<td>Nijiboer and Dierenfeld, 2011</td>
</tr>
<tr>
<td><em>Thamnocalamus spp.</em></td>
<td></td>
<td>Nijiboer and Dierenfeld, 2011</td>
</tr>
<tr>
<td><em>Chimonobambusa spp.</em></td>
<td></td>
<td>Nijiboer and Dierenfeld, 2011</td>
</tr>
<tr>
<td><em>Qiongzhuea spp.</em></td>
<td></td>
<td>Nijiboer and Dierenfeld, 2011</td>
</tr>
</tbody>
</table>

During the growing season, some grasses translocate their energy from the leaf part into the stem to protect its nutrients from herbivores (Miler and Straile, 2010). This could explain why free glucose levels increase in the culm during the spring and summer. The increased lignification accompanied by increased silica accumulation could also be another strategy used by the plant to protect nutrients, as silica is considered unpalatable by pandas (Schaller et al., 1985). These strategies most likely act as an anti-herbivory defense by the plant during the growing season (Ito and Saki, 2009; Bezemer and van Dam, 2005; Hanley et al., 2007).
**Pandas Primarily Consume Cell Wall Components**

Cellulose, hemicellulose, and lignin make up the majority of plant cell wall components (Vahouny, 1987). Cellulose is a glucose polymer connected by β 1-4 linkages (Clarke, 1997; Vahouny, 1987). Hemicellulose is also a β 1-4 linked polymer, but its basic component is not glucose, but it can be made by several different monosaccharides (Vahouny, 1987). Lignin is another complex polymer, but it is made of aromatic alcohols. Host enzymes can degrade none of these components (Vahouny, 1987). The β 1-4 linkages of cellulose and hemicellulose can be degraded by microbial digestion, and hemicellulose can be degraded in an alkali environment. Lignin, however, is not as easily digested; some microbial digestion has been found, but this ability is extremely rare (Vahouny, 1987; Fang et al., 2012).

**Physical Adaptations**

Both panda species have adapted to their bamboo diet through several physical adaptations. Their GIT has undergone slight changes when compared to other carnivores, but major adaptations have occurred in relation to their jaw structure and their radial sesamoid. The giant and red panda have a unique jaw mechanism and canine morphology (Christiansen, 2008) including expanded zygomatic arches and associated muscles that give them the ability to masticate their fibrous bamboo diet (Edwards et al., 2006; Christianssen and Wroe, 2007). Their enhanced jaw structure gives them the ability to efficiently masticate bamboo (Jin et al., 2007; Sacco and van Valkenburgh, 2004; Edwards et al., 2006; Christianssen and Wroe, 2007).

Evolved separately, pandas have also adapted the presence of a “pseudo-thumb” to enhance foraging ability and subsequent consumption of bamboo (Delisle and
Strobeck 2005; Fulton and Strobek 2006; Salesa et al., 2006). This “pseudo-thumb” is an extension of the radial-sesamoid bone and the first metacarpal bone in giant pandas (Edwards et al., 2006; Endo et al., 1996; Endo et al., 2000; Endo et al., 2003). Unlike the giant panda, the sesamoid bone of red pandas is not attached to the first metacarpal. Although similar, the giant and red pandas pseudo-thumb operate differently, as the giant pandas utilize double pincer-like functional units and hammock-like muscles to forage bamboo (Endo et al., 2001), whereas the red panda’s pseudo-thumb acts as a supporting bar, and the flexing angle during manipulation action is smaller in the red panda when compared to the giant panda (Endo et al., 2008).

**Taxonomic Status**

Assumed to be closely related, the giant panda was previously placed in the same subfamily, Ailurinae, as the red panda (Groves, 2011). However, there was a long disagreement over whether the giant panda should be classified with bears, raccoons, or in its own family. A study by O’Brien et al. (1985) using molecular methods directed the proper taxonomic placement of giant pandas in Ursidae (Figure 1.4), and this placement has been further confirmed by Flynn et al. (2000), Yu et al. (2004), and Yu et al. (2011). These studies also indicated that the red panda diverged from ursids at the same time as other procyonids. With the separation of giant pandas from other bears approximately 15-20 Ma, and red pandas separated from other procyonids approximately 38 Ma (O’Brien et al., 1985).
Figure 1.4  Phylogenetic position of the giant panda and red panda

Adapted from Wei et al., (2012) a) phylogenetic tree based on immunological, DNA-DNA hybridization and isozyme; b) maximum-likelihood phylogenetic tree based on combined.

Past Microbiome Research

With such uniquely herbivorous carnivores, their gut microbiota is of interest. There have been several studies to characterize giant panda GIT microbial communities using both culture-dependent and independent methods, but only one study has been conducted on red pandas (Table 1.3). In characterizing the gut microbiota of red pandas, Kong et al. (2014) determined that captive and wild red pandas have significantly different microbial communities. Using 16S rRNA pyrosequencing, they found that wild red pandas have a much more diverse bacterial community than captive pandas (Kong et al., 2014).
Table 1.3  Gastrointestinal tract microbial studies on giant and red pandas.

<table>
<thead>
<tr>
<th>Species</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Giant Panda</strong></td>
<td></td>
</tr>
<tr>
<td>Hirayama et al. (1989)</td>
<td>Traditional culturing technique</td>
</tr>
<tr>
<td>Wei et al. (2007)</td>
<td>16S rDNA (TGGE)</td>
</tr>
<tr>
<td>Zhu et al. (2011)</td>
<td>16s rRNA sequencing</td>
</tr>
<tr>
<td>Williams et al. (2012)</td>
<td>Anaerobic traditional culturing technique</td>
</tr>
<tr>
<td>Fang et al. (2012)</td>
<td>16S rDNA clone library</td>
</tr>
<tr>
<td>Tun et al. (2014)</td>
<td>16S rRNA and ITS1 pyrosequencing</td>
</tr>
<tr>
<td>Jiang et al. (2014)</td>
<td>Genome sequencing of <em>Klebsiella oxytoca</em> isolated from feces</td>
</tr>
<tr>
<td>Zhao et al. (2015)</td>
<td>feces</td>
</tr>
<tr>
<td><strong>Red Panda</strong></td>
<td></td>
</tr>
<tr>
<td>Kong et al., (2014)</td>
<td>RNAseq of <em>Bacillus subtilis</em> isolated from feces</td>
</tr>
<tr>
<td></td>
<td>16S rRNA pyrosequencing</td>
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</table>

Hirayama et al. (1989) used traditional culturing methods to provide the first characterization of giant panda GIT microbiota, finding that the most common isolates were members of *Streptococcus* and Enterobacteriaceae, that obligate anaerobes were found in much lower numbers than traditional herbivores, and that fastidious anaerobes were not present (Hirayama et al., 1989). Williams et al. (2012) carried out another culture-dependent study monitoring the effect of a changing bamboo preference on the GIT microbiota of giant pandas, finding that their changing diet significantly affected *Bacteroides* and *Lactobacillus* species (Williams et al., 2012).

Using culture independent methods, Wei et al. (2007) found that organisms phylogenetically related to *Escherichia coli* to be the operational taxonomic unit (OTU) in highest relative abundance using 16S rDNA-based temperature gradient gel.
electrophoresis (TGGE) profiling of the V3 region. Zhu et al. (2011), using 16S rRNA sequencing, found evidence of a cellulose metabolism by the GIT microbiome, with thirteen OTUs closely related to known groups of Clostridium capable of degrading cellulose. Tun et al. (2014) used 16S pyrosequencing to characterize bacterial and fungal GIT communities of adult and geriatric giant pandas. In this study, they found differences in the microbial communities inhabiting adult and geriatric pandas, with the Actinobacteria, in particular, only being found in adults (Tun et al., 2014). They also found developmental differences in the fungal community, with differences in the Sordariomycetes in adult pandas to Saccharomycetes in geriatric pandas. Geriatric pandas also displayed higher levels of Candida tropicalis, a potentially probiotic fungus (Tun et al., 2014).

Other studies have been conducted to analyze the ability of giant pandas to utilize their highly fibrous diet. Fang et al. (2012) found evidence of lignin oxidation by their gut microbiota, and Jiang et al. (2014) and Zhao et al. (2015) examined cellulose degradation pathways in Klebsiella oxytoca and Bacillus subtilis, respectively, isolated from feces of giant pandas.

**Functional Aspects Of The Herbivore Gastrointestinal Tract**

The GIT plays an important role in the acquisition of nutrients for the body through digestive processes, but it also functions as a major part of host health through the modulation of the host-symbiont relationship (Hooper, Midtvedt, and Gordon, 2012). This is a delicate balance. To facilitate proper host health, the gut microbiota must also be balanced to aid in digestion and to engage in its role in normal structure and function of the GIT.
**Gastrointestinal Tract Functions**

*Transport and Digestion of Feeds*

The gastrointestinal tract (GIT) allows for feeds to be transported from mouth to rectum during the digestive process (Williams, 2009), but one of most important role of the GIT is digestion of feedstuffs. The digestive process is summarized in Table 1.4 for monogastric, ruminant, and hindgut fermenters (Prosser and DeVillez, 1991; Moir, 1964; Langer and Snipes, 1989; Hume and Sakaguchi, 1989). This process varies between physiological-type. For most animals, the digestive process begins with the mouth. The mouth utilizes mechanical digestion through mastication (excluding ruminants), and in some animals, the mouth releases amylase in the saliva to begin starch degradation (Prosser and DeVillez, 1991). From the mouth, ingesta travels to the stomach via the esophagus.

In ruminants, the reticulo-rumen is the first section of the stomach that ingesta enters (Moir, 1964; Phillipson and Ash, 1964). There, microbial fermentation of carbohydrates, proteins, and fatty acids occurs (Prosser and DeVillez, 1991; Walker, 1964). Starch and cellulose are converted into volatile fatty acids (VFAs), true protein and non-protein nitrogen (NPN) are converted into microbial protein, and lipids are converted into glycerol and fatty acids (Prosser and DeVillez, 1991; Walker, 1964; Garton, 1964). For monogastric animals, regardless of hind-gut fermentation status, hydrochloric acid and pepsin (pepsinogen—cleaved to form pesin) is released in the stomach. In ruminants, this process is carried out in the abomasum (Prosser and DeVillez, 1991, Sineshchekow, 1964).
The stomach empties into the small intestine, the area where protein degradation is continued. Pancreatic enzymes (trypsin, chymotrypsin, and carboxypeptidase) and enzymes from the small intestine are released to degrade proteins (ruminants: microbial protein and bypass protein; non-ruminants: true protein) into di/tripeptides and free amino acids (Kato, Katoh, and Barej, 1989; Prosser and DeVillez, 1991). Hindgut fermenters lack the opportunity to convert NPN into microbial protein for protein digestion, as protein digestion occurs prior to microbial fermentation. In non-ruminants, carbohydrate digestion begins with the digestion of starch into glucose, and fatty acids are also digested here (Prosser and DeVillez, 1991).

Table 1.4 Digestion of feeds

<table>
<thead>
<tr>
<th>Location</th>
<th>Monogastric</th>
<th>Ruminant</th>
<th>Hind-gut</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouth</td>
<td>Mastication and starch digestion*</td>
<td>Add moisture/buffer</td>
<td>Mastication</td>
</tr>
<tr>
<td>Stomach</td>
<td>Release of HCl and pepsin begin protein digestion, blends digesta</td>
<td>Reticulo-rumen: microbial fermentation of carbohydrates, true protein, NPN, and lipids, vitamin synthesis</td>
<td>Release of HCl and pepsin begin protein digestion, blends digesta</td>
</tr>
<tr>
<td>Small intestine</td>
<td>Carbohydrate digestion, protein digestion, FA digestion</td>
<td>Microbial protein (\rightarrow) AAs</td>
<td>Starch, FA, and protein digestion</td>
</tr>
<tr>
<td>Large intestine</td>
<td>Some microbial fermentation</td>
<td>Bypass protein (\rightarrow) AAs</td>
<td>Cecum: microbial degradation of fiber, vitamin synthesis</td>
</tr>
</tbody>
</table>

*For animals that produce amylase in saliva. HCl-Hydrochloric acid, TAG-triacyl glycerides, FA-fatty acid, NPN-non-protein nitrogen, AA-amino acid, n/a-not applicable.

The large intestine does not serve a major role in digestion of ruminants. However, in non-ruminants, this location is the epicenter for fiber digestion. This occurs
in small amounts in animals lacking a cecum, but in those with a cecum, complex carbohydrates are digested through microbial fermentation into VFAs (Prosser and DeVillez, 1991).

*Absorption of Nutrients*

The absorption process for ruminants and non-ruminants is summarized in Table 1.5 (Prosser and DeVillez, 1991; Moir, 1964; Langer and Snipes, 1989; Hume and Sakaguchi, 1989). In ruminants, the stomach is the first site of host absorption (Sineshchekov, 1964). VFAs produced through microbial fermentation are absorbed through the wall of the rumen, and water is absorbed in the omasum (Sineshchekov, 1964; Prosser and DeVillez, 1991).

The stomach does not serve as an absorption site for non-ruminants. Nutrient absorption in non-ruminants begins in the small intestine, where products of starch and lipid digestion, including glycerol, are absorbed (Prosser and DeVillez, 1991). In all types, products from protein (true and microbial), and fatty acid digestion are absorbed (Webb and Bergman, 1989; Prosser and DeVillez, 1991). Free fatty acids can be absorbed either directly through the mucosa via carrier systems or through the hepatic portal vein. The small intestine does have the ability to absorb di/tripeptides where they can be passed into blood circulation. Also, fat-soluble vitamins and minerals are absorbed in the small intestine (Prosser and DeVillez, 1991).

The large intestine serves primarily as an absorption site with the exception of cecum housing microbial fermentation in hindgut fermenters (Prosser and DeVillez, 1991). In all three types, water, water-soluble vitamins, and minerals are absorbed in the
large intestine. In hindgut fermenters, VFAs from fiber digestion are absorbed in the cecum (Prosser and DeVillez, 1991).

Table 1.5 Absorption of nutrients.

<table>
<thead>
<tr>
<th>Location</th>
<th>Monogastric</th>
<th>Ruminant</th>
<th>Hindgut</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>n/a</td>
<td>Reticulo-rumen: VFAs</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>through rumen wall</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Omasum: water absorption</td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
<td>Glucose(from starch), glycerol and FAs, AAs, fast-</td>
<td>FA, AA</td>
<td>Glucose (from starch), FA, AA, fat-</td>
</tr>
<tr>
<td></td>
<td>soluble vitamins, and minerals</td>
<td>Fat-soluble vitamins and</td>
<td>soluble vitamins and</td>
</tr>
<tr>
<td></td>
<td></td>
<td>minerals</td>
<td>minerals</td>
</tr>
<tr>
<td>Large intestine</td>
<td>Water, water-soluble vitamins, and minerals</td>
<td>Water, water-soluble vitamins,</td>
<td>Water, water-soluble vitamins and</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and minerals</td>
<td>minerals; VFAs via cecum</td>
</tr>
</tbody>
</table>

Host-Symbiont Interactions in Digestion

Hosts rely heavily on their GIT microbiota for digestion of highly fibrous diets (Prosser and DeVillez, 1991, Varel and Yen, 2007, Dehority, 2003). Not only are microbes involved in degrading complex carbohydrates, they are also involved in protein digestion and nitrogen (N) acquisition (Prosser and DeVillez, 1991; Dehority, 2003; Hungate, 1966). The energy flow of the host-symbiont digestion is summarized in Figure 1.5. The total energy supplied from the diet as determined by a bomb calorimeter is considered the gross energy of the diet (Pond et al., 2005). Gross energy consists of everything that is considered digestible and indigestible. Indigestible energy, also referred to as the fecal energy, is comprised of all undigested feeds, metabolic products, and anything that is not available for digestible energy (Pond et al., 2005).
Digestible energy consists of the total amount of energy available for absorption by the host. This value includes all energy found in the diet minus the energy excreted (Pond et al., 2005). In herbivores, two main groups that undergo digestion are carbohydrates and proteins, although lipids are also degraded through microbial action (Hungate, 1966; Dehority, 2003).

**Microbes Degrade Complex Carbohydrates**

A significant host-symbiont relationship revolves around the degradation of complex carbohydrates like cellulose, hemicellulose and pectin (Baldwin; 1964; Hungate, 1966; Dehority, 2003). No vertebrates and only a few invertebrates have the ability to degrade these carbohydrates without the aid of microbial communities. Therefore, vertebrate herbivores rely on the microbes residing in their GIT for degradation of their highly fibrous feedstuffs. The microbial communities found residing in the rumen and cecum consist of anaerobic bacteria, fungi, and protozoa (Walker, 1964; Hungate, 1966; Dehority, 2003). These communities collectively partake in cross-feeding, one type of organism breaks a substrate down to a certain point, and then another organism further degrades it (Dehority, 2003). This process is extremely beneficial to the host, as microbial degradation will, in essence, digest the complex carbohydrates for them with no host input.

Microbes utilize these complex carbohydrates and their simpler monomers to meet their energy needs for maintenance and growth. Most carbohydrates undergo glucose fermentation for production of ATP (Baldwin, 1964; Hungate, 1966; White, 2007). The end products of typical microbial metabolic pathways are volatile fatty acids (VFA) such as acetate (2C), propionate (3C), and butyrate (4C) (Hungate, 1966; White,
These VFAs produced as end products during microbial fermentation can be absorbed through the rumen or cecal wall and used by the host as a form of energy (Hungate, 1966). This is a mutually beneficial process because VFAs act as inhibitors in the negative feedback loop for microbes (Hungate, 1966; White, 2007). When the host absorbs the VFAs as an energy source for themselves, they are also allowing further microbial fermentation to take place, a benefit for both themselves and the microorganism.

By-products of these fermentative reactions are gaseous energy (methane) and heat (Pond et al., 2005). The rumen is such a reduced environment, that anaerobic fermentation must take place (Hungate, 1966). In this environment, \( \text{O}_2 \) is not present; therefore, \( \text{CO}_2 \) must serve as an electron acceptor. Pyruvate is converted into lactate, not Acetyl-CoA, via lactate dehydrogenase and is then further converted to a VFA, propionate (Baldwin, 1964; Hungate, 1966; White, 2007). This process produces gaseous methane, and eructation of methane must occur or ruminants will bloat which is extremely dangerous (Hungate, 1966). These fermentation reactions also produce heat, which is another form of energy released by the system.

Microbial populations vary according to location within the rumen and also by diet. Therefore, different populations will be found in ruminants on high forage vs. high grain (starch) diet (Hungate, 1966). These varying populations will also produce varying amounts of VFA products (Pond et al., 2005).

**Ruminal Microbes Ferment Proteins**

The bacterial communities present in the rumen ferment the majority of protein that enters, unless it is a protected protein (called by-pass protein because it by-passes
microbial fermentation) (Blackburn, 1964; Hungate, 1966). Not only can these bacteria utilize proteins, they can also use non-protein nitrogen (NPN) (Hungate, 1966, White, 2007). This is extremely useful for ruminant animals, but less so for non-ruminants, as they would require a higher quality diet because they cannot utilize NPNs because their fermentation processes happen post-small intestine. Microbes utilize both nitrogen sources to create nucleic acids and amino acids for their genomic material as well as protein.

Nitrogen quality is extremely important, as it also affects microbial populations cellulolytic ability. That is why diets must be balanced in terms of carbohydrate complexity and nitrogen source quality. Lower quality nitrogen sources, like urea (NPN) require a carbon skeleton to make amino acids (Blackburn, 1964; Hungate, 1966). Therefore, if you feed bovines a low quality nitrogen source, then you must feed them a less complex carbohydrate to rapidly provide carbon skeletons for amino acid formation. The reverse is also true, if you feed them a high quality nitrogen source (like soybean meal, an escape protein), then the cow could be fed a more complex carbohydrate source (hay, cellulose) because it would not require carbon skeletons rapidly (Hungate, 1966). If not properly managed, urea can be absorbed and excreted via urine (Hungate, 1966). This removal of nitrogen residues from metabolism make up the urinary energy lost during digestion and absorption of energy by the host.
Figure 1.5  Summarized energy flow of host-symbiont digestion in a) ruminants and b) hindgut fermenters.
Hosts can take advantage of microbial fermentation of true proteins and NPN because any microbe that leaves the rumen to pass into the abomasum/omasum and the small intestine can now degrade the protein, whether it be microbial protein (from true protein or NPN) or protected proteins (by-pass protein) (Hungate, 1966). These proteins will be digested and absorbed by the host in the small intestine either as di/tripeptides or free amino acids either directly to blood or through a carrier system (Blackburn, 1964; Prosser and DeVillez, 1991).

Host Intermediary Metabolism

Metabolizable energy is considered the total amount of energy that can be available to tissues for metabolism. Up to this point, the microbial populations have primarily degraded all the products to a form that can be utilized by the host, and these products have been absorbed. The transport of these products requires use of the blood stream, and in particular, the hepatic portal vein for amino acids.

The VFAs produced by microbial populations within the rumen and cecum (acetate, propionate, and butyrate) can either be used for fatty acid synthesis or be converted into glucose (Prosser and DeVillez, 1991). Acetate and butyrate are considered ketogenic fatty acids, and will be converted into Acetyl-CoA. Propionate will be converted into Succinyl-CoA and then to glucose (via oxaloacetate) using gluconeogenesis via the Krebs cycle. Butyrate is most commonly either converted to acetate in the rumen or to ketone bodies. Acetate is rapidly metabolized by the body; once oxidized, it functions as respiratory CO₂, with the unoxidized form contributing to fatty acid synthesis (Hungate, 1966). All amino acids are considered gluconeogenic with the exception of leucine and isoleucine. These two amino acids are exclusively
ketogenic, and cannot be converted to glucose. The conversion of gluconeogenic amino acids to glucose occurs through different intermediates in the Krebs cycle.

**GIT-associated Immune Function**

The GIT is considered the largest lymphoid organ in the body due to its immense surface area (Forchielli and Walker, 2005). One of the main reasons the GIT serves as an important lymphoid tissue is that foreign material enters through the gut and is processed and absorbed. Therefore, it is an area that is highly monitored by the immune system.

The lymphoid organ is made up of the gut associated lymphoid tissue (GALT). This tissue modulates the host-commensal relationship and is diagrammed in Figure 1.6. It is a well-established fact that a normally structured and functioning GIT requires the colonization of commensal microorganisms (Forchielli and Walker, 2005). The commensal organisms play a large role in the development of the immune system following initial colonization, but it also helps aid innate immunity through modulation.

Determining the difference between commensals and pathogens through a process called immune homeostasis is an important function of the GALT (Forchielli and Walker, 2005). The physical separation of microbiota from the epithelial surface of the intestines is one of the fundamental mechanisms that maintains host-bacterial homeostasis. Intestinal epithelial cells serve as the interface between the commensal microbiota and GALT (Forchielli and Walker, 2005). This relationship is mutually beneficial, as some microbes, such as *Bacteroides thetaiotamicron* and *Bifidobacterium bifidum* can utilize glycans produced by intestinal epithelial cells (IEC) as a carbon source and the presence of these microbes keeps invading pathogens away from the mucosal surface (Koropatkin, Cameron, and Martens, 2012). Also, commensal microbes stimulate the mature B-
lymphocytes via the TLR signaling pathway to produce intestinal Immunoglobulin A (IgA) antibodies which can block microbial antigens to maintain immune homeostasis (Liu and Rhoads, 2013).

Figure 1.6 Interaction between gut-associated lymphoid tissue (GALT) and microbial communities within the gastrointestinal tract.

**Diet Affects Gut Microbes**

The effect of diet of gut microbiota has been examined in many species, including that of the giant and red panda (Amato et al., 2014; Scott et al., 2013; Nicholson et al., 2012; Bolnick et al., 2014; Kong et al., 2014; Williams et al., 2012). The substrates that enter the GIT are fermented into various VFAs, which in turn can alter the GIT environment, leading to proliferation of certain commensal bacteria (Forchielli and Walker, 2005). A changing diet is an influential factor in shifting microbial populations
(Buddington and Sunvold, 1998; Collins and Gibson, 1999; Williams et al., 2012). By changing the availability of dietary substrates, proportions of GIT microorganisms and their fermentation products will also change; this is evidenced by a decrease in proportion of pathogens accompanied by an increase in beneficial microorganisms following an increase in fermentable fiber in the diet of canines (Buddington and Sunvold, 1998; Varel and Pond, 1985; Varel and Yen, 1997; Zentek et al., 2003).

As one of the most important gut microbial product, VFAs function not only as energy currency, but also in modulating immune function (Hooper et al., 2012). They affect a wide range of host processes such as energy utilization, host-microbe signaling, and control of colonic pH (Flint et al., 2012; Hooper et al., 2012). These processes in turn have subsequent effects on the overall microbial composition, gut motility, and epithelial cell proliferation. As two of the primary VFAs, both acetate and propionate are carried in the bloodstream to different organs to serve as substrates for oxidation, lipid synthesis, and energy metabolism, particularly by hepatocyte cells which use propionate for gluconeogenesis (Prosser and DeVillez, 1991).

Butyrate is primarily produced by clostridia, eubacteria, and roseburia microbes, and it plays a significant role in regulating neutrophil function and migration inhibiting inflammatory cytokines (Louis and Flint, 2009). The presence of butyrate increases expression of tight junction proteins in colon epithelia and can be directly used there to produce ketone bodies and carbon dioxide. Changes in diet can result in a change in butyrate production, which can lead to a loosening of the tight junctions between gut epithelial cells and an accumulation of bacterial components in the hepatic portal vein with downstream effects on inflammation (Louis and Flint, 2009).
Dietary changes can also lead to dysbiosis, including conditions such as inflammatory disease and metabolic dysfunction (Xu et al., 2013). Obesity and diabetes are considered metabolic syndromes, and have both been linked to GIT microbiota (Musso, Gambino, and Cassader, 2010). Microbial communities in accordance to IECs work to control the storage and expenditure of energy (Xu et al., 2013). Several studies have been carried out in gnotobiotic mice have shown that the fecal transplant material affects the weight status of the mouse. When transplanted with feces from a lean mouse, the mouse maintains a healthy weight, but when a lean mouse is transplanted feces from an obese mouse, they themselves become obese (Turnbaugh et al., 2006). These obese mice also had a 50% reduction in relative abundance of the Bacteroidetes and an increase of the Firmicutes. The microbial communities residing in an obese mouse were found to be more efficient at extracting and storing energy from substrates than lean individuals (Turnbaugh et al., 2006, Payne et al., 2011).

Shifts in microbial communities, in addition to host genetic factors and immune function, also play a role inflammatory bowel disease (IBD) such as ulcerative colitis (UC) and Crohns disease (CD). Particularly in CD, where the GIT microbiota are directly involved in the triggering of the immune system to initiate the inflammatory response (Seksik et al., 2006), and the mucin-associated bacteria are thought to be more involved in this process than the fecal-associated (Swindsinski et al., 2005). A decrease in total abundance of the fecal-associated bacteria, in addition to lowered Firmicutes diversity, but an increase in diversity of Actinobacteria and Proteobacteria was observed in IBD patients (Sokol et al., 2006; Manichanh et al., 2006; Frank et al., 2007). Butyrate-producing bacteria were also reduced (Frank et al., 2007), and this loss of butyrate
production is significant because IECs receive energy from this SCFA. Reduced function of IECs due to lack of energy source could play a role in the inflammatory events that define IBD.

**16S rRNA Next-Generation Sequencing Technologies**

Sequencing, in general, has truly enlightened the field of microbiology. As we now understand, only approximately 1% of microorganisms can be cultured. Therefore, by using traditional culture-based approaches our knowledge of existing microbes was limited prior to sequencing technologies. If single culture sequencing advanced the field, the advent of parallel sequencing has exploded it. Traditional sequencing methods were only able to sequence one organism at a time. With parallel sequencing, also referred to as next-generation sequencing (NGS), multiple microorganisms can be sequenced concurrently, or in parallel.

The importance of NGS technology is apparent when one thinks of the number of organisms that not only reside in the biosphere ($4-6 \times 10^{30}$) (Wooley, Godzik, and Friedberg, 2010; Vieites et al., 2009), but also those that reside as commensals on and within our bodies ($10^{14}$) (Qin et al., 2010). Microbes have been found in every habitat on Earth and are essential to life, and they play key roles in nutrient sequestration and carbon cycling, amongst many other roles (Wooley, Godzik, and Friedberg, 2010). Since microbes do not live alone as individual species but as a large community of organisms, it is important that they be studied as such. NGS technologies allow for this to happen. DNA is obtained directly from the environment, so whole communities can be elucidated.
Figure 1.7  Parallel sequencing workflow

Adapted from Loman et al. (2012).
In this dissertation, two technologies are used: Roche 454 pyrosequencing and Illumina MiSeq. Each technology has its advantages and disadvantages, and researchers should use caution when selecting the platform to meet their needs (Table 1.6). Regardless of platform, these technologies all utilize similar procedures (Figure 1.7).

Table 1.6 Comparison of next-generation sequencing platforms.

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Chemistry</th>
<th>Output (Gb)</th>
<th>Read number (M)</th>
<th>Read length (bp)</th>
<th>Run time (hr)</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>MiSeq (Illumina)</td>
<td>Reversible terminator</td>
<td>0.3-15</td>
<td>25</td>
<td>2x300</td>
<td>5-55</td>
<td>Cost-effective, short run times, Minimal hands on time</td>
<td>Randomly generated base call errors</td>
</tr>
<tr>
<td>GS Junior + (Roche)</td>
<td>Pyrosequencing</td>
<td>0.70</td>
<td>0.70</td>
<td>700</td>
<td>18</td>
<td>Long read lengths</td>
<td>Appreciable hands on time, High homopolymer error rates</td>
</tr>
</tbody>
</table>

Sample Acquisition and Nucleic Acid Preparation

Sequencing begins with sample collection, and this is also where the first set of biases can be introduced beginning with the total number of samples collected. In this case, sampler bias can be problematic not only in sample selection, but also in creating batch effects (Taub, Bravo, Irizarry, 2010; Schmeisser, Steel, and Streit, 2007). Rochelle et al. (1994) compared several different sample collection protocols and determined that the way the sample was collected affected the microbial diversity found in samples. If samples from anaerobic environments were stored aerobically for 24 hours prior to freezing, the beta- and gamma-Proteobacteria dominated sequences. However, if they were stored anaerobically, samples were represented by the alpha-Proteobacteria, and if
they were frozen within a 2-hour period, they found the highest sequence diversity (Rochelle et al., 1994). These results indicate that the status of the cells before nucleic acid extraction has an effect on the microbial diversity observed.

From the sample, an extraction must be conducted. There are several methods for extracting nucleic acids, and method selection can bias community analysis. DNA quality and quantity are highly varied depending on the method selected. To begin an extraction, cells must be lysed to release DNA. Different lysis techniques can affect overall quality and yield of the DNA. If cell lysis technique is not efficient, the template DNA may not give an honest representation of the microbial community (i.e. cell wall structure). However, using a more efficient lysis technique (bead beating/sonication) to generate greater yield can decrease quality by shearing of DNA which can be problematic downstream due to short fragments creating chimeric products downstream (Schmeisser, Steele, and Streit, 2007; Yuan et al., 2012; Wintzingerode et al., 1997).

Yuan et al. (2012) examined six commonly used methods to analyze a mock community for yield, DNA shearing, representation of microbial community, and reproducibility. For greatest yield, they determined that using the phenol-chloroform-isoamyl alcohol generated the highest yield in all but one sample (n=11/12), and this method generated significantly higher than yields of commercially available kits. Shearing was found in all samples, but was less in those that utilized a non-physical disruption technique, indicating that method also affects DNA quality (Yuan et al., 2012).

**Template Amplification and Enrichment**

Following DNA extraction, template DNA is amplified using primers specific for the task. Polymerase chain reaction (PCR) also introduces another layer of bias into the
process, as some sequences are more favorable to be amplified than others. For quantitative abundance measures several assumptions must be made: i) all molecules are equally accessible to primer hybridization, ii) primer-template hybrids form with equal efficiencies, iii) extension efficiency of DNA polymerase is the same for all templates, and iv) limitations by substrate exhaustion equivalently affect the extension of all templates. These assumptions are difficult to overlook, as many universal primer sets contain degeneracies that can influence primer-template hybrid formation (Wintzingerode et al., 1997). For instance, if bacterial community analysis is required, a 16s rRNA primer set must be used. Selection of primer sets introduces bias as neither the 454 or MiSeq has read lengths that can span the entire variable region. Therefore, a primer set that spans several regions should be implemented. It must also be noted that some “universal” primers will only produce a subset of the true spectrum of different species (Huson et al., 2009). Also, DNA concentration, genome size, and G+C content, has also been observed affecting PCR (Wintzingerode et al., 1997).

During amplification, adaptors or barcodes are attached to sequences, thus labeling sequences to the sample from which they came. A library must be constructed, so samples undergo equimolar pooling, and technician error can introduce bias in this step. Depending on the NGS platform samples may undergo another amplification step. Roche 454 pyrosequencing utilizes emulsion PCR and enrichment, whereas Illumina/Solexa platforms use a solid-phase bridge amplification (Loman et al., 2012).

**Sequencing**

Both NGS platforms utilize a sequencing-by-synthesis design. Illumina platforms perform Solexa chemistry that is driven by reversible termination of sequencing products.
Termination is avoided in the 454 chemistries; this platform instead flows a single dNTP across the template. The 454 approach takes advantage of pyrosequencing (light emission following an enzyme cascade created by the presence of pyrophosphate).

Each platform has its own drawbacks (Table 1.6). The 454 GS Junior is also prone to mistakes in homopolymeric tracks. Whereas the Illumina base-call errors are not randomly distributed across cycle positions in sequenced reads (Taub, Bravo, and Irizarry, 2010) but overall the MiSeq platform has the highest throughput per run, lowest error rate and a user-friendly workflow.

Sequence coverage values can have an impact on the analysis. For instance, low coverage in identical or highly similar genomic regions produces biased measurements that can remove a region from consideration (Taub et al., 2010). Also, it has been shown that there is a significant relationship between G+C content and sequence coverage; therefore, adjusting G+C content will increase precision (Taub et al., 2010), whereas AT regions typically have very low coverage (Harismendy et al., 2009).

**Data Analysis**

After generating so much metadata, the next thing to decide how to best analyze it. Each platform delivers data in different formats. This can be especially problematic later when comparing data generated on different platforms. Also, there is some indication that depending on what pipeline you select for data analysis (Mothur, QIIME, etc.) different results are found.

The pipeline for data analysis will differ, so different steps are required; however, two basic approaches are available for high-throughput analysis: reads can be i) aligned to known reference sequences or ii) subjected to *de novo* assembly. The choice of
approach is usually dictated by read length; alignment approach is better for short reads, this method is useful for biological application such as documenting genetic variation in genomes of highly related strains (Loman et al., 2012). Alignment can become problematic when there are reads from repetitive regions or from regions of genomes that are missing from reference database.

False negatives may occur if the relevant genomic material may be removed during sequence filtering because the sequence homologs have never been deposited into databases (Wooley, Godzik, and Friedberg, 2010). Databases are constructed using known organisms, therefore some organisms will be absent from these databases due to the fact they have yet to be discovered. Database selection can also bias sequencing results. For example, if sequences were aligned to the SILVA database, many OTUs may be labeled and unknowns compared to if the same sequences were aligned to the GreenGenes databases. SILVA databases are far more stringent than GreenGenes. Therefore, depending on database selection, you may report different results.

**Justification**

Previously, the exact phylogenetic relationship between the two panda species has been unclear, but with the advent of molecular sequencing technologies, these two animals are now known to be very distantly related to each other, with their divergence from a common ancestor estimated to be approximately 38 Ma (O’Brien et al., 1985; Yu et al., 2004; Yu et al., 2011; Salesa et al., 2006). Although taxonomically distinct, these two panda species have several things in common, primarily their almost exclusively bamboo diet, but also their similar physical adaptations to their diet and their sympatry in some locations (Roberts and Gittleman, 1984; Davis, 1964; Schaller et al., 1985; Qi et al.,
2009). What was unknown was if their similarities also extended to their gut microbiota. As discussed previously, the relationship between diet and gastrointestinal microbial communities has been examined in many species, including both panda species (Kong et al., 2014; Williams et al., 2012). However, until now, there has been no comparison between these obligate bamboo foragers. This dissertation’s aim is to not only investigate key areas of interest regarding both giant and red pandas individually using next-generation sequencing technologies, but also compare these uniquely herbivorous carnivores to gain an understanding of the role their diet and their gut microbiota play in these threatened species.
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CHAPTER II
SPECIALIZED BAMBOO DIET SELECTS FOR SIMPLIFIED GUT MICROBIOME
IN RED PANDAS (AILURUS FULGENS)

Abstract

Red pandas (Ailurus fulgens) are unique in that they are physiologically a carnivore yet consume an exclusively herbivorous diet. Gastrointestinal tract (GIT) microbiota present in herbivores play an essential role in the digestion of their plant-based diet as well as influencing host health and immunity. Therefore, the GIT microbiome of a taxonomically defined carnivore with a unique herbivorous diet is of interest. Here, we examine the GIT bacterial microbiota of four ex-situ red pandas using next-generation 16S rRNA pyrosequencing of fecal samples. On average, the GIT communities were dominated by the phylum Firmicutes, with lesser contributions from the Proteobacteria, Actinobacteria, Bacteroidetes, Fusobacteria, SC4, Tenericutes and TM7. The GIT microbiota displayed low diversity, with each sample dominated by one operational taxonomic unit (OTU). However, the dominating sequence varied in individuals between two OTUs, an unclassified Lactobacillales and a Clostridium species, and all individuals contained representatives of both sequences and others, suggesting a shared core community. Using the weighted and unweighted UniFrac metric, age, sex, and geographical location of red pandas significantly affected the gut microbiota. Cubs had significantly higher levels of the orders Lactobacillales and
Enterobacterales and significantly lower levels of Clostridiales compared to adult red pandas (all, \( P<0.05 \)). Geographical location also significantly affected relative sequence abundance of Lactobacillales and Clostridiales (\( P<0.05 \)). These findings contribute to a better understanding of this herbivorous carnivore’s GIT microbiome and the role that age, sex, and geographical location may play in determining GIT bacterial communities.

**Introduction**

The red panda (*Ailurus fulgens*), also known as the lesser panda, is native to the Himalayas and China (Choudhury 2001; Wei et al., 1999a). They are considered a vulnerable species (IUCN, 2008) and protected as a category II species under the Wild Animal Protection Law of China and CITES (1995). As the only member of the family Ailurodae and a member of the superfamily Musteloidea, they are most closely related to members of Mustelidae (weasels, otters, martens, and badgers), Procyonidae (raccoons, coatis, kinkajous, olingos, ringtails and cacomistles), and Mephitidae (skunks) (Flynn et al., 2000).

Importantly, red pandas are classified as carnivores but exhibit a highly specialized herbivorous diet composed primarily of bamboo, a highly cellulose-rich plant material. In the wild, red pandas have a diet consisting of approximately 90% bamboo leaves (Wei et al., 1999a; Nijiboer & Dierenfeld, 2011; Yonzon & Hunter, 1989; Wei et al., 199b; Reid, Hu, & Huang, 1991). During the spring, they supplement their diet with bamboo shoots, and in the summer, they consume fruits (Wei et al., 1999a; Wei et al., 1999b; Reid, Hu, & Huang, 1991; Johnson, Schaller, and Hu, 1988). In captivity, red pandas are fed a diet that mimics the wild diet in bamboo and fruit composition with the
addition of a commercially prepared high-fiber biscuit that ensures a stable nutrient supply (Nijiboer & Dierenfeld, 2011).

The red panda gastrointestinal tract (GIT) is structurally similar to other carnivores (Davis, 1964; Roberts & Gittleman, 1984). Specifically, red pandas have a reduced length of small intestine, an increased relative surface area of the large intestine, and a smaller liver (Davis, 1964). Moreover, unlike other herbivores, they have a simple stomach, lack a cecum, and have shorter digesta transit times on the order of ten hours or less (Nijiboer & Dierenfeld, 2011; Davis, 1964; Roberts & Gittleman, 1984; Bleijenberg & Nijiboer, 1989; Van Soest, 1996). These anatomical features are distinct from herbivores, which have highly developed rumens and ceca that favor plant biomass degradation. In particular, the GIT of herbivores are thought to promote the retention of microbes specialized for the breakdown and fermentation of plant biomass.

Together, these observations raise the question as to how red pandas have adapted to an herbivorous diet in the presence of a carnivorous GIT. One hypothesis is that red pandas do not possess a GIT microbiota capable of plant biomass degradation, as it had also been posited for the giant panda (Van Soest, 1996; Hirayama et al., Wei et al; Senshu et al., 2007; Dierenfeld et al., 1982), a phylogenetically distinct mammal that also specializes on bamboo (Schaller et al., 1985; Flynn et al., 2000). Like red pandas, giant pandas are considered carnivores but consume a strictly herbivorous diet (Schaller et al., 1985; Edwards et al., 2006). Recent work has revealed that giant pandas are capable of plant biomass degradation highlighted by the detection of cellulases and hemicellulases belonging to the Clostridia and Bacilli (Zhu et al., 2011), and a study by Kong et al. (2014) indicates that adult red panda GIT microbiota may also have cellulolytic
capabilities; however there is no information regarding the effect of age or sex on these microbial communities.

In this study, fecal samples from four captive red pandas were analyzed using 454 pyrosequencing of the 16S rRNA gene yielding the first description of any Musteloidea gut microbiome to date. Differences in the gut bacterial community were compared across age, sex, and geographical location to determine the effects of these factors on the structure and composition of the red panda GIT microbiota. These data provide the first insight into the effect of age and sex on the red pandas’ GIT microbiota and will aid in establishing a framework for understanding their peculiar biology.

Materials and Methods

Ethics Statement

Prior to sampling, approval was granted from the Memphis Zoological Society and the Smithsonian National Zoological Park Institutional Animal Care and Use Committee (IACUC) for use of animals in this non-invasive study. Red panda fecal samples from the Memphis Zoo were collected under a signed biomaterials request form, and no IACUC was needed as the project was viewed as non-invasive by the institution.

Study animals

This study consisted of a young male, “Justin” (Studbook number: 1219, Date of birth (DOB) July 1, 2012), a young female, “Lucille” (Studbook number: 1215, DOB June 21, 2012), an adult male, “Pete” (Studbook number: 1107, DOB June 25, 2011), all housed at the Memphis Zoological Society, and an adult female, “Leo Mei” (Studbook number: 0521, DOB June 10, 2005), housed at National Zoological Park-Front Royal.
Justin and Lucille are housed together; therefore, samples labeled as “Cubs” belong to Justin or Lucille.

**Sample Collection**

We collected fresh fecal samples (n=17) from red pandas immediately following defecation. All samples were flash-frozen in liquid N\textsubscript{2}, transported on dry ice, and stored at −80°C prior to processing.

**DNA Extraction**

Total genomic DNA was extracted via mechanical disruption and hot/cold phenol extraction following Stevenson et al.’s protocol (2007) with the exception that 25:24:1 phenol:chloroform:isoamyl alcohol was used in place of phenol:chloroform at all steps. DNA was quantified using a Qubit Fluorometer (Invitrogen) and stored at −20°C.

**Two-step PCR**

In this study, primers and a two-step PCR were selected to minimize chloroplast contamination and amplify the V5-V8 region of the 16S rRNA (Hanshew et al., 2013). Reactions were done in triplicate containing 5.0-10 ng template DNA, 0.25 μL Herculase II DNA polymerase (Agilent Technologies, Santa Clara, CA), 1.0 nM dNTPs, 0.5 μL DMSO, 5.0μL Herculase II buffer, 300nM of forward (799F-5’-AACMGGATTAGATA CCCKG-3’) and reverse primers (1392R-5’ACGGGCGGTGTGTRC-3’) and water to a final reaction volume of 25 μL. PCR conditions were as described previously (Hanshew et al., 2013). Triplicate reactions were pooled, and PCR products were concentrated and purified using a PureLink® PCR Purification Kit (Invitrogen) following the manufacturer’s protocol. Purified products were used for gel extraction; the ~600 bp 16S
band was excised from a 1.0 % low-melt agarose gel (National Diagnostics, Atlanta, GA) using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA), and gel-extracted products were quantified with a Qubit Fluorometer (Invitrogen).

In the second step, primers with A- and B- adapters suitable for Lib-L Titanium 454 pyrosequencing along with a 5 bp barcode for each sample were used (799mod6F-5’-CMGGATTAGATACCCKGGT-3’ and 1392R) (Hanshew et al., 2013). Samples underwent the same PCR conditions as for the preceding step except reactions were done in duplicate with template DNA concentrations of 0.5-5.0 ng and cycle number reduced to 10 cycles. Duplicate reaction products were pooled and loaded onto a 1.0 % low-melt agarose gel, and the 16S band (~700bp) was extracted. Gel-extracted products were quantified with a Qubit Fluorometer and pooled in equimolar concentrations. The pool was diluted to 10-6 DNA molecules/µL for use in sequencing.

**Pyrosequencing**

The pooled amplicons were sequenced following manufacturer’s protocols (Roche Applied Science) for Titanium sequencing on a Roche 454 GS Junior Titanium sequencer with an initial emulsion PCR ratio of 0.80 molecules of DNA per bead.

**Data Analysis**

Sequences were processed using the program MOTHUR v.1.29.2 (Schloss et al., 2009) with default parameters unless stated otherwise. 16S rRNA reads were assigned to samples based on their sample-specific barcodes, and poor quality sequences were removed. The *shhh.flows* command, an implementation of the AmpliconNoise algorithm (Quince et al., 2011), was used, and sequences were trimmed and filtered based on
sequence quality (pdiffs=2, bdiffs=0, maxhomop=6, minlength=250). Unique sequences were determined and aligned against the SILVA 16S rRNA gene alignment database (Pruesse et al., 2007). Chimeras (chimera.uchime) and sequences identified as members of Eukaryota, Archaea and Cyanobacteria lineages were removed, and 16S rRNA sequences were classified using the GreenGenes reference database (De Santis et al., 2006) with a bootstrap value cutoff of 60. The following analyses were performed within MOTHUR: Good’s coverage (Good, 1953), Morista-Horn index (Horn, 1966), nonmetric multidimensional scaling (NMDS, iters =2,000,000) (Shepard, 1966), Principal Coordinate Analysis (PCoA) (Gower, 1966), Berger-Parker diversity metric (Maguran, 1988), Simpson diversity metric (Simpson, 1949), Shannon diversity metric (Pielou, 1975), and UniFrac (Lozupone et al., 2006).

**Statistical analysis**

All statistical analyses of taxonomic groups were carried out in SAS 9.3 statistical software (Cary, NC) and data are expressed as the mean ± SE and considered significant if P < 0.05. Differences in mean relative sequence abundance for both age and sex were observed via t test. Using the folded-F statistic in the equality of variance test, we found the assumption of equal variances to be violated in two taxonomic groups; therefore, we used an approximate version, the Satterthwaite-adjusted t test, to compensate for unequal variances. In MOTHUR, both the weighted and unweighted UniFrac metrics were used to analyze differences in microbial communities based upon host groups. To ensure true significance, samples were also randomly assigned into groups. Significance was tested at α=0.05.
Results

Sequence coverage and diversity metrics

For all samples, we generated a total of 118,360 (105,904 high-quality) sequences using 16S rRNA pyrosequencing (Table 2.1). A Good’s coverage value of > 0.99 (Table S1) and a leveling off of generated rarefaction curves (Figure S1) for each sample indicated that sequencing was adequate to detect the majority of bacterial diversity present in all samples. A 95 % operational taxonomic unit (OTU) analysis corresponding to genus-level classification identified 78 unique OTUs across all samples with values ranging from 13 to 37 per sample (Table 2.1).

Table 2.1 Number of sequences, estimated coverage, diversity and OTU richness in each sample.

<table>
<thead>
<tr>
<th>Sample</th>
<th>16S rRNA reads</th>
<th>Community Richness</th>
<th>Community Diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>High-quality</td>
<td>Berger-Parker</td>
</tr>
<tr>
<td>Justin (n=4)</td>
<td>25498</td>
<td>20376</td>
<td>0.71 ± 0.069</td>
</tr>
<tr>
<td>Lucille (n=1)</td>
<td>8256</td>
<td>8092</td>
<td>0.86</td>
</tr>
<tr>
<td>Cubs (n=4)</td>
<td>35478</td>
<td>25993</td>
<td>0.76 ± 0.038</td>
</tr>
<tr>
<td>Pete (n=5)</td>
<td>38822</td>
<td>33860</td>
<td>0.85 ± 0.086</td>
</tr>
<tr>
<td>Leo Mei (n=3)</td>
<td>18137</td>
<td>17583</td>
<td>0.90 ± 0.088</td>
</tr>
</tbody>
</table>

Mean values with standard error given when n>1.

Each individual sample’s microbiota displayed relatively low diversity and was dominated by a single OTU. Across all fecal samples, the calculated Berger-Parker index values ranged from 0.55 to 0.99, with Leo Mei (adult female) having the highest and Justin (young male) the lowest mean Berger-Parker value (Table 2.1). Pete (adult male) had the lowest and Justin the highest mean Shannon’s diversity index value with values ranging from 0.04 to 0.93 across all fecal samples (Table 2.1). For the inverse-Simpson’s
diversity index, values ranged from 1.2 to 1.7 across all fecal samples with Leo Mei
having the lowest and Justin the highest mean value (Table 2.1).

Sequences from fecal samples represented 8 phyla, with 96 ± 1.6 % of the
sequences belonging to the Firmicutes, 3.8 ± 1.6 % belonging to the Proteobacteria and
with < 0.2 % of sequences belonging to the Actinobacteria, Bacteroidetes, Fusobacteria,
SC4, Tenericutes, and TM7. Classes with > 1.0 % relative sequence abundance were the
Bacilli (50 ± 9.0 %), the Clostridia (46 ± 9.6 %) and the Gammaproteobacteria (3.3 ± 1.7
%). Orders with appreciable representation were the Lactobacillales (50 ± 8.8 %), the
Clostridiales (46 ± 9.6 %) and the Enterobacteriales (3.0 ± 1.7 %), and all other orders
were found in less than one percent relative sequence abundance (Figure 2.2a). At the
family and genus level, 50 % and 46 % of sequences, respectively, were also annotated.

Red pandas have a shared core community

The majority of the sequences in all samples belonged to two OTUs, an
unclassified Lactobacillales (50 ± 8.8 %) and a member of Clostridium genera (45 ± 9.6
%), and these OTUs were found in all samples. Other OTUs that were found in all
individuals were an unclassified Enterobacteriaceae (1.9 ± 1.6 %), a Jeotgalicoccus
species (0.56 ± 0.52 %), an unclassified Actinomycetales (0.13 ± 0.075 %), a
Pseudomonas spp. (0.12 ± 0.060 %), and a Faecalibacterium spp. (0.025 ± 0.10 %). In
total, shared OTUs accounted for 98 ± 0.0047 % of sequences within each samples.

The red panda microbiota differs according to age

Nonmetric multidimensional scaling (NMDS) clustering analysis (Figure 2.1a), as
well as both weighted and unweighted UniFrac analyses (P<0.001) revealed slight age-
related differences between the gut microbiota of adult and cub red pandas. Weighted and unweighted UniFrac analyses of individuals randomly assigned to two groups did not result in significant differences. Similar clustering trends were seen in three-dimensional principal coordinate analysis (PCoA) using the unweighted UniFrac metric (Figure S2a). Although not statistically different, red panda cubs had a more diverse microbiota than adults as determined by a higher Shannon’s and inverse Simpson’s diversity indices (Table 2.1). Specifically, cubs have a significantly higher relative sequence abundance of the orders Lactobacillales and Enterobacteriales in addition to a significantly lower relative sequence abundance of Clostridiales (73 ± 6.0 %, 5.9 ± 2.9 %, and 19 ± 7.2 %, respectively) than adult red pandas (23 ± 12 %, 0.25 ± 0.21 %, and 75 ± 12 %, respectively) (Figure 2.2b; Table 2.1). The two OTUs in highest abundance, the unclassified Lactobacillales and the Clostridium spp., also vary significantly with respect to age (P=0.0069 and P=0.0030, respectively).

![Figure 2.1](image.jpg)

Figure 2.1  Total bacterial community structure differs by age and sex by three-dimensional nonmetric multidimensional scaling (NMDS).

NMDS analysis of 95 % OTUs was completed comparing all individual red panda samples by a) age and b) sex. NMDS visualizations were viewed from multiple angles, and the appropriate angle is displayed to best denote clusters (lowest stress: 0.2211, R-square: 0.5754).
Figure 2.2   Relative sequence abundance of taxa at the order level

Notes: Shown by a) individual mean values for each panda, b) sex, and c) age
Stacked bar graphs of sequence abundance for taxonomic orders with > 1.0 % are shown
for red pandas.

The red panda microbiota differs according to sex

Based on weighted and unweighted UniFrac metrics, we found that there were
significant differences in the GIT microbial communities between male and female red
pandas (P<0.001). Again, these analyses did not yield significance when individuals
were randomly assigned to two groups. While males and females did not create fully
distinct clusters when analyzed using NMDS, groupings did show some differences
between sexes (Figure 2.1b). This trend was also present in a PCoA using the
unweighted UniFrac metric (Figure A.2b). Although not significantly different, males
were found to have a more diverse microbiota than females by both Shannon’s and
inverse-Simpson’s diversity indices (Table 2.1). We also found no significant differences
in relative sequence abundance at the taxonomic level of order with respect to sex (Table
2.2). However, males did have greater relative sequence abundance of Lactobacillales
and Enterobacterales and lower relative sequence abundance of Clostridiales and
Campylobacterales (47 ± 12 %, 2.5 ± 2.1 %, 49 ± 13 %, 0.050 ± 0.040 %, respectively)
than females (26 ± 20 %, 0.24 ± 0.18 %, 70 ± 21 % and 1.8 ± 1.6 %, respectively) (Figure 2.2c).

Table 2.2 P-values of significantly different orders for age and sex.

<table>
<thead>
<tr>
<th></th>
<th>Lactobacillales</th>
<th>Clostridales</th>
<th>Enterobacterales</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.0015(^{p*})</td>
<td>0.0010(^{p*})</td>
<td>0.0308(^{s*})</td>
</tr>
<tr>
<td>Sex</td>
<td>0.3682(^{p})</td>
<td>0.3943(^{p})</td>
<td>0.3139(^{s})</td>
</tr>
</tbody>
</table>

P-values for relative sequence abundance at the order levels at the \(\alpha=0.05\). Post-hoc tests were Pooled\(^{p}\) and Satterthwaite\(^{s}\). Significant values are indicated by *.

Discussion

In this study, we investigated the GIT microbiota of captive red pandas using high-throughput 16S rRNA pyrosequencing of fecal samples. Herbivores have significantly different microbial communities from carnivores (Ley et al., 2008), and specialized herbivores, such as ruminants, depend upon a defined community of plant-degrading microbes for the breakdown of fibrous feeds (Van Soest, 1996). Red pandas, like the giant panda, are herbivorous carnivores that have adapted to their diet through several foraging enhancements (Wei et al., 1999b, Van Soest, 1996; Zhang et al., 2007; Wei et al., 2000; Loeffler, Montali, & Rideout, 2006) but have not adapted their GIT (Nijiboer & Dierenfeld, 2011; Roberts & Gittleman, 1984; Schaller et al., 1985; Bleijenberg & Nijiboer, 1989; Loeffler, Montali, & Rideout, 2006). Their unconventional biology offers an important glimpse into the adaptations required in the gut bacterial community for nutritional acquisition in a GIT lacking adaptations seen in other herbivores.

With such a uniquely herbivorous carnivore, it is unknown whether red pandas’ GIT microbiota are more similar to that of an herbivore or a carnivore. Together, the
phyla Firmicutes and Bacteroidetes typically dominate in the mammalian gut of both herbivores and carnivores. However, we found that for red pandas, the Firmicutes dominated, and the members of the Proteobacteria were found second highest in relative sequence abundance, and all other phyla, including the Bacteroidetes, were found in < 0.2%. Herbivores have a much higher microbial diversity within their GIT (Ley et al., 2008). The koala, a hindgut fermenter, had a Shannon diversity index value of approximately 5.5, and the Firmicutes were observed in highest abundance, followed by the Bacteroidetes and the Synergistetes, with the Fusobacteria and the Proteobacteria in lesser abundances (Barker et al., 2013). As a foregut fermenter, forage-fed bovines, on average, have a Shannon diversity index value of 7.49, and their GIT microbiota are also dominated by the Firmicutes with lesser contributions by the TM7, the Actinobacteria, the Proteobacteria, the Bacteroidetes, and the Verrucomicrobia (Kim et al., 2013).

When compared to herbivores, carnivores have a much lower microbial diversity in the GIT. Domesticated canines and felines have Shannon diversity index values of 1.76 and 2.18, respectively (Handl et al., 2013). In a study by Handl et al. (2013) the Firmicutes were found in highest abundance in canine and felines GIT microbiota; however, the second most prevalent phylum varied. The Bacteroidetes were observed second highest in relative sequence abundance in canines and third in felines, while the Actinobacteria were observed in second highest relative sequence abundance in felines and third in canines.

It appears that the red panda is unlike either classical example of herbivores and carnivores. The red panda has much lower microbial diversity than observed in carnivores (Handl et al., 2013), and the phyla representations were not similar to either
the herbivores or carnivores (Kim et al., 2013, Barker et al., 2013; Handl et al., 2013). However, when compared to another herbivorous carnivore, the giant panda, red pandas have very similar low microbial diversity as observed by the average Shannon diversity index value (Tun et al., 2014). Like the red panda, the Firmicutes are found in highest relative abundance in giant pandas with the Proteobacteria found in second highest, with lesser contributions by Bacteroidetes and Actinobacteria (Zhu et al., 2011, Tun et al., 2014). Therefore, red pandas appear to be most similar to giant pandas in terms of GIT microbiota.

Our analysis of the red panda GIT microbiome at the OTU level revealed a core community defined by seven OTUs. These included two that were found in high abundance and five in very low abundances (< 2.0 %). The two high abundance OTUs are an unclassified Lactobacillales and a Clostridium species, which dominated the GIT microbiome and were found in all samples. The Lactobacillales could represent a key component of GIT microbiota due to their presence as commensal organisms in the GIT of other mammals (Reid & Burton, 2002; Salvetti et al., 2013). Importantly, the Lactobacillales include lactic acid bacteria (LAB) (Makarova et al., 2006) that are known for their ability to ferment a wide range of short-chain fatty acids which serve as a primary energy source for red pandas (Salvetti et al., 2013; Warnell, Crissey, & Oftedal, 1989). Members of the Clostridiales, including Clostridium spp., have cellulosylytic properties (Burrell et al., 2004; Sleat, Mah, & Robinson, 1984; Varel, Yen, & Kreikemeir, 1995) capable of adhering to plant particulate and degrading fibrous material (Burrel et al., 2004; Shiratori et al., 2006). Thus, these bacteria could be involved in the degradation of the red pandas’ high fiber diet.
Similarly, species belonging to the phyla Firmicutes and, in particular, members of the *Lactobacillus* and *Clostridium* genera, were found in Kong et al.’s study (2014). Members of these genera have been reported in the GIT of giant pandas in both culture-dependent and independent studies (Hirayama et al., 1989; Wei et al., 2007; Williams et al., 2013; Zhu et al., 2011). Zhu et al. (2011) found that 61 % of the total 16S rRNA sequences recovered from giant panda feces belonged to the class Clostridia (includes genus *Clostridium*) and 23 % of total sequences belonged to the class Bacilli (includes order Lactobacillales). These values are similar to what we observed for adult red pandas (75 ± 12 % and 23 ± 12 %, respectively), and with both panda species having an almost exclusively bamboo diet, these data suggest that feed acts as a driving force of the gut microbial community.

However, the two core OTUs in high abundance, the unclassified Lactobacillales and the *Clostridium* spp., displayed significant differences with respect to age and geography. Since one of these two OTUs dominate in all samples, these microorganisms may fulfill different roles in the gut community, and factors such as age or geography may determine which OTU dominates the GIT microbiota.

We also found that the GIT microbiome differed significantly with respect to age (Figure 2.1a). This may be due to a diet difference, as cubs consume milk replacer prior to shifting to an adult bamboo diet. Red panda cubs displayed significantly higher levels of the Lactobacillales and significantly lower levels of Clostridiales than adults. This difference is similar to changes seen in mice when the Lactobacillales dominate the GIT microbiome during breastfeeding, and after weaning, levels of Lactobacillales decrease, and Clostridiales become more prominent in the colon (Hasegawa et al., 2010). It is
possible that the members of the Lactobacillales present in our study include the LAB, which are initial colonizers and may help cubs maintain active immunity following the loss of passive immunity acquired from colostrum, as observed in bovine calves (Malmuthuge et al., 2013). As red pandas age, they must shift to a plant-based diet, and members of the Clostridiales increase possibly aiding in the degradation of fibrous materials (Burrell et al., 2004; Shiratori et al., 2006).

Sex was also found to significantly affect the red panda gut microbiome (Figure 1b). However, there were no significant differences in relative sequence abundance at any taxonomic order (Table 1). Sex-related differences in gut microbiota have been observed in humans, mice, and chimpanzees (Li et al, 2007, Koren, et al., 2012; Mueller et al., 2006; Campbell 2012, Degnan 2012). These observed differences have been attributed to sex-related differences in the foraging behavior of chimpanzees (degnan, 2012), an interaction with genotype in mice (Campbell, 2012), and the reproductive status (estrus, gestation, or lactation) of female bovines and humans (Koren et al., 2012; Wang et al., 2012). In our study, the male and female red pandas were offered the same diet, and none of our females were in estrus, in gestation or lactating. Therefore, our small sample size may have created artificial levels of significance in UniFrac, and a larger sample size is required to discern any true difference.

Previous work on red pandas suggested that they were unable to harbor microbial populations in their GIT capable of fermenting fibrous feedstuffs due to the short retention time of their ingesta (Nijiboer & Dierenfeld, 2011; Belijenberg & Nijiboer, 1989; Warnell, Crissey, & Oftedal, 1989). However, work by Clemens & Stevens (1979) determined that raccoons, which are omnivorous carnivores and members of the
superfamily Musteloidea, also lack a compartmentalized foregut or sacculated colon, which suggests a long retention time may not be required for the microbial degradation of feed. Moreover, Zhu et al. (2011) observed cellulose-metabolizing microorganisms in giant pandas despite a short ingesta time. In our study, we found that the red pandas had extremely low diversity but contained a seven-OTU core community. This indicates that red pandas do, in fact, harbor a GIT microbial community. Their specialized bamboo diet in the absence of herbivorous GIT adaptations may have selected for such low diversity. Therefore, with a similar GIT physiology and specialized microbiota, red pandas may also microbial degraders of fibrous feed in a manner similar to raccoons and giant pandas. In addition to determining their distinct core community, this study uncovered differences in the red panda gut microbiota with respect to age and sex, and has provided framework for future work on this vulnerable and uniquely herbivorous carnivore.
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CHAPTER III

DIETARY CHANGES DURING WEANING SHAPE THE GUT MICROBIOTA OF RED PANDAS (*AILURUS FULGENS*)

Abstract

Mammalian herbivores have adaptations to utilize their plant-based diets including a modified gastrointestinal tract (GIT) and symbiotic microbiota involved in digestion and host health maintenance. Red pandas (*Ailurus fulgens*) are herbivorous carnivores that subsist almost entirely on bamboo without the specialized GIT common to other herbivores. The GIT microbiota is of further importance, as red pandas suffer from high infant mortality that has been attributed to failure to meet nutritional requirements. To gain insight into their GIT microbiota establishment, we examined two cubs during weaning using next-generation 16S rRNA pyrosequencing. Across all weaning stages, the GIT microbial community displayed low diversity and was dominated by the phylum Firmicutes with lesser contributions from the Proteobacteria. A core community was found across weaning stages and included an unclassified Lactobacillales, a *Clostridium* sp., and an unclassified Enterobacteriaceae. Community structure analysis showed that although the GIT microbiota is established early in red pandas, subsequent dietary changes during weaning further shape the community and are correlated with the presence of new bacterial species. This work is the first analysis of red panda cubs GIT
microbiota during weaning and provides a framework for understanding how diet and host microbiota impact the development of these threatened animals.

**Introduction**

Microorganisms play a fundamental role in the survival of their animal hosts (McFall-Ngai et al., 2013). Specifically, the gastrointestinal tract (GIT) microbiota maintains the host’s immune system through modulation of normal GIT function and pathogen exclusion as well as contributes to nutrient and energy acquisition (Flint et al., 2012; Hooper et al., 2002). Gut-associated microbes are particularly important in herbivores where they are wholly responsible for the breakdown of dietary plant matter into accessible nutrients (Bergman, 1990). Many herbivores have evolved a number of physiological adaptations, such as a rumen and slow digesta transit time, to promote and enhance this microbial fermentation. An unusual exception is the red panda (*Ailurus fulgens*), which is able to survive on entirely plant-derived materials without such adaptations. Specifically, red pandas are members of Carnivora but consume an exclusively herbivorous diet (Loeffler, 2011). Unlike other typical herbivores, they have the GIT of a carnivore (Davis, 1964) with a simple stomach, no cecum, (Davis, 1964; Roberts and Gittleman, 1984) and a shorter digesta transit time on the order of ten hours or less (Bleijenberg and Nijboer, 1989; Nijboer and Dierenfeld, 2011).

Recent work on the GIT microbiota of both wild and captive adult red pandas found that the microbes present in the gut have some cellulolytic capabilities (Kong et al., 2014). This indicates that like other herbivores, gut microorganisms in red pandas play an important role in nutrient acquisition. However, there is little information regarding the acquisition of these microbial communities in juvenile red pandas as they are weaned
from a milk-based diet to bamboo. Without the accompanying change in GIT morphology seen in other herbivores, it is unclear how and when red pandas acquire the fibrolytic organisms necessary for survival as an adult.

This is particularly important for captive red pandas, as cubs born in North American institutions have high first year mortality (approximately 50 %), which has been attributed to many husbandry factors including failing to meet nutritional requirements (Loeffler, 2011). This is extremely problematic as red pandas are considered a vulnerable species with a declining population of 10,000 individuals (IUCN 2008), and ex situ breeding programs are not as successful as expected (Loeffler, 2011). Studying a threatened species is extremely difficult, as there are limited individuals. Therefore, gaining an understanding of the developing GIT microbiota in young red pandas may lead to new insights for husbandry of captive red pandas.

In this study, the gut bacterial microbiotas of two captive red panda cubs were tracked through weaning using 454 pyrosequencing of the 16S rRNA gene of fecal material. Gut bacterial communities at different weaning stages (pre-weaning, during-weaning, and post-weaning) were compared to characterize succession into an adult-like microbiota. We also correlated these data to diet to determine its effect on the development of the red panda GIT microbiota during weaning. These data provide the first insight into young red pandas GIT microbiota and establishes a framework for understanding their peculiar biology as herbivorous carnivores.
Materials and Methods

Ethics statement

Red panda fecal samples from the Memphis Zoo were collected under a signed biomaterials request form, and no IACUC was needed as the project was viewed as non-invasive by the institution.

Study animals

All animals used in this study were housed at the Memphis Zoological Society, Memphis, TN, USA. This study consisted of two hand-reared red pandas: a young male, “Justin” (Studbook number: 1219, Date of birth (DOB) July 1, 2012), and a young female, “Lucille” (Studbook number: 1215, DOB June 21, 2012), both housed at the Memphis Zoological Society. Animals were co-housed and therefore, samples could not be attributed to a specific individual and were pooled as weaning stages.

Sample Collection

Fresh fecal samples (n = 3) were collected for each weaning stage from red pandas following defecation. All samples were flash-frozen in liquid N\textsubscript{2}, transported on dry ice, and stored at −80 °C prior to processing. Sample collection times occurred in coordination with changes in diet composition at pre-weaning (Stage 1: milk only), during the weaning transition (Stage 2: milk and leaf eater diet; bamboo introduction) and post-weaning (Stage 3: bamboo and leaf eater diet) (Table C.1).

DNA Extraction

Total genomic DNA from fecal samples was extracted via mechanical disruption and hot/cold phenol extraction following Stevenson et al.’s protocol (2007) with the
exception that 25:24:1 phenol:chloroform:isoamyl alcohol was used in place of phenol:chloroform at all steps. DNA was quantified using a Qubit Fluorometer (Invitrogen) and stored at −20°C following extraction.

**Two-step PCR**

A two-step PCR was used to minimize chloroplast contamination and amplify the V5-V8 region of the 16S rRNA (Hanshew et al., 2013). The initial PCR was performed with forward (799F-5’-AACMGGATTAGATACCCKG-3’) and reverse primers (1392R-5’ACGGGCGGTGTGTRC-3’) designed to yield separate bacterial and chloroplast products that could be purified by gel extraction. Reactions were done in triplicate containing 5.0 - 10 ng DNA, 0.25 μL Herculase II DNA polymerase (Agilent Technologies, Santa Clara, CA), 2 % DMSO, dNTPS to 1.0 nM, and primers to 300 nM. PCR conditions were as described previously (Hanshew et al., 2013). Triplicate reactions were pooled, and PCR products were concentrated and purified using a PureLink® PCR Purification Kit (Invitrogen) following the manufacturer protocol. Purified products were used for gel extraction; the ~600 bp 16S band was excised from a 1.0 % low-melt agarose gel (National Diagnostics, Atlanta, GA) using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA), and gel-extracted products were quantified with a Qubit Fluorometer (Invitrogen).

In the second PCR, primers with A- and B- adapters suitable for Lib-L Titanium 454 pyrosequencing along with a 5 bp barcode for each sample were used (799mod6F-5’-CMGGATTAGATACCCKGGT-3’ and 1392R) (Hanshew et al., 2013). Samples underwent the same PCR conditions as in the first PCR, except reactions were done in duplicate with template DNA concentrations of 0.5 - 5.0 ng and the cycle number was
reduced to 10 cycles. Duplicate reaction products were pooled and loaded onto a 1.0% low-melt agarose gel, and the 16S band (~700 bp) was extracted. Gel-extracted products were quantified with a Qubit Fluorometer and pooled in equimolar concentrations. The pool was diluted to 10⁻⁶ DNA molecules/μL for use in sequencing.

**Pyrosequencing**

The pooled amplicons were sequenced following the manufacturer’s protocols (Roche Applied Science) for Titanium sequencing on a Roche 454 GS Junior Titanium sequencer with an initial emulsion PCR ratio of 0.8 molecules of DNA per bead. All sequences were deposited into the National Center for Biotechnological Data’s Short Read Archive under Accession Number SRP051780.

**Data Analysis**

Sequences were processed using the program MOTHUR v.1.29.2 (Schloss et al., 2009) with default parameters unless stated otherwise. 16S rRNA reads were assigned to samples based on their sample-specific barcodes, and poor quality sequences were removed. The shhh.flows command, an implementation of the AmpliconNoise algorithm (Quince et al., 2011), was used, and sequences were trimmed and filtered based on sequence quality (pdiffs=2, bdiffs=0, maxhomop=6, minlength=250). Unique sequences were determined and aligned against the SILVA 16S rRNA gene alignment database (Pruesse et al., 2007). Chimeras (chimera.uchime) and sequences identified as members of *Eukaryota, Archaea* and *Cyanobacteria* lineages were removed, and 16S rRNA sequences were classified using the GreenGenes reference database (De Santis et al., 2006) with a bootstrap value cutoff of 60. The following analyses were performed within
Mothur: Good’s coverage (Good, 1953), Morista-Horn index (Horn, 1966), nonmetric multidimensional scaling (NMDS, iters =2,000,000) (Shepard, 1966), Principal Coordinate Analysis (PCoA) (Gower, 1966), Berger-Parker diversity metric (Magurran, 1988), Simpson diversity metric (Simpson, 1949), Shannon diversity metric (Pielou, 1975), and UniFrac (Lozupone et al., 2007).

Statistical Analysis

All statistical analyses were carried out in SAS 9.3 statistical software (Cary, NC) and data are expressed as the mean ± SE and considered significant if P < 0.05. Differences in mean relative sequence abundance for the three weaning periods were observed using the general linearized model (PROC GLM). In Mothur, both the weighted and unweighted UniFrac metrics were used to analyze differences in microbial communities based upon host groups. To ensure true significance, samples were also randomly assigned into groups and tested.

Results

Sequence coverage and diversity metrics

For all samples, we generated a total of 69,232 16S rRNA sequences using 454 pyrosequencing (Table 3.1), and 54,461 of these were deemed high-quality. A Good’s coverage value of > 0.99 (Table 3.1) and a leveling off of rarefaction curves (Figure S2) indicated that sequencing was adequate to detect the majority of bacterial diversity present in all samples. A 95 % operational taxonomic unit (OTU) analysis corresponding to genus-level classification (Schloss and Handelsman, 2005) identified 44 unique OTUs across all samples with values ranging from 5 to 17 OTUs per sample (Table 3.1).
Table 3.1  Number of sequences, estimated coverage, diversity and OTU richness.

<table>
<thead>
<tr>
<th>Sample</th>
<th>16S rRNA reads</th>
<th>Community Richness</th>
<th>Community Diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>High-quality</td>
<td>Berger-Parker</td>
</tr>
<tr>
<td>Total</td>
<td>69232</td>
<td>54461</td>
<td>0.76 ± 0.038</td>
</tr>
<tr>
<td>Stage 1 (n=3)</td>
<td>17667</td>
<td>13076</td>
<td>0.68 ± 0.089</td>
</tr>
<tr>
<td>Stage 2 (n=3)</td>
<td>29698</td>
<td>22005</td>
<td>0.83 ± 0.044</td>
</tr>
<tr>
<td>Stage 3 (n=3)</td>
<td>20224</td>
<td>19380</td>
<td>0.78 ± 0.028</td>
</tr>
</tbody>
</table>

Mean values with standard error given when n>1.

Each sample’s microbiota displayed low diversity and was dominated by a single OTU (Berger-Parker 0.68 to 0.83, Table 3.1). Samples obtained during weaning (Stage 2) had the lowest mean Shannon’s diversity index, while those sampled pre-weaning (Stage 1, Table 3.1) had the highest. The mean Shannon’s diversity index ranged from 0.55 to 0.72 across all stages (Table 3.1). The inverse-Simpson’s diversity index ranged from 1.4 to 1.8 across all samples with Stage 2 having the lowest and Stage 1 the highest mean value (Table 3.1).

Analysis of sequences from samples revealed the presence of four phyla, with 94 ± 2.9 % belonging to the Firmicutes, 6.1 ± 2.9 % to the Proteobacteria, and < 1.0 % each to the Actinobacteria and Bacteroidetes. At lower taxonomic classifications, bacterial classes with > 1.0 % relative sequence abundance included the Bacilli (74 ± 6.2 %), Clostridia (19 ± 7.2 %), and Gammaproteobacteria (6.0 ± 2.9 %). At the order level, those with > 1.0 % representation were the Lactobacillales (73 ± 6.0 %), Clostridiales (19 ± 7.2 %), Enterobacteriales (5.9 ± 2.9 %), and Bacillales (1.1 ± 0.65 %, Figure 3.1). At the family and genus level, 36 and 21 % of sequences, respectively, were annotated.
Red pandas cubs have a shared core bacterial community

To determine if there were specific OTUs common to both red pandas across all weaning stages, we analyzed all samples together. We found a total of eight OTUs across all samples (Table S4) and three of these had high sequence counts including an unclassified Lactobacillales (73 ± 6.0 %), a member of the genus *Clostridium* (19 ± 7.4 %), and an unclassified Enterobacteriaceae (5.9 ± 2.9 %). The other five OTUs were classified as belonging to the genus *Jeotgalicoccus* (1.1 ± 0.65 %), an unclassified Lactobacillales (0.26 ± 0.18 %), a *Pseudomonas* species (0.12 ± 0.052 %), a *Flavobacterium* (0.048 ± 0.044 %), and an unclassified Actinomycetales (0.042 ± 0.018 %). In total, these shared OTUs accounted for 99 ± 0.35 % of sequences within each sample.
The young red panda microbiota differs according to weaning stage

To determine if GIT microbiota differed across weaning stages, we performed a three-dimensional principal coordinate analysis (PCoA) using the weighted UniFrac metric (Figure 3.2) and found weaning stage-related differences in total bacterial communities. In particular, during weaning (Stage 2), samples clustered less closely and were more variable than those within either Stage 1 or 3. Similar trends were also observed when this analysis was performed with a three-dimensional nonmetric multidimensional scaling (NMDS) clustering approach (Figure B.2). Unweighted and weighted UniFrac comparisons between groups revealed that all weaning stages were significantly different from one another (Table 3.2). Weighted and unweighted UniFrac analyses of individuals randomly assigned to two groups did not result in significant differences. There were no significant differences in the relative abundances of bacterial families with respect to weaning stage. Across all stages, sequences in the family Lactobacillales dominated. Although not significant, the Clostridiales and Enterobacteriales varied over the three weaning stages, while the Bacillales decreased over time (Figure 1).

Table 3.2 P-values of significantly different bacterial communities using the weighted and unweighted UniFrac metrics.

<table>
<thead>
<tr>
<th>Weaning stage comparison</th>
<th>Weighted</th>
<th>Unweighted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1-3</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2-3</td>
<td>&lt;0.001</td>
<td>0.015</td>
</tr>
</tbody>
</table>
Figure 3.2  Three-dimensional principal coordinate analysis of microbial diversity using the weighted UniFrac metric for weaning stages.

Note: The percentage of variation is explained by PC1, PC2, and PC3 and indicated on the axes (total: 90.35 %).

A shift in diet during weaning correlates to the presence of new OTUs

Of the total forty-four OTUs found across all samples (Figure 3.3), eight defined the core community for young red pandas, while sixteen were present in more than one weaning stage. Although there were no significant differences in the relative abundances of specific OTUs between weaning stages, seven OTUs disappeared entirely following the shift from a milk-based diet (Stage 1) to the introduction of a leaf-eater and bamboo diet (Stage 2), while fifteen new OTUs appeared (Figure 3.3; Table 3.3). Of these fifteen OTUs, four OTUs (a *Ruminococcus* sp., a *Faecalibacterium* sp., an unclassified *Lachnospiraceae*, and a *Clostridium* sp.) were still present after the shift to a completely leaf-eater and bamboo diet (Stage 3) while ten new OTUs appeared in this weaning stage (Figure 3; Table S4).
Figure 3.3  A comparison of the number of OTUs shared between the three weaning stages.

Notes: OTUs were defined at the 95% similarity level corresponding to the genus taxonomic level.
Table 3.3  Core microbiota associated with weaning stages.

<table>
<thead>
<tr>
<th>OTU</th>
<th>Phylum</th>
<th>Lowest taxonomic classification</th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>009</td>
<td>Actinobacteria</td>
<td>Actinomyctaelles</td>
<td>□</td>
<td>■</td>
<td>□</td>
</tr>
<tr>
<td>008</td>
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<td>□</td>
<td>□</td>
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<tr>
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<td>Clostridium</td>
<td>■</td>
<td>■</td>
<td>■</td>
</tr>
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<td>□</td>
</tr>
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<td>Firmicutes</td>
<td>Lactobacillales</td>
<td>■</td>
<td>■</td>
<td>■</td>
</tr>
<tr>
<td>006</td>
<td>Firmicutes</td>
<td>Lactobacillales</td>
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<td>□</td>
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<td>■</td>
<td>■</td>
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<td>□</td>
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</tr>
</tbody>
</table>

■ denotes that all samples within a stage contained the specified OTU
☐ denotes ≤ 2 samples within each stage contained the specified OTU.
Discussion

The establishment of an adult gut microbiota is vital for the survival of herbivores as they rely on these communities for nutrient extraction from their plant-based diets. Weaning, and the resulting anatomical changes that occur in developing herbivores like ruminants, are thought to drive the establishment of these gut communities and enable the host’s survival as an adult (Jami et al., 2013; Koenig et al., 2011; Rey et al., 2013). Red pandas are an exception to this as they are herbivorous carnivores that lack such specialized GIT adaptations. Thus, they likely develop their gut microbiota differently than other herbivores and other physiological adaptations developed during weaning may aid in this GIT microbial development. Moreover, challenges related to nutritional inadequacies may contribute to the high cub mortality of red pandas in captivity (Loeffler, 2011), and investigating the establishment of the GIT microbiota may provide insight into this problem. To this end, we characterized the GIT bacterial microbiota of captive red panda cubs across weaning using high-throughput 16S rRNA pyrosequencing.

Overall, we found that the young red panda GIT had low diversity (Table S2) and was dominated by bacteria in the phyla Firmicutes with lesser contributions from the Proteobacteria; this is consistent with previous reports on captive adult red pandas (Kong et al., 2014). Across all weaning stages, the GIT microbiota of red panda cubs had an abundant core community defined by eight OTUs (>96 % in all samples), and there were no significant differences in specific taxa or OTUs between stages. Thus, it appears that the microorganisms necessary for the red panda’s survival on its adult leaf-based diet are present prior to weaning and as early as one month of age in healthy individuals.
posit that initial colonization by these organisms likely occurs during birth or very early in life through maternal contact, similar to humans (Mackie et al., 1999; Vallès et al., 2014; Vaishampayan and Kuehl, 2010), and failure to acquire early communities may contribute to cub mortality. However, further work detailing the microbiota at these early stages of life is necessary to fully elucidate this mechanism.

Interestingly, red pandas maintain a core community of eight OTUs throughout all stages of weaning, yet low abundance OTUs are shown to appear and disappear throughout sampling periods, as bacterial succession was observed during the red panda weaning process. Succession models describe a period of species loss due to competition, followed by an increase in community stability (Connel and Slatyer, 1977). After the introduction of solid feed, a total of fifteen OTUs appear during weaning (Stage 2) (Table 3.3). We also did not observe any period of stability, as eleven of the OTUs that appear in Stage 2 disappear by Stage 3 and are accompanied by the appearance of ten new OTUs (Table 3.3). These results indicate that the introduction to solid feeds causes a disturbance to the GIT microbiota and prevents the formation of a stabilized bacterial community.

Although the red panda cubs’ core community post-weaning does not completely resemble that of an adult, similar results have been observed in humans (Vallès et al., 2014), suggesting that succession may not have been completed during the final sampling period. Also, as more fibrous substrates are introduced, cellulolytic microorganisms are selected to occupy the gut and breakdown plant-based feeds, as observed by the appearance of a Ruminococcus species and a Clostridium species. Both of these genera are known for containing cellulolytic members, indicating that the weaning process may be selecting microbes capable of digesting the fibrous diet consumed by red pandas.
While the same core OTUs consistently dominated the red panda gut microbiota, the overall community structure changed over time as indicated by PCoA (Figure 3.2) and UniFrac analyses (Table 3.2). Differences in the total bacterial microbiota of red pandas across weaning were likely caused by non-significant changes in several taxa. For example, the Lactobacillales tended to increase across weaning (Figure 3.1) and remained at higher abundances than those found in adult captive red pandas (Kong et al., 2014). The Lactobacillales, especially lactic acid bacteria, are common members of the mammalian GIT (Reid and Burton, 2002; Salvetti et al., 2013) and serve dual purposes by fermenting numerous milk components (Makarova et al., 2006) into short-chain fatty acids that serve as an energy source for the host (Salvetti et al., 2013; Warnell et al., 1989) as well as helping to maintain active immunity following the loss of passive immunity acquired from colostrum (Malmuthuge et al., 2013). As the red pandas in our study were recently weaned, both roles likely contribute to the continued high abundance of Lactobacillales in the GIT of young red pandas, and we expect the levels of Lactobacillales to decrease as the animals age. Alternatively, this dissimilarity between captive North American cubs and Chinese adults (Kong et al., 2014) may be due to a number of differences in environment or diet as affected by geography or institution husbandry practices.

We found that bacteria in the order Clostridiales, which were dominated by the family Clostridiaceae, decreased in Stage 2 but then increased somewhat in Stage 3 (Figure 3.1), though not to the level seen in a previous study of Chinese adult red pandas (Kong et al., 2014). The Clostridiaceae have a wide range of metabolic abilities, including those that are proteolytic, saccharolytic, and cellulolytic members (Wiegel et
al., 2006) and have been reported previously in other pre-weaning herbivores (Koening et al., 2011; Rey et al., 2013; Fonty et al., 1987). We speculate that changes in the abundance of the Clostridiales in developing red pandas are due to changes in available dietary substrates with Stage 2 serving as a transition between the milk-degrading proteolytic and saccharolytic species and the bamboo-degrading cellulolytic species. Moreover, the overall lower abundance of these bacteria in cubs, relative to adult red pandas (Kong et al., 2014), may be due to the absence of fibrolytic species that have not yet colonized the gut of recently weaned cubs or may be due to geographical and environmental differences in Chinese red pandas and those housed in North American institutions.

Overall, our study demonstrates that the GIT bacterial community of developing red pandas was significantly different with respect to weaning stage, but no single taxonomic group was responsible for these differences. Specifically, the core GIT microbiota is established early in red pandas but changes in diet during weaning, and possibly physiological changes post-weaning, further shape the GIT community by driving the relative abundances of already present taxa. Our work also revealed a succession of microbes that appear based on weaning stage, and we propose that this is due to the shift in diet that occurs as these red pandas transition from a milk-based to plant-based diet. Due to the observed early establishment of core microbiota in two healthy red pandas used in our study, proper development and cub survival may rely on the initial colonization of these core microbial communities.
References


CHAPTER IV
THE RED PANDAS’ (AILURUS FULGENS) GASTROINTESTINAL TRACT
BACTERIAL COMPOSITION CHANGES SEASONALLY

Abstract

As a specialized herbivore, the diet of the red panda (Ailurus fulgens) consists almost exclusively of bamboo. Most herbivores exhibit a digestive tract with modifications and gastrointestinal (GIT) microbial populations to enable digestion of plant materials. However, red pandas lack the physical adaptations seen in most herbivores, but there is some evidence that a cellulolytic GIT microbiota may exist in red pandas. To maximize their diet, red pandas exhibit a feeding strategy by changing their bamboo preference. To gain insight into the seasonal effect on GIT bacterial communities, we examined twelve red pandas throughout four seasons using next-generation 16s rRNA sequencing. Across all seasons, we found a community with low diversity dominated by members of the Firmicutes and the Proteobacteria, with lesser contributions by the Actinobacteria. A core community of 193 OTUs was found, with only twenty-one OTUs found > 1.0 % relative abundance in any sample. Significant differences in overall community structure were seen using the UniFrac analyses (P<0.001), but no distinct clusters were observed via three-dimensional PCoA. However, significant differences with respect to season were also observed at various taxonomic levels. This work is the first analysis of seasonal variation in red panda GIT microbiota,
and provides the foundation in understanding how their changing diet affects the microbial communities and impacts the overall health of these vulnerable species.

**Introduction**

As an herbivorous carnivore, red pandas (*Ailurus fulgens*) are members of Carnivora but consume an almost exclusively herbivorous diet. Their diet is comprised primarily of bamboo leaves, a highly cellulose-rich plant material, and in the wild, 90% of red pandas diet consists of bamboo leaves (Wei et al., 1999a, Nijiboer and Dierenfeld, 2011; Yonzon and Hunter, 1989; Wei et al., 1999b; Reid, Hu, and Huang, 1991; Johnson, Schaller, and Hu, 1988). The red pandas bamboo diet leads to an interesting diet paradox; although their diet is herbivorous, their gastrointestinal tract (GIT) is structurally similar to other carnivores (Davis, 1964, Roberts and Gittleman, 1984; Schaller et al., 1985). Specifically, red pandas anatomical features are distinct from other herbivores, as they lack foregut and hindgut adaptations found in most herbivores. Red pandas do exhibit a reduced length of small intestine, an increased relative surface area of the large intestine, and a smaller liver (Davis, 1964). In particular, the GIT of herbivores is thought to promote the retention of microbes specialized for the breakdown and fermentation of plant biomass, and Kong et al.’s (2014) initial description of the red pandas GIT microbiota indicates some evidence of cellulosytic capabilities, which is fitting for their herbivorous diet.

Consuming the most nutritious feeds available, red pandas diet changes seasonally. During the spring, they supplement their diet with bamboo shoots, and in the summer, they consume fruits (Wei et al., 1999a, Wei et al., 1999b, Reid, Hu, and Huang, 1991; Johnson, Schaller, and Hu, 1988; Zhang et al., 2009). In captivity, red pandas are
fed a diet that mimics the wild diet in bamboo and fruit composition with the addition of a commercially prepared high-fiber biscuit that ensures a stable nutrient supply (Nijiboer and Dierenfeld, 2011). Also, nutritive values of bamboo change seasonally (Schaller et al., 1985; Christian et al., 2014), and these changes can be considered an influential factor in shifting microbial populations (Buddington and Sunvold, 1998; Collins and Gibson, 1999; Williams et al., 2012; Yokoyama et al., 1988). Diet shifts significantly affected the GIT microbiota of giant pandas (Ailuropoda melanoleuca) (Williams et al., 2012), but it is unknown how seasonal changes affect red pandas’ microbial populations. In this study, the gut microbiotas of twelve captive red pandas were tracked through the year using next-generation sequencing technology. Microbial communities were compared to determine the effect of season on the red panda GIT microbiome. These data provide the first characterization of how the seasonal variation impacts red panda GIT microbiota.

**Materials and Methods**

**Study Animals**

Animals used in this study were housed at the Memphis Zoological Society (n=3), Memphis, TN, USA and the Smithsonian Conservation Biology Institute (n=9). The Smithsonian National Zoological Park Institutional Animal Care and Use Committee (IACUC) granted approval for use of animals in this non-invasive study. Samples from the Memphis Zoo were collected under a signed biomaterials request form, and no IACUC was need as the project was viewed as non-invasive by the institution. Individuals involved in this study can be found in Table C.1.
Sample Collection

Fresh fecal samples (n = 3) were collected for each season from red pandas following defecation. All samples were flash-frozen in liquid N\textsubscript{2}, transported on dry ice, and stored at −80 °C prior to processing. Seasonal collection times occurred as follows: Fall (September 22-December, 20, 2013), Winter (December 21, 2013-March, 20, 2014), Spring (March 21-June 20, 2014), and Summer (June 21-September 21, 2014).

DNA Extraction

Total genomic DNA from fecal samples was extracted via mechanical disruption and hot/cold phenol extraction following Stevenson et al.’s protocol (2007) with the exception that 25:24:1 phenol:chloroform:isoamyl alcohol was used in place of phenol:chloroform at all steps. DNA was quantified using a Qubit Fluorometer (Invitrogen) and stored at −20 °C following extraction.

Library Preparation & Sequencing

16S rRNA amplification and sequence indexing

Library preparation was carried out following manufacturers recommendations (Illumina, 2013). In brief, an amplicon PCR targeted the 16S rRNA gene (V3-V4) using a forward (V3-4F, TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGN GGCGW GCAG) and reverse (V3-4R, GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACAGG ACTACHVGGGTATCTAATCC) primer in a 25-μL reaction with 1X KAPA HiFi Hot Start Ready Mix (Kapa Biosystems), 0.2 mM each primer, and 1-10 ng DNA. Amplification conditions were as follows: 95 °C for 3 min, 25 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final elongation of 72 °C for 5 min. PCR products
were purified via gel extraction (Zymo Gel DNA Recovery Kit; Zymo, Irvine, CA) using a 1 % low melt agarose gel (National Diagnostics, Atlanta, GA). Purified products underwent an indexing 25μL-PCR reaction (1x KAPA HiFi Hot Start Ready Mix, 0.2mM indices, and 5μL of purified product) with the same reaction conditions as amplicon PCR except reduction of cycles to 8.

Library quantification and sequencing

The final index PCR product underwent gel extraction (Zymo Gel DNA Recovery Kit; Zymo, Irvine, CA), and the final purified product concentration was determined by Qubit. Samples were pooled yielding an equimolar 4 nM pool. Following manufacturer’s protocol, sequencing was conducted on an Illumina MiSeq reagent kit V3 (600 cycles), as described previously (Illumina, 2013).

Data Analysis

Sequence analysis

Sequence analysis was carried out using the program Mothur v.1.31.2 following the MiSeq SOP (Kozich et al., 2013). In brief, contigs were formed from 16s rRNA reads, and poor quality sequences were removed. Sequences were trimmed and filtered based on quality (maxambig=0, minlength=250, maxlength=600). Unique sequences were determined and aligned against the SILVA 16S rRNA gene alignment database (Pruesse et al., 2007). Chimeras (chimera.uchime) and sequences identified as members of Eukaryota, Archaea, and Cyanobacteria lineages were removed. Sequences were classified using the SILVA database with a bootstrap value cutoff of 80. The following analyses were carried out in Mothur: Good’s coverage (Good, 1953), Morista-Horn index
(Horn, 1966), Principal Coordinate Analysis (PCoA) (Gower, 1966), Berger-Parker diversity metric (Magurran, 1988), Simpson diversity metric (Simpson, 1949), Shannon diversity metric (Pielou, 1975), and UniFrac (Lozupone et al., 2007).

**Statistical Analysis.**

All statistical analyses were carried out in SAS 9.3 software (Cary, NC) and data are expressed as the mean ± SE and considered significant if P < 0.05. To determine which taxonomic groups are important in defining microbial communities, Step-wise analysis was used (PROC STEPDISC). Following stepwise analysis, groups found to be significant were included in the discriminant analysis (PROC DISCRIM) to determine factors important for proper classification of seasonal samples.

**Results**

**Sequence coverage and diversity metrics**

Overall, we generated a total of 4,672,674 (2,793,845 high-quality) 16S rRNA sequences. A Good's coverage value of >0.99 (Table 4.1) combined with leveling of rarefaction curves (Figure C.1) indicated that the majority of bacterial diversity was detected in all samples via sequencing. Using a 97 % operational taxonomic unit (OTU) analysis corresponding to genus-level classification (Schloss and Handelsman, 2005), we identified 2176 unique OTUs in total, with only 193 OTUs found >1.0 % relative abundance in any sample (Table 4.1).

The sample diversity was very low overall, as all seasons had low diversity (Berger-Parker: 0.53 ± 0.022, Shannon: 1.8 ± 0.095, inverse-Simpson: 5.7 ±0.69). Berger-Parker diversity index values ranging from 0.44± 0.041 for the winter season and
0.61 ± 0.038 for the spring season. Winter samples had the highest Shannon and inverse-Simpson diversity indices (2.2 ± 0.19 and 8.0 ± 1.5, respectively), and the spring season samples had the lowest Shannon and inverse-Simpson (1.3 ± 0.13 and 3.1 ± 0.58, respectively) (Table 4.1).

### Table 4.1   Number of sequences, estimated coverage, diversity and OTU richness.

<table>
<thead>
<tr>
<th>Sample</th>
<th>16S rRNA reads</th>
<th>Community Richness</th>
<th>Community Diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>High-quality</td>
<td>Berger-Parker</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Number of unique OTUs</td>
<td>Good's coverage</td>
</tr>
<tr>
<td>Total</td>
<td>4,672,674</td>
<td>0.53 ± 0.022</td>
<td>1.8 ± 0.095</td>
</tr>
<tr>
<td>Summer (n=29)</td>
<td>958,330</td>
<td>0.55 ± 0.049</td>
<td>1.7 ± 0.20</td>
</tr>
<tr>
<td>Fall (n=26)</td>
<td>946,525</td>
<td>0.50 ± 0.47</td>
<td>1.8 ±0.20</td>
</tr>
<tr>
<td>Winter (n=35)</td>
<td>1,416,423</td>
<td>0.44 ± 0.041</td>
<td>2.2 ± 0.19</td>
</tr>
<tr>
<td>Spring (n=35)</td>
<td>1,351,396</td>
<td>0.61 ± 0.038</td>
<td>1.3 ± 0.13</td>
</tr>
</tbody>
</table>

Mean values and standard error given. OTUs present represent those found > 1.0% in any sample.

Twenty-eight phyla were observed in red panda fecal samples, with only eight Phyla represented in greater than >1.0 % in any sample, including the Firmicutes (55 ± 2.7 %), the Proteobacteria (32 ± 2.5 %), the Actinobacteria (6.3 ± 1.0 %), the Bacteroidetes (2.8 ± 0.53 %), the Chloroflexi (0.64 ± 1.0 %), the Fusobacteria (0.34 ± 0.20 %), the TM7 (0.18 ± 0.040 %), and the Planctomycetes (0.040 ± 0.022 %). Overall, there were seventy classes represented, with only nineteen classes found in > 1.0 % relative abundance in any sample, including the Gammaproteobacteria (28 ± 2.5 %), the Clostridia (24 ± 2.7 %), the Bacilli (23 ± 2.3 %), the Erysipelotrichia (11 ± 1.8 %), the Actinobacteria (5.9 ± 0.95 %), the Alphaproteobacteria (2.1 ± 0.41 %), the Epsilonproteobacteria 2.0 ± 0.71 %), the Flavobacteriia (1.2± 0.36 %), the Sphingobacteriia (1.1 ± 0.23 %), the Thermomicrobia (0.63 ± 0.18 %), the Betaproteobacteria (0.55 ± 0.15 %), the Bacteroidia (0.53 ± 0.29 %), the Fusobacteriia
(0.34 ± 0.20 %), the Acidimicrobiia (0.23 ± 0.15 %), an unclassified TM7 (0.18 ± 0.040 %), the Thermoleophiila (0.13 ± 0.077 %), the Planctomycetacia (0.040 ± 0.022 %), the Negativicutes (0.036 ± 0.013 %), and the Cytophagia (0.029 ± 0.015 %).

Figure 4.1  Relative sequence abundance at the order taxonomic level for each season.

A total of 131 orders were found in all samples; however, there were forty orders found in >1.0 % in any sample, but only ten were found in >1.0 % in all samples, including the Clostridiales (23 ± 2.7 %), the Lactobacillales (20 ± 2.3 %), the Enterobacteriales (19 ± 2.4 %), Erysipelotrichales (12 ± 1.9 %), the Pseudomonadales (7.4 ± 1.1 %), the Micrococcales (4.7 ± 0.84%), the Bacillales (2.5 ± 0.56 %), the Campylobacterales (1.9 ± 0.70 %), the Flavobacteriales (1.2 ± 0.36 %), the
Sphingobacteriales (1.1 ± 0.23 %) (Figure 4.1). At the family and genus level, 98 % and 77 % of sequences were successfully annotated, respectively.

**Red pandas have a shared core community**

Samples from all seasons were analyzed together to determine if there were specific OTUs common to red pandas throughout the seasons. In total, we found a total of 159 OTUs shared by the four seasons, with only twenty-one OTUs represented by > 1.0 % relative abundance in any sample (Figure 4.2, Table 4.2). Six OTUs were found in high sequence abundance including a member of the *Escherichia-Shigella* genus (19 ± 2.4 %), a *Streptococcus* species (12 ± 2.2 %), a *Turicibacter* species (9.4 ± 1.8 %), an unclassified member of the Clostridiaceae family (7.8 ± 4.1 %), a *Sarcina* species (5.7 ± 1.5 %), and a member of the *Psychrobacter* genus (5.3 ± 0.94 %) (Table 4.2).

Other OTUs found in lower abundance were classified as two members of the *Enterococcus* genus (3.1 ± 0.93 % and 0.68 ± 0.29 %), two unclassified members of the Clostridiaceae family (4.4 ± 1.2 % and 1.6 ± 0.66 %), two *Clostridium* species (3.2 ± 0.73 % and 0.59 ± 0.26 %), a member of the *Helicobacter* genus (2.0 ± 0.71 %), an unclassified member of the Peptostreptococcaceae family (1.5 ± 0.51 %), a member of the *Brachybacterium* genus (1.4 ± 0.60 %), an *Arthrobacter* species (0.95 ± 0.20 %), a member of the *Eremococcus* genus (0.82 ± 0.23 %), an *Atopostipes* species (0.51 ± 0.13 %), a *Trichococcus* species (0.41 ± 0.11 %), a member of the *Cetobacterium* genus(0.26 ± 0.17 %), and an unclassified member of the Nocardioidaceae family (0.18 ± 0.048 %).

In total, these shared OTUs accounted for 80 ± 4.6 % of sequences within each sample. Of these twenty-one OTUs, two were found to be important in determining seasonal sample, including the *Escherichia-Shigella* species (OTU 01) and the *Streptococcus*
species (OTU 03) (Figure C.1). Both OTUs decreased from summer to fall sampling.

The *Escherichia-Shigella* species continued to decrease through the winter sampling and increased in the spring, but the *Streptococcus* species increased through the winter and spring sampling (Figure S2).

![Venn diagram showing the number of OTUs shared between the four seasons](image)

**Figure 4.2** A comparison of the number of OTUs shared between the four seasons.

Notes: OTUs were defined at the 97% similarity level corresponding to the genus taxonomic level.
Table 4.2  Relative abundance of shared OTUs found at >1.0 %.

<table>
<thead>
<tr>
<th>OTUs</th>
<th>Average</th>
<th>Summer</th>
<th>Fall</th>
<th>Winter</th>
<th>Spring</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Actinobacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU 10 Arthrobacter</td>
<td>0.95±0.20</td>
<td>0.85±0.51</td>
<td>1.3±0.26</td>
<td>1.4±0.32</td>
<td>0.28±0.18</td>
</tr>
<tr>
<td>OTU 12 Brachybacterium</td>
<td>1.4±0.60</td>
<td>0.67±0.45</td>
<td>1.5±0.30</td>
<td>1.2±0.38</td>
<td>2.2±2.0</td>
</tr>
<tr>
<td>OTU 40 Nocardioidaceae-unclassified</td>
<td>0.18±0.048</td>
<td>0.22±0.12</td>
<td>0.19±0.037</td>
<td>0.26±0.12</td>
<td>0.057±0.039</td>
</tr>
<tr>
<td><strong>Firmicutes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU 02 Turicibacter</td>
<td>9.4±1.8</td>
<td>4.2±2.1</td>
<td>11±2.2</td>
<td>11±3.8</td>
<td>11±4.0</td>
</tr>
<tr>
<td>OTU 04 Clostridiaceae-unclassified</td>
<td>7.8±4.1</td>
<td>0.36±0.14</td>
<td>6.0±1.2</td>
<td>2.2±1.1</td>
<td>21±15</td>
</tr>
<tr>
<td>OTU 05 Sarcina</td>
<td>5.7±1.5</td>
<td>0.86±0.56</td>
<td>3.4±0.66</td>
<td>10±3.6</td>
<td>6.7±3.7</td>
</tr>
<tr>
<td>OTU 08 Clostridiaceae-unclassified</td>
<td>4.4±1.2</td>
<td>3.3±2.5</td>
<td>4.6±0.91</td>
<td>8.6±3.0</td>
<td>0.92±0.50</td>
</tr>
<tr>
<td>OTU 09 Clostridium</td>
<td>3.2±0.73</td>
<td>3.0±1.5</td>
<td>4.2±0.82</td>
<td>3.2±1.4</td>
<td>2.6±0.79</td>
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<tr>
<td>OTU 07 Enterococcus</td>
<td>3.1±0.93</td>
<td>5.7±2.9</td>
<td>5.4±1.1</td>
<td>1.6±0.81</td>
<td>0.84±0.46</td>
</tr>
<tr>
<td>OTU 03 Streptococcus</td>
<td>12±2.2</td>
<td>16±4.3</td>
<td>1.7±0.33</td>
<td>5.8±2.8</td>
<td>24±5.7</td>
</tr>
<tr>
<td>OTU 16 Clostridiaceae-unclassified</td>
<td>1.6±0.66</td>
<td>1.4±0.74</td>
<td>0.16±0.032</td>
<td>4.0±2.2</td>
<td>0.43±0.29</td>
</tr>
<tr>
<td>Peptostreptococcaceae-unclassified</td>
<td>1.5±0.51</td>
<td>0.42±0.22</td>
<td>0.64±0.13</td>
<td>1.5±0.47</td>
<td>3.0±1.7</td>
</tr>
<tr>
<td>OTU 13 Eremococcus</td>
<td>0.82±0.23</td>
<td>1.5±0.67</td>
<td>1.3±0.25</td>
<td>0.26±0.10</td>
<td>0.44±0.19</td>
</tr>
<tr>
<td>OTU 15 Enterococcus</td>
<td>0.68±0.29</td>
<td>1.1±0.52</td>
<td>1.3±0.26</td>
<td>0.35±0.18</td>
<td>0.17±0.068</td>
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<tr>
<td>OTU 14 Atopostipes</td>
<td>0.51±0.13</td>
<td>0.58±0.29</td>
<td>0.27±0.053</td>
<td>0.59±0.27</td>
<td>0.55±0.30</td>
</tr>
<tr>
<td>OTU 46 Clostridium</td>
<td>0.59±0.26</td>
<td>0.19±0.090</td>
<td>1.0±0.20</td>
<td>0.37±0.25</td>
<td>0.80±0.68</td>
</tr>
<tr>
<td><strong>Fusobacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU 30 Cetobacterium</td>
<td>0.26±0.17</td>
<td>0.088±0.072</td>
<td>0.092±0.018</td>
<td>0.25±0.25</td>
<td>0.54±0.54</td>
</tr>
<tr>
<td><strong>Proteobacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU 01 Escherichia-Shigella</td>
<td>19±2.4</td>
<td>34±6.2</td>
<td>24±4.7</td>
<td>5.9±2.6</td>
<td>15±3.6</td>
</tr>
<tr>
<td>OTU 06 Psychrobacter</td>
<td>5.3±0.94</td>
<td>2.7±1.4</td>
<td>6.0±1.2</td>
<td>7.0±2.4</td>
<td>5.4±1.6</td>
</tr>
<tr>
<td>OTU 11 Helicobacter</td>
<td>2.0±0.71</td>
<td>0.086±0.059</td>
<td>3.6±0.71</td>
<td>3.9±1.7</td>
<td>0.54±0.38</td>
</tr>
</tbody>
</table>

Mean values with standard error given.

**Seasonal effects on gut microbiota**

To determine if red panda GIT microbiota differed across seasons, we performed a weighted and unweighted UniFrac comparisons and found the seasons differed significantly from each other (P<0.001). We also conducted three-dimensional principal coordinate analyses (PCoA) using the weighted and unweighted UniFrac metric, yet no season clustered separately. However, when individual profiles were examined, seasonal variation was observed (Figure C.2-15). Significant differences were also observed at various taxonomic levels. At the phyla level, we found that the Firmicutes and the Actinobacteria differed significantly over the seasons (Table 4.3; Figure C.2). From initial sampling in the summer, the Firmicutes decreased into the fall sampling, and then...
increased, displaying an increase in mean relative abundance through the winter and spring sampling periods (Figure C.2). The Actinobacteria displayed the inverse relationship, as mean relative abundance increased through the fall and winter, and then decreased during the spring sampling.

Table 4.3 Rankings of significantly important taxonomic groups.

<table>
<thead>
<tr>
<th>Ranking</th>
<th>Group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phyla</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Firmicutes</td>
<td>0.0021</td>
</tr>
<tr>
<td>2</td>
<td>Actinobacteria</td>
<td>0.0110</td>
</tr>
<tr>
<td>Class</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Gammaproteobacteria</td>
<td>0.0004</td>
</tr>
<tr>
<td>2</td>
<td>Sphingobacteriia</td>
<td>0.0063</td>
</tr>
<tr>
<td>3</td>
<td>Bacilli</td>
<td>0.0321</td>
</tr>
<tr>
<td>Order</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Enterobacteriales</td>
<td>0.0001</td>
</tr>
<tr>
<td>2</td>
<td>Lactobacillales</td>
<td>0.0024</td>
</tr>
<tr>
<td>3</td>
<td>Sphingobacteriales</td>
<td>0.0100</td>
</tr>
<tr>
<td>4</td>
<td>Bacilli-unclassified</td>
<td>0.0221</td>
</tr>
<tr>
<td>5</td>
<td>NKB5</td>
<td>0.0300</td>
</tr>
<tr>
<td>Family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Micrococcales-unclassified</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>2</td>
<td>Enterobacteriaceae</td>
<td>0.0004</td>
</tr>
<tr>
<td>3</td>
<td>Propionibacteriaceae</td>
<td>0.0005</td>
</tr>
<tr>
<td>4</td>
<td>Sphingobacteriaceae</td>
<td>0.0077</td>
</tr>
<tr>
<td>5</td>
<td>Lactobacillales-unclassified</td>
<td>0.0446</td>
</tr>
<tr>
<td>6</td>
<td>Streptococcaceae</td>
<td>0.0377</td>
</tr>
<tr>
<td>7</td>
<td>Carnobacteriaceae</td>
<td>0.0390</td>
</tr>
<tr>
<td>OTU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td><em>Escherichia-Shigella</em> (OTU 01)</td>
<td>0.0025</td>
</tr>
<tr>
<td>2</td>
<td><em>Streptococcus</em> (OTU 03)</td>
<td>0.0066</td>
</tr>
</tbody>
</table>

At the class level, the Gammaproteobacteria, the Bacilli, and the Sphingobacteriia were found significantly different with respect to season (Table 4.3). The mean relative abundance of the Gammaproteobacteria decreased from summer to winter and increased
during the spring sampling (Figure 4.4). The Bacilli displayed a decreased in mean relative abundance from summer to fall but increased during the winter and spring seasons (Figure 4.4). Inversely, the Sphingobacteriia decreased in mean relative abundance from the summer to winter seasons but increased in the spring (Figure 3).

![Figure 4.3](image)

Figure 4.3 Three-dimensional principal coordinate analysis of microbial diversity using the unweighted UniFrac metric for all seasons.

Notes: The percentage of variation is explained by PC1, PC2, and PC3 are indicated on the axes (total: 13.01 %).

Five orders were also found significantly different (Table 4.3) including the Lactobacillales, Enterobacteriales, Sphingobacteriales, and unclassified-Bacilli, and the NKB5 (Figure C.3). Seven families, including an unclassified Micrococcales, the
Enterobacteriaceae, the Propioibacteriaceae, the Sphingobacteriaceae, an unclassified Lactobacillales, the Streptococcaceae, and then Carnobacteriaceae, were also found significantly different with respect to season (Table 4.3; Figure C.4).

![Graph showing percent relative abundance of significantly important classes across all seasons.]

Figure 4.4  Percent relative abundance of significantly important classes across all seasons.

**Discussion**

The homeostatic relationship between host and bacterial communities within the GIT is important to host survival as the microbiota play a major role not only in GIT function, but also in immune function and energy metabolism (McFall-Ngai, 2013; Bergman, 1990). Several factors have been shown to shape the GIT microbiota of mammals, such as taxonomy, diet, host genotype, and seasonality (Ley et al., 2008; Amato et al., 2014; Scott et al., 2013; Nicholson et al., 2012; Bolnick et al., 2014;
Williams et al., 2012; Khachatryan et al., 2008; Davenport et al., 2014; Bailey et al., 2010). In our study, we examined twelve red pandas throughout all four seasons to determine any changes in GIT microbiota associated with season. Overall, we found the bacterial community of red pandas to be dominated by the Firmicutes and the Proteobacteria regardless of season, with lesser contributions by the Actinobacteria. These findings are similar to those previously described Kong et al., (2014). Like the red panda, the giant panda (Ailuropoda melanoleuca) is an obligate bamboo forager (Schaller et al., 1985), and significant differences in their GIT microbiota have been observed with respect to seasonal changes in part preference (Williams et al., 2012).

In our study of red pandas, we found several taxonomic groups to vary with respect to season. In particular, we found season affected the Firmicutes and the Actinobacteria significantly, with both phyla displaying high mean relative abundance in the winter season (Figure C.2). Similar results were also observed in a study of humans, and like our results, the winter season also had the highest level of diversity (Table 4.1) (Davenport et al., 2014). In the Siberian hamster (Phodopus sungorus), relative abundance of the Firmicutes and the Proteobacteria varied significantly with respect to photoperiod (Bailey, 2010). Like red pandas, the hamster also had significantly lower relative abundance of the Firmicutes in the summer season (Table 4.3), and although not significant in red pandas, both the hamster and red panda displayed higher relative abundance of the Proteobacteria in the summer season. In giant pandas, the Streptococcus and the Lactobacillus genera changed significantly over time (Williams et al., 2012), and in red pandas, we found the Streptococcaceae family and the
Lactobacillales order also varied significantly with respect to season (Table 4.3; Figure C.4).

Analyzing all seasons together, we found a core community represented by twenty-one OTUs. This core community comprised only 80 ± 4.6 % of the total sequences found in red pandas regardless of season, with season-specific OTUs ranging from 16-29 (Table 4.1; Figure 4.2), and this value is far less than observed in a previous study of red pandas. Using step-wise analysis, we found that two members of the core community, an Escherichia-Shigella species (OTU 01) and a Streptococcus species (OTU 03) did change significantly overtime (Table 2; Table 3). The Escherichia-Shigella species (OTU 01) dominated the bacterial community of the summer and fall seasons, but was reduced in the winter and spring, and the Streptococcus species (OTU 03) dominated the winter season (Figure C.1; Table 4.3).

We found significant differences in GIT microbiota using UniFrac analyses and at various taxonomic levels (Table 4.3). However, we did not observe any distinct seasonal clustering with three-dimensional principal coordinate analysis (Figure 4.3). The lack of distinct clustering may be due to the stability of the red panda diet, as they primarily only consume the leaf portion year round. Wei et al. (1999) found the overall gross energy of leaves consumed by red pandas to not change significantly throughout the year, yet digestibility varied significantly. Specifically, red pandas had the highest digestive efficiency in the summer and fall months and the lowest in the winter (Wei et al., 1999). Interestingly, the season with the highest efficiency corresponds to periods of highest lignin accumulation in bamboo leaves (Schaller et al., 1985), and, therefore, we would have expected lower digestibility. Silica, an indigestible compound, is also found in
higher concentrations in bamboo leaves in the summer and winter seasons (Schaller et al., 1985). Silica negatively affects coliforms (Namkung et al., 2004), and its higher presence in the winter months may correlate to the significantly lower levels of the *Escherichia-Shigella* species and the Enterobacteriaceae family (Figures C.1; Figure C.4; Table 3.3). Indigestible substrates, such as silica and lignin, can impact bacterial fermentation within the GIT, and can subsequently alter bacterial communities and digestive efficiency (Gibson and MacFarlane, 1995).

High levels of coliforms, such as the *Escherichia* genus and members of the Enterobacteriaceae family, both found in relative high abundance in red pandas, have been associated with increased levels of intestinal pathogens (Lupp et al., 2007; Stecher et al., 2007), and the Enterobacteriaceae, in particular, have been observed creating an environment suitable for pathogen colonization, as the presence of the enteric pathogen takes advantage of host inflammation and reduces commensal organisms (Pedron and Sansonetti, 2008; Stecher and Hardt, 2008). As red pandas are prone to bacterial overgrowth (Loeffler, 2011), these findings indicate that seasonal changes in GIT microbiota could alter the host-microbiome symbiotic relationship into dysbiosis, leading to the overgrowth observed in red pandas.

Although we did not find distinct seasonal differences in bacterial populations using PCoA, we did find significant differences in with respect to season overall using UniFrac analyses and at differing taxonomic levels. These data, combined with differences of season-specific OTUs indicate that red panda GIT microbiota do exhibit seasonal shifts, and these shifts are possibly due to changes in nutritive levels within their bamboo diet. As a vulnerable species, this work allows for a better understanding of how
their diet affects the GIT microbiota, and subsequently the health of the red panda, laying the foundation for further inquiries into this unique species.
References


Gibson G, and MacFarlane GT. 1995. Human Colonic Bacteria: Role in nutrition, physiology, and pathology. CRC Press, Boca Raton, USA.


CHAPTER V

16S rRNA SEQUENCING UNCOVERS DISTINCT GUT MICROBIOTA OF OBLIGATE BAMBOO FORAGERS, THE GIANT PANDA (*Ailuropoda melanoleuca*) AND THE RED PANDA (*Ailurus fulgens*)

Abstract

Both giant pandas (*Ailuropoda melanoleuca*) and red pandas (*Ailurus fulgens*) are taxonomically classified and physiologically similar to a carnivore but consume almost an exclusively bamboo diet. Pandas have evolved to their bamboo diet through the development of a pseudo-thumb, an enhanced jaw structure, and a unique diet selection behavior. However, neither panda has adapted to a more herbivore-like gastrointestinal tract (GIT). Within a typical herbivores’ GIT, microbiota play a major role in the digestion of its plant-based diet as well as host health and immunity. Therefore, the GIT microbiome of these uniquely herbivorous carnivores is of interest. Here, we examine the GIT bacterial microbiota of two adult giant pandas and two adult red pandas using next-generation 16S rRNA sequencing of fecal samples. On average, the fecal communities of both giant and red pandas were dominated by the phylum Firmicutes (98 ± 0.51 %) with lesser contributions by the Proteobacteria (1.2 ± 0.48 %). However, distinct clusters in panda GIT microbial communities were found using both the weighted and unweighted UniFrac metric, three-dimensional principal coordinate analysis and
nonmetric dimensional scaling, indicating that microbial communities differ with respect to panda species. This study is the first to compare the two species, and lays the foundation for further studies to reveal the effect that shapes GIT microbiota in pandas.

**Introduction**

Both giant pandas (*Ailuropoda melanoleuca*) and red pandas (*Ailurus fulgens*) are taxonomically classified and physiologically similar to a carnivore, but as obligate bamboo foragers, they consume almost an exclusively bamboo diet. In addition, giant and red pandas are distantly related phylogenetically (O’Brien et al., 1985; Flynn et al., 2000; Yu et al., 2004; Yu et al., 2011), and have both evolved adaptations to their highly fibrous diet independently of each other (Groves, 2011).

Adaptations to their bamboo diet include the extension of their radial sesamoid, creating a pseudo-thumb for foraging strategy improvement (Delisle and Strobeck 2005; Fulton and Strobeck, 2006; Salesa et al., 2006; Endo et al., 1996), an enhanced jaw structure for bamboo mastication (Christiansen, 2008; Christiansen and Wroe, 2007), and a unique diet selection behavior to ensure higher quality diet (Schaller et al., 1985; Hansen et al., 2010; Tarou et al., 2006). However, neither panda has adapted to a more herbivore-like gastrointestinal tract (GIT), with both pandas exhibiting a GIT similar to a carnivore with a simple stomach and no cecum, yet they do exhibit a reduced length of small intestine, an increased relative surface area of the large intestine, and a smaller liver (Davis, 1964).

Within a typical herbivores’ GIT, microbiota play a major role in the digestion of its plant-based diet as well as host health and immunity (Flint et al., 2012; Hooper et al., 2002). What is unknown is if the pandas’ similarities extend to their GIT microbiota.
Previous research to establish the driving factor in gut microbial communities indicate that individuals are more likely to resemble animals they are closely related to (Ley et al., 2008). However, diet also drives GIT microbial communities (Amato et al., 2014; Scott et al., 2013). Here, we examine the GIT bacterial microbiota of two adult giant pandas and two adult red pandas using next-generation 16S rRNA sequencing.

**Materials and Methods**

**Study Animals**

Animals in this study were housed at the Memphis Zoological Society, Memphis, TN, USA. Samples were collected under a signed biomaterials request form, and no IACUC was need as the project was viewed as non-invasive by the institution. Individuals were two giant pandas, an adult female, “YaYa,” (studbook number: 507), an adult male, “LeLe,” (studbook number 466), two red pandas, an adult female, “Lucille,” (studbook number: 1215), and an adult male, “Justin,” (studbook number: 1219). Giant pandas diet consisted of bamboo leaf and culm portions, and red panda diet consisted of leaf eater diet, bamboo leaf, and applesauce (Musselmans).

**Sample Collection**

Fresh fecal samples for each giant panda (n=5) and red panda (n = 3) were collected. All samples were flash-frozen in liquid N₂, transported on dry ice, and stored at −80°C prior to processing.

**DNA Extraction**

Total genomic DNA from fecal samples was extracted via mechanical disruption and hot/cold phenol extraction following Stevenson et al.’s protocol (2007) with the
exception that 25:24:1 phenol:chloroform:isoamyl alcohol was used in place of phenol:chloroform at all steps. DNA was quantified using a Qubit Fluorometer (Invitrogen) and stored at −20°C following extraction.

**Library Preparation & Sequencing**

*16S rRNA amplification and sequence indexing*

Library preparation was carried out following manufacturers recommendations (Illumina, 2013). In brief, an amplicon PCR targeted the 16S rRNA gene (V3-V4) using a forward (V3-4F, TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGN GGCW GCAG) and reverse (V3-4R, GTCTCGTGGGCTCGGAGATGTGTATAAGACAGG ACTACHVGGGTATCTAATCC) primer in a 25-μL reaction with 1X KAPA HiFi Hot Start Ready Mix (Kapa Biosystems), 0.2 mM each primer, and 1-10 ng DNA. Amplification conditions were as follows: 95 °C for 3 min, 25 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final elongation of 72 °C for 5 min. PCR products were purified via gel extraction (Zymo Gel DNA Recovery Kit; Zymo, Irvine, CA) using a 1 % low melt agarose gel (National Diagnostics, Atlanta, GA). Purified products underwent an indexing 25μL-PCR reaction (1x KAPA HiFi Hot Start Ready Mix, 0.2mM indices, and 5μL of purified product) with the same reaction conditions as amplicon PCR except reduction of cycles to 8.

*Library quantification and sequencing*

The final index PCR product underwent gel extraction (Zymo Gel DNA Recovery Kit; Zymo, Irvine, CA), and the final purified product concentration was determined by Qubit Fluorometer (Invitrogen). Samples were pooled yielding an equimolar 4 nM pool.
Following manufacturer’s protocol, sequencing was conducted on an Illumina MiSeq reagent kit V3 (600 cycles), as described previously (Illumina, 2013).

**Data Analysis**

*Sequence analysis*

Sequence analysis was carried out using the program Mothur v.1.31.2 following the MiSeq SOP (Kozich et al., 2013). In brief, contigs were formed from 16s rRNA reads, and poor quality sequences were removed. Sequences were trimmed and filtered based on quality (maxambig=0, minlength=250, maxlength=600). Unique sequences were determined and aligned against the SILVA 16S rRNA gene alignment database (Pruesse et al., 2007). Chimeras (chimera.uchime) and sequences identified as members of Eukaryota, Archaea, and Cyanobacteria lineages were removed. Sequences were classified using the SILVA database with a bootstrap value cutoff of 80. The following analyses were carried out in Mothur: Good’s coverage (Good, 1953), Morista-Horn index (Horn, 1966), Principal Coordinate Analysis (PCoA) (Gower, 1966), Berger-Parker diversity metric (Magurran, 1988), Simpson diversity metric (Simpson, 1949), Shannon diversity metric (Pielou, 1975), and UniFrac (Lozupone et al., 2007).

**Statistical Analysis.**

All statistical analyses were carried out in SAS 9.3 software (Cary, NC) and data are expressed as the mean ± SE and considered significant if P < 0.05. To determine which taxonomic groups are important in defining microbial communities, Step-wise analysis was used (PROC STEPDISC). Following stepwise analysis, groups found to be
significant were included in the discriminant analysis (PROC DISCRIM) to determine classifications based on taxonomic groups.

Results

Sequence Coverage and Diversity Metrics

Using Illumina paired-end sequencing, we generated a total of 536,427 (315,460 high-quality) 16S rRNA sequences (Table 5.1). A Good’s coverage value of > 0.99 (Table 5.1) and a leveling off of rarefaction curves indicated that sequencing was adequate to detect the majority of sequences in our samples (Figure D.1). A 97 % operational taxonomic unit (OTU) analysis corresponding to genus level classification (Schloss and Handelsman, 2005) identified 432 OTUs across all samples, with 232 and 282 OTUs found within the giant and red panda, respectively (Table 5.1) (Figure 5.1).

<table>
<thead>
<tr>
<th>Sample</th>
<th>16S rRNA reads</th>
<th>Community Richness</th>
<th>Community Diversity</th>
<th>Number of OTUs</th>
<th>Good’s coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>High-quality</td>
<td>Berger-Parker</td>
<td>Shannon</td>
<td>Inverse-Simpson</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>536,427</td>
<td>315,460</td>
<td>0.57 ± 0.024</td>
<td>1.3 ± 0.070</td>
<td>2.4 ±0.10</td>
</tr>
<tr>
<td>Giant panda (n=10)</td>
<td>374,668</td>
<td>194,566</td>
<td>0.57 ± 0.035</td>
<td>1.2 ±0.053</td>
<td>2.3 ± 0.12</td>
</tr>
<tr>
<td>Red panda (n=6)</td>
<td>161,759</td>
<td>120,894</td>
<td>0.57 ± 0.027</td>
<td>1.4 ±0.16</td>
<td>2.6 ± 0.17</td>
</tr>
</tbody>
</table>

Mean values with standard error given.

All samples displayed low diversity, with both giant and red pandas’ GIT microbiota being dominated by a single OTU (Berger-Parker: 0.57 ± 0.035 and 0.57 ± 0.027, respectively) (Table 5.1). Similar values were also observed via the Shannon diversity index (1.2 ± 0.053 and 1.4 ± 0.016, respectively) and the inverse-Simpson diversity index (2.3 ± 0.12 and 2.6 ± 0.17, respectively) (Table 5.1).
Figure 5.1  A comparison of the number of OTUs shared between giant and red pandas.

Notes: OTUs were defined at the 97% similarity level corresponding to the genus taxonomic level.

From all samples sequenced, ten phyla were observed with 98 ± 0.51 % represented by the Firmicutes, lesser contributions by the Proteobacteria (1.2 ± 0.48), and all other phyla were found at less than 1.0 % relative abundance. At the class level, only five were found at > 1.0 % relative abundance in any sample including the Clostridia (70 ± 5.7 %), the Erysipelotrichia (24 ± 5.2 %), the Bacilli (5.0 ± 2.0 %), the Gammaproteobacteria (1.1 ± 0.49 %), and the Actinobacteria (0.34 ± 0.20 %). Bacterial orders, including the Clostridiales (70 ± 5.8 %), the Erysipelotrichales (24 ± 5.2 %), Lactobacillales (4.9 ± 1.9 %), the Pasteurellales (0.66 ± 0.48 %), the Enterobacteriales (0.35 ± 0.11 %), and the Micrococcales (0.25 ± 0.15 %), were the only orders found in > 1.0 % relative abundance. At the family and genus level, 99.8 % and 68 % were successfully annotated, respectively (Figure 5.2). Three OTUs were found > 1.0 % in any sample only in giant pandas including an unclassified Pasteurellaceae (1.0 ± 0.72 %),
an unclassified Clostridiaceae (0.66 ± 0.12%), and a Streptococcus species (0.41 ± 0.19 %). Two OTUs were found only in red pandas >1.0 % were a Jeotgalicoccus species (0.29 ± 0.29 %) and a member of the Lactococcus genus (0.27 ± 0.19 %).

Figure 5.2 Relative abundance of the taxonomic order
a) family and b) genus for both giant and red pandas.

**Shared Core Community across Pandas**

Analyzing giant and red panda samples together, we determined that they share eighty-two OTUs (Figure 5.1). Of these, only twelve OTUs were found in > 1.0 % relative abundance in any sample (Table 5.2). Eight OTUs were found in high relative abundance, including a member of the *Clostridium* genus (35 ± 7.4 %), a *Turicibacter* species (23 ± 5.1 %), three unclassified Clostridiaceae OTUs (14 ± 6.0 %, 4.4 ± 2.4 %, and 8.3 ± 4.8 %), a *Sarcina* species (3.2 ± 1.7 %), a *Streptococcus* species (3.4 ± 1.9 %). Other OTUs found in lower abundance were two unclassified members of the *Pastuerellaceae* family (0.62 ± 0.46 % and 0.31 ± 0.18 %), two *Streptococcus* species
(0.11 ± 0.068 %, and 0.11 ± 0.068 %), and a member of the *Clostridium* genus (0.25 ± 0.14 %).

Although both pandas’ species share the twelve OTUs, they have different dominant OTUs within their individual microbial communities. The dominant OTU found in the giant panda, a *Clostridium* species, was found in significantly lower abundance in the red panda (55 ± 4.6 and 0.40 ± 0.29 %) (Table 5.3), whereas the dominant OTU in the red panda is an unclassified member of the Clostridiaceae family (38 ± 10 %), but also found in low abundance in the giant panda (0.0021 ± 0.0014 %).

Table 5.2 Relative abundance of shared OTUs found at > 1.0 %.

<table>
<thead>
<tr>
<th>Firmicutes</th>
<th>Giant Panda</th>
<th>Red Panda</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RSA</td>
<td>Ranking</td>
<td>RSA</td>
</tr>
<tr>
<td>OTU 17 <em>Streptococcus sp.</em></td>
<td>35 ± 15</td>
<td>1</td>
<td>22 ± 5.0</td>
</tr>
<tr>
<td>OTU 02 <em>Turicibacter sp.</em></td>
<td>30 ± 5.3</td>
<td>2</td>
<td>10 ± 8.6</td>
</tr>
<tr>
<td>OTU 08 <em>Streptococcus sp.</em></td>
<td>4.9 ± 2.9</td>
<td>3</td>
<td>0.99 ± 0.81</td>
</tr>
<tr>
<td>OTU 07 Peptostreptococcaceae-unclassified</td>
<td>4.2 ± 0.71</td>
<td>4</td>
<td>0.37 ± 0.17</td>
</tr>
<tr>
<td>OTU 01 <em>Clostridium sp.</em></td>
<td>0.36 ± 0.36</td>
<td>5</td>
<td>0.40 ± 0.29</td>
</tr>
<tr>
<td>OTU 12 <em>Streptococcus sp.</em></td>
<td>0.41 ± 0.19</td>
<td>6</td>
<td>0.0014 ± 0.0014</td>
</tr>
<tr>
<td>OTU 19 <em>Streptococcus sp.</em></td>
<td>0.18 ± 0.11</td>
<td>7</td>
<td>0.0070 ± 0.0026</td>
</tr>
<tr>
<td>OUT 05 <em>Sarcina sp.</em></td>
<td>0.0075 ± 0.0037</td>
<td>8</td>
<td>8.5 ± 3.8</td>
</tr>
<tr>
<td>OTU 04 Clostridiaceae-unclassified</td>
<td>0.0065 ± 0.0024</td>
<td>9</td>
<td>12 ± 5.4</td>
</tr>
<tr>
<td>OTU 18 Peptostreptococcaceae-unclassified</td>
<td>0.0026 ± 0.0013</td>
<td>10</td>
<td>0.83 ± 0.43</td>
</tr>
<tr>
<td>OTU 03 Clostridiaceae-unclassified</td>
<td>0.0021 ± 0.0014</td>
<td>11</td>
<td>38 ± 10</td>
</tr>
<tr>
<td>OTU 22 <em>Clostridium sp.</em></td>
<td>0.0017 ± 0.0011</td>
<td>12</td>
<td>0.67 ± 0.31</td>
</tr>
<tr>
<td>OTU 06 Clostridiaceae-unclassified</td>
<td>0.0011 ± 0.00081</td>
<td>13</td>
<td>22 ± 11</td>
</tr>
</tbody>
</table>

Mean values with standard error given. RSA: relative sequence abundance.

**Gut microbiota differs according to panda species**

To determine if GIT microbiota differed between panda species, we performed a three-dimensional principal coordinate analysis (PCoA) using the weighted and unweighted UniFrac metric (Figure 5.3a; Figure D.2) and found panda species-related differences in overall bacterial communities. Similar trends were also observed through a
three-dimensional nonmetric dimensional scaling (NMDS) clustering approach (Figure 5.3b). Unweighted and weighted UniFrac comparisons between groups also revealed that the total GIT microbiota were significantly different from each other (P<0.001).

Table 5.3  Rankings of significantly important taxonomic groups

<table>
<thead>
<tr>
<th>Rank</th>
<th>Group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phyla</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Actinobacteria</td>
<td>0.045</td>
</tr>
<tr>
<td>Class</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Clostridia</td>
<td>0.0416</td>
</tr>
<tr>
<td>2</td>
<td>Actinobacteria</td>
<td>0.0004</td>
</tr>
<tr>
<td>Order</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Clostridiales</td>
<td>0.00416</td>
</tr>
<tr>
<td>2</td>
<td>Micrococcales</td>
<td>0.0003</td>
</tr>
<tr>
<td>Family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Peptostreptococcaceae</td>
<td>0.0139</td>
</tr>
<tr>
<td>2</td>
<td>Clostridaceae</td>
<td>0.0008</td>
</tr>
<tr>
<td>3</td>
<td>Pasteurellacea</td>
<td>0.0341</td>
</tr>
<tr>
<td>Genus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Peptostreptococcaceae-unclassified</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>2</td>
<td>Clostridium</td>
<td>0.0085</td>
</tr>
<tr>
<td>OTU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Clostridium species (OTU 01)</td>
<td>0.0051</td>
</tr>
<tr>
<td>2</td>
<td>Streptococcus species (OTU 12)</td>
<td>0.0082</td>
</tr>
</tbody>
</table>

Significant differences were also observed at the different taxonomic levels (Table 5.3). At the phyla level, the Actinobacteria differed significantly with respect to panda species, with the red panda having a larger relative abundance when compared to the giant panda (0.847 ± 0.48 % and 0.044 ± 0.015 %, respectively). The Clostridia and the Actinobacteria classes also were significantly different (Table 5.3). Red pandas had a significantly higher relative abundance than the giant panda (85 ± 11 % and 61 ± 4.9 %, respectively). Of the seven orders analyzed, only two were found to be important in
determining differences in GIT microbial communities in pandas. These orders included the Clostridiales and the Micrococcales, as the Micrococcales order is found in higher relative abundance in the red panda than that of the giant panda (0.64 ± 0.37 % and 0.025 ± 0.011 %). Three families also were found to be significantly different with respect to the giant and red panda. The Peptostreptococcaeae (4.3 ± 0.72 % and 1.3 ± 0.56 %, respectively) and a subsequent unclassified genus were found in higher relative abundance in the giant panda when compared to the red panda. Also the Clostridiaceae (57 ± 4.6 % and 83 ± 12 %), and the Clostridium genus (55 ± 4.6 % and 1.4 ± 0.65 %) were also found significantly different. Interestingly, the Pasteurellaceae was also found as an important family, as it was only observed in the giant panda (1.1 ± 0.75 % and 0%, respectively).

Figure 5.3 Analysis of microbial diversity

a) three-dimensional principal coordinate analysis using the weighted UniFrac metric for both pandas and b) three-dimensional nonmetric multidimensional scaling.
Notes: PCoA (total percentage of variation for PC1, PC2, and PC3, as indicated on the axes: 82.2 %), and NMDS analysis(lowest stress: 0.243, R-square: 0.458).
Discussion

Bacterial communities play a vital role in the host survival (McFall-Ngai, 2013). In particular, the GIT microbiota of herbivores maintains a homeostatic relationship with the host by modulating normal gut and immune functions and degrading fibrous substrates to generate energy for the host (Bergman, 1990). Several studies have shown that diet is the driving force that shapes GIT microbial communities (Amato et al., 2014; Scott et al., 2013; Nicholson et al., 2012; Bolnick et al., 2014), but in a zoo-wide assessment consisted of sixty mammalian species including traditional herbivores, carnivores, and pandas by Ley et al., (2008), taxonomic placement was found to be more important than diet. Our two panda species, the giant and red panda, are anomalies, as they are taxonomically classified as carnivores, yet exhibit an exclusively herbivorous diet. Although the pandas were included in Ley et al.'s (2008) study, no other mustelid was studied. Therefore, we do not know whether the two panda species will be more similar due to their diet.

Pandas are taxonomically distantly related (O'Brien et al., 1985). Giant pandas are members of the Ursidae, a family that contains other bears (Yu et al., 2004; Yu et al., 2011), whereas the red panda is classified as a member of the Musteloidae superfamily, containing members such as weasels and otters (Flynn et al., 2000). Pandas may be taxonomically distinct, but they share similarities other than their name. They both consume bamboo almost exclusively, and they have adapted to their bamboo diet in similar ways. Unlike a typical herbivore, both pandas have a relatively short GIT, more like their carnivorous relatives, as they have a simple stomach and lack a cecum. However, they do have slight GIT adaptations. The length of their small intestine is
reduced; they have an increased surface area of their large intestine and a smaller liver (Davis, 1964). Other physical adaptations include their enhanced jaw structure that allows them the increased force required to crush bamboo (Christiansen, 2008; Christiansen and Wroe, 2007) and an extension of the radial sesamoid, known as a "pseudo-thumb," that increases their efficiency for foraging bamboo (Delisle and Strobeck 2005; Fulton and Strobeck, 2006; Salesa et al., 2006; Endo et al., 1996).

Previous work indicates that panda GIT microbiota have a cellulose metabolism; therefore, they are capable of digesting their bamboo diet (Zhu et al., 2011; Kong et al., 2014). Zhu et al.'s (2011) study found that giant pandas GIT microbiota were related to organisms that digest cellulose, finding sequence homologues to genes coding for cellulase and β-glucosidase. These observations were also observed by Kong et al. (2014), finding that 20% of the OTUs found in red pandas were related to known cellulose metabolizers.

In our study, we found the two panda GIT microbiomes contain little diversity with few OTUs found > 1.0% in any sample. Although they share a core community of twelve OTUs, the communities associated with each panda are significantly different overall (Figure 5.3) and at various taxonomic levels (Table 3.3). Both pandas GIT microbiotas are dominated by the Firmicutes with contributions from the Proteobacteria, as observed in previous studies (Zhu et al., 2011; Kong et al., 2014), yet their bacterial communities are each dominated by a different OTU. These data indicate that although they share a core community, their microbial communities overall are significantly different.
What is even more interesting is the fact that individual OTUs that are found in high abundance in one panda are found in such low relative abundance in the other panda species. The dominant OTU in the giant panda GIT microbiome, a \textit{Clostridium} species, was found in very low abundance in the red panda (55 ± 4.6 % and 0.41 ± 2.9 %). This is also true for the red panda’s dominant OTU, an unclassified member of the \textit{Clostridiaceae} (38 ± 10 and 0.0021 ± 0.0014 %). This relationship was also observed with the other OTUs found in high relative abundance in red pandas, including two unclassified members of the \textit{Clostridiaceae} (22 ± 11 % and 0.0011 ± 0.00081 %; 12 ± 5.4 % and 0.0065 ± 0.0025 %, respectively) and a \textit{Sarcina} species (8.5 ± 3.8 % and 3.2 ± 1.7 %, respectively). For giant pandas, the OTU found second in relative abundance is a \textit{Turicibacter} species, an OTU also found in relatively high abundance in red pandas (30 ± 5.3 % and 10 ± 8.6 %, respectively). Interestingly, the \textit{Turicibacter} and \textit{Sarcina} genera have been associated with potential opportunistic pathogens (Bosshard, Zbinden, Altwegg 2002; Lam-Himlin et al., 2009), not degradation of fibrous diets. As both the giant and red panda suffer from gastrointestinal disease in captivity (Qiu and Mainka, 1993; Loeffler, 2006; Phillipa and Ramsay, 2001; Nijiboer and Dierenfeld, 2011), it is interesting to find such high relative abundances of these groups.

What is unknown is whether the differences we observed are due to their different taxonomic placement, or due to another factor. One factor that could have an effect is their different bamboo consumption behaviors. Although they both consume an almost exclusively bamboo diet, giant and red pandas display differences in part preference (Tarou et al., 2006; Roberts and Gittleman, 1984; Wei et al., 2000). Giant pandas undergo changes in part preference throughout the year (Schaller et al., 1985; Hansen et
al., 2010; Williams et al., 2012), but they will consume the leaf, culm, and shoot portion. Red pandas, however, will not consume the culm portion and only consume the leaf portion with the addition of shoots in the spring (Wei et al., 1999a; Wei et al., 1999b; Reid, Hu, and Huang, 1991; Johnson, Schaller, and Hu, 1988; Zhang et al., 2009). Their difference in part preference may play a role in shaping their distinct microbial communities, as different bamboo parts contain different nutritive values (Christian et al., 2015).

Overall, the GIT microbiota of pandas appear to be different, although they share core microbiota. However, without comparing these to other members of their respective families (Urisidae and Musteliodae), we cannot determine whether taxonomy or diet shapes microbial communities within the gastrointestinal tract. This study is the first to compare the two species, and lays the foundation for further studies to reveal the driving force of GIT microbiota in pandas.
References


CHAPTER VI

COMPARING NORMAL AND MUCOSAL STOOLS FROM GIANT PANDAS

(*AILUROPODA MELANOLEUCA*) REVEAL DIFFERING BACTERIAL COMMUNITIES

**Abstract**

A change in diet and subsequent changes in the gastrointestinal tract (GIT) microbial community can result in dysbiosis, often occurring with hosts experiencing inflammatory conditions. Giant pandas (*Ailuropoda melanoleuca*) undergo dramatic feeding shifts and experience episodes of chronic GIT distress, with symptoms including abdominal pain, loss of appetite and the excretion of mucous stools. To gain insight into the cause of these mucous excretions, we examined two giant pandas during these episodes using next-generation 16S rRNA sequencing of both normal and mucous stools. We found that both sample types have low diversity overall, and are dominated by the Firmicutes and the Proteobacteria, with lesser contributions by the Bacteroidetes and the Actinobacteria. A core community was found across both sample types, including nineteen OTUs found in > 1.0 % relative abundance. Of these nineteen OTUs, five were found to be significantly different between sample-type and included an unclassified Pasteurellaceae, a *Pseudomonas* species, a *Sporosarcina* species, and two *Streptococcus* species. Also, the overall community was found to be significantly different with respect to sample type as observed by three-dimensional PCoA, NMDS, and UniFrac analyses.
This work is the first to characterize the microbiota associated with mucous excretions in giant pandas and acts as the first step to elucidating the mechanism behind an issue that affects the welfare of these endangered species.

**Introduction**

The host-symbiont relationship within the gastrointestinal tract is critical, as these symbionts play a fundamental role not only in the digestion of fibrous substrates, but also maintaining the host immune system through the modulation of normal GIT function and the maintenance of host-bacterial homeostasis (Flint et al., 2012; Hooper et al., 2002). Normally, commensals associate with the gastrointestinal associated lymphoid tissue to exclude pathogens and produce short chain fatty acids (SCFA) that can be used as an energy source for the host (Johansson, Larsson, and Hansson, 2011; Flint et al., 2012). The intestinal epithelial cells (IEC) use SCFAs to increase expression of tight junction proteins, thus increasing the barrier to pathogens (Louis et al., 2009; Brown et al., 2002).

When this relationship shifts toward dysbiosis, the host can experience an inflammatory response, and, if unchecked, this can develop into a chronic condition (Fava et al., 2011). A change in diet can subsequently change microbial populations, as evidenced in cases of dysbiosis-induced inflammatory conditions such as irritable bowel disease (IBD) in humans (Huda-Faujan et al., 2010; McOrist et al., 2008). In these conditions, IBD patients observed significant changes in diversity and relative abundance of different bacterial groups (Frank et al., 2007; Sokol et al., 2006; Manichanh et al., 2006).

Giant pandas are uniquely herbivorous carnivores, and annually undergo a dramatic shift in eat behavior (Schaller et al., 1985; Tarou et al., 2006; Hansen et al., 2012).
2010), resulting in significant changes in GIT microbial communities (Williams et al., 2012). Also, as an endangered species, these bears suffer greatly from gastrointestinal tract disorders both ex situ and in situ (Qiu and Mainka, 1993; Loeffler, 2006). In particular, captive giant pandas undergo chronic GIT distress, with bouts of abdominal discomfort and loss of appetite, resulting in the expression of a mucous excretion (mucoid) (Edwards et al., 2006; Loeffler, 2006). Previous efforts to reduce these mucoids by increasing their diet’s protein concentration resulted in an increase of occurrence of mucoids (Janssen et al., 2006; Edwards et al., 2006), indicating that giant pandas require high-fiber diets to maintain host-microbiota homeostasis. However, even with their high fiber diet, captive pandas still have mucoids. It is believed that these mucous excretions are associated with pathogenic organisms, as they have been weakly linked to these disturbances (Loeffler, 2006). Using next-generation sequencing technology, this study will be the first to characterize the microbiota associated with mucous excretions in two giant pandas and to compare these bacterial communities to normal fecal samples collected pre- and post-mucous excretions.

**Materials and Methods**

**Study Animals**

The two giant pandas (“YaYa, studbook number: 507, and “LeLe,” studbook number: 466) used in this study were housed at the Memphis Zoological Society, Memphis, TN, USA. Samples were collected under a signed biomaterials request form, and no IACUC was need as the project was viewed as non-invasive by the institution.
**Sample Collection**

Fresh fecal (n = 18) and mucous excretion (n= 5) samples were collected. All samples were transported on dry ice, and stored at −80°C prior to processing.

**DNA Extraction**

Total genomic DNA from fecal samples was extracted via mechanical disruption and hot/cold phenol extraction following Stevenson et al.’s protocol (2007) with the exception that 25:24:1 phenol:chloroform:isoamyl alcohol was used in place of phenol:chloroform at all steps. DNA was quantified using a Qubit Fluorometer (Invitrogen) and stored at −20°C following extraction.

**Library Preparation & Sequencing**

*16S rRNA amplification and sequence indexing*

Library preparation was carried out following manufacturer’s recommendations (Illumina, 2013). In brief, an amplicon PCR targeted the 16S rRNA gene (V3-V4) using a forward (V3-4F, TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGN GGCWGCGAG) and reverse (V3-4R, GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACTACHVGGGTATCTAATCC) primer in a 25-μL reaction with 1X KAPA HiFi Hot Start Ready Mix (Kapa Biosystems), 0.2 mM each primer, and 1-10 ng DNA. Amplification conditions were as follows: 95 °C for 3 min, 25 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final elongation of 72 °C for 5 min. PCR products were purified via gel extraction (Zymo Gel DNA Recovery Kit; Zymo, Irvine, CA) using a 1 % low melt agarose gel (National Diagnostics, Atlanta, GA) or a PureLink® Pro96 PCR Purification Kit (Invitrogen, Carlsbad, CA). Purified products underwent an
indexing 25 μL-PCR reaction (1x KAPA HiFi Hot Start Ready Mix, 0.2 mM indices, and 5 μL of purified product) with the same reaction conditions as amplicon PCR except reduction of cycles to 8.

**Library quantification and sequencing**

The final index PCR product underwent gel extraction (Zymo Gel DNA Recovery Kit; Zymo, Irvine, CA), and the final purified product concentration was determined by Qubit Fluorometer (Invitrogen). Samples were pooled yielding an equimolar 4 nM pool. Following manufacturer’s protocol, sequencing was conducted on an Illumina MiSeq reagent kit V3 (600 cycles), as described previously (Illumina, 2013).

**Data Analysis**

**Sequence analysis**

Sequence analysis was carried out using the program Mothur v.1.34.1 following the MiSeq SOP (Kozich et al., 2013). In brief, contigs were formed from 16s rRNA reads, and poor quality sequences were removed. Sequences were trimmed and filtered based on quality (maxambig=0, minlength=250, maxlength=600). Unique sequences were determined and aligned against the SILVA 16S rRNA gene alignment database (Pruesse et al., 2007). Chimeras (chimera.uchime) and sequences identified as members of Eukaryota, Archaea, and Cyanobacteria lineages were removed. Sequences were classified using the SILVA database with a bootstrap value cutoff of 80, and OTUs found with <2 sequences were removed. The following analyses were carried out in Mothur: Good’s coverage (Good, 1953), Morista-Horn index (Horn, 1966), Principal Coordinate Analysis (PCoA) (Gower, 1966), nonmetric dimensional scaling (NMDS,
Behavior analysis of bamboo consumption

The study of bamboo consumption behavior at the Memphis Zoo has been ongoing since the fall of 2003 and was conducted as previously described (Hansen et al., 2010). In brief, behavior data were collected in 20-min periods in 30-s increments while the bear was feeding on bamboo using an ethogram focusing on foraging behaviors. These behaviors were divided into four main consumption categories: leaf, culm (stalk), other plant part (i.e. shoot or branch) and unknown plant part. For each month, the total consumption behaviors were quantified by time spent consuming specific parts and each individual behavior was expressed as a percentage of the total consumption behaviors.

Statistical Analysis.

All statistical analyses were carried out in SAS 9.3 software (Cary, NC) and data are expressed as the mean ± SE and considered significant if P < 0.05. To determine which taxonomic groups are important in defining microbial communities, Step-wise analysis was used (PROC STEPDISC). Following stepwise analysis, groups found to be significant were included in the discriminant analysis (PROC DISCRIM) to determine factors important for proper classification of sample type.
Results

Bamboo Consumption Behavior

Dramatic eating behavioral shifts were observed in both pandas, and the mean monthly observed bamboo consumption behavior can be seen in Figure 1. In general, the bears consumed more culm than leaf throughout the year, but a shift to a higher proportion of leaf consumption can be observed in the months of August and September (Figure 6.1). Interestingly, the pandas consumed negligible amounts of leaf in May and increased their leaf portion to its highest relative proportion in August. Following this peak, leaf consumption steadily declined through December (Figure 6.1).

Figure 6.1 Mean monthly proportions of bamboo consumption behavior observed for leaf, culm and other displayed as a percentage of total feeding observations by giant pandas.
Sequence Coverage and Diversity Metrics

For all samples, we generated a total of 363,549 (254,014 high quality) 16S rRNA sequences using Illumina MiSeq paired-end sequencing (Table 6.1). A Good’s coverage value of $>0.95$ (Table 6.1) and a leveling off of rarefaction curves (Figure E.1) indicated that sequencing was adequate to detect the majority of bacterial diversity present in all samples. A 97% operational taxonomic unit (OTU) analysis corresponding to genus-level classification (Schloss and Handelsman, 2005) identified 1772 unique OTUs across all samples with values ranging from 505 to 288 OTUs per sample type (Table 6.1).

Table 6.1  Number of sequences, estimated coverage, diversity and OTU richness.

<table>
<thead>
<tr>
<th>Sample</th>
<th>16S rRNA reads</th>
<th>Community Richness</th>
<th>Community Diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>High-quality</td>
<td>Berger-Parker</td>
</tr>
<tr>
<td>Total</td>
<td>363,549</td>
<td>254,014</td>
<td>0.55 ± 0.046</td>
</tr>
<tr>
<td>Mucoid (n=5)</td>
<td>115,227</td>
<td>88,075</td>
<td>0.44 ± 0.086</td>
</tr>
<tr>
<td>Normal (n=18)</td>
<td>248,322</td>
<td>165,939</td>
<td>0.57 ± 0.0053</td>
</tr>
</tbody>
</table>

Mean values and standard error given.

The sample diversity varied, as some samples, particularly normal fecal samples displayed low diversity with some samples dominated by a single OTU (Berger-Parker 0.53-0.95) (Table 6.1). However, some normal fecal samples had higher diversity than others (Berger-Parker 0.28-0.47). Mucoid samples displayed much higher diversity overall when compared to normal samples, (Berger-Parker: $0.44 ± 0.086$ and $0.57 ± 0.0053$, respectively) (Table 6.1). Mucoid samples also had a higher Shannon and inverse-Simpson diversity index ($2.0 ± 0.30$ and $4.4 ± 1.1$, respectively) than did normal fecal samples ($1.3 ± 0.13$ and $2.9 ± 0.34$, respectively) (Table 6.1).

Seventeen phyla represented sequences from all samples analyzed, with $58 ± 6.8\%$ belonging to the Firmicutes, $40 ± 6.7\%$ belonging to the Proteobacteria, and $1.8 ± 1.0\%$ belonging to the Actinobacteria.
% belonging to the Bacteroidetes, and all others found at less than 1.0 % relative abundance. Bacterial classes with > 1.0 % were the Gammaproteobacteria (38 ± 6.7 %), the Clostridia (30 ± 5.6 %), the Bacilli (19 ± 4.1 %), the Erysipelotrichia (9.2 ± 2.3 %), the Flavobacteria (1.6 ± 0.97 %), and the Betaproteobacteria (1.4 ± 0.91 %). At the order level, those with > 1.0 % representation were the Enterobacteriales (36 ± 6.8 %), the Clostridiales (30 ± 5.6 %), the Lactobacillales (19 ± 4.1 %), the Erysipelotrichales (9.2 ± 2.3 %), the Flavobacteriales (1.6 ± 0.97 %), the Neisseriales (1.3 ± 0.91 %), and the Pasteurellales (1.4 ± 0.89 %) (Figure 6.2). At the family and genus level, 99 and 91 % of sequences, respectively, were annotated.

Figure 6.2 Relative sequence abundance at the order level for each sample-type.
**Shared Core Community across Sample-type**

To determine if there were specific OTUs common to both mucoid and normal fecal types, we analyzed all samples together. We found a total of 177 OTUs common to both samples, with only nineteen OTUs represented by > 1.0 % relative abundance in any sample (Table 6.2, Figure 6.3). Five OTUs were found in high sequence abundance belonging to the genus *Escherichia-Shigella* (36 ± 6.8 %), *Clostridium* (25 ± 4.9 %), *Turicibacter* (9.2 ± 2.3 %), *Leuconostoc* (8.1 ± 2.9 %), and *Streptococcus* (5.2 ± 2.2 %). The other OTUs were classified belonging to an unclassified Peptostreptococcaceae (4.6 ± 1.1 %), a member of the genus *Weisella* (1.8 ± 0.81 %), four members of *Streptococcus* (1.5 ± 1.0 %, 0.31 ± 0.24 %, 0.16 ± 0.14 %, and 0.13 ± 0.074 %), an unclassified Pasteurellaceae (1.4 ± 0.89 %), an unclassified Neisseriaceae (1.3 ± 0.91 %), a member of *Bergeyella* (0.57 ± 0.41 %), a *Lactococcus* species (0.36 ± 0.33 %), a member of the *Pseudomonas* genus (0.18 ± 0.18 %), a member of the *Sporosarcina* genus (0.080 ± 0.077 %), an *Acinetobacter* species (0.074 ± 0.068 %), and a member of the *Granulicatella* genus (0.066 ± 0.060 %). In total, these shared OTUs accounted for 98 ± 0.98 % of sequences within each sample. Of these nineteen-shared OTUs, only five were found to be important in determining sample type, including: an unclassified Pasteurellaceae, and members of the genera *Pseudomonas*, *Sporosarcina*, and *Streptococcus* (Table 6.3).
Table 6.2  Relative abundance of shared OTUs found at > 1.0 %.

<table>
<thead>
<tr>
<th></th>
<th>Mucoid RSA</th>
<th>Ranking</th>
<th>Normal RSA</th>
<th>Ranking</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteroidetes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU 11 Bergeyella</td>
<td>2.6 ± 1.8</td>
<td>8</td>
<td>0.0051 ± 0.0049</td>
<td></td>
</tr>
<tr>
<td><strong>Firmicutes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU 2 Clostridium</td>
<td>35 ± 15</td>
<td>1</td>
<td>22 ± 5.0^2</td>
<td>2</td>
</tr>
<tr>
<td>OTU 3 Turicibacter</td>
<td>12 ± 7.0</td>
<td>3</td>
<td>8.6 ± 2.4</td>
<td>4</td>
</tr>
<tr>
<td>OTU 9 Streptococcus</td>
<td>6.7 ± 4.2</td>
<td>4</td>
<td>0.010 ± 0.0052</td>
<td>12</td>
</tr>
<tr>
<td>OTU 5 Peptostreptococceae-unclassified</td>
<td>6.0 ± 2.4</td>
<td>6</td>
<td>4.3 ± 1.2</td>
<td>6</td>
</tr>
<tr>
<td>OTU 25 Sporosarcina</td>
<td>0.36 ± 0.36</td>
<td>10</td>
<td>0.00041 ± 0.00041</td>
<td>17</td>
</tr>
<tr>
<td>OTU 49 Granulicatella</td>
<td>0.29 ± 0.28</td>
<td>12</td>
<td>0.0024 ± 0.0024</td>
<td>15</td>
</tr>
<tr>
<td>OTU 32 Streptococcus</td>
<td>0.23 ± 0.18</td>
<td>13</td>
<td>0.11 ± 0.085</td>
<td>11</td>
</tr>
<tr>
<td>OTU 10 Streptococcus</td>
<td>0.15 ± 0.088</td>
<td>14</td>
<td>6.6 ± 2.8</td>
<td>5</td>
</tr>
<tr>
<td>OTU 51 Streptococcus</td>
<td>0.039 ± 0.035</td>
<td>16</td>
<td>0.19 ± 0.19</td>
<td>10</td>
</tr>
<tr>
<td>OTU 21 Lactococcus</td>
<td>0.042 ± 0.029</td>
<td>15</td>
<td>0.45 ± 0.43</td>
<td>8</td>
</tr>
<tr>
<td>OTU 4 Leuconostoc</td>
<td>0.016 ± 0.010</td>
<td>17</td>
<td>10 ± 3.6</td>
<td>3</td>
</tr>
<tr>
<td>OTU 12 Weissella</td>
<td>0.004 ± 0.0024</td>
<td>18</td>
<td>2.3 ± 1.0</td>
<td>7</td>
</tr>
<tr>
<td>OTU 15 Streptococcus</td>
<td>0.0026 ± 0.0017</td>
<td>19</td>
<td>0.40 ± 0.31</td>
<td>9</td>
</tr>
<tr>
<td><strong>Proteobacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU 1 Escherichia-Shigella</td>
<td>13 ± 6.8</td>
<td>2</td>
<td>43 ± 8.1</td>
<td>1</td>
</tr>
<tr>
<td>OTU 6 Pasteurallaceae-unclassified</td>
<td>6.2 ± 3.7</td>
<td>5</td>
<td>0.0026 ± 0.0024</td>
<td>14</td>
</tr>
<tr>
<td>OTU 7 Neisseriaaceae-unclassified</td>
<td>5.9 ± 3.9</td>
<td>7</td>
<td>0.010 ± 0.0098</td>
<td>13</td>
</tr>
<tr>
<td>OTU 13 Pseudomonas</td>
<td>0.84 ± 0.83</td>
<td>9</td>
<td>0.00081 ± 0.00081</td>
<td>16</td>
</tr>
<tr>
<td>OTU 24 Acinetobacter</td>
<td>0.34 ± 0.32</td>
<td>11</td>
<td>0.00039 ± 0.00039</td>
<td>18</td>
</tr>
</tbody>
</table>

Mean values with standard error given. RSA-Relative sequence abundance

Figure 6.3  A comparison of the number OTUs shared between sample-types.

Notes: OTUs were defined at a 97 % similarity corresponding to the genus taxonomic level.
Fecal Microbiota Differs According to Sample-type

To determine if GIT microbiota differed across fecal type, we performed a three-dimensional principal coordinate analysis (PCoA) using the weighted (Figure E.4) and unweighted UniFrac metric (Figure 4a) and found sample type-related differences in total bacterial communities. These results were confirmed with a three-dimensional nonmetric dimensional scaling (NMDS) clustering approach (Figure 4b). In particular, normal fecal samples clustered more closely together than did mucoid samples. Unweighted and weighted UniFrac comparisons between groups also revealed that fecal types were significantly different from one another (P<0.0001). Of the 111 OTUs that were specific to normal fecal samples, none were found >1.0 % in any normal sample (Figure 7.3). However, five OTUs of the 328 OTUs specific to mucoids were found > 1.0 % in any sample.

Figure 6.4 Three-dimensional analysis

Notes: a) principal coordinate analysis of microbial diversity using the weighted UniFrac metric and b) nonmetric multidimensional scaling analysis examining bacterial community differences PCoA: and the percentage of variation is explained by PC1, PC2, and PC3 are indicated on the axes (total: 20.32 %), NMDS: (lowest stress: 0.2745, R-square: 0.3978).
Sample-type Correlates to Different Taxonomic Groups

Using step-wise analysis, taxonomic groups were identified as significantly important in determining the microbial community of giant panda fecal samples (Table 6.3). Two phyla, the Bacteroidetes and the Actinobacteria, were found to be important. Both phyla were represented in higher abundance in mucoid samples (7.6 ± 3.9 % and 0.91 ± 0.68 %, respectively) when compared to normal fecal samples (0.16 ± 0.041 % and 0.13 ± 0.028 %, respectively). Following these phyla throughout the course of sampling, we observed marked changes in these groups overtime, as an increase in sequence abundance is observed during mucoid excretion in both the male (days 26 and 35) and female (day 32) giant panda (Figure E.2).

Similar observations were also seen at the class level, where the Actinobacteria, Sphingobacteriia, Alphaproteobacteria, Fusobacteria, Flavobacteriia, Bacilli, and Erysipelotrichia were all deemed as important taxonomic groups (Table 6.3). The Actinobacteria, Sphingobacteriia, Alphaproteobacteria, Fusobacteria, and Flavobacteriia, were found to increase during mucoid sampling in both male and female giant pandas (Figure E.3). However, the Bacilli class was observed to decrease during mucoid excretions in the male giant panda (days 15, 36, 34, and 35), but in the female, Bacilli abundance dropped pre- (days 17, 31) and post-mucoid (day 33), but was higher during the day of mucous excretion (day 32) (Figure E.3). Also unlike the male, Erysipelotrichia displayed a decline throughout sampling of the female, and did not display the increase during mucus excretion (Figure E.3).
Table 6.3  Rankings of significantly important taxonomic groups.

<table>
<thead>
<tr>
<th>Ranking</th>
<th>Group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phyla</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Bacteroidetes</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>2</td>
<td>Actinobacteria</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td><strong>Class</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Flavobacteriia</td>
<td>0.0024</td>
</tr>
<tr>
<td>2</td>
<td>Alphaproteobacteria</td>
<td>0.0001</td>
</tr>
<tr>
<td>3</td>
<td>Sphingobacteriia</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>4</td>
<td>Actinobacteria</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>5</td>
<td>Fusobacteria</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>6</td>
<td>Bacilli</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>7</td>
<td>Erysipelotrichia</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td><strong>Order</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Pasteurellales</td>
<td>0.0026</td>
</tr>
<tr>
<td>2</td>
<td>Burkholderiales</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>3</td>
<td>Xanthomonadales</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>4</td>
<td>Lactobacillales</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td><strong>Family</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Moraxellaceae</td>
<td>0.0007</td>
</tr>
<tr>
<td>2</td>
<td>Pasteurellacea</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>3</td>
<td>Clostridaceae</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td><strong>OTU</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Pasteurellaceae-unclassified</td>
<td>0.0025</td>
</tr>
<tr>
<td>2</td>
<td><em>Pseudomonas</em></td>
<td>0.0066</td>
</tr>
<tr>
<td>3</td>
<td>Sporosarcina</td>
<td>0.0016</td>
</tr>
<tr>
<td>4</td>
<td>Streptococcus (OTU 9)</td>
<td>0.0051</td>
</tr>
<tr>
<td>5</td>
<td>Streptococcus (OTU 15)</td>
<td>0.0082</td>
</tr>
</tbody>
</table>

At the family level, Moraxellaceae, Pasteurellacea, and Clostridaceae were found as important groups, and all were found at higher relative abundance in mucoid samples than normal fecal samples (0.98 ± 0.44 % and 0.068 ± 0.017 %; 6.2 ± 3.6 % and 0.023 ± 0.021 %; 34 ± 14 % and 22 ± 4.9 %, respectively) (Figure 6.5). The family level was also observed as the best taxonomic level to determine sample-type based on relative abundance of these three families with an overall classification rate of 85 %. Only three
samples were misclassified, a mucoid sample (male, day 34) and two normal fecal samples (female, days 1 and 31).

Figure 6.5  Percentage relative abundance of the significantly important families For both the a) male and b) female giant panda.

Discussion

The maintenance of the host-symbiont relationship is important to survival, especially in herbivores. Hosts depend on these microbial communities not only for
energy through the degradation of fibrous feedstuffs, but also in maintaining host
immunity through GIT-microbiota homeostasis (Flint et al., 2012; Hooper). Any
alteration to this homeostasis can result in dysbiosis, which can have profound effects on
host health (Huda-Faujan et al., 2010; McOrist et al., 2008). It is believed that the giant
pandas’ diet plays a role in the production of mucus stools, a symptom of excruciating
GIT distress observed in giant pandas (Janssen et al., 2006; Edwards et al., 2006). Giant
pandas undergo a dramatic change in diet (Figure 6.1) (Schaller et al., 1985; Tarou et al.,
2006; Hansen et al., 2010; Williams et al., 2012), and the subsequent changes in GIT
microbial communities (Williams et al., 2012) may be the cause of these episodes. These
excretions have also been weakly linked to pathogenic organisms (Loeffler, 2006);
therefore, we characterized the bacterial microbiota associated with normal and mucous
stools to determine differences using high-throughput 16s rRNA sequencing.

Overall, we found that all giant panda samples had low diversity (Table 1) and
were dominated by the phyla Firmicutes and the Proteobacteria, with lesser contributions
from the Bacteroidetes. Across both sample types, the microbial communities had a core
community that was defined by 177 OTUs, with only nineteen found at > 1.0 % in any
sample (>98 % in all samples) (Table 6.2). Of these nineteen-shared OTUs, only five
were found to be important in determining sample type (Table 6.3), indicating that these
significant OTUs that occur in mucoids are also present in normal samples.

The overall community structure was different as indicated by PCoA, NMDS,
(Figure 6.4; Figure E.4) and UniFrac analyses (P<0.001), and the differences in the
bacterial microbiota were also seen in different taxonomic groups, as observed by
discriminant analyses (Table 6.3). As mucosa- and fecal-associated microbial
communities have been shown to differ in humans, calves, and chickens (Malmathuge et al., 2012; Zoentendal, 2005), it is unknown whether the overall community differences are due to the difference in sample type. However, the same OTUs consistently dominate the microbiota of both sample types, albeit in different relative abundances, indicating that the communities contain the same core OTUs, as this would not be expected if differences were only attributed to bacterial association with mucosa or feces.

Similar to mucosa-associated communities, we found mucoids to have higher diversity than that of normal feces (Table 6.1), and a higher relative abundance of the certain phyla. In particular, there was a significant difference observed in the Bacteroidetides, as the relative abundance of this phyla increased during mucous excretions. Mucosa-associated communities typically have higher levels of these phyla when compared to fecal-associated communities (Malmathuge et al., 2012; Zoentendal et al., 2002). In our sampling, the relative abundances of the Flavobacteriia class and the Pasteurellaceae family were found as important taxa and were significantly different (p=0.0024 and p<0.001, respectively) with respect to sample type. Members of these groups are known commensals, especially those that are associated with the mucosal lining of animals (Jakobsson et al., 2014; Kuhnert and Christensen, 2008).

However, some of our findings are not consistent with the changes in microbiota being related to differences in sample-type. For example, we observed a significant decrease in Lactobacillales in mucoids when compared to normal feces (Table 6.3). The Lactobacillales contain members of the lactic acid bacteria (LAB) and are a part of the mucosa-associated community that plays a major role in host-microbial homeostasis (Reid and Burton, 2002; Salvetti et al., 2013), and LAB have been reduced in those
suffering from IBD (Swidsinski et al., 2002). If our sampling were only detecting sample-type differences, we would expect to find an increase in this group. Interestingly, we also found a significant increase in the Actinobacteria phyla and the Fusobacteria class (Table 6.3), as both have been associated with IBD in humans (LePage et al., 2011, Ohkusa et al., 2003, Ohkusa et al., 2009). Also, three of the five OTUs specific to mucoids found > 1.0 % relative abundance are typically mucosal-associated commensals but also opportunistic pathogens; therefore it is unknown whether their appearance only in mucoids indicates their role.

It is believed that the pandas’ diet plays a role in the occurrence of mucous stools, as previous efforts to enhance the nutritional value of their diet through the increase in protein and decrease in fiber exacerbated the problem (Edwards et al., 2006). Giant pandas undergo a dietary shift in the spring from a leaf to culm diet and in the summer from an almost exclusively culm diet to leaf (Figure 6.1) (Hansen et al., 2010, Williams et al., 2012). Although mucoids have been observed throughout the year, they have been more frequently observed in the summer months (Williams, 2011), and this dietary shift could have profound effects on the GIT microbiota. Liu et al. (2014) reported caecal mucosal injury in goats when their diet decreased in fiber content. In this study, goats were sampled after the diet change, and found significant differences in the bacterial community, mucosal morphology, and caecal environment with respect to diet (Liu et al., 2014). With the appearance of mucoids following a diet shift in giant pandas, the similarities cannot be ignored. It is possible that the diet change observed in giant pandas may also lead to mucosal injury and subsequent expulsion of mucosa. However, giant pandas are a well-protected, endangered species, and working with species like these is
extremely difficult. We cannot determine the exact mechanism leading to mucus excretions in giant pandas, as sampling the GIT mucosa from a healthy giant panda could not be conducted.

Overall, our work reveals a shared core-community, but an overall significantly different microbiota associated with mucoids and normal feces of giant pandas. With this evidence of differing microbial communities, combined with the physical symptoms of the GIT distress in giant pandas, we propose that a dietary shift leading to dysbiosis may be responsible for mucoid episodes. As the first work to characterize the mucoid-associated microbiota in giant pandas, it also serves as the first step to elucidating the mechanism behind an issue that affects the overall health of these endangered species.
References


Johansson ME, Larsson JM, Hansson GC. 2011. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host–microbial interactions. Proceedings of the national academy of sciences108:4659–4665.


Williams, CL. (2011). The effect of dietary changes on microbial populations within the gastrointestinal tract of the giant panda (Ailuropoda melanoleuca) [electronic resource]. Mississippi State: Mississippi State University, 2011.


APPENDIX A

CHAPTER II SUPPLEMENTARY MATERIAL
Supplementary Figures

Figure A.1  Rarefaction results based on operational taxonomic units (OTUs) for all fecal samples.

Figure A.2  Three-dimensional principal coordinate analysis of microbial diversity using the unweighted UniFrac metric

Notes: For a) age and b) sex-related differences.
The percentage of variation is explained by PC1, PC2, and PC3 are indicated on the axis.
APPENDIX B

CHAPTER III SUPPLEMENTARY MATERIAL
Supplementary Table

Table B.1  Diet composition during each sampling period.

<table>
<thead>
<tr>
<th>Weaning Stage</th>
<th>Date</th>
<th>Age (days)</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>8/08/2012</td>
<td>38</td>
<td>Milk replacer (Esiblac)</td>
</tr>
<tr>
<td>Stage 2</td>
<td>10/29/2012</td>
<td>120</td>
<td>Milk replacer, leaf eater diet (Mazuri), bamboo introduced</td>
</tr>
<tr>
<td>Stage 3</td>
<td>2/12/2013</td>
<td>226</td>
<td>Bamboo, leaf eater diet</td>
</tr>
</tbody>
</table>

Supplementary Figures

Figure B.1  Rarefaction results based on 95 % operational taxonomic units (OTUs) for all fecal samples.
Figure B.2  Three-dimensional NMDS analysis examining bacterial community differences of weaning stages

(lowest stress: 0.1655, R-square: 0.6319).
APPENDIX C

CHAPTER IV SUPPLEMENTARY MATERIAL
### Supplementary Table

Table C.1 Complete list of individuals in the study.

<table>
<thead>
<tr>
<th>Studbook number</th>
<th>Name</th>
<th>Location</th>
<th>Sex</th>
<th>Date of birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>1107</td>
<td>Pete</td>
<td>Memphis Zoo</td>
<td>Male</td>
<td>6/25/11</td>
</tr>
<tr>
<td>1219</td>
<td>Justin</td>
<td>Memphis Zoo</td>
<td>Male</td>
<td>7/1/12</td>
</tr>
<tr>
<td>1215</td>
<td>Lucille</td>
<td>Memphis Zoo</td>
<td>Female</td>
<td>7/25/12</td>
</tr>
<tr>
<td>9704</td>
<td>Panya</td>
<td>NZP-Front Royal</td>
<td>Female</td>
<td>6/09/97</td>
</tr>
<tr>
<td>0308</td>
<td>Rocco</td>
<td>NZP-Front Royal</td>
<td>Male</td>
<td>6/25/03</td>
</tr>
<tr>
<td>0814</td>
<td>Regan</td>
<td>NZP-Front Royal</td>
<td>Female</td>
<td>7/01/08</td>
</tr>
<tr>
<td>0521</td>
<td>Leo Mei</td>
<td>NZP-Front Royal</td>
<td>Female</td>
<td>6/10/05</td>
</tr>
<tr>
<td>1010</td>
<td>Angus</td>
<td>NZP-Front Royal</td>
<td>Male</td>
<td>6/23/10</td>
</tr>
<tr>
<td>1128</td>
<td>Yanhua</td>
<td>NZP-Front Royal</td>
<td>Female</td>
<td>7/05/11</td>
</tr>
<tr>
<td>1203</td>
<td>Sherman</td>
<td>NZP-Front Royal</td>
<td>Male</td>
<td>6/22/12</td>
</tr>
<tr>
<td>0707</td>
<td>Shama</td>
<td>NZP-Front Royal</td>
<td>Female</td>
<td>7/01/07</td>
</tr>
<tr>
<td>1235</td>
<td>Rusty</td>
<td>NZP-Front Royal</td>
<td>Male</td>
<td>7/11/12</td>
</tr>
</tbody>
</table>

### Supplementary Figures

![Rarefaction results based on 97 % operational taxonomic units (OTUs) for all fecal samples.](image)

Figure C.1 Rarefaction results based on 97 % operational taxonomic units (OTUs) for all fecal samples.
Figure C.2  Relative sequence abundance at the phyla level for each season.

Figure C.3  Relative sequence abundance at the class level for each season.
Figure C.4 Three-dimensional principal coordinate analysis for Lucille
Using the a) weighted and b) unweighted UniFrac metric.
Figure C.5  Three-dimensional principal coordinate analysis for Justin
Using the a) weighted and b) unweighted UniFrac metric.
Figure C.6  Three-dimensional principal coordinate analysis for Pete
Using the a) weighted and b) unweighted UniFrac metric.
Figure C.7  Three-dimensional principal coordinate analysis for Leo Mei
Using the a) weighted and b) unweighted UniFrac metric.
Figure C.8  Three-dimensional principal coordinate analysis for Panya
Using the a) weighted and b) unweighted UniFrac metric.
Figure C.9  Three-dimensional principal coordinate analysis for Reagan
Using the a) weighted and b) unweighted UniFrac metric.
Figure C.10  Three-dimensional principal coordinate analysis for Rocco Using the a) weighted and b) unweighted UniFrac metric.
Figure C.11  Three-dimensional principal coordinate analysis for Sherman
Using the a) weighted and b) unweighted UniFrac metric.
Figure C.12  Three-dimensional principal coordinate analysis for Yanhua
Using the a) weighted and b) unweighted UniFrac metric.
Figure C.13  Three-dimensional principal coordinate analysis for Angus
Using the a) weighed and b) unweighted UniFrac metric.
Figure C.14  Three-dimensional principal coordinate analysis for Rusty
Using the a) weighted and b) unweighted UniFrac metric.
Figure C.15  Three-dimensional principal analysis for Shama

Using the a) weighted and b) unweighted UniFrac metric.
Figure C.16  Percentage relative abundance of important shared OTUs throughout four seasons.

Figure C.17  Percentage relative abundance of the significantly important phyla throughout the four seasons.
Figure C.18  Percentage relative abundance of significantly important orders throughout the four seasons.

Figure C.19  Percentage relative abundance of significantly important families throughout the four seasons.
APPENDIX D

CHAPTER V SUPPLEMENTARY MATERIAL
Figure D.1 Rarefaction results based on 97% operational taxonomic units (OTUs) for all samples.
Figure D.2 Relative sequence abundance at the class level for panda comparisons.

Figure D.3 Relative sequence abundance at the order level for panda comparison.
Figure D.4  Three-dimensional principal coordinate analysis of microbial diversity using the unweighted UniFrac metric for panda comparison.

Notes: The percentage of variation is explained by PC1, PC2, and PC3 are indicated by the axes (total: 32.5 %).
APPENDIX E

CHAPTER VI SUPPLEMENTARY MATERIAL
Supplementary Figures

Figure E.1  Rarefaction results based on 97% operational taxonomic units (OTUs) for all samples.
Figure E.2  Relative sequence abundance at the phyla level for each sample-type.

Figure E.3  Relative sequence abundance at the class level for each sample-type.
Figure E.4  Three-dimensional principal coordinate analysis of microbial diversity using the unweighted UniFrac metric.

Notes: the percentage of variation is explained by PC1, PC2, and PC3 are indicated on the axes (total: 20.32 %).
Figure E.5  Percent relative abundance of the significantly important phyla for both the a) male and b) female giant panda.
Figure E.6  Percent relative abundance of significantly important classes for both the a) male and b) female giant pandas.
Figure E.7  Percent relative abundance of the significantly important orders For both the a) male and b) female giant panda.
APPENDIX F
DATA ANALYSIS SUPPLEMENTARY MATERIAL
Sequencing Analysis

Pyrosequencing batch file example:

```bash
sffinfo(sff=plate1.sff, flow=t, trim=t, fasta=t)

trim.flows(flow=plate1.flow, oligos=red1.txt, pdiffs=2, bdiffs=0, fasta=T, minflows=450,
            maxflows=450)

shhh.flows(file=plate1.flow.files)

trim.seqs(fasta=plate1.shhh.fasta, name=plate1.shhh.names, oligos=red1.txt, pdiffs=2,bdiffs=0,
           maxhomop=6, minlength=250, flip=T)

sffinfo(sff=plate2.sff, flow=t, trim=t, fasta=t)

trim.flows(flow=plate2.flow, oligos=red2.txt, pdiffs=2, bdiffs=0, fasta=T, minflows=450,
            maxflows=450)

shhh.flows(file=plate2.flow.files)

trim.seqs(fasta=plate2.shhh.fasta, name=plate2.shhh.names, oligos=red2.txt, pdiffs=2,bdiffs=0,
           maxhomop=6, minlength=250, flip=T)

merge.files(input=plate1.shhh.trim.fasta-plate2.shhh.trim.fasta, output=red.panda.fasta)

merge.files(input=plate1.shhh.trim.names-plate2.shhh.trim.names, output=red.panda.names)

merge.files(input=plate1.shhh.groups-plate2.shhh.groups, output=red.panda.groups)

count.groups(group=red.panda.groups)

unique.seqs(fasta=Red.panda.fasta, name=Red.panda.names)

align.seqs(fasta=Red.panda.unique.fasta, reference=silva.all.fasta, flip=T)

screen.seqs(fasta=Red.panda.unique.align, name=Red.panda.names, group=Red.panda.groups,
            minlength=250, end=41790)

filter.seqs(fasta=Red.panda.unique.good.align, vertical=T, trump=.)

unique.seqs(fasta=Red.panda.unique.good.filter.fasta, name=Red.panda.good.names)
```
pre.cluster(fasta=Red.panda.unique.good.filter.unique.fasta,
          name=Red.panda.unique.good.filter.names, group=Red.panda.good.groups, diffs=2)

chimera.uchime(fasta=Red.panda.unique.good.filter.unique.precluster.fasta,
               name=Red.panda.unique.good.filter.unique.precluster.names,
               group=Red.panda.good.groups, processors=1)

remove.seqs(accnos=Red.panda.unique.good.filter.unique.precluster.uchime.accnos,
            fasta=Red.panda.unique.good.filter.unique.precluster.fasta,
            name=Red.panda.unique.good.filter.unique.precluster.names,
            group=Red.panda.good.groups)

classify.seqs(fasta=Red.panda.unique.good.filter.unique.precluster.pick.fasta,
              template=nogap.all.fasta, taxonomy=silva.all.silva.tax, cutoff=60, processors=1)

remove.lineage(fasta=Red.panda.unique.good.filter.unique.precluster.pick.fasta,
               name=Red.panda.unique.good.filter.unique.precluster.pick.names,
               group=Red.panda.good.pick.groups,
               taxonomy=Red.panda.unique.good.filter.unique.precluster.pick.silva.wang..taxonomy,
               taxon=Archaea;-Bacteria;Cyanobacteria;-Eukaryota;-unknown;)

system(copy Red.panda.unique.good.filter.unique.precluster.pick.pick.fasta Red.panda.final.fasta)

system(copy Red.panda.unique.good.filter.unique.precluster.pick.pick.names
       Red.panda.final.names)

system(copy Red.panda.good.pick.pick.groups Red.panda.final.groups)

summary.seqs(fasta=Red.panda.final.fasta, name=Red.panda.final.names)

count.groups(group=Red.panda.final.groups)

dist.seqs(fasta=Red.panda.final.fasta, output=lt)

cluster(phylip=Red.panda.final.phylip.dist, name=Red.panda.final.names, method=nearest,
        cutoff=0.25)
make.shared(list=Red.panda.final.phylip.nn.list, group=Red.panda.final.groups, label=0.05)
classify.seqs(fasta=Red.panda.final.fasta, group=Red.panda.final.groups,
              name=Red.panda.final.names, template=nogap.all.fasta, taxonomy=silva.all.silva.tax,
              cutoff=60, processors=2)
classify.otu(list=Red.panda.final.phylip.nn.list, taxonomy=Red.panda.final.silva.wang..taxonomy,
              group=Red.panda.final.groups, name=Red.panda.final.names, label=0.05, cutoff=60,
              basis=otu, probs=F)
rarefaction.single(shared=Red.panda.final.phylip.nn.shared, groupmode=F, label=0.05)
summary.single(shared=Red.panda.final.phylip.nn.shared, label=0.05, calc=coverage-
              bergerparker-chao-ace-shannon-simpson)
tree.shared(shared=Red.panda.final.phylip.nn.shared, subsample=T, iters=1000,
           calc=morisitahorn, label=0.05)
clearcut(phylip=Red.panda.final.phylip.dist)
sub.sample(fasta=Red.panda.final.fasta, group=Red.panda.final.groups,
           name=Red.panda.final.names, persample=T, size=1605)
dist.seqs(fasta=Red.panda.final.subsample.fasta, output=lt)
cluster(phylip=Red.panda.final.subsample.phylip.dist, name=Red.panda.final.subsample.names,
       method=nearest, cutoff=0.25)
making.shared(list=Red.panda.final.subsample.phylip.nn.list,
             group=Red.panda.final.subsample.groups, label=0.05)
pca(shared=Red.panda.final.subsample.phylip.nn.shared)

unifrac.unweighted(tree=Red.panda.final.phylip.tre, name=Red.panda.final.names,
                    group=Red.panda.final.groups, distance=lt, random=t, processors=1)
unifrac.weighted(tree=Red.panda.final.phylip.tre, name=Red.panda.final.names,
group=Red.panda.final.groups, distance=lt, random=t, processors=1)
unifrac.unweighted(tree=Red.panda.final.phylip.tre, name=Red.panda.final.names,
group=Red.panda.final.groups, distance=lt, processors=1, random=f, subsample=t)
pcoa(phylip=Red.panda.final.phylip.1.unweighted.ave.dist)
unifrac.weighted(tree=Red.panda.final.phylip.tre, name=Red.panda.final.names,
group=Red.panda.final.groups, distance=lt, processors=1, random=f, subsample=t)
pcoa(phylip=Red.panda.final.phylip.tre1.weighted.ave.dist)
ccluster(phylip=red.panda.final.pick.phylip.nn.list, name=red.panda.final.pick.names,
method=nearest, cutoff=0.25)
make.shared(list=red.panda.final.pick.phylip.nn.list, group=red.panda.final.pick.groups,
label=0.05)
Dist.shared(shared=red.panda.final.phylip.nn.shared)
nmds(phylip=red.panda.final.pick.phylip.nn.jclass.0.05.lt.dist, iters=2000000, maxdim=3)
MiSeq batch file example:

```sh
make.contigs(file=RPseasonal_16S.txt, processors=12)

summary.seqs(fasta=RPseasonal_16S.trim.contigs.fasta)

screen.seqs(fasta=RPseasonal_16S.trim.contigs.fasta,
            group=RPseasonal_16S.contigs.groups, maxambig=0, minlength=250,
            maxlength=600, processors=12)

unique.seqs(fasta=RPseasonal_16S.trim.contigs.good.fasta)

count.seqs(name=RPseasonal_16S.trim.contigs.good.names,
           group=RPseasonal_16S.contigs.good.groups)

summary.seqs(fasta=current, count=current)

align.seqs(fasta=RPseasonal_16S.trim.contigs.good.unique.fasta,
           reference=~/.silva/silva.nr_v119.align, flip=T, processors=12)

summary.seqs(fasta=current, count=current)

screen.seqs(fasta=RPseasonal_16S.trim.contigs.good.unique.align,
            count=RPseasonal_16S.trim.contigs.good.count_table,
            summary=RPseasonal_16S.trim.contigs.good.unique.summary, start=6388,
            end=25316, maxhomop=8, processors=12)

summary.seqs(fasta=current, count=current)

filter.seqs(fasta=RPseasonal_16S.trim.contigs.good.unique.good.align, vertical=T,
            trump=., processors=12)

unique.seqs(fasta=RPseasonal_16S.trim.contigs.good.unique.good.filter.fasta,
            count=RPseasonal_16S.trim.contigs.good.good.count_table)
```

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summary.seqs(fasta=current, count=current)

count.groups(count=current)

split.abund(fasta=RPseasonal_16S.trim.contigs.good.unique.good.filter.unique.precluster.
pick.pick.fasta,
    count=RPseasonal_16S.trim.contigs.good.unique.good.filter.unique.precluster.uc
    hime.pick.pick.count_table, cutoff=10)

system(cp
    RPseasonal_16S.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.
abund.fasta RPseasonal_16S.final.fasta)

system(cp
    RPseasonal_16S.trim.contigs.good.unique.good.filter.unique.precluster.uchime.pi
ck.pick.abund.count_table RPseasonal_16S.final.count_table)

count.groups(count=RPseasonal_16S.final.count_table)

dist.seqs(fasta=RPseasonal_16S.final.fasta, output=lt, processors=12)

cluster(phylip=RPseasonal_16S.final.phylip.dist,
    count=RPseasonal_16S.final.count_table, method=nearest, cutoff=0.25)

make.shared(list=RPseasonal_16S.final.phylip.nn.unique_list.list,
    count=RPseasonal_16S.final.count_table, label=0.03)

summary.single(shared=RPseasonal_16S.final.phylip.nn.unique_list.shared, label=0.03,
    calc=coverage)

rarefaction.single(shared=RPseasonal_16S.final.phylip.nn.unique_list.shared,
    groupmode=F, label=0.03)
summary.single(shared=RPseasonal_16S.final.phylip.nn.unique_list.shared, label=0.03, calc=coverage-bergerparker-chao-ace-shannon-simpson)

tree.shared(shared=RPseasonal_16S.final.phylip.nn.unique_list.shared, subsample=T, iters=1000, calc=morisitahorn, label=0.03)

clearcut(phylip=RPseasonal_16S.final.phylip.dist)

count.groups(count=RPseasonal_16S.final.count_table)

sub.sample(fasta=RPseasonal_16S.final.fasta, count=RPseasonal_16S.final.count_table, persample=T, size=9934)

dist.seqs(fasta=RPseasonal_16S.final.subsample.fasta, output=lt)

cluster(phylip=RPseasonal_16S.final.subsample.phylip.dist, count=RPseasonal_16S.final.subsample.count_table, method=nearest, cutoff=0.25)

make.shared(list=RPseasonal_16S.final.subsample.phylip.nn.unique_list.list, count=RPseasonal_16S.final.subsample.count_table, label=0.03)

pca(shared=RPseasonal_16S.final.subsample.phylip.nn.unique_list.shared)

unifrac.unweighted(tree=RPseasonal_16S.final.phylip.tre, count=RPseasonal_16S.final.count_table, distance=lt, random=t, processors=12)

unifrac.weighted(tree=RPseasonal_16S.final.phylip.tre, count=RPseasonal_16S.final.count_table, distance=lt, random=t, processors=12)

unifrac.unweighted(tree=RPseasonal_16S.final.phylip.tre, count=RPseasonal_16S.final.count_table, distance=lt, processors=12, random=f, subsample=t)

pcoa(phylip=RPseasonal_16S.final.phylip.1.unweighted.ave.dist)
unifrac.weighted(tree=RPseasonal_16S.final.phylip.tre,
               count=RPseasonal_16S.final.count_table, distance=lt, processors=12, random=f,
               subsample=t)

pcoa(phylip=RPseasonal_16S.final.phylip.tre1.weighted.ave.dist)
Statistical Analysis

Example t-test code

dm 'LOG; CLEAR; OUTPUT;CLEAR';
OPTIONS PS=55 LS=85;
DATA RED PANDA;
  length id $4;
  INPUT ID $ SEX $ AGE $ GEO $ TIME LACTO CLOST ENTERO bac camp actino pseudo flavo burk sphing sphingomon gammaproteo bacteroid rhodobact gem un erysipel firmicute sc4 acid bdell bifido caulo tm fuso rhizo soliru bacill;
datalines;
;
"DATA"
RUN;

PROC UNIVARIATE alpha=.05 NORMAL;
VAR LACTO CLOST ENTERO ;
run;/*
proc ttest sides=2 alpha=.05 h0=0;
title "two sample t-test example";
class age;
var LACTO CLOST ENTERO bac camp actino pseudo flavo burk sphing sphingomon gammaproteo bacteroid rhodobact gem un erysipel firmicute sc4 acid bdell bifido caulo tm fuso rhizo soliru bacill; run; /*proc ttest sides=2 alpha=.05 h0=0;
title "two sample t-test example";
class geo;
var LACTO CLOST ENTERO bac camp actino pseudo flavo burk sphing sphingomon gammaproteo bacteroid rhodobact gem un erysipel firmicute sc4 acid bdell bifido caulo tm fuso rhizo soliru bacill; run;*/
DATA RP_ALL;
  length id $4;
  INPUT ID $ SEX $ AGE $ GEO $ TIME LACTO CLOST ENTERO bac camp actino pseudo flavo burk sphing sphingomon gammaproteo bacteroid rhodobact gem un erysipel firmicute sc4 acid bdell bifido caulo tm fuso rhizo soliru bacill;
datalines;
;
"DATA"
run;
proc ttest data=rp_ALL sides=2 alpha=.05 h0=0;
title "two sample t-test example";
class sex;
var LACTO CLOST ENTERO bac camp actino pseudo flavo burk sphing sphingomon gammaproteo bacteroid rhodobact gem un erysipel firmicute sc4 acid bdell bifido caulo tm fuso rhizo soliru bacill; run;
Example PROC GLM code

```
dm 'LOG; CLEAR; OUTPUT; CLEAR';
OPTIONS PS=55 LS=85;
DATA REDPANDA;
   length id $4;
   INPUT ID $ Time age $ sex $ LACTO CLOST ENTERO Staph Lachno
   Helico Actino Pseudo;
datalines;

   "DATA"

RUN;

PROC GLM;
CLASS TIME;
MODEL LACTO CLOST ENTERO staph lachno helico actino pseudo = TIME;
LSMEANS TIME/PDIFF LINES;
RUN;
```
Step-wise and Discriminant Analysis

OPTIONS PS=55 LS=85;
DATA Seasonal_phyla;
INPUT ID $ TYPE $ Actinobacteria Bacteroidetes
   Candidate_division_TM7 Chloroflexi Firmicutes Fusobacteria
   Planctomycetes Proteobacteria;
CARDS;

“DATA”

RUN;
PROC MEANS SUM MEAN CSS MAXDEC=3 FW=10;
VAR Actinobacteria Bacteroidetes Candidate_division_TM7
    Chloroflexi Firmicutes Fusobacteria Planctomycetes
    Proteobacteria;
CLASS TYPE;
RUN;
PROC STEPDISC slentry=0.1 slstay=0.1;
class TYPE;
var Actinobacteria Bacteroidetes Candidate_division_TM7
    Chloroflexi Firmicutes Fusobacteria Planctomycetes
    Proteobacteria;
run;
PROC DISCRIM pool=test crosslisterr listerr crossvalidate manova;
class Type;
var Firmicutes Actinobacteria ;
run;
quit;
Rarefaction

As sample sizes are not always equal, rarefactions aid to cope with the difficulty by working out the number of species that would be expected in samples of a standard size, the formula:

\[ E(S) = \sum \left(1 - \left(\frac{N - n_i}{n}\right)\right) \]

\[ \text{G.1} \]

Where:

- \( E(S) \) = the expected number of species in the rarefied sample
- \( n \) = standardized sample size
- \( N \) = the total number of individuals recorded in the sample to be rarefied
- \( N_i \) = the number of individuals in the \( i^{th} \) species in the sample to be rarefied

Indices based on the proportional abundances of species

Information statistic indices

Shannon diversity index

As an information statistic index, the Shannon diversity index (H’) assumes that individuals are randomly sampled from an ‘indefinitely large,’ that is an effectively infinite population. The index also assumes that all species are represented in the sample. Typical index values fall between 1.5 and 3.5, with rare observations surpassing 4.5.

The index is calculated with the formula:

\[ H' = -\sum p_i \ln p_i \]

\[ \text{G.2} \]

Where: \( p_i \) = the proportion of individuals found in the \( i^{th} \) species.

Dominance Measures
**Simpson diversity index**

As a dominance measure, the Simpson diversity index (D) gives the probability of any two individuals drawn at random from an infinitely large community belonging to different species, as defined by the equation:

\[ D = \sum p_i^2 : \sum \left( \frac{n_i (n_i - 1)}{N(N-1)} \right) \]

**Where:** \( n_i = \) the number of individuals in the ith species

\( N = \) the total number of individuals

As D increases, diversity decreases; therefore the Simpson index is usually expressed as 1 - D or 1/D. Simpson’s index is heavily weighted towards the most abundant species in the sample while being less sensitive to species richness.

**Berger-Parker index**

The Berger-Parker index (d) is an intuitively simple dominance measure with the virtue of being easy to calculate. The Berger-Parker index expresses the proportional importance of the most abundant species, with the formula:

\[ d = \frac{N_{\text{max}}}{N} \]

**Where:** \( N = \) total number of individuals

\( N_{\text{max}} = \) the number of individuals in the most abundant species