Exploring factors governing the gut microbiome of Japanese macaques

ニホンザルにおける腸内細菌叢の変動要因

Lee Wan Yi
李婉儀

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Chapter 1 General Introduction

1.1 Overview on mammalian gut microbiome

The mammalian gastrointestinal tract harbors a complex ecosystem that made up of a variety of microorganisms, collectively known as the gut microbiome. By estimation, gut microbiome encodes about three million genes, 150 times larger than that of the human genome (Qin et al., 2010). The gut microbiome has been shown actively involved in multiple physiological processes of the mammals, ranging from immune system to behavior (Clayton, Gomez, et al., 2018; Round & Mazmanian, 2009; Rowland et al., 2018). Especially for the herbivorous and omnivorous mammals, its contribution to energy harvest from the fibrous foods has been the most noted and well-studied (Lambert, 1998; Mackie, 2002; Stevens & Hume, 1998; Yamauchi & Iwasa, 1995). While plant materials make up an indispensable part of the mammals' daily diet, mammals lack the ability to directly extract nutrient and energy. In particular, the digestion of the plant materials is hindered by the fiber in the plant cell walls, since mammals themselves do not produce the essential digestive enzyme for breaking down the fiber (Theander, Westerlund, Åman, & Graham, 1989). Instead, mammals rely on the gut microbiome to process the indigestible plant materials. Through fermentation, the gut microbes transform fiber and the other indigestible materials into short-chain fatty acids (SCFAs) and other nutrients, which are then absorbed by the host animals (Bugaut, 1987; Bugaut & Bentéjac, 1993; den Besten et al., 2013). For animals that depend on plant material as the main component of their diet, gut microbiome and its digestive function are vital for their survival (Lambert, 1998; Mackie, 2002; Stevens & Hume, 1998).

The selection pressure on harboring a gut microbiome in turn facilitates morphological adaptations of the herbivorous/omnivorous mammals. In general, there are two forms of fermentation – foregut and hindgut fermentation – depending on where the main fermentation chamber situates (Stevens & Hume, 1998). For foregut fermenters, fermentation is carried out in an enlarged, compartmentalized stomach. Foods were first fermented in the modified stomach chamber before the enzymatic digestion by the hosts. This is commonly seen in the ruminants such as deer and cattle, as well as in the nonruminants such as hippos, sloths and macropod marsupials. Whereas for hindgut fermenters such as horses and rhinoceros, fermentation activity mainly occurs in the enlarged colon or cecum. After being ingested, food materials go through enzymatic digestion in the stomach, after which the remaining undigested part enters the hindgut for fermentation. For either strategy, the duration that foods are retained in the gut (food retention time) determines the degree of the microbial fermentation, as time available for fermentation and absorption of SCFA increases with retention time (Clauss, Jürgen Streich, Schwarm, Ortmann, & Hummel, 2007; Edwards & Ullrey, 1999; Stevens & Hume, 1998; Yamauchi & Iwasa, 1995). In respect to this, foregut fermenting mammals generally display longer retention time than the hindgut fermenting mammals, enabling them to more efficiently exploit diet of lower quality (Clauss et al., 2007, 2008; Edwards & Ullrey, 1999; Hume & Sakaguchi, 1991).

1.2 Nonhuman primates and their gut microbiome

Nonhuman primates (hereafter primates) consume a wide range of plant materials, including high-quality foods like fruits and low-quality foods like mature leaves and barks. Although high-quality foods are preferentially consumed, availability of high-

quality foods varies across time and habitats. This is when the low-quality foods come into play. Low-quality foods typically contain high fiber and thus require extra processing effort (Marshall & Wrangham, 2007). Similar to the other mammals, primates depend on gut microbiome to regularly exploit the low-quality, fibrous food materials (Lambert, 1998). Interestingly with the order Primate, both forms of fermentation exist. Facilitated by their enlarged, compartmentalized stomach, members of subfamily Colobinae are the only primate taxon capable of foregut fermentation. In contrast to the foregut fermenting Colobinae, most primate species are hindgut fermenters, using an enlarged cecum or colon as the primary fermentation chamber.

In the past few decades, primates and their gut microbiome have drawn researchers' attention. This is not only because of its implication on anthropology, but also because of its value in understanding mammalian ecology. For example, howler monkeys (*Alouatta pigra*) derive energy through gut microbiome and produced SCFAs during the extreme dietary shifts (Amato, Leigh, et al., 2015) and across different life stages (Amato et al., 2014). With limited changes in activity budget, howlers have relied greatly on gut microbiome in "compensating" the nutritional demand. Not limiting to the howler monkey, the contribution of gut microbiome to host nutrition, especially in food-scarce seasons, has been widely observed in the wild primates, including western lowland gorillas (Hicks et al., 2018; Sharma et al., 2020), saddleback tamarin (Garber, Mallott, Porter, & Gomez, 2019), Verreaux's sifakas (Springer et al., 2017), Tibetan macaques (Sun et al., 2016; Xia et al., 2021) and gelada (Baniel et al., 2021). Together, these studies reveal how gut microbiome contributes the host nutrition, while suggesting the potential role of gut microbiome in

facilitating primate dietary flexibility. Despite the contributions of gut microbiome, mechanism shaping primate gut microbiome remains obscure. Such knowledge on primate gut microbiome can offer critical perspective in understanding primate feeding ecology.

1.3 Japanese macaques and their feeding ecology

Japanese macaques (*Macaca fuscata*) are undoubtedly one of the best-studied primates – since 1948, there have been extensive studies on the feeding ecology of Japanese macaques based on the free-ranging and captive individuals (Yamagiwa, 2010). They distribute widely across Japanese archipelago from as north as Shimokita Peninsula (41°N) to as south as Yakushima Island (30°N), inhabiting a range of habitats including cool-temperate deciduous forests, warm-temperate evergreen forest and alpine grassland (Tsuji, 2010). Highly flexible, Japanese macaques also thrive well in habitats associated with human activities, such as captivity, farmlands and monkey parks (Muroyama & Yamada, 2010; Yamagiwa, 2010). Thriving in various habitats, Japanese macaques are astonishingly flexible in their feeding behavior, making them the most suitable study subject for host-gut microbiome relationship in primates.

Living at the northern limits of the primate global range, Japanese macaques inhabit the marginal habitat for the primates. Compared to the tropical forests, temperate forests are characterized by lower fruit production, as well as stronger but predictable seasonality (Hanya & Aiba, 2010; Hanya, Tsuji, & Grueaer, 2013). As high-quality foods, fruits and seeds are preferentially consumed by the macaques whenever available. When fruits and seeds are scarce, the macaques feed on mature

leaves and barks, contrasting with the tropical primates which fallback to fig syconia and young leaves (Hanya et al., 2011; Tsuji, Hanya, & Grueter, 2013). Within the species of Japanese macaques, there is a clear difference in fallback foods between the cool-temperate deciduous forests and warm-temperate evergreen forests. Macaques inhabiting the cool-temperate deciduous forest feed more on barks and dormant buds, whereas those in warm-temperate evergreen forest feed more on the mature leaves (Agetsuma & Nakagawa, 1998; Hanya, 2004a; D. A. Hill, 1997; Tsuji, Ito, Wada, & Watanabe, 2015). In any habitat type, consumption of fibrous, low-quality foods is necessary for the macaques to survive food-scarce seasons. In some areas, fiber-rich foods make up as much as 45% of annual feeding time of Japanese macaques (Hanya, 2004a). Even though their dependence on fibrous foods is comparable to that of the colobus (Hanya et al., 2011), Japanese macaques do not exhibit anatomical specialization for folivory as do the colobus. The gut microbiome may play an important role in macaques' adaptation to these marginal habitats, along with other adaptations like foraging behavior and fat deposition (Hamada, Hayakawa, Suzuki, Watanabe, & Ohkura, 2003; Kurihara, Kinoshita, Shiroishi, & Hanya, 2020).

The studies so far have revealed how macaques flexibly adapt to dietary variation across habitats and seasons through their foraging behaviors. The gut microbiome of Japanese macaques (and other primates) has yet to be investigated in depth (Clayton, Gomez, et al., 2018). Understanding of macaques' gut microbiome will not only offer basic information to the feeding ecology of Japanese macaques but also provide insights to primate's radiation from tropical to temperate regions.

1.4 Outline of this thesis

Using Japanese macaques, this thesis aims to advance our understandings for the primate gut microbiome. In particular, I investigate the ecological factors shaping gut microbiome of Japanese macaques at the individual and population level.

- Chapter 2 is dedicated to exploring the variation of gut microbiome within the individual between two gut sites – the stomach and colon. Through this chapter, I aim to understand how gut microbiome adapts to different digestive organ, and how this process may influence the distribution pattern and functions of gut microbes within an individual.
- Chapter 3 aims to compare the gut microbiome among different populations of Japanese macaques. Categorizing macaque populations into captive, provisioned, crop-raiding and wild, I compared the gut microbiome of the macaques from each category. Influencing by the macaques' interaction with humans living close by, these populations vary in their diet quality. In this chapter, I aim to understand how long-term dietary changes may influence the primate gut microbiome composition.

Finally, Chapter 4 provides a summary of the findings in Chapters 2 and 3. I will discuss the implications of this thesis in understanding the mechanism shaping primate gut microbiome. This chapter will also point out the implication of this thesis towards feeding ecology of Japanese macaques, a primate species inhabiting marginal habitats, and how this knowledge applies to other primate taxa. Finally, I will talk about some prospects to advance our understanding in the field.

Chapter 2 Stomach and colonic microbiome of wild Japanese macaques

Abstract

Within the gastrointestinal tract, the physiochemical microenvironments are highly diversified among the different stages of food digestion. Accordingly, gut microbiome composition and function vary at different gut sites. In this study, we examine and compare the compositional and functional potential between the stomach and colonic microbiome of wild Japanese macaques (*Macaca fuscata yakui*) living in the evergreen forest of Yakushima Island. We find a significantly lower microbial diversity in the stomach than in the colon, possibly due to the stomach's acidic and aerobic environment, which is suboptimal for microbial survival. According to past studies, the microbial taxa enriched in the stomach are aero- and acid-tolerant. By functional prediction through PICRUSt2, we reveal that the stomach microbiome is more enriched in pathways relating to the metabolism of simple sugars. On the contrary, the colonic microbiota is more enriched with fiber-degrading microbes, such as those from Lachnospiracea, Ruminococcaceae and *Prevotella*. Our study shows a clear difference in the microbiome between the stomach and colon of Japanese macaques in both composition and function. This study provides a preliminary look at the alpha diversity and taxonomic composition within the stomach microbiome of Japanese macaques, a hindgut-fermenting non-human primate.

Introduction

Along the GI tract, the microbiome typically diversifies in relation to the digestive functions (mechanical, chemical, and microbial breakdown) and corresponding physiochemical environment at different gut sites (Gu et al., 2013; Hillman, Lu, Yao, & Nakatsu, 2017; D. Li, Chen, Zhao, Zhang, & Chen, 2019). For example, the microbial community in the upper GI tract is likely suited to the breakdown of simple sugars and proteins, while the microbiome in the lower GI tract is likely suited to complex plant polysaccharides. In addition, how different microbes adapt to the physiochemical environment at different gut sites may determine the acquisition/colonization mechanism of the gut microbiome (Merrell, Goodrich, Otto, Tompkins, & Falkow, 2003; Seedorf et al., 2014; Vega, 2019).

Many studies on the gut microbiome-host relationship have focused on the colonic microbiome, which plays a major role in fermentation. In the anaerobic environment of the colon, gut microbes carry out fermentation to transform food materials into short-chain fatty acid and other nutrients, serving as energy and nutritional source for the hosts. It is estimated that the colon alone contains over 70% of the bacteria residing in the body (in the case of humans (Jandhyala et al., 2015)). Compared to the other GI sites, which usually require invasive sampling, it is possible to study the colonic microbiome non-invasively using fecal samples. Therefore, despite the potential differences among GI sites, the gut microbiome studies have mainly focused on the microbial community in the colon/hindgut of animals (Clayton et al., 2019).

Compared with the colon, the stomach, which carries out chemical digestion, presents a different environment for most bacteria, including its low-pH environment

and short transit time. In past studies on humans and animals (captive rats, swine, mice, baboons and red-shanked doucs), bacterial diversity in the upper sections of the GI tract, such as the stomach, tends to be lower than that in the lower sections, such as the colon (Clayton et al., 2019; Stevens & Hume, 1998). Moreover, the function of microbes in the stomach is potentially different from that in the colon. For example, pathways related to environmental information processing increases in the upper GI tract of house mice, suggesting an active material exchange between gut microbes and the digestive organ (D. Li et al., 2019).

Despite the environmental differences between the stomach and colon, there have been few studies devoted to the stomach microbiome. An understanding of the stomach microbiome is, however, important in providing insights into how the animals acquire gut microbes and how the microbes distribute to the lower GI tract. Mammals are generally born with a sterile GI, and thus they acquire gut microbes from the environment. Even after acquisition, microbes vary in their ability to establish a population under various physiochemical environments across the GI tract. While some studies have pointed out the difference in microbiome between the stomach/foregut and colon/hindgut of the animals, the study subjects have only been a few species of nonhuman primates (NHPs), mostly with a focus on the captive foregut-fermenting species (e.g. red-shanked doucs (Amato, Metcalf, et al., 2016; Clayton et al., 2019), black and white colobus monkeys, and langurs (Amato, Metcalf, et al., 2016)). Despite the fact that most NHPs are hindgut fermenters, there is clearly a lack of knowledge on the diversity and distribution of microbial communities within the hindgut fermenting NHPs. Such knowledge would provide basic information regarding the gut microbiome of the hindgut fermenting NHPs. Furthermore,

comparing NHP gut microbiome of different fermentative strategies would improve our understanding of the special digestive adaptations of the foregut-fermenters and thus the evolutionary trajectory of primate feeding strategy.

In this work, we studied wild Japanese macaques (*Macaca fuscata yakui*) inhabiting warm-temperate evergreen forest in Yakushima Island, Japan, to understand the spatial difference in the gut microbiome between the stomach and colon. Japanese macaques feed on a considerable amount of mature leaves and other fibrous foods to survive the food-scarce seasons (Hanya, 2004a; Hanya, Noma, & Agetsuma, 2003; D. A. Hill, 1997; Kurihara et al., 2020). They spend approximately 35% of their annual feeding time on fibrous leaves and shoots (D. A. Hill, 1997). Of this, the neutral detergent fiber content of the major food leaves could be as high as 42% (Hanya, Kiyono, Takafumi, Tsujino, & Agetsuma, 2007). It is therefore critical to understand how the gut microbiome contributes to the macaques' nutrition while considering the macaques' intake of fibrous food items.

In this study we aimed (1) to examine and compare the microbiome compositions of Japanese macaques at two different gut sites, the stomach and colon, and then (2) to infer and compare the functions of the gut microbiome at different gut sites. Our hypothesis is that the stomach microbiome will be less diverse and related to environmental information processing and simple sugar metabolism, while the colonic microbiome will be more diverse and enriched with pathways involving fiber digestion. This study aims to improve our understanding of the hindgut-fermenting NHPs' gut microbiome, while focusing on the filtering effect imposed by different GI sites on the microbiome diversity and function.

Methods

Sample collection

We collected stomach content and colon samples from a total of 13 individual macaques inhabiting the coastal area of Yakushima Island, Japan (30°N, 131°E): males and females from each of the three troops (Umi A, Umi B and Umi C) during July 11-14, 2017, May 27-30, 2018, and September 25-28, 2019 (Table 2.1). We sampled each monkey only once. In 2017, we only collected colonic samples: one male and one female from Umi A and one female from Umi B. In 2018, we collected both stomach and colon samples from one male and one female from each of the three troops. In 2019, we collected stomach and colon samples from one male and one female from Umi A and Umi C. These individuals were captured for the purpose of attaching GPS collars. One of the co-authors (A. Kaneko), as a vet, darted the animals with VARIO 1V ® Telinject and anesthetized them with 40 or 60 mg of ketamine, 0.2 or 0.3 mg of medetomidine, 1 or 1.5 mg of midazolam, and 0.5 or 0.75 mg of atipamezole, assuming that body mass is 8 or 12 kg for adult females or males, respectively. We determined the amount of anesthetic based on data from previous captures for this population and the guidelines set by Primate Research Institute, Kyoto University (Cizauskas, 2008; Primate Research Institute, Kyoto University (KUPRI), 2010). After immobilization, we sampled stomach content by inserting a Nelaton catheter from the mouth into the stomach. For colonic (rectal) microbiome, we swabbed an 8-cm sterile cotton swab into the anus. We stored all the samples in 1-ml lysis buffer (0.5% SDS, 100 mM EDTA (pH 8.0), 100 mM Tris-HCl (pH 8.0), and 10 mM NaCl) at room temperature. We obtained permission for the capture of macaques and entry to the study sites from the Yakushima Forest Ecosystem Conservation

Center, Kagoshima Prefecture, and the Ministry of Environment, Japan, adhering to the legal requirements of Japan. We followed the approved capture and sampling protocol by the Field Research Committee of Primate Research Institute, Kyoto University (KUPRI) (#2017-009, #2018-002 and #2019-006). The entire project, including capture and sampling, followed the Guidelines for Field Research of KUPRI and the American Society of Primatologists Principles for Ethical Treatment of Non-Human Primates.

Sample storage, DNA purification, 16S rRNA amplification and sequencing

Our method followed Hayakawa et al. (2018) with slight modification. After bead-beating and centrifuging at 20,000 x *g* for 1 min, we mixed each sample with 1000 µl InhibitEX buffer of the QIAamp DNA Stool Mini Kit (QIAGEN GmbH, Hilden, Germany), then centrifuged the samples at 20,000 x g for 1 min. After that, we mixed 600 µl of the supernatant with 25 µl proteinase K and 600 µl Buffer AL. We followed the manufacturer's protocols to purify the fecal DNA. Using the Qubit dsDNA HS Assay Kit and a Qubit fluorometer (Thermo Fisher Scientific), we then estimated the DNA concentration for each sample. We amplified the V3-V4 region of the 16S rRNA gene with primers as follows: S-D-Bact-0341-b-S-17 (forward) 5′-CCT ACG GGN GGC WGC AG-3′ and S-D-Bact-0785-a-A-21 (reverse) 5′-GAC TAC HVG GGT ATC TAA TCC-3′ (Klindworth et al., 2013). To improve chastity in the Illumina platform, we fused these primers with the specific overhang adapters 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG-[3-6-mer Ns]-[forward primer]-3′ and 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G-[3-6-mer Ns]-[reverse primer]-3′, where

the 3-6-mer Ns (NNN, NNN N, NNN NN, or NNN NNN) were in the same quantity (Lundberg, Yourstone, Mieczkowski, Jones, & Dangl, 2013).

We purified the PCR product using Agencourt AMPure XP beads (Beckman Coulter, Inc., Carlsbad, CA, USA). Using the Illumina Nextera XT Index Kit, we attached specific dual indices and sequencing adapters to each amplicon by PCR. To make the pooled sequencing library, we mixed the PCR products at the same amount of DNA (2 ng/sample). Using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., La Jolla, CA, USA), we then estimated the fragment size distribution of the library. After diluting the library to 15 pM, we carried out the sequencing run with 30% PhiX spike-in on an Illumina MiSeq sequencing platform using the MiSeq Reagent Kit v3 (600 cycles). The read lengths from the MiSeq run were 301 bp (forward sequences), 8 bp (forward indices), 8 bp (reverse indices), and 301 bp (reverse sequences). We deposited the raw data in the DDBJ database with accession number DRA009571.

Data analysis

We processed the raw sequences with QIIME2-2019.10 (Bolyen et al., 2019). After demultiplexing according to the barcodes, we implemented quality control, denoising, chimera removal, and generation of amplicon sequence variants using the DADA2 pipeline (Callahan et al., 2016). The pipeline filtered out one stomach sample, UMI11, and one colonic sample, UMI21, due to low sequencing quality. We then determined phylogeny of the denoised amplicon specific variants (ASVs) using the q2-fragment insertion. To assign the taxonomy of the ASVs, we used QIIME2 naïve Bayes feature classifier trained against the Greengenes 13_8 reference database. Before analysis, we excluded ASVs classified as mitochondria or chloroplast from the dataset.

We plotted the rarefaction curves using the "ggrare" function of R package *ranacapa* (Kandlikar et al., 2018). To explore the functional difference between gut sites, we predicted the Kyoto Encyclopedia of Genes and Genome Orthology (KO) pathways through phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt2) (Langille et al., 2013) following guidelines at https://github.com/picrust/picrust2/wiki. By default, PICRUSt2 excluded all ASVs with the nearest sequenced taxon index (NSTI) value > 2 from the output. The average NSTI value of our dataset was 0.1901 ± SD 0.1805.

We performed statistical analyses in R v 3.6.1 with an alpha level of 0.05, with R packages *phyloseq* (McMurdie & Holmes, 2013), *vegan* (Oksanen et al., 2019), and *microbiome* (Lahti & Shetty, 2012). For analysis, we transformed the dataset to compositional abundance (i.e., % of total sequences per sample) using the "transform" function in package *microbiome.* We calculated alpha diversity through the "alpha" function in package *microbiome*. To test the effect of gut sites in alpha diversity (observed richness and Shannon index), we used the pairwise Wilcoxon rank sum test with P-adjustment using the false discovery rate (FDR) method. For beta diversity (weighted and unweighted UniFrac), we constructed principal coordinate analysis (PCoA) plots based on unweighted and weighted UniFrac distances calculated using ASVs. We then used the PERMANOVA test with the "adonis" function in package *vegan* (permutation = 999). To detect the bacterial taxa and KO pathways that were significantly different between stomach and colonic microbiota (log linear discriminant analysis (LDA) score > 2.0, $P < 0.05$), we carried out linear discriminant analysis of effect size (LEfSe) with the default parameters (Segata et al., 2011) available at

http://huttenhower.sph.harvard.edu/galaxy/. We conducted the LEfSe on bacterial taxa at level 6 (i.e., genus level).

Results

Sequencing result and basic characteristics of stomach and colonic microbiome

After quality filtering, we acquired 1,296,806 reads from 9 stomach and 12 colonic samples of Japanese macaques (Table 2.1). For the stomach samples, the average reads obtained per sample was 15,448 ± SD 13,119. For the colon samples, the average reads obtained per sample was 100,081 ± SD 118,889. The rarefaction plot for the samples showed that the sequencing depth was sufficient (Figure 2.1).

The stomach and colon did not share any ASVs. In the colon, the 1290 ASVs uncovered were from 14 phyla, 23 classes, 29 orders, and 46 families. The top three abundant phyla of the colonic microbiome were Firmicutes (74.48 ± SD 9.90 %), Bacteroidetes (12.04 ± SD 10.12 %), and Proteobacteria (4.61 ± SD 3.24 %) (Figure 2.2; Supplementary Table 2.2). In contrast, the top three abundant phyla of the stomach microbiota were Proteobacteria, Firmicutes, and Bacteroidetes, making up 70.07 ± SD 17.09 %, 20.79 ± SD 13.16 %, and 1.96 ± SD 2.29 % of the stomach microbiome (Figure 2.2; Supplementary Table 2.2). The 240 ASVs uncovered in the stomach were from 6 known phyla, 12 classes, 17 orders, and 21 families.

Alpha and beta diversity differed significantly between stomach and colon

Alpha diversity indices (observed richness and Shannon index) were significantly higher in the colon than in the stomach (pairwise Wilcoxon signed rank-sum test, P-adjustment by FDR: observed richness: V=27, p = 0.0313, Figure 2.3a;

Shannon index: V=28, p = 0.0156, Figure 2.3b). The average observed richness and Shannon index of the stomach microbiome were 30 ± SD 9.23 and 2.92 ± SD 0.29, respectively. On the contrary, average observed richness and Shannon index of the colonic microbiome were 119.55 ± SD 109.88 and 4.16 ± SD 0.86.

Principal coordinates analysis plot (PCoA) plots based on unweighted (Figure 2.4a) and weighted UniFrac distance (Figure 2.4b) revealed that samples form two distinctive clusters based on the gut site. In both plots, the colon samples were more scattered compared with the stomach samples. Adonis tests also suggested a significant effect of gut sites to the gut microbiome (Adonis: unweighted UniFrac: R^2 = 0.1029, p = 0.001; weighted UniFrac: R^2 = 0.2408, p = 0.001). Microbiota of the stomach and colon were different in both composition and abundance. The difference in microbial composition between gut sites overrode the difference caused by seasonal variation and/or identity of the individuals (i.e., troop and sex). We did not find any effect of the troop (Adonis: unweighted UniFrac: R^2 = 0.1144, p = 0.310; weighted UniFrac: R^2 = 0.1306, p = 0.249) or sex of the individuals (Adonis: unweighted UniFrac: R^2 = 0.0567, p = 0.354; weighted UniFrac: R^2 = 0.0308, p = 0.831). Since we collected the samples at different seasons/times of different years, unweighted UniFrac, but not on weighted UniFrac, was marginally significantly related to the year of collection (Adonis: unweighted UniFrac: R^2 = 0.0671, p = 0.048; weighted UniFrac: R^2 = 0.0620, p = 0.313). Close examination of datasets containing only stomach or colon samples, however, suggested little difference based on the year of collection (Adonis: stomach: R^2 = 0.1533, p = 0.298; colon: R^2 = 0.1023, p = 0.318). This marginal effect may be a result of the small sample size.

Taxonomy-based comparison between stomach and colonic microbiome

Through the LEfSe test, we detected the bacterial genera whose relative abundance differs significantly between the colonic and stomach microbiomes of Japanese macaques. In total, 133 genera were significantly enriched at specific gut sites (LEfSe: log LDA score > 2.0, $p < 0.05$; Figure 2.5; Table 2.3). Of these taxa, 26 were enriched in the stomach, including orders Pasteurellales and Enterobacteriales (class Gammaproteobacteria), Lactobacillales and Gemellales (class Bacilli), Neisseriales (class Betaproteobacteria), and Fusobacteriales (class Fusobacteriia). In the colon, 107 genera were enriched, mainly from phyla Verrucomicrobia, Tenericutes, and Bacteroidetes and orders Clostridiales (phylum Firmicutes) and Bacteroidales (phylum Bacteroidetes) (Figure 2.6). In particular, the top 15 enriched genera were mostly from families Ruminococcaceae and Lachnospiraceae of the order Clostridiales.

Predicted functional difference between stomach and colonic microbiome

Overall, PICRUSt2 identified 154 KO pathways (average NSTI: 0.1901 ± SD 0.1805) (Douglas et al., 2019). Based on LEfSe analysis, we defined 75 differentially abundant pathways between the stomach and colon (LEfSe: log LDA score > 2.0, $f <$ 0.05). Among these, 36 pathways were enriched in the colon and 39 were enriched in the stomach (Figure 2.7; Table 2.4). Most of the differentially abundant pathways (54/75) were related to metabolism. Specifically, the top enriched metabolic pathways in the colon microbiome were related to the metabolism of multiple nutrients such as terpenoids, polyketides, amino acid and glycan. Other than the metabolic pathways, multiple pathways related to cellular processes and genetic information processing

were also enriched in the colonic microbiome. On the other hand, the stomach microbiome was especially enriched with metabolic pathways related to carbohydrates e.g. ascorbate and aldarate metabolism and citrate cycle. Furthermore, pathways related to the metabolism of other amino acids, e.g. glutathione metabolism, were enriched in the stomach microbiome. Other than metabolic pathways, stomach microbiome was also enriched in pathways related to environmental information processing, such as the phosphotransferase system and ABC transporters.

Discussion

Stomach microbiome is less diverse than colonic microbiome

Our study found that wild Japanese macaques' stomach microbiome was less diverse than their colonic microbiome, supporting findings in the previous studies on mammals (red-shanked doucs (Clayton et al., 2019), Abert's and fox squirrels (Reed, Pigage, Pigage, Glickman, & Bono, 2019), and pikas (H. Li et al., 2017)). Such a difference in diversity revealed the strong effect exerted on the microbiota by the physiochemical environment in the stomach. The stomach generally has a rapid flow of low-pH gastric acid, causing strong disturbance for the survival and growth of microbes (Lambert, 1998; Savage, 1977). As a result, the stomach not only has lower microbial diversity but also may have lower microbial biomass than the colon. The indigenous microbes in such an environment are likely have a tolerance to the acidic and aerobic environment in the stomach and could reproduce rapidly as a way to maintain an active population in the stomach. Though not able to colonize the stomach, some microbes presumably could pass through the stomach and eventually colonize the lower GI tract, such as the colon. The colon provides a rather different environment

for bacterial growth: it is characterized by an anaerobic and neutral-to-alkaline condition. Together with the extended transit time, microbes are able to establish populations and form complex interactions within the colon (Müller et al., 2019; Roager et al., 2016). In the case of humans, the half-emptying time of the colon (ca 400 min) could double that of the stomach (ca 165 min) (Camilleri et al., 1989). The physiochemical environment and fast transit of the stomach may present as a bottleneck for bacterial growth, "selecting" the gut microbes passing down to the lower GI tract. However, the gut microbes may then be able to establish a population once they pass through.

Taxonomic difference between stomach and colon microbiome

As adaptive characteristics to the acidic and aerobic conditions, the stomach microbiome is enriched by acid- and aero-tolerant microbes. Our results revealed that Proteobacteria were especially abundant in the stomach (70.07%) in comparison with their proportion in the colon, which is just 4.61%. Unlike the majority of gut microbes, Proteobacteria are often facultatively anaerobic, and thus are competitive in surviving in the oxic environment of the stomach (Moon, Young, Maclean, Cookson, & Bermingham, 2018; Shin, Whon, & Bae, 2015). By LEfSe analysis, we also identified Lactobacillales enriched in the stomach microbiome. In addition to their ability to withstand an oxic condition, they are also acid-tolerant, which may allow the species to flourish in the stomach (Walter, 2008). Residing in the epithelial surface of the stomach, Lactobacillales species are able to maintain a community even under the continuous disturbance of gastric acid (Savage, 1977; Walter, 2008). As opposed to the stomach microbiome, we found colonic microbiota enriched in anaerobic microbes

that actively involved in fiber degradation. For example, families Lachnospiracea and Ruminococcaceae and genus *Prevotella* were more abundant in the colon. These bacterial taxa are active plant degraders with key carbohydrate-active enzymes, sugar transport mechanisms, and metabolic pathways (Biddle, Stewart, Blanchard, & Leschine, 2013; Chen et al., 2017). The presence of fiber-degrading bacterial taxa such as families Lachnospiracea and Ruminococcaceae and genus *Prevotella* corroborates the major role of colonic microbiota as fiber fermenters. Nevertheless, the absolute abundance of these bacterial taxa would possibly be higher in the colonic microbiome if the biomass of the stomach microbiome were really low.

Interestingly, we found that the bacterial taxa enriched in the stomach were related to the oral cavity in other mammals, including humans. For example, genera *Veillonella* and *Streptococcus*, the oral nitrate-reducing bacteria, are common in the mouth or throat of feral horses and humans (Abranches et al., 2019; Doel, Benjamin, Hector, Rogers, & Allaker, 2005; Meyer et al., 2010). Hence, the community we observed in the stomach may have represented the transient microbes that were swallowed during food intake of Japanese macaques. Japanese macaques usually store food in their cheek pouch for an extended duration (Yumoto, Noma, & Maruhashi, 1998). The microbes in the oral cavity may colonize the food surface before the macaques actually swallow the food. This partly supports the notion that the gut microbes enter from the oral cavity but then the GI sites “selects” out a part through the varied physiochemical environments. It would be interesting to further study how the microbes transfer from the oral cavity to the stomach and the lower GI tract.

Functional difference between stomach and colon microbiome

According to the functional prediction by PICRUSt2, the main functional differences between the stomach and colonic microbiomes were related to metabolism. Such differences may be related to the different digestive roles of the stomach and the colon. The stomach microbiome was more enriched in the metabolic pathways involving carbohydrates, especially simple sugar. For example, we found glycolysis/gluconeogenesis and citrate cycle (TCA) enriched in the stomach microbiome. The microbes may utilize part of the simple sugar that was not digested by the enzyme in the stomach. However as mentioned above, the stomach microbiome may be less abundant and less diverse. While the stomach microbiome may have functions supplementing the digestive role of the stomach, the overall effect remains limited. On the other hand, metabolic pathways related to terpenoids, polyketides, amino acid and glycan increased in the colonic microbiome. Glycan biosynthesis and metabolism are also abundant in the gut microbiome of Tibetan macaques during winter (Sun et al., 2016). These pathways are related to the digestion of glycan produced by the breakdown of cellulose and hemicellulose. Since the colonic microbiome is the main fermentation site, it makes sense that the enriched pathways are related to the digestive efficiency of the fibrous foods eaten by the macaques. Overall, the differentially enriched pathways implied that the microbial communities in both gut sites are equipped to supplement the digestive functions of these gut sites.

Stomach microbiome of Japanese macaques compared to foregut microbiome of colobus

Compared with the foregut-fermenting NHPs, the relative difference in diversity between the gut sites was great in this study (Table 2.5). Given the biases caused by varied sampling and analysis methods across studies (Asangba et al., 2019; Hayakawa, Sawada, et al., 2018), we only made comparisons of diversity across different host species in the form of stomach to colon ratio, instead of the absolute number of ASVs or any other index. In our study, the observed richness of the macaques' stomach microbiome is nearly a quarter that of the colonic microbiome. On the other hand, the red-shanked douc's foregut microbiome is about half as diverse as the hindgut microbiome (Clayton et al., 2019). Again, this may be due to the difference in gut physiology between foregut- and hindgut-fermenting animals. The colobines are anatomically unique in having evolved a large, sacculated foregut for extended fermentation (Matsuda, Chapman, & Clauss, 2019). Compared to the hindgut fermenters like Japanese macaques in the present study, the foregut of the colobines is relatively alkaline for the optimal fermentation condition (Lambert, 1998). The relatively alkaline stomach environment of colobines may allow a more diverse foregut microbiota and thus maximize energy harvest from their nutritionally poor folivory-based diet. Despite the biases caused by variations in sampling, storage and analysis methods across studies, the relative difference in alpha diversity indices between the foregut- and hindgut-fermenting NHPs is apparent. However, again, the current study remains preliminary, and further studies, including more species and a larger sample size, would greatly improve our knowledge of the stomach/foregut microbiome of NHPs overall.

In comparing composition at the phylum level, the top two dominant phyla in the stomach microbiome of wild Japanese macaques and the foregut of captive red-shanked doucs (Clayton et al., 2019) were Proteobacteria and Firmicutes, different from those of the wild proboscis monkeys (Hayakawa, Nathan, et al., 2018) studied, which are dominated by Firmicutes and Bacteroidetes. Notably, the dominance of Firmicutes and Bacteroidetes rather than Proteobacteria is a more common pattern found in the colonic microbiome of mammals including NHPs (Amato, Leigh, et al., 2015; Clayton, Gomez, et al., 2018; Lee, Hayakawa, Kiyono, Yamabata, & Hanya, 2019; Ley, Lozupone, Hamady, Knight, & Gordon, 2008). As mentioned above, Proteobacteria are competitive in surviving the relatively oxygen-abundant environment of the stomach. The foregut of wild proboscis monkeys may present an environment similar to the colon, thus harboring a colonic microbiome-like community. Alternatively, the enriched Proteobacteria found in the stomach/foregut microbiome of Japanese macaques and red-shanked doucs may be replaced by functionally redundant microbial species from the phyla Firmicutes and Bacteroidetes in the foregut microbiome of the proboscis monkeys. The difference between the foregut microbiomes of the two colobines may be related to the simplified captive diet that includes more easily digestible foods. The foregut microbiome of captive proboscis monkeys was less diverse than and compositionally different from that of the wild proboscis monkeys which forage on diverse types of plants (Hayakawa, Nathan, et al., 2018). Hence, the foregut of the captive red-shanked doucs may be different from that of proboscis monkeys through divergence in macronutrient intake. To clarify the general pattern of the dominant phyla and species in the stomach/foregut microbiome as well as the related factors, data from more species and a larger sample size are

needed. In the present study, the stomach microbiome composition of Japanese macaques was marginally related to the dietary variation across seasons. However, our examination of the effect of seasons, sex and other host factors remains preliminary due to the limited sample size. It would be interesting to carry out a detailed study to examine the response of the foregut/stomach microbiome to environmental factors.

Conclusion

Overall, the stomach and colonic microbiome of the Japanese macaques are distinctive from each other in diversity, composition and function. Compared with the foregut-fermenting NHPs, the stomach of hindgut-fermenting NHPs potentially present a harsher physiochemical environment for microbial acquisition and survival. Our result revealed the filtering effect imposed by different GI sites on the gut microbiome, shedding light on how microbes adapt to different physiochemical GI environments and distribute along the GI tract.

Author contribution statements

W. Lee was responsible for performing the molecular experiments and data analysis as well as writing the manuscript. T. Hayakawa was responsible for the molecular experiments and reviewing the manuscript. Y. Kurihara, M. Hanzawa, A. Sawada, A. Kaneko, Y. Morimitsu, T. Natsume, S. Aisu, T. Ito and T. Honda were involved in sample collections. G. Hanya contributed to the design of the study, sample collection and writing of the manuscript.

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Chapter 3 Gut microbiota composition of Japanese macaques associates with extent of human encroachment

Abstract

In recent decades, human-wildlife interaction and associated anthropogenic food provisioning has been increasing due to fast population growth and urban development. Noting the role of the gut microbiome in host physiology like nutrition and health, it is thus essential to understand how human-wildlife interactions and availability of anthropogenic food in habitats can affect an animal's gut microbiome. This study therefore set out to examine the gut microbiota of Japanese macaques (*Macaca fuscata*) with varying accessibility to anthropogenic food and the possibility of using gut microbiota as indicator for macaques' reliance on anthropogenic food. Using 16S rRNA gene sequencing, we described the microbial composition of macaques experiencing different types of human disturbance and anthropogenic food availability– captive, provisioned, crop-raiding and wild. In terms of alpha diversity, our results showed that observed richness of gut microbiota did not differ significantly between disturbance types but among collection sites, whereas Shannon diversity index differed by both disturbance types and sites. In terms of beta diversity, captive populations harbored the most distinctive gut microbial composition, and had the greatest difference compared to wild populations. Whereas for provisioned and crop-raiding groups, the macaques exhibited intermediate microbiota between wild and captive. We identified several potential bacterial taxa at different taxonomic ranks whose abundance potentially could help in assessing macaques' accessibility to anthropogenic food. This study revealed the flexibility of the gut microbiome of

Japanese macaques and provided possible indices based on the gut microbiome profile in assessing macaques' accessibility to/reliance on anthropogenic foods.

Introduction

The gut microbiome is the community of microorganisms residing in the gastrointestinal tract and is actively involved in many aspects of host physiology such as energy harvest, nutrition (Amato et al., 2014; Amato, Leigh, et al., 2015), behavior (Zheng et al., 2016) and immune system response (Round & Mazmanian, 2009). While the gut microbiome strongly influences hosts' digestive efficiency and health, host diet in turn affects the gut microbiome (Groussin et al., 2017; Ley, Hamady, et al., 2008; Muegge et al., 2011). Host diet affects the metabolic activities of the gut microbes by providing different substrates and nutrition, thus influencing the composition and functions of the gut microbiome. Many studies based on feeding experiments have revealed that gut microbiota is related to the types and the macronutrient profile of food, as exemplified by the distinct human and NHP gut microbial communities in response to Western and non-Western diets (Amato, Yeoman, et al., 2015; De Filippo et al., 2010). In particular, humans who consume Western diets which is low in fiber, high in protein and fat exhibited increased *Bacteroides*, whereas those who consumed non-Western diets had increased abundance of *Prevotella*. Compared with feeding based experiments of humans and lab animals, wild animals exhibit even wider dietary variation. Food sources of wild NHPs vary temporally and spatially, in relation to the local climate, habitat type, plant phenology and so on. Corresponding to temporal and spatial dietary difference, composition and function of NHP gut microbiome were found to vary across seasons and habitats (Amato, Martinez-Mota, et al., 2016; Baniel et al., 2021; Hicks et al., 2018;

Sun et al., 2016; Zhao et al., 2018). A notable example of diet-gut microbiome relationship across seasons was revealed by Amato et al. (2015). This study showed that gut microbiome shifts in composition across seasons and functions to compensate for seasonal reduction in howler energy intake. Because the host-gut microbiome relationship has evolved in the natural environments, studies on wild, free-ranging animals will allow more thorough understanding of the role of environmental factors in this relationship.

On the global scale, human disturbance like agriculture and tourism have been increasingly affecting ecology and behavior of NHPs (Fuentes & Hockings, 2010; C. M. Hill & Webber, 2010). In particular, such human disturbance made anthropogenic food available to NHP via directly provisioning or crop-raiding thus could easily influence foraging behavior and nutritional intake of the NHPs (C. M. Hill, 2017; Ilham, Rizaldi, Nurdin, & Tsuji, 2016; Sha & Hanya, 2013). In some cases, anthropogenic food could constitute as much as 70% of NHP's total diet (Ilham et al., 2016). Whereas for captive individuals, the diet is managed by the keepers and is predominantly composed of commercial monkey chow for ease of management (Dierenfeld, 1997; Jaman & Huffman, 2008). From a nutritional aspect, monkey chow and the food enhancement from cropland and tourism tend to have lower fiber and higher digestible carbohydrates and energy value than wild foods (Clayton, Al-Ghalith, et al., 2018; Riley, Tolbert, & Farida, 2013). Such dietary shift likely induces significant changes in gut microbiome composition. Indeed, previous studies have revealed a general pattern of NHP gut microbiome composition becomes altered with decreased dietary diversity in captive environments (Clayton, Al-Ghalith, et al., 2018; Clayton et al., 2016; Hayakawa, Nathan, et al., 2018; McKenzie et al., 2017). Likewise, the gut microbiome

of captive NHPs is less diverse and shows signs of humanization, converging toward the modern human microbiome (Clayton et al., 2016). However, captivity is not the only human activities influencing NHPs. To further understand how the gut microbiome of wild animals could be affected by anthropogenic activities, we examined the gut microbiome of NHPs under several human-disturbed habitats with varying availability of anthropogenic food.

In this sense, the *Macaca* genus serves as a suitable study subject due to their extensive distribution and proximity to humans (Priston & McLennan, 2013). In the present study, we focused on Japanese macaques (*Macaca fuscata*), an endemic primate species widely distributed in Japanese archipelago. Human-monkey interactions come in varying forms in Japan, including but not limited to conditions of captivity, provisioning, and crop-raiding (C. M. Hill & Webber, 2010; Nakagawa, Nakamichi, & Sugiura, 2010; Yamagiwa & Hill, 1998). Human-disturbed Japanese macaques may similarly influence the gut microbiome with the associated environmental and dietary shifts. In the wild, Japanese macaques mainly feed on plant parts like leaves, flowers, fruits, buds and bark but the proportion of each food item differs by seasons and regions (Tsuji, 2010). For example, fruits are the primary food for macaques inhabiting the Yakushima lowland (D. A. Hill, 1997) whereas for macaques in the Yakushima highland, leaves are the most consumed food (Hanya, 2004a). On the contrary, captive, provisioned, and crop-raiding macaques feed on anthropogenic foods, e.g., commercial monkey chows and crops with varying proportion among populations. At the extreme, captive macaques are completely dependent on anthropogenic food because they are limited by the enclosures or cages. Although researchers have noted the effect of human disturbance and anthropogenic

food availability on NHPs' behavior, previous studies rarely examine varying degrees of human disturbance on single species, Japanese macaques hence are suitable study subjects since they are commensal with humans in many of their habitats.

Here, we aim to understand how disturbance and associated anthropogenic food enhancement may affect the gut microbiome profile of Japanese macaques. Specifically, we described and compared the gut microbiota of macaques with different accessibility to anthropogenic food under different human disturbance types, i.e., wild, provisioned, crop-raiding and captive. With this data set, we also examined the bacterial taxa whose relative abundance is associated with the anthropogenic food availability in habitats. With reference to previous studies (Amato, Yeoman, et al., 2015; Amato et al., 2013; Clayton, Al-Ghalith, et al., 2018; Clayton et al., 2016; McKenzie et al., 2017), we contrasted the patterns observed in Japanese macaques with other primate species. As an outcome of their diverse, fiber-rich diet, we hypothesized that the gut microbiome of wild macaques would be more diverse and enriched in microbes specialized for fiber digestion. Whereas for the anthropogenic food-enhanced macaques i.e., captive, crop-raiding and provisioned macaques, their gut microbiota would be less diverse and distinctive from that of the wild macaques based on the availability of anthropogenic food in habitats.

Methods

Collection of fecal samples from Japanese macaques

Fecal samples were collected from Japanese macaques. Based on human disturbance types or diet the populations experienced, the monkeys were categorized as wild, provisioned, crop-raiding, or captive (Table 3.1; Table 3.2). Samples from wild

macaques were collected from free-ranging groups in highland and lowland areas of Yakushima Island, Kagoshima Prefecture, Japan (30°N, 131°E) in August 2013 and May 2017 respectively. Samples from provisioned macaques were collected from free ranging monkeys in Koshima Islet, Miyazaki prefecture (31°22′N, 131°26′E) in April 2017 and Shodoshima, Kagawa prefecture, Japan in May 2017. Samples from crop-raiding macaques were collected from free-ranging groups in Suzuka, Mie prefecture (N34° 55' E136° 28') in July 2017. Japanese macaques in Shodoshima are intensively provisioned 3-4 times a day to make visible to visitors (Leca, Gunst, & Huffman, 2008). On the other hand, provisioning in Koshima is relatively limited in frequency and quantity, which occurs about 2 times a week, and the macaques spent similar amount of time foraging for natural foods (Go, 2009; Leca et al., 2008). Samples from captive macaques were collected from individuals living in individual cages and individuals living as a group in enclosures at Primate Research Institute, Kyoto University (PRI). The diet of captive individuals is composed of monkey chow and minor food items like sweet potatoes (Jaman & Huffman, 2008; Jaman, Huffman, & Takemoto, 2010). For each site, ten fecal samples were randomly collected. During sampling, we also collected fecal samples from unknown individuals with unknown age-sex class, since our goal is to predict macaques' reliance on anthropogenic food irrespective of age and sex.

Sample storage, DNA purification, 16S rRNA amplification and sequencing

Our method followed Hayakawa et al. (2018) with slight modification. All fecal samples (N=70) were collected immediately after defecation using sterilized cotton swab, then stored in 1-ml lysis buffer (0.5% SDS, 100 mM EDTA (pH 8.0), 100 mM

Tris-HCl (pH 8.0), and 10 mM NaCl), where the lysis buffer provided an appropriate storage medium for bacterial DNA as well as easy to handle and cost-effective. After bead-beating and centrifuged at 20,000 x *g* for 1 min, each fecal sample was mixed with1000 µl InhibitEX buffer of the QIAamp DNA Stool Mini Kit (QIAGEN GmbH, Hilden, Germany). The mixture was centrifuged at 20,000 x g for 1 min, 600 µl of the supernatant was mixed with 25 µl proteinase K and 600 µl Buffer AL and followed by the manufacture's protocols to purify the fecal DNA. DNA concentration was estimated with Qubit dsDNA HS Assay Kit and a Qubit fluorometer (Thermo Fisher Scientific). We amplified the V3-V4 region of 16S rRNA gene with primers as follows: S-D-Bact-0341-b-S-17 (forward) 5′-CCT ACG GGN GGC WGC AG-3′ and S-D-Bact-0785-a-A-21 (reverse) 5′-GAC TAC HVG GGT ATC TAA TCC-3′ (Klindworth et al., 2013). PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, Inc., Carlsbad, CA, USA). Using the Illumina Nextera XT Index Kit, specific dual indices and sequencing adapters were attached to each amplicon by PCR. Products were mixed in the same amount of DNA concentrations to form the pooled sequencing library. Fragment size distribution of the library was estimated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., La Jolla, CA, USA). The library was diluted to 15 pM and subjected to a sequencing run and 30% PhiX spike-in on an Illumina MiSeq sequencing platform using the MiSeq Reagent Kit v3 (600 cycles). The read lengths from the MiSeq run were 301 bp (forward sequences), 8 bp (forward indices), 8 bp (reverse indices), and 301 bp (reverse sequences). The data have been deposited in the DDBJ database with accession number DRP005397. This research was approved by Primate Research Institute with permission number 2017-161-07 and conducted in accordance to Primate Research Institute's Guideline for Animal Health and Welfare.

Our research is complied with the American Society of Primatologists Principles for the Ethical Treatment of Non-Human Primates.

Data analysis

Raw sequences were processed following steps described in Hayakawa et al. (2018) using software Claident v0.2.2016.4.7 and QIIME2. Demultiplexed sequences with quality score <30 were discarded, then merged using PEAR v0.9.3 (http://sco.h-its.org/exelixis/web/software/pear/) with setting *p* 0.0001 and *u* 0). To pick operational taxonomic units (OTUs), read sequences were clustered at 97% cutoff similarity level. For taxonomic identification, OTUs were assigned through the ribosomal database project (RDP) classifier at 50% confidence threshold with GreenGenes v13_8 as the reference database. The sequencing read set of each sample was rarefied to the minimum read number among the analyzed samples (13,100). Rarefaction curves were plotted and slope of rarefied curves for each sample were checked using "rarecurve" and "rareslope" function of R package *vegan*. Statistical analyses were performed in R Version 3.4. Rarefied dataset was analyzed without pruning any bacterial taxa. Alpha diversity and beta diversity were calculated using R package *phyloseq*. We analyzed the differences of alpha diversity indexes between groups using dunn's test. To construct a phylogenetic tree of the OTUs, we used the built-in function align-to-tree-mafft-fasttree of QIIME2. For multivariate analysis of microbiome composition, we constructed non-metric multidimensional scaling (NMDS) by Bray-Curtis and principal coordinate analysis (PCoA) plots by weighted and unweighted UniFrac indices through *phyoseq* in R. To find the indicator bacterial taxa for the level of human disturbance experienced by the macaques, we analyzed the data set at

different taxonomic rank using regression random forest model through R packages *randomForest* and *caret*. In order to confirm the reliability of picked bacterial taxa in identifying disturbance level experienced by the macaques, we also employed leave-one-out validation.

Results

General characteristics of gut microbiome of Japanese macaques

After removing samples with rarefaction curve slope < 0.01 (one Yakushima highland, one Yakushima lowland, one cage, one Koshima), we detected totally 2125 OTUs at 97% sequence similarity in the remaining 66 samples (rarefaction curve slope: 0.0021-0.0085, Figure 3.1). OTUs identified were from 35 phyla, 74 classes, 109 orders and 165 families. Average observed OTU richness was 362 ± 64 /sample, ranging from 193-461 OTUs (Figure 3.2; Table 3.2). The average unclassified rates of OTUs were 0.08% at the phylum level and 32.6% at the genus level. At the phylum level, Firmicutes and Bacteroidetes the gut microbiome of Japanese macaques by 59.95% and 29.50% (Figure 3.3, Table 3.3). The other dominant phyla were Proteobacteria (4.53%) and Spirochaetes (2.19%) and Verrucomicrobia (0.98%). At the genus level, *Prevotella* accounts for 20.70%, followed by *Faecalibacterium* (7.98%) and *Oscillospira* (7.31%).

Variation of gut microbiota among different human disturbance types: Alpha diversity

Overall, indices for alpha diversity, observed richness and Shannon diversity index, showed different patterns. OTU richness did not differ by disturbance types (Kruskal-Wallis chi-squared = 0.7619, df = 3, $p = 0.86$; Figure 3.2a). Compared to that, Shannon

diversity index differs by disturbance types (Kruskal-Wallis chi-squared = 8.5960, df = 3, $p = 0.04$; Figure 3.2b). Between different disturbance types, Shannon diversity index of captive macaques' gut microbiome was significantly lower than that of the wild macaques (Dunn's rank sum test corrected by Bonferroni, captive vs. wild, $p = 0.0275$). Except for that, no other pairwise comparisons showed any significant difference.

Variation of gut microbiota among different human disturbance types: Beta diversity

According to multivariate analysis based on NMDS plot by Bray-Curtis and PCoA plots by unweighted UniFrac, individuals from the same collection sites always possessed more similar microbial communities (Figure 3.4). We performed PERMANOVA tests to assess the degree of variation explained by disturbance type and collection site. Site where samples were collected was a good predictor for gut microbial community (PERMANOVA, Bray-Curtis, $R^2 = 0.4926$, $p < 0.001$; unweighted UniFrac, $R^2 = 0.4529$, $p < 0.001$), whereas disturbance type explained less of the variation (PERMANOVA, Bray-Curtis, $R^2 = 0.3576$, $p < 0.001$; unweighted UniFrac, $R^2 = 0.3284$, $p < 0.001$). In NMDS plot and PCoA plot based on unweighted UniFrac, samples with different human disturbance level/accessibility to anthropogenic food were separated on the first dimension, in the order PRI cage = PRI enclosure > Shodoshima > Suzuka > Koshima > Yakushima lowland > Yakushima highland. Samples from individuals living in cages and enclosures clustered together and were distinct from the other individuals. On the contrary, samples from the Yakushima lowland and highland were situated at the farthest end, away from the captive cluster. Provisioned and crop-raiding individuals occupied the intermediate position of captive and wild. On the second dimension of NMDS plot, samples with similar accessibility

to anthropogenic food but from different sites were distinguishable by second dimension of NMDS2 but not by PC2. Yakushima highland samples were separated from Yakushima lowland, and samples from PRI cages were separated from those collected in PRI enclosures. The difference between the NMDS plot and PCoA plot by unweighted UniFrac may be related to phylogenetic closeness of OTUs shared by cage and enclosure samples that could not be detected through NMDS plot. In PCoA plot based on weighted UniFrac, only captive samples were weakly clustered, despite the significant difference by sites and disturbance type revealed by PERMANOVA (weighted UniFrac, site, R^2= 0.4187, $p < 0.001$; disturbance, R^2= 0.2872, $p < 0.001$; Figure 3.4).

Gut bacterial taxa related to availability of anthropogenic food to macaques

To find the potential bacterial indicator, we analyzed using random forest model and check if the picked bacterial taxa could correctly predict the disturbance level of the samples. Specifically, the levels are captive-cage and enclosure, intensively provisioned-Shodoshima, crop-raiding-Suzuka, less provisioned-Koshima, wild-Yakushima lowland and wild-Yakushima highland (Figure 3.5). Overall, accuracy of indicator taxa from lower taxonomic ranks is higher than those from higher taxonomic ranks. Models using families (OOB rate =3.03%, accuracy = 1.00, Kappa = 1.00) predicts the food reliance level better than orders (OOB rate = 10.61%, Accuracy = 1.00, Kappa = 1.00), classes (OBB rate = 15.15%, Accuracy=1.00, Kappa=1.00) and phyla (OBB rate = 22.73%, Accuracy=1.00, Kappa=1.00). To identify macaques' accessibility to anthropogenic food, information at lower taxonomic rank may serve good indicator, since they may provide more diet-specific features. Whereas as when

we use information from higher taxonomic ranks, some characteristics of the gut bacteria may be overlooked. In our dataset, were identified class Bacilli, class Chloroplast, order Lactobacillales and family Bacteroidaceae to be the most important indicator taxa, with mean decrease in accuracy higher than 0.04 (Supplementary table 3.4, Figure 3.5).

Several indicators used in previous studies on gut microbiome, i.e. Firmicutes to Bacteroidetes ratio, Chloroplast, *Bacteroides* and *Prevotella* showed varying response to human disturbance and anthropogenic food enhancement. For the Firmicutes to Bacteroidetes ratio, macaques from wild populations had the highest value, followed by provisioned and crop-raiding, with the lowest in captive populations (Kruskal-Wallis rank sum test chi-squared = 21.2245, df = 3, $p < 0.0001$; Dunn's rank sum test corrected by Bonferroni, Captive vs. Crop-raiding, $p = 0.0514$; Captive vs. Provisioned, $p = 0.0362$; Captive vs. Wild, $p < 0.0001$; Crop-raiding vs. Provisioned, $p = 1.000$; Crop-raiding vs. Wild, $p = 0.4412$; Provisioned vs. Wild, $p = 0.1096$; Figure 3.3). We also examined reads classified as Chloroplast, as it is used as an indicator of host fiber intake (Clayton, Al-Ghalith, et al., 2018; Clayton et al., 2016). Despite the intriguingly high abundance in Koshima samples, abundance of Chloroplast is negatively related to availability of anthropogenic food for the macaques, with captive samples with significantly low Chloroplast abundance (Kruskal-Wallis rank sum test chi-squared = 34.2052, df = 3, $p < 0.0001$; Dunn's rank sum test corrected by Bonferroni, Captive vs. Crop-raiding, $p = 0.0048$; Captive vs. Provisioned, $p < 0.0001$; Captive vs. Wild, $p < 0.0001$; Crop-raiding vs. Provisioned, $p = 0.6495$; Crop-raiding vs. Wild, $p = 1.000$; Provisioned vs. Wild, $p = 1.000$; Figure 3.6). For *Prevotella*, one of the dominant human gut microbial genera, significantly higher abundance was

found in captive individuals, while crop-raiding, provisioned and wild individuals had similar abundance (Kruskal-Wallis chi-squared = 15.2951, df = 3, $p < 0.001$; Dunn's rank sum test corrected by Bonferroni, Captive vs. Crop-raiding, $p = 0.0171$; Captive vs. Provisioned, $p = 0.0205$; Captive vs. Wild, $p = 0.0011$; Crop-raiding vs. Provisioned, $p = 1.000$; Crop-raiding vs. Wild, $p = 1.000$; Provisioned vs. Wild, $p = 1.000$; Figure 3.7). Whereas for *Bacteroides*, another dominant human gut microbial genus, wild harbored the highest abundance, followed by captive macaques, and the lowest abundance was found in crop-raiding and provisioned macaques (Kruskal-Wallis chi-squared = 15.295, df = 3, $p = 0.002$; Dunn's rank sum test corrected by Bonferroni, Captive vs. Crop-raiding, $p = 0.0007$; Captive vs. Provisioned, $p = 0.0003$; Captive vs. Wild, $p = 0.2127$; Crop-raiding vs. Provisioned, $p = 1.000$; Crop-raiding vs. Wild, $p < 0.0001$; Provisioned vs. Wild, $p < 0.0001$; Figure 3.7). However, *Bacteroides* generally were not abundantly present in the gut of Japanese macaques; the provisioned and crop-raiding individuals had low or sometimes no presence of *Bacteroides* spp.

Discussion

General characteristics of gut microbiome profile of Japanese macaques

At the phylum level, the gut microbiome of Japanese macaques was dominated by Firmicutes and Bacteroidetes. Microbes from phyla Proteobacteria and Spirochaetes were detected with lower abundance. At the genus level, *Prevotella* was the most dominant. Our findings of gut microbiome composition were consistent with previous studies on Japanese macaque gut microbiome (Hayakawa et al., 2018; Ma et al., 2014). In previous studies, Firmicutes and Bacteroidetes were the most abundant phyla constituting approximately 90% of the Japanese macaques' gut.

Likewise, they also found Spriochaetes and Proteobacteria at minor abundance. However, previous studies on Japanese macaques focused on captive samples mostly (n=2 from Hayakawa et al. (2018); n=97 from Ma et al. (2014)) while limited samples were from wild, free-ranging individuals (n=2 from Hayakawa et al. (2018)).

Overall, the gut microbiome profile of Japanese macaques is similar with other macaques but different from great apes and humans. Firmicutes and Bacteroidetes are the two most dominant phyla in the mammalian gut (Ley, Hamady, et al., 2008). In this sense, Japanese macaques and other primate species including humans are similar, since Firmicutes and Bacteroidetes make up a great proportion of the gut microbiome. For hosts from the *Macaca* genus, Firmicutes, Bacteroidetes, Proteobacteria, and Spirochaetes are the four most abundant bacterial phyla detected; in addition to Japanese macaques, these bacterial phyla also constitute a major part of the gut microbiota for captive *M. mulatta* (McKenna et al., 2008; Yasuda et al., 2015), captive *M. fascicularis* (X. Li et al., 2018), and wild *M. thibetana* (Sun et al., 2016).

Compared with macaques, the four most common phyla in gut microbiota of great apes, i.e. bonobo, chimpanzees, and gorillas are Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria (Moeller et al., 2013). Similarly in humans, these four bacterial phyla constitute the majority of gut microbiota but the relative abundance varies with dietary habits (Arumugam et al., 2011; Bäckhed, Ley, Sonnenburg, Peterson, & Gordon, 2005; De Filippo et al., 2010). Human, non-human apes, and macaque gut microbiome is distinctive in the presence of phyla Spirochaetes, which tends to be rare in human and non-human ape guts. Also, genus *Bacteroides* is considered a major component of the human gut microbiota, but only minor in the macaque gut. Instead, *Prevotella*, another dominant bacterial genus for the human gut,

is the most dominant genus for macaques. This difference was also noted by McKenna et al. (2008), who compared the gut microbiome profile of rhesus macaques with that of humans.

Effect of anthropogenic food availability on the gut microbiome of Japanese macaques: similarity with other species

Our result showed that anthropogenic food availability in habitats of Japanese macaques and the associated dietary change was correlated with altered gut microbiota. A gradual change of gut microbiome composition was detected from macaques heavily relied on anthropogenic food (captive) to those relied on natural foods (wild). However, gut microbial diversity did not necessarily decrease along with increasing availability of anthropogenic food in disturbed conditions; Shannon diversity index differed between captive and wild populations, but observed richness was similar among disturbance types.

Within the three different types of human disturbance we examined, captivity poses the most contrasting diet from the wild environment. Animals in captivity tend to have a simple and low-fiber diet, so as the captive individuals in present study which feed mainly on commercial monkey chow. In addition to dietary change, other environmental factor relating to human disturbance that may be related to altered composition in gut microbiome, such as hygiene, home range, social contact, and geography (Clayton, Al-Ghalith, et al., 2018; Clayton et al., 2016). This is corroborated by the most distinctive gut microbial community of our captive individuals living in either cage or enclosure. Recent research comparing captive and wild mammals also detected a general pattern of composition shift in captive animals (Clayton, Al-Ghalith,

et al., 2018; Clayton et al., 2016; McKenzie et al., 2017). These studies attributed the shifts to the reduced diet diversity and fiber intake. Compared to diet of captive individuals, diet of free-ranging individuals is more diverse and fiber-rich.

Our dataset further supports that the gut microbiota of Japanese macaques is related to the specific diet macaques consumed under different disturbance types. For example, we found chloroplast abundance, an indirect indicator of plant intake, more enriched in macaques less disturbed by human activities. As reported in red shanked doucs, abundance of chloroplast is also positively related to the wildness of doucs' lifestyle; chloroplast was barely observed in captive douc populations, while a considerable amount was detected in wild populations (Clayton et al., 2016). Difference in chloroplast abundance therefore may reflect the macaques' intake of fibrous food in different conditions. In PRI, captive macaques are fed predominantly with easily-digestible monkey chow, and sometimes minor food items like sweet potatoes (Jaman & Huffman, 2008; Jaman et al., 2010). Every 100 g of monkey chow contains approximately 44.5 g soluble non-nitrogen matter, 28.2 g crude protein, 9.5 g crude lipid, 8.2 g water, 2.5 g crude fiber and 2.5 g crude ash (Jaman et al., 2010). In contrast to that, the average NDF of major food leaves consumed by wild macaques were around 42% (Hanya et al., 2007). In the wild, such kinds of fiber-rich food is an important part of the macaques' daily diet (Hanya, 2004a, 2004b, 2010). Yakushima highland macaques spent 45% of annual feeding time on fiber-rich food items (Hanya, 2004a), and the lowland macaques spent around 35% (D. A. Hill, 1997). Presumably, the provisioned and crop-raiding macaques consume a mixed diet of agricultural crops and forest foods, with differing proportions between sites. Within provisioned samples, the diet of Koshima macaques resembles that of wild macaques since provisioning

was restricted to twice a week (Go, 2009; Leca et al., 2008; Tsuji et al., 2015). Relative to Koshima macaques, Shodoshima macaques are intensively provisioned by visitors and staff of the monkey park, for about 3-4 times per day (Leca et al., 2008). On the contrary, the diet of crop-raiding macaques is rarely studied. Some studies suggested that crop-raiding events were highly related with food availability in the forest; in food scarce seasons like summer and winter, macaques rely more on human settlements and crops (Ueda, Kiyono, Nagano, Mochizuki, & Murakami, 2018; Yamada & Muroyama, 2010). Our crop-raiding samples were collected in early July, hence the macaques may have feed on crops.

Another exemplary bacterial indicator revealing the relationship between diet and the gut microbiome may be the Firmicutes and Bacteroidetes ratio. A negative relationship was found between the Firmicutes and Bacteroidetes ratio and the availability of anthropogenic food for the macaques: the highest ratio was found in wild Japanese macaques, intermediate in crop-raiding and provisioned, and lowest in captive macaques. In our dataset, the increasing abundance of Firmicutes microbes in wild macaques' gut were mainly accounted by microbes from families Lachnospiracea, Ruminococcaceae and Peptococcaceae. In particular, microbes from Lachnospiracea and Ruminococcaceau play role as active plant degraders with identified key carbohydrate-active enzymes, sugar transport mechanisms, and metabolic pathways (Biddle et al., 2013). A positive relationship between host fiber intake and the abundance of Lachnospiraceae and Ruminococcaceae was also found in Sifakas (Springer et al., 2017) and black howler monkeys (Amato, Leigh, et al., 2015).

Interestingly within undisturbed populations from Yakushima, we also found a higher ratio in highland macaques which consume a large amount of fiber-rich food throughout the year (Hanya, 2004a). Similarly within wild rhesus macaque populations, macaques from high altitude regions exhibited an elevated ratio possibly as an adaptation to fiber-rich diet and increased energy consumption in high altitude (Zhao et al., 2018). Hence, the ratio may be related to the fruit and fiber consumption of mammals, including Japanese macaques in this case, in different environments. Considering Firmicutes and Bacteroidetes are commonly present in most mammals, the ratio could be a suitable indicator for of not only the macaques but also other wild animals. However, there is still no direct test for the causal relationship between the Firmicutes to Bacteroidetes ratio and fermentative ability of gut microbiome yet. For example, *in vitro* digestibility assay for testing fermentative capacity on same food item may be good option for further research.

Nevertheless, the Firmicutes to Bacteroidetes ratio does not completely mirror the trend for Chloroplast abundance, the indirect measure for fiber intake (Clayton, Al-Ghalith, et al., 2018; Clayton et al., 2016). For example, Koshima macaques, which had exceptionally enhanced abundance of Chloroplast, did not necessarily hold higher Firmicutes to Bacteroidetes ratio. It is possible that the Koshima macaques acquire chloroplast from other sources. Koshima macaques have been previously reported to use fish as a food source; the elevated ratio may be related to ingestion of herbivorous fish (Sullam et al., 2012; Watanabe, 1989). Nevertheless, our reasoning is limited as we did not collect detailed dietary data around the time of sample collection. To unravel the diet-gut microbiome relationship of Japanese macaques, further studies on the gut microbiome combining detailed dietary data is required.

Effect of anthropogenic food availability on the gut microbiome of Japanese macaques: contrasts with other species

Aside from similar patterns we detected in Japanese macaques and other mammals, alpha diversity and relative abundance of some gut bacterial taxa of Japanese macaques showed unexpected response towards inclusion of anthropogenic food in diet. Comprehensive research of captive, semi-captive and wild NHPs suggested that alpha diversity of NHPs' gut microbiota was significantly reduced by the dietary shift associated with provisioning (Clayton, Al-Ghalith, et al., 2018; Clayton et al., 2016). Although we did detect lower Shannon diversity index in captive than wild populations, both observed richness and Shannon diversity did not show a decreasing trend along with the anthropogenic food availability in the habitat. In terms of indicator bacterial taxa, our wild populations had higher abundance of genus *Bacteroides*, which was found more enriched in other humanized NHPs (Clayton et al., 2016; Jia et al., 2018).

These differential patterns in the macaque gut microbiome may be associated with the species-specific response of Japanese macaques to inclusion of anthropogenic food in daily diet. Host traits such as host taxonomy, foraging ecology and gut physiology could result in deviating responses in gut microbiome even towards similar environmental stimuli (Amato, Martinez-Mota, et al., 2016; McKenzie et al., 2017). In particular, host taxonomy plays an important role in determining the set of gut microbiome harbored by the species and thus response may vary across NHP species (Amato, Yeoman, et al., 2015; Ley, Hamady, et al., 2008; McCord et al., 2014; McKenzie et al., 2017). In a study encompassing 41 species of mammals across six

orders, reduced alpha diversity of gut microbiome in captivity is not a universal phenomenon (McKenzie et al., 2017). Among the 11 mammalian families investigated, six families showed no significant change in alpha diversity, while four had significantly decreased and one had significantly increased diversity in captivity. And again, relative abundance of bacterial taxa changes in different ways with regards to the host species. Comparison between two closely related howler monkey species by Amato et al. (2016) also revealed that a small difference in host genetics could result in differential responses of the gut microbiome; despite sharing many microbial genera, mantled howler monkeys had gut microbiota more resistant to dietary shifts than black howler monkeys. Our study subject, the Japanese macaques, may be another example suited to human-disturbed habitats by having taxonomically diverse but functionally redundant microbes. In our study, the gut of captive macaques maintained gut microbial diversity similar to that of wild macaques, which may sustain macaques even in the suboptimal condition. It is possible that unrelated gut microbial taxa perform a similar function as a result of convergent evolution (Groussin et al., 2017; Muegge et al., 2011). Consequently, specific bacterial taxa may vary in response due to the potential difference in cross-feeding and competition at lower taxonomic levels.

On top of host taxonomy, host foraging ecology and gut morphology could lead to species specific response in gut microbiome. Compared with folivorous NHPs examined in previous studies, e.g. red-shanked doucs (Clayton, Al-Ghalith, et al., 2018; Clayton et al., 2016) and black howler monkeys (Amato et al., 2013), reliance of Japanese macaques on gut microbiome to digest the fiber-rich plant materials may not be as high. Though their diet contains fibrous food items, Japanese macaques are not strictly herbivorous but feed on more nutritious foods such as fruits and nuts

whenever available (Hanya, 2004a). Indeed, some folivorous NHP species like doucs develop an enlarged gut for extended fermentation (Lambert, 1998; Matsuda et al., 2019), but this is not the case for Japanese macaques which are caeco-colic/hindgut fermenters. For foregut fermenters, the food items arrive at the fermentation chamber undigested, leaving more nutrients available to the gut microbes. Opposite to that, caeco-colic/ hindgut fermenters absorb all the digestible components from food before the fermentation. As gut morphology determines the host digestive physiology, even the same food item could have a different impact on the gut microbiome depending on the gut morphology of the hosts (Lambert, 1998; Ley, Hamady, et al., 2008).

Limitations

In this study, we used the terms “availability of/accessibility to anthropogenic food” and “disturbance type” in categorizing and describing macaque populations. Such terms may be vague because the pattern we observed here is not solely attributed to diet, but the synergy of multiple environmental factors like geography, home range and social interactions. For examples, captive populations generally had distinctive gut microbiota with other free-ranging populations. Aside from diet, the distinctive gut microbiota harbored by captive populations may be attributed to reduced contact with potential microbes due to limited home range and social interaction. Despite the presence of confounding factors, we believe that the dietary change caused by human disturbance is one of the major elements leading to the difference in gut microbiota presented in this study.

However, another problem in this study is that the sampling sites and populations experiencing different human disturbance level are confounded. For example, two

groups for wild population were only gathered in Yakushima Island, and captive samples were all from Primate Research Institute only. The gut microbiota may be more similar to each other because they are from close site, not only because of the difference in disturbance level and anthropogenic food availability among sites. Again, we argue that the effect of sites is weak, though not completely negligible. If effect of sites were greater, then gut microbiota of both wild groups from Yakushima Island should be more similar. Yet in our study, Yakushima lowland samples are in fact more similar to Koshima samples than to Yakushima highland. To unravel the diet-gut microbiome relationship of Japanese macaques, further studies including more sites is highly recommended.

In addition to the above-mentioned points, we were not able to analyze possible sex and age effect. It is supported by multiple studies that individuals from different age-sex classes have different crop-raiding tendencies. Depending on age-sex class of the individuals, the quantity and types of anthropogenic food may be differentially consumed.

Conclusion

Overall, our results demonstrated that it is possible to predict animals' degree of reliance on anthropogenic food through gut microbiota, but one should always pay attention to species-specific response of the animals' gut microbiota. Even genetically closely related species could exhibit distinctive responses due to a species trait, severity of disturbance, and characteristics of gut microbes. This suggests that the picked bacterial taxa in this study may only be applicable to Japanese macaques but not to other species. In some degree, the gut microbiome can provide a general picture

of human disturbance as our data did reveal a gradual change of gut microbial community along anthropogenic food availability in macaques' habitat. Despite that, one should consider carefully if alpha diversity and relative abundance of certain gut microbial taxa can be used in assessment. Additionally, the differential responses exhibited by Japanese macaques may also mean that more cautions should be taken when using NHP models for inferring the host-gut microbiome relationship of humans. This is especially true when considering unique human physiological adaptations and dietary shifts across evolutionary time, which may lead to further deviation of response in the gut microbiome.

Author contribution statements

W. Lee was responsible for collecting samples, performing the molecular experiments and data analysis, as well as writing the manuscript. M. Kiyono and G. Hanya conceived the framework of this study. N. Yamabata collected the samples. G. Hanya contributed to the design of the study and writing of the manuscript. All authors provided critical feedback and helped shape the research and manuscript.

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Chapter 4 General Discussion

4.1 Overview of the findings

By facilitating digestion of otherwise indigestible foods, gut microbiome plays a crucial role in feeding ecology of the herbivorous/omnivorous mammals including the primates. As a primate species living in the marginal habitats, Japanese macaques serve an interesting study subject because of their high dependence on fiber-rich foods. Using Japanese macaque as a model, this thesis examined the ecological factors shaping primate gut microbiome. Focusing on the individual level, Chapter 2 reveals that composition and function differ between different gut sites of individual Japanese macaques. This chapter presents how physiochemical condition at each gut site "selects" gut microbes. Examining at the population level, Chapter 3 demonstrates that gut microbiome of Japanese macaque changes in association with the populations' access to anthropogenic foods. This chapter shows the close link between macaques' diet and their gut microbiome. Taken together, the presented chapters allow a more thorough understanding of how gut microbiome of Japanese macaques is acquired and established. Here in Chapter 4, I will focus on how these two studies infer about the nature of primate gut microbiome. With reference to the previous studies on Japanese macaques, I will also discuss how the presented chapters offer insight into the feeding ecology of Japanese macaques and other primates.

4.2 Implications on the nature of primate gut microbiome

Born sterile, primates and other mammals acquire microbes from their surrounding environments. Upon acquisition, microbes go through a series of

environmental filters before they establish a stable population/community. Here, the presented chapters revealed that the primate gut microbiome is filtered by (1) physiochemical environment in the gut and (2) the host foraging behavior. While the described mechanism also applies to the other mammals, this section will mainly discuss from the perspective of primates.

Some microbes fail to colonize due to the mismatch between their survival requirement and the physical conditions in the hosts. Stomach and duodenum have been regarded as the major environmental filters due to the antimicrobial effects of gastric acids and bile acids (Donohue et al., 2019; Hillman et al., 2017; Ridlon, Kang, Hylemon, & Bajaj, 2014). Through Chapter 2, I presented how physiochemical condition at the stomach and colon "selects" microbes. Especially, stomach environment is characterized by low pH of around 2, in contrast to a pH of around 7 in the mouth and esophagus where microbes enter (Di Pilato et al., 2016). Through constant flush of low pH gastric acid and oxic environment, macaques' stomach may have "selected" out the microbes that could proceed to the colon/cecum. On contrary, colobus have evolved a different set of environmental filters in their stomach/foregut to facilitate foregut fermentation (Lambert, 1998). For example, the foregut of the colobines is relatively alkaline and has greater surface area for the optimal fermentation condition (Lambert, 1998). Whereas for Japanese macaques and other hindgut-fermenting primate species, colon is the major fermentation chamber, where the physiochemical condition supports the establishment of gut microbiome. The strong effect imposed by gut environment is further supported by the clear difference in gut microbiome between the foregut and hindgut fermenting primates (Amato et al., 2019; Ley, Hamady, et al., 2008).

Furthermore, primates impose environmental filters on gut microbiome by their foraging behavior. Basically, gut microbes feed on the foods/nutrients ingested by their hosts. And so, the ability of microbes to utilize dietary substrates would determine their abundance in the gut. For example, in Chapter 3, undisturbed macaques are more enriched in Lachnospiraceae and Ruminococcaceae, microbial families known as active plant degraders (Biddle et al., 2013). Among these undisturbed macaque populations inhabiting Yakushima Island, overall fermentative ability of gut microbiome proves to be higher in leaf-eating highland macaques than in fruit-eating lowland ones (Hanya et al., 2020). Not only affect the relative abundance of certain microbes, diet also affects alpha diversity of the primate gut microbiome. Using red shanked doucs, Clayton et al. (2018) presented a direct relationship between ingested plant diversity and primate gut microbial diversity. While in this thesis I only presented the effect of long-term diet, short-term dietary change could also induce changes in primate gut microbiome. In fact, seasonal variation in gut microbiome has been widely observed in multiple primate species (Amato, Leigh, et al., 2015; Baniel et al., 2021; Hicks et al., 2018; Sun et al., 2016). Similar to Japanese macaques, Ruminococcaceae becomes more enriched in gut microbiome of black howler monkeys, in response to reduced energy intake (Amato, Leigh, et al., 2015).

Overall, these findings have implied the governing role of host-specific traits over primate gut microbiome composition and function. Although here I showed their effect independently, these two factors (with the others) indeed interplay to affect gut microbiome. Given the variation in gut physiology and/or foraging behavior across and within the primate species, a flexible gut microbiome may serve as a “tailor-made” solution for different survival challenges.

4.3 Implications on the feeding ecology of Japanese macaques

Temperate habitats are considered marginal for the primates, who originate from the tropics. For primates living in temperate habitats, it is critical to have the ability to cope with the strong seasonality in food availability. Although Japanese macaques do not exhibit morphological adaptations such as foregut of the colobus, fiber-rich foods constitute a nonnegligible part of their diet (Hanya et al., 2011; Tsuji et al., 2013). This contrasts with the tropical macaques who spend over 50% of annual feeding time on fruits (Hanya, 2004b). Combining this thesis with previous research, Japanese macaques may have adapted to the temperate forests by improving their processing ability for fallback food through gut microbiome, while metabolizing the fat deposited from eating fruits and seeds.

Supplementing digestive function for fiber, gut microbiome facilitates nutrition harvest of Japanese macaques during food-scarce season. Comparing gut microbiome of different macaque populations, Chapter 3 and Hanya et al (2020) together proved that gut microbiome of macaques consuming more fibrous diet is more enriched in the fiber-degrading microbes and has better fermentative ability for leaves. Nevertheless, Japanese macaques overall have limited physiological/morphological adaptations for fiber fermentation considering their gut capacity (Hanya, 2010; Sawada, Sakaguchi, & Hanya, 2011). For example, when fed with diet containing ca. 14% NDF, the Japanese macaques have food retention time of 47.5 hours. Compared with other hindgut fermenting primates, their retention time is slightly longer than their tropical macaques, *M. fascicularis* (36.9 hours), but considerably shorter than the orangutans which have bigger body and gut size (124.7

hours) (Chang, Su, & Lee, 2016; Sawada et al., 2011). Considering from the physiological perspectives, Japanese macaques may indeed implement a strategy for increasing food intake rather than for increasing fermentation efficiency (Clauss et al., 2007, 2008; Stevens & Hume, 1998; Yamauchi & Iwasa, 1995).

In addition to their reliance on gut microbiome, Japanese macaques adopt other strategies to survive the food-scarce seasons. One noted strategy is fat deposition (Hamada et al., 2003; Kurihara et al., 2020). Before food-scarce seasons, Japanese macaques store fat by ingesting food in excess of their daily requirement. In both warm- and cool- temperate forests, fruit and seed intake results in greatest energy intake of Japanese macaques in the year (Iwamoto, 1982; Kurihara et al., 2020; Tsuji, 2010). Subsequently when eating mature leaves and other fibrous foods, Japanese macaques partially fulfill their energy demand with the stored fat.

4.4 Implications on the feeding ecology of other primate species

With its flexible, gut microbiome has played a key role in primate by facilitating the adoption of low-quality diet. Especially, this thesis offers insights into the role of gut microbiome in the adaptive radiation of hindgut fermenting primates to the marginal habitats. Howler monkeys, which was introduced in Chapter 1, represent another example of the hindgut-fermenting, folivorous primates. Howler monkey gut microbial composition and production of SCFAs shifted with diet across season and forest types, suggesting the buffer effect of gut microbiota against nutritional stress (Amato, Leigh, et al., 2015; Amato, Martinez-Mota, et al., 2016). In a similar sense, rhesus macaques living in high-altitude harbor a gut microbiome more enriched in metabolism-related

pathways than those living in low-altitude areas, as an adaption to low-quality diet and climate in alpine environment (Wu et al., 2020; Zhao et al., 2018).

This thesis also demonstrates the combined strategy of Japanese macaques against seasonal but predictable dietary fluctuation - improving processing ability for fallback food through gut microbiome, while metabolizing the fat deposited from eating fruits and seeds. Similar strategy may also apply to other hindgut fermenting primates living in other types of seasonal habitats. Even though evidence on fat deposition and metabolization is limited for other species of primates, orangutans are one of the few exceptions. In lowland dipterocarp forests where orangutans inhabit, fruit availability could vary dramatically year to year due to the unpredictable masting events (Hanya et al., 2013; Sakai, 2002). In response, they store fats by spending 100% of feeding time on fruits during masting season. Outside of masting season, they metabolized fat while switching diet to ingest barks and leaves (Knott, 1998). Considering their diet consisting of barks, leaves and other fibrous foods, gut microbiome may play a critical role for their survival. However at present, it remains unknown of how gut microbiome may have a role in their feeding ecology. As thus, further studies regarding the dietary plasticity provided by primate gut microbiome are warranted for more other species.

4.5 Future prospective

While offering some insight, these findings constitute only fragments of the complex mechanisms shaping primate gut microbiome. Phylosymbiosis has been proposed as the main mechanism shaping the host-gut microbiome relationship, where the gut microbiota similarity mirrors the host phylogeny. Such pattern could arise through intimate co-evolution between the hosts and microbes, and the strict

transmission of microbes within a host lineage such as vertical transmission of microbes from mother to offspring (Groussin et al., 2017). For a more holistic picture of mechanisms underlying primate gut microbiome, future works examining phylosymbiosis within and across primate species are warranted. For example, in the case of Japanese macaques, some gut microbes of particular importance may have co-evolved along with macaques' expansion throughout the Japanese archipelago.

This thesis has focused on how hosts select gut microbes through their physiology and behavior, but ultimately what matters for the wild animals is the ecological consequence of gut microbiome. An understanding on the gut microbiome function would be more straightforward. Shotgun metagenomics has been used to provide insight into the community function. However, analysis using metagenomic data has been complicated and costly. Another example would be the *in vitro* digestibility conducted by Lambert and Fellner (2012) and Hanya et al. (2020). By simulating the fermentation process of the food items, this method directly shows the digestive ability of gut microbiome at the system level.

From the perspective of macaque feeding ecology, there is more in their gut microbiome remained to be explored. So far, published research on wild macaques' gut microbiome has only compared populations with different long-term diet quality (Hanya et al (2020) and Chapter 3 of this study). However as mentioned in the previous sections, seasonality has been a major challenge for macaques' survival and thus it is critical to closely examine how the gut microbiome may respond to seasonality. In fact, seasonal variation has been widely reported in the gut microbiome of primate species, such as great apes (Hicks et al., 2018) and howler monkeys (Amato, Leigh, et al., 2015). Such changes in gut microbiome may provide a buffer

against seasonal changes in diet intake. For example, gut microbiome of howler monkeys produced more SCFAs during the extreme diet shifts (Amato, Leigh, et al., 2015). Moreover, as mentioned in Chapter 1, there is a clear difference in fallback foods between the warm-temperate and cool-temperate forests (Agetsuma & Nakagawa, 1998; Hanya, 2004a; D. A. Hill, 1997; Tsuji et al., 2015). Compared to the warm-temperate forests, cool-temperate forests, on the other hand, are challenging due to lower primary production and longer food-scarce season (Hanya et al., 2003). Gut microbiome of individuals inhabiting the cool-temperate forest may provide more evidence on how gut microbiome facilitate primate radiation towards temperate region.

4.6 Conclusion

An understanding of the ecological processes shaping gut microbiome is fundamental for the primate ecology. Using Japanese macaques as a model, this thesis described the factors shaping primate gut microbiome at individual, and population scale. Within the individual, gut microbiome is shaped by the physiochemical environment at the gut sites. Contrasting with the foregut of colobus, stomach environment of the hindgut-fermenting primates generally harbors gut microbiome of low diversity and biomass. For hindgut-fermenting primates, colon is the main fermentation chamber supporting a compositionally and functionally diverse microbiome. Across populations of single host species, primate gut microbiome is closely linked to long-term diet quality of the hosts. Facilitating exploitation of low-quality foods, gut microbiome provide buffer against the dietary challenges encountered by its hosts. Examining gut microbiome of Japanese macaques, this

thesis contributes to a better understanding of the feeding ecology of Japanese macaques and primates overall.

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Figures

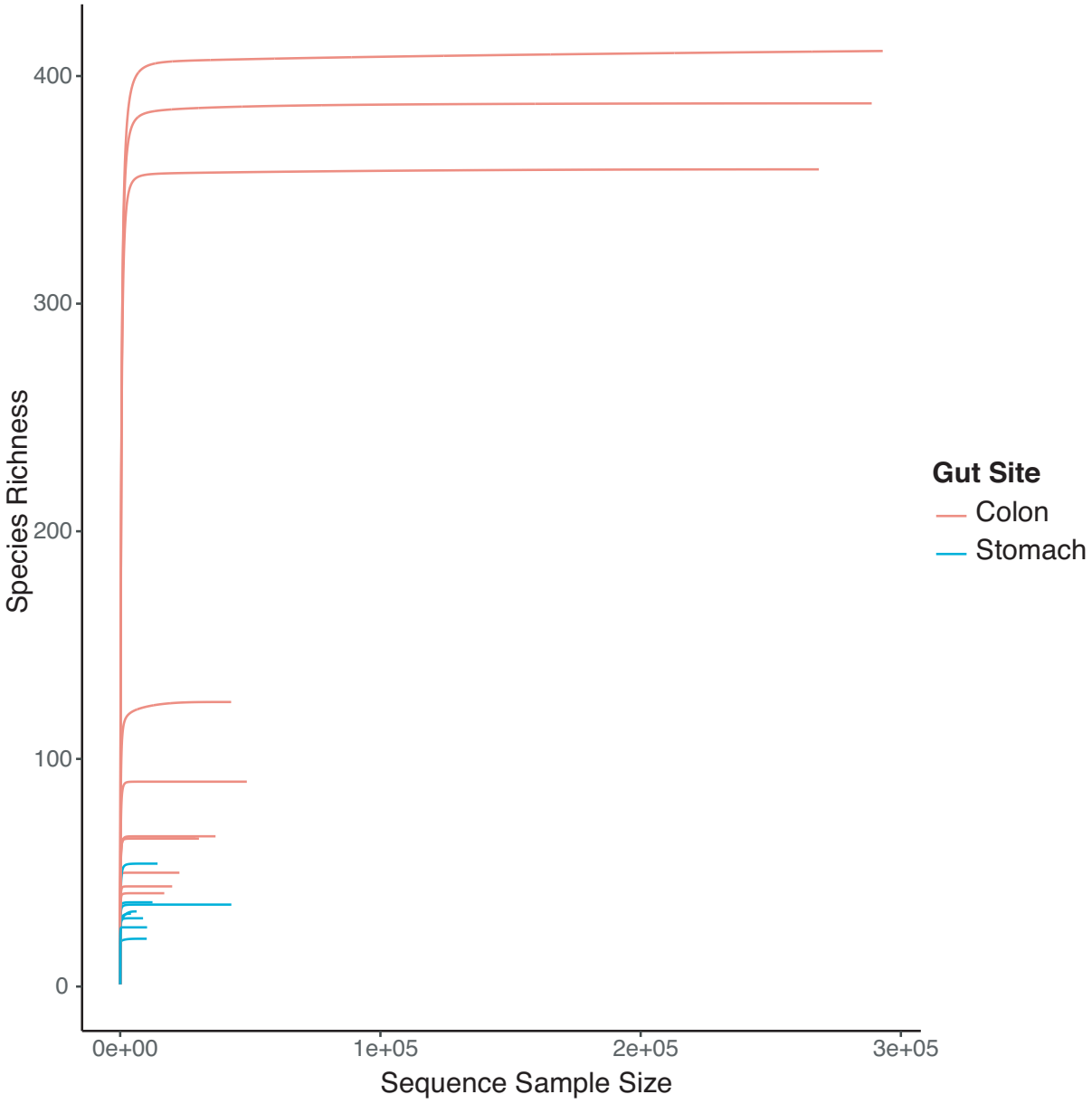

Figure 2.1 Rarefaction curve of stomach and colonic samples

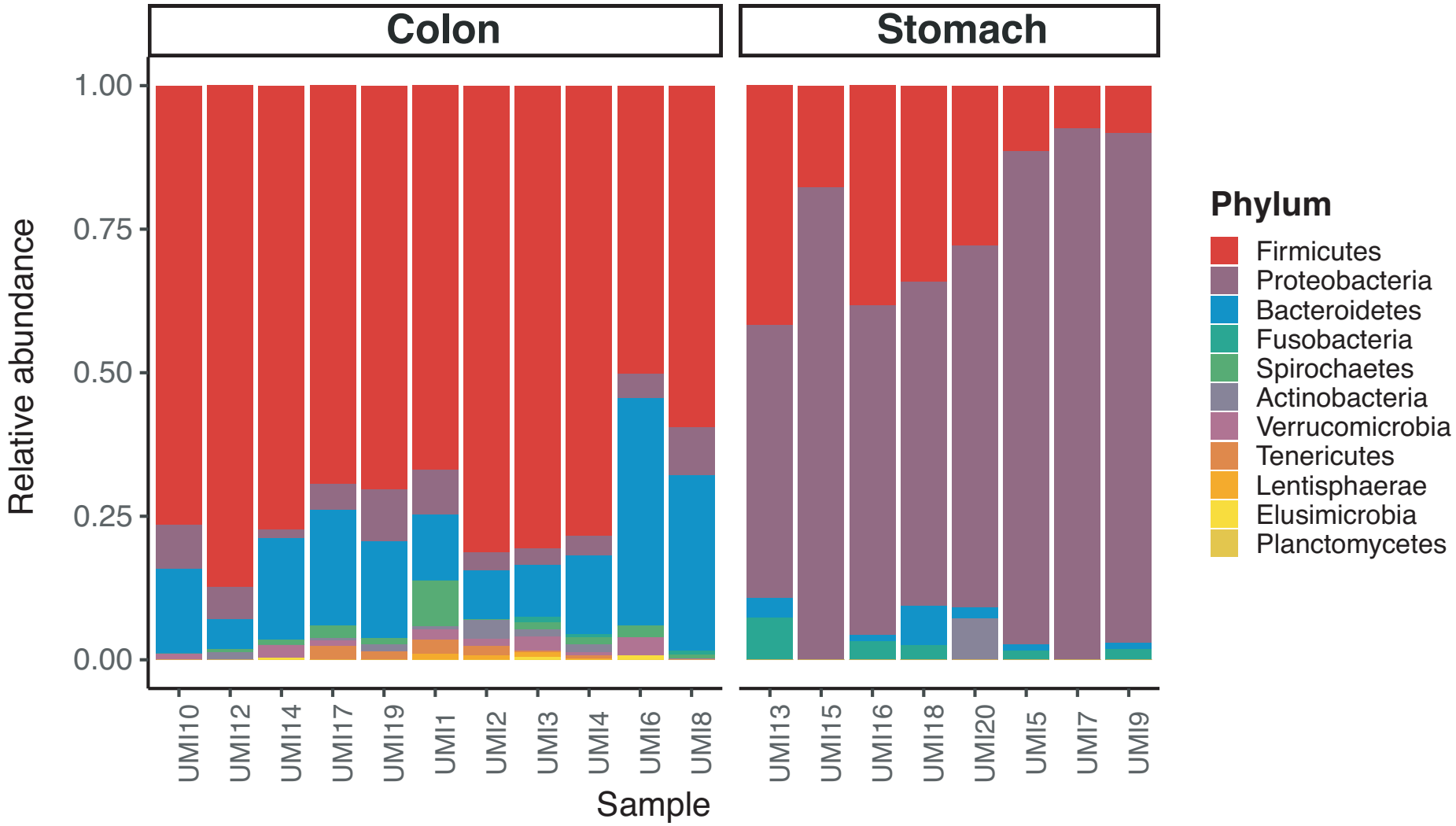

Figure 2.2 Relative abundance of gut bacterial taxa at phylum level (% of total sequences per sample)

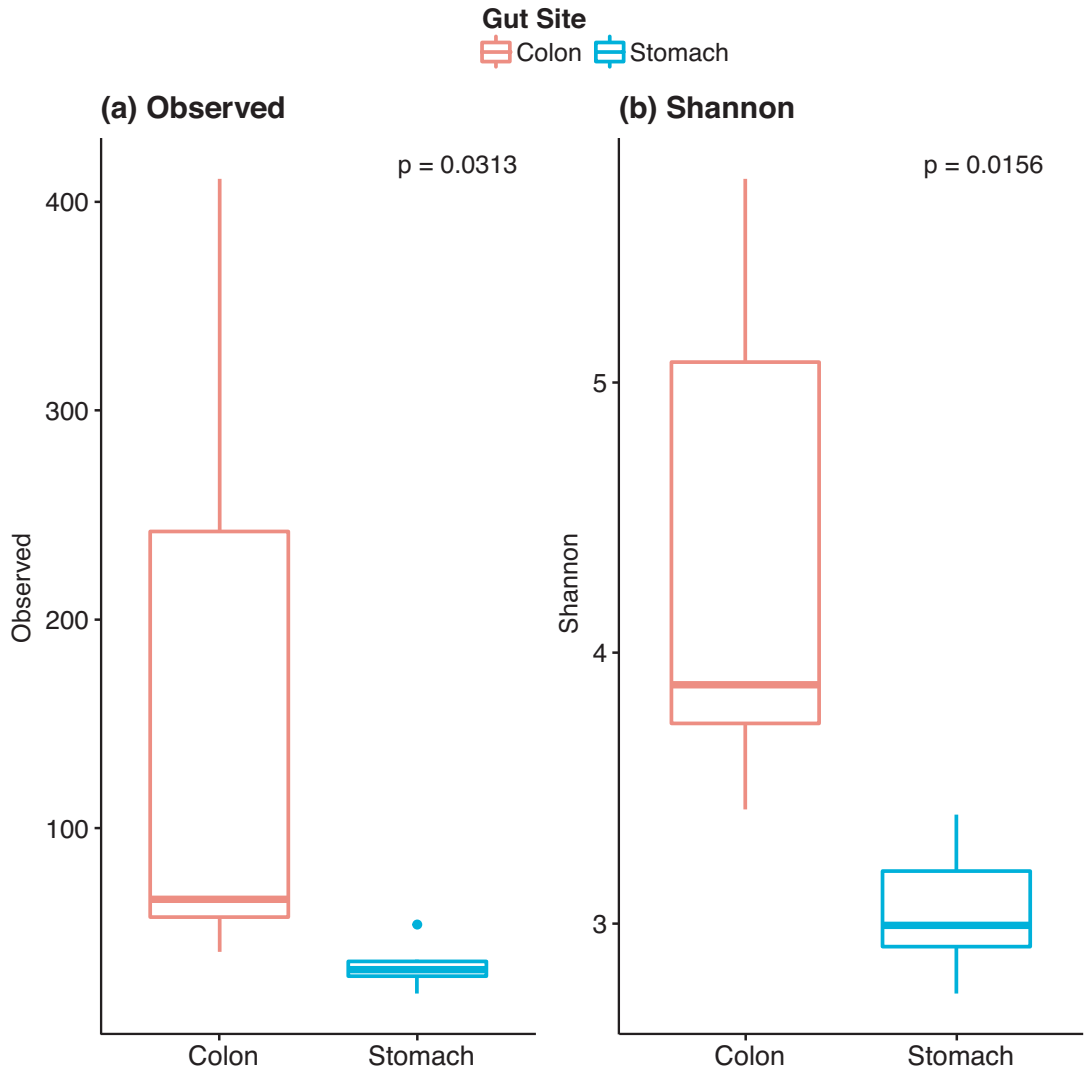

Figure 2.3 (a) Observed richness and (b) Shannon diversity index of stomach and colonic microbiomes of Japanese macaques

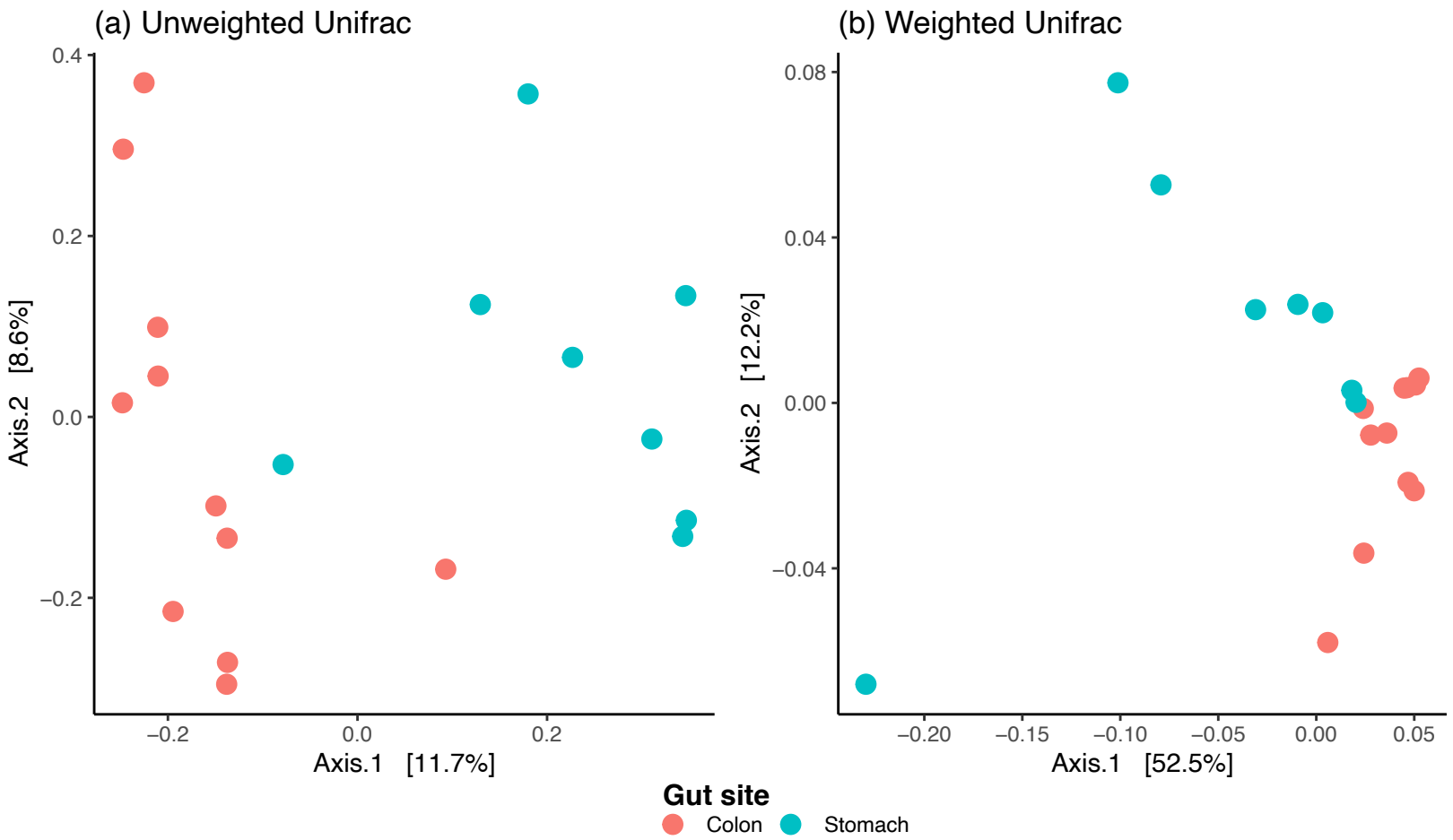

Figure 2.4 Principal coordinate analysis plots based on (a) unweighted and (b) weighted UniFrac distance for macaques' gut bacterial communities

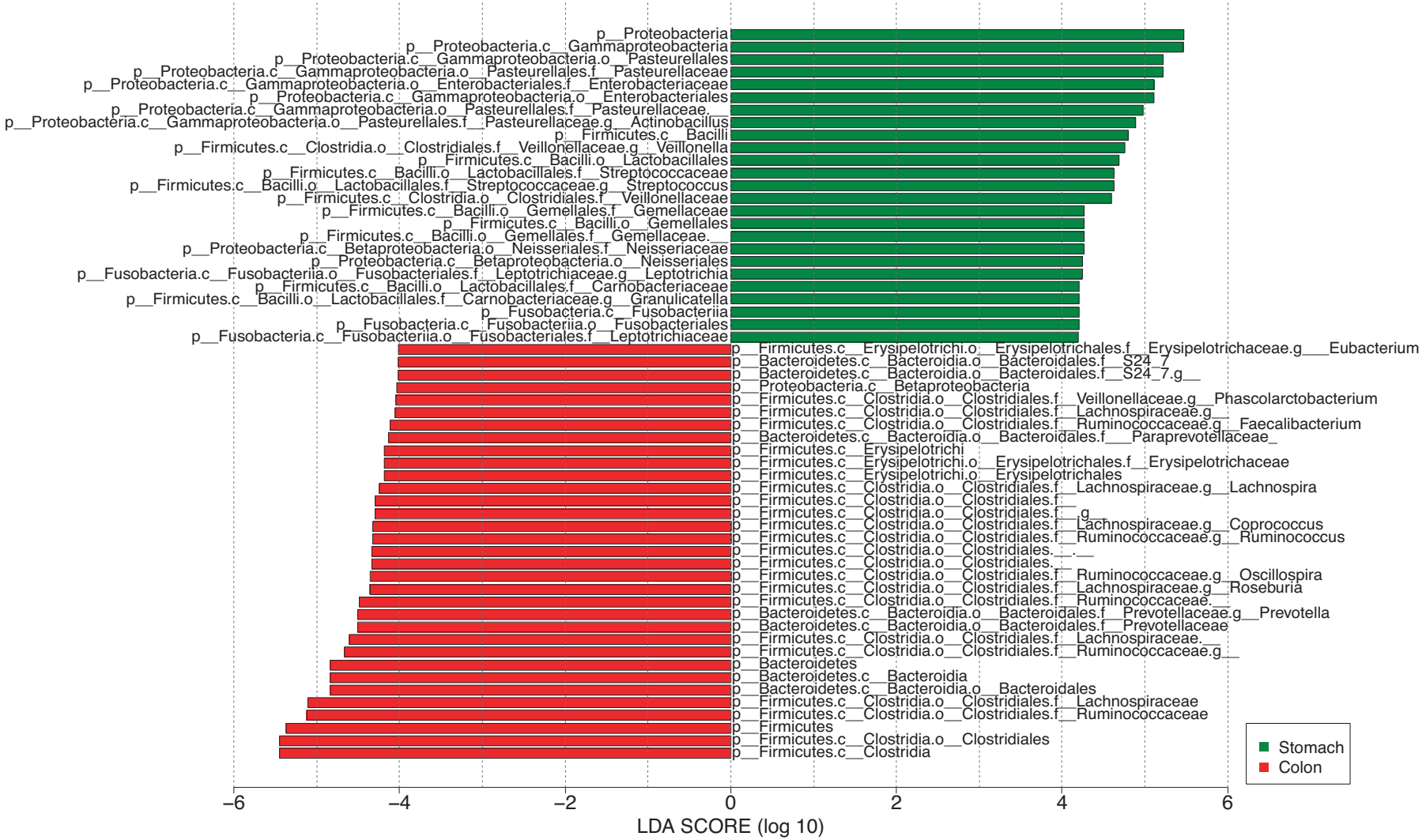

Figure 2.5 Gut microbial genera differentially abundant in the stomach and colonic microbiome. Plot showing the histogram of linear discriminant analysis (LDA) scores computed for differentially abundant bacterial genera (log LDA score > 5.0, p <0.05)

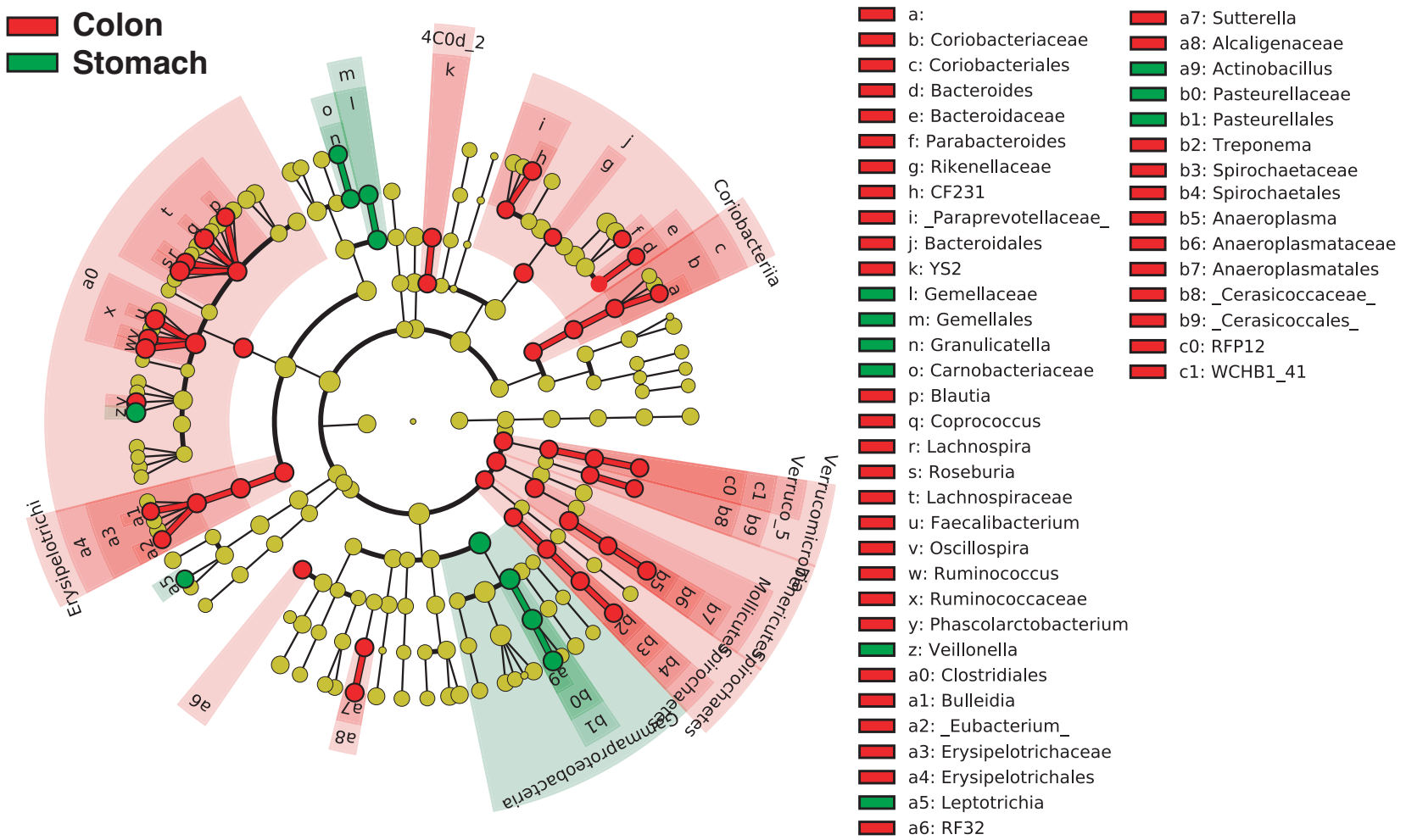

Figure 2.6 Cladogram plotted from LEfSe showing the taxonomic levels represented by rings with phyla in the outermost the ring and genera in the innermost ring. Each circle is a member within that level. Those taxa in each level are colored by the gut sites in which the taxa are more abundant (log LDA score >2.0, p <0.05)

Figure 2.7 Histogram of LDA scores computed for differentially abundant Kyoto Encyclopedia of Genes and Genome Orthology (KO) pathways in the stomach and colonic microbiome (log LDA score > 2.0, $p < 0.05$)

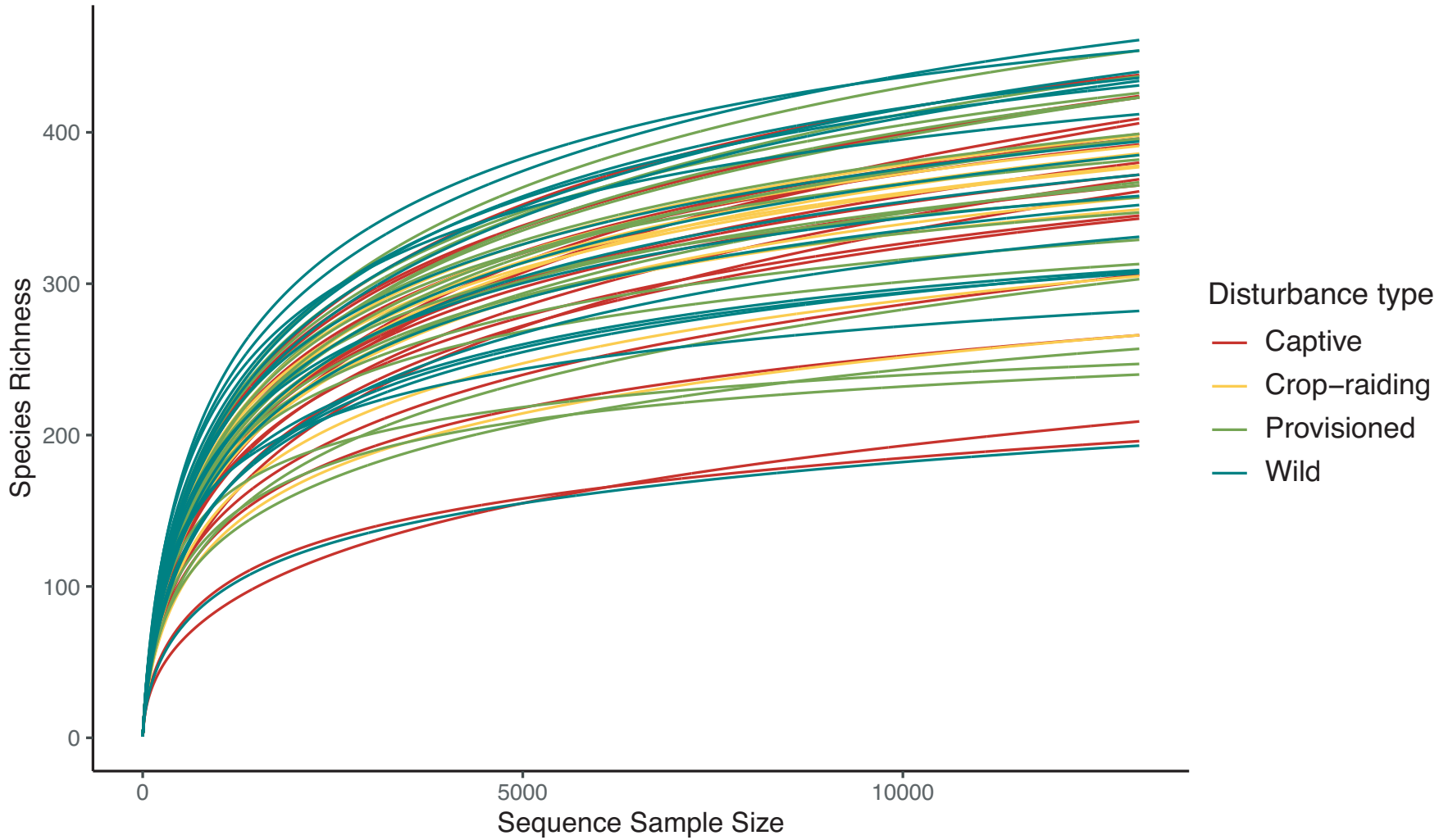

Figure 3.1 Rarefaction curves colored by disturbance types

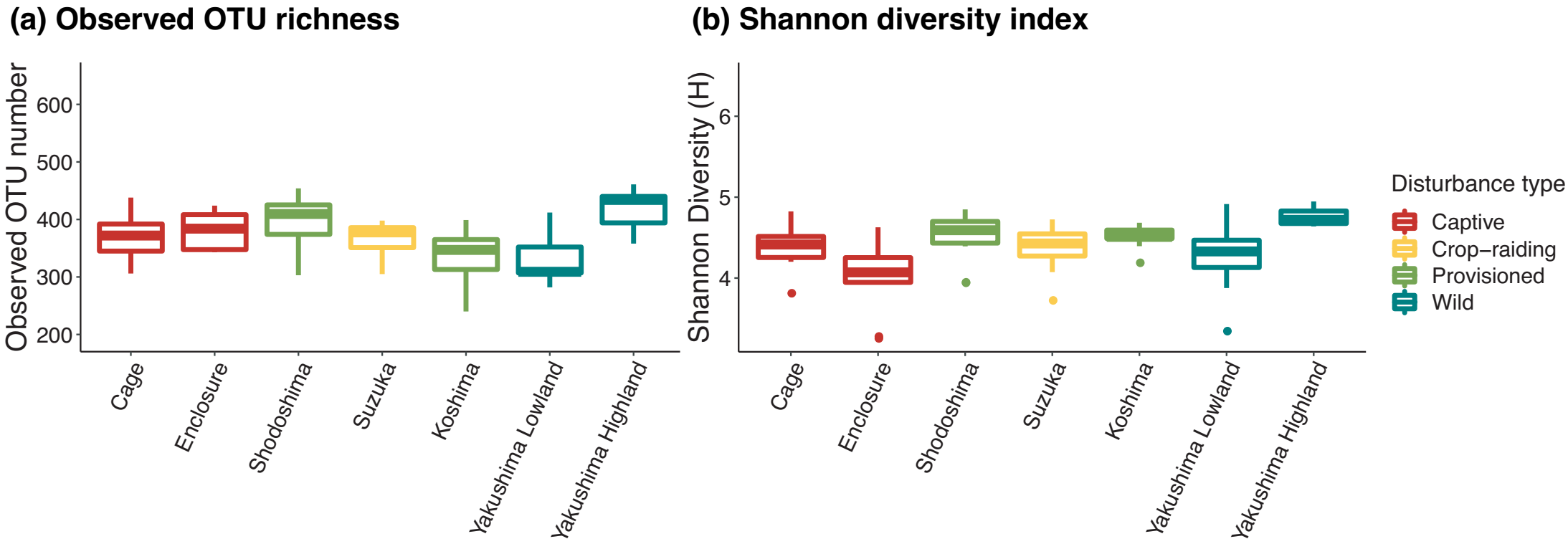

Figure 3.2. (a) Observed OTU richness and (b) Shannon diversity index of Japanese macaques. Color indicates human disturbance type

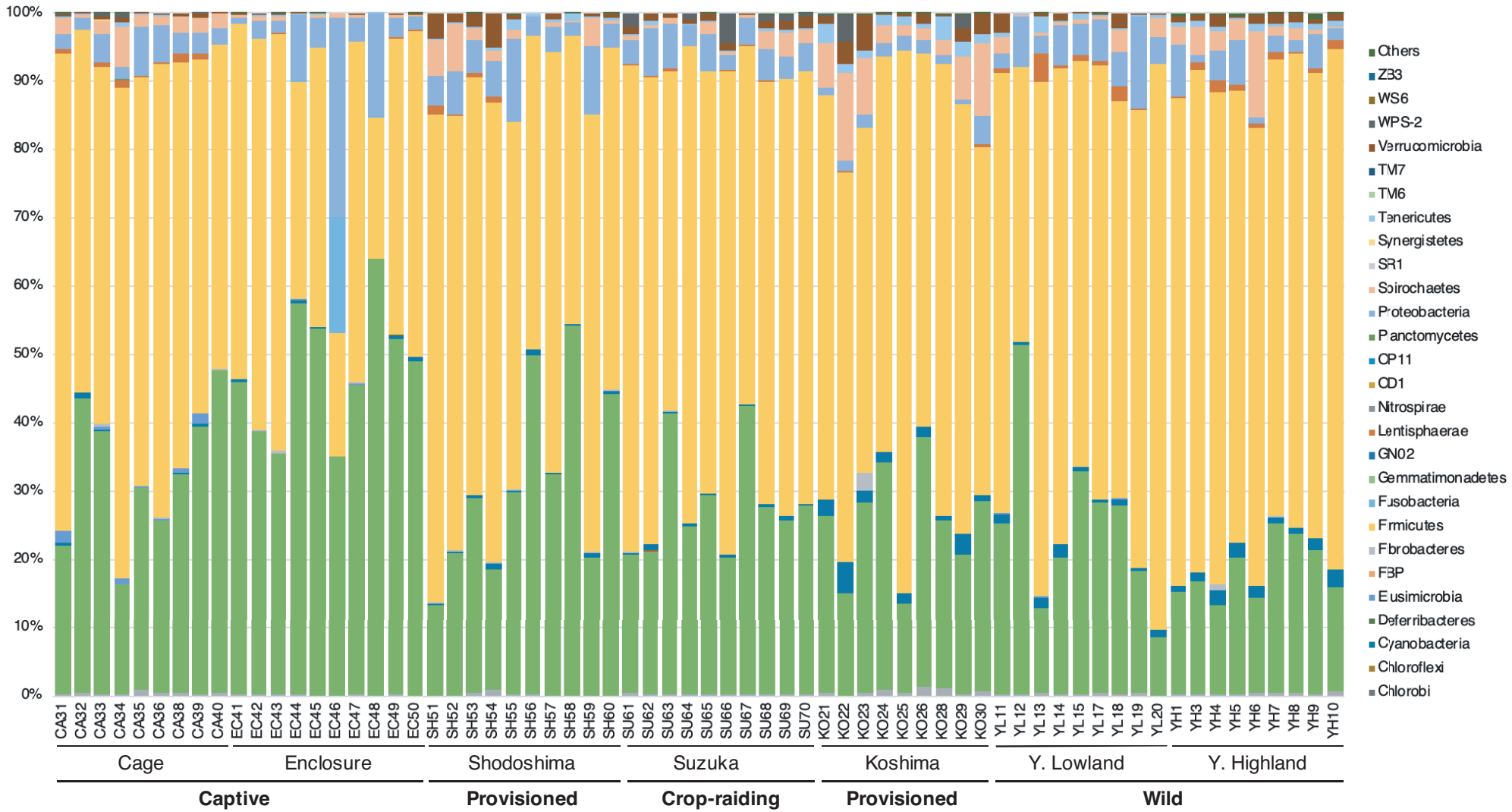

Figure 3.3 Relative abundance of gut bacterial taxa at phylum level. Abbreviation represents the collection sites

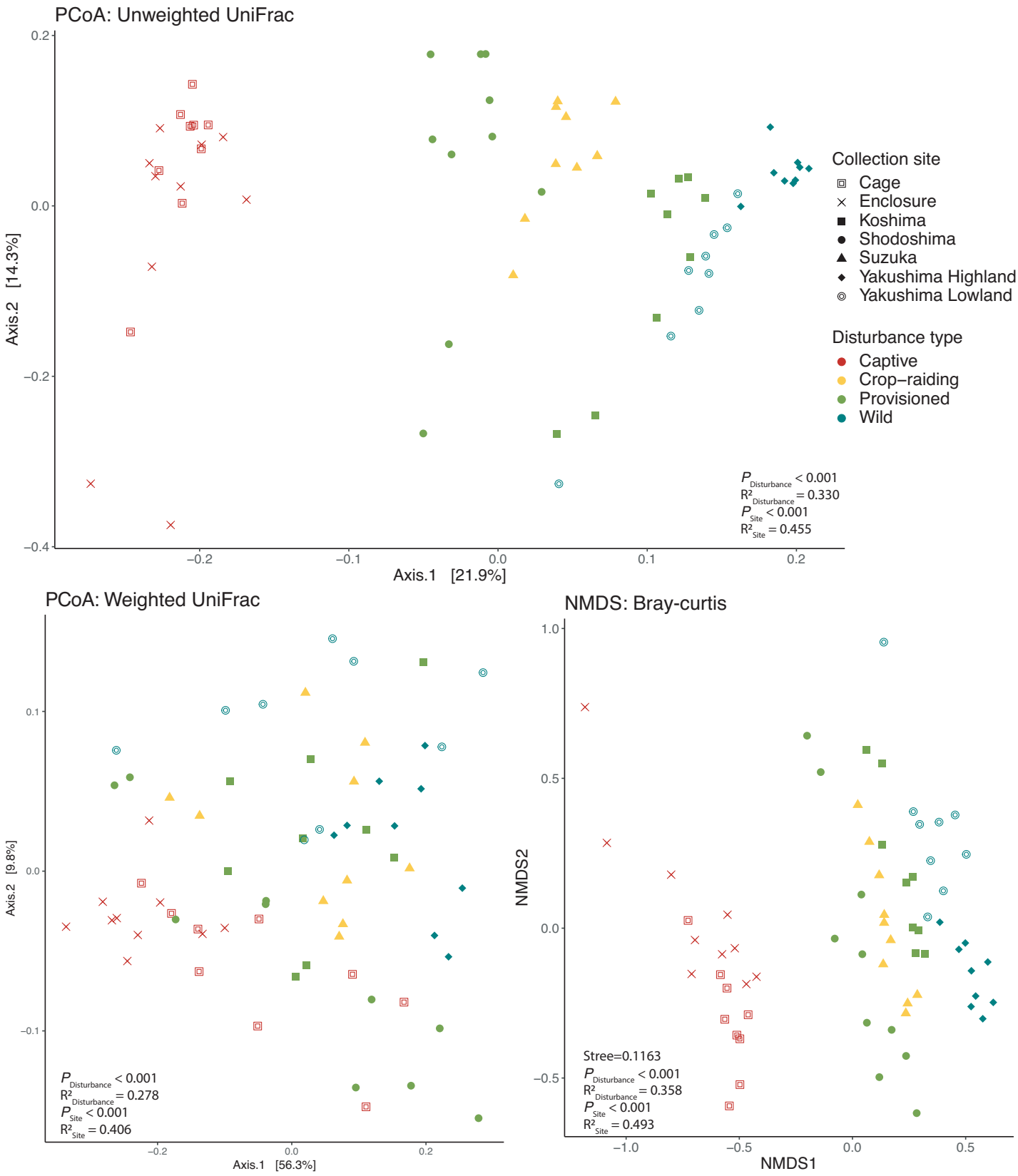

Figure 3.4 NMDS and PCoA plots based UniFrac distance for macaques' gut bacterial communities

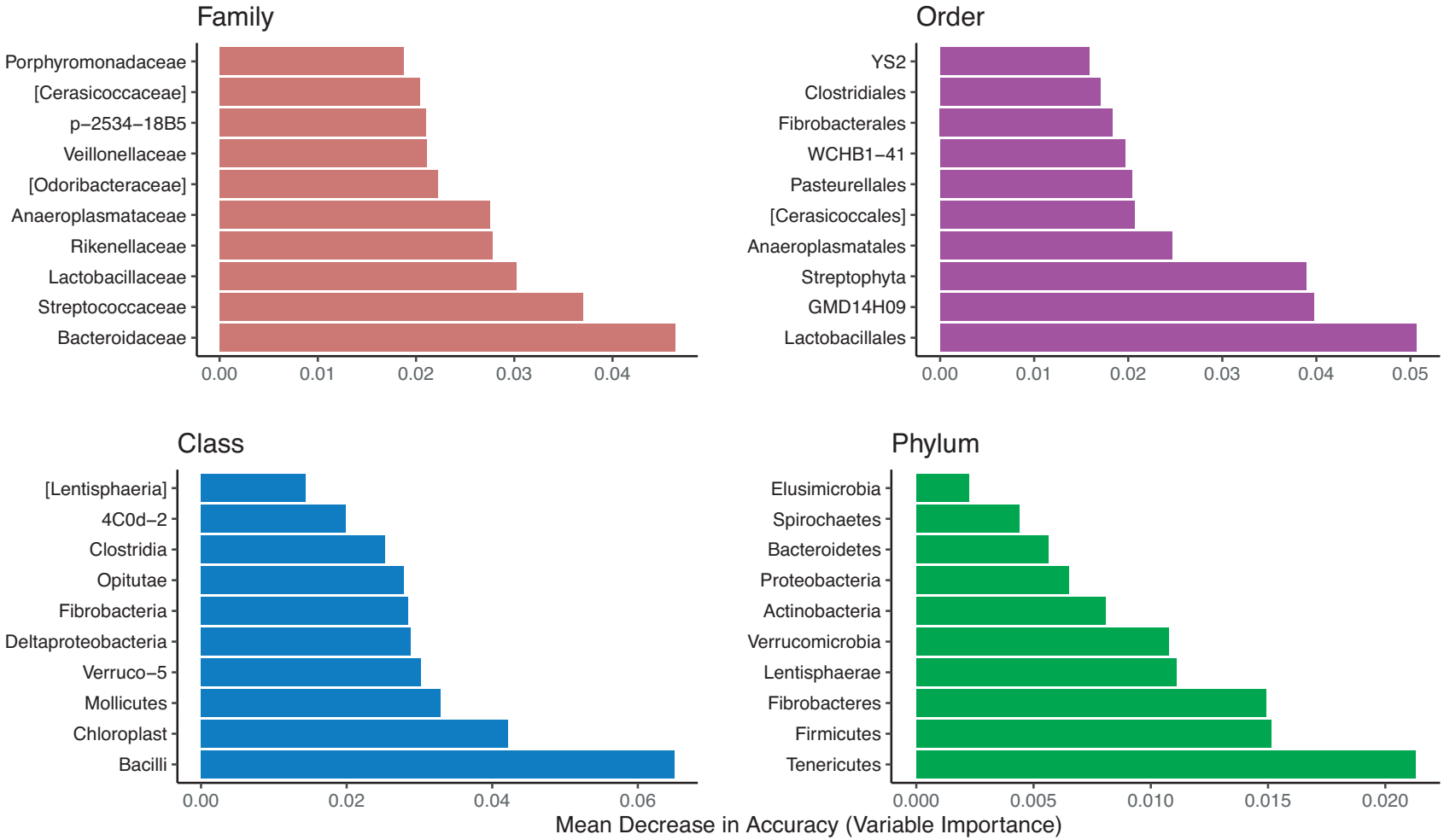

Figure 3.5 Top ten bacterial taxa important in assessing macaques' reliance on anthropogenic food at each taxonomic rank (phylum, class, order, family)

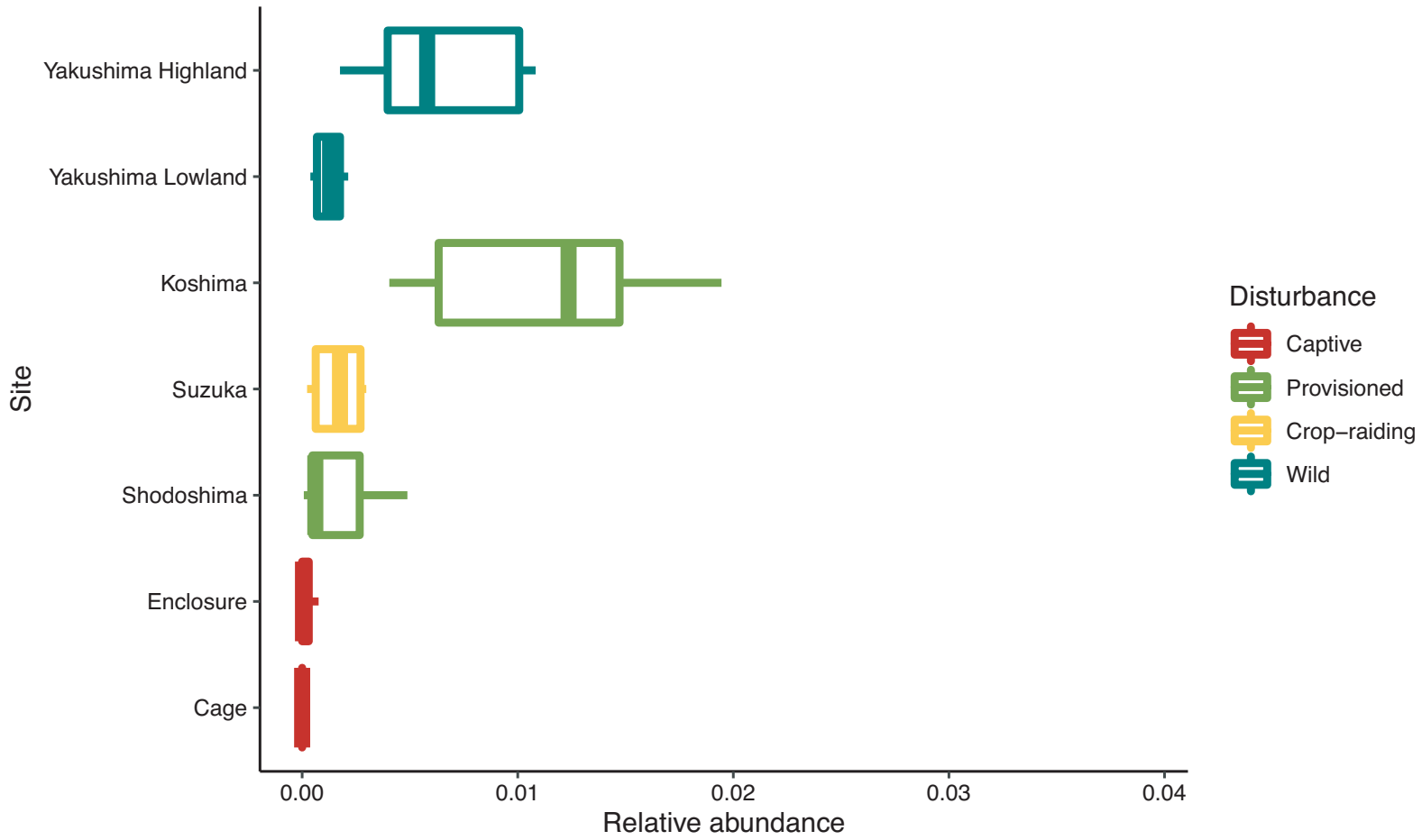

Figure 3.6 Relative abundance of Chloroplast

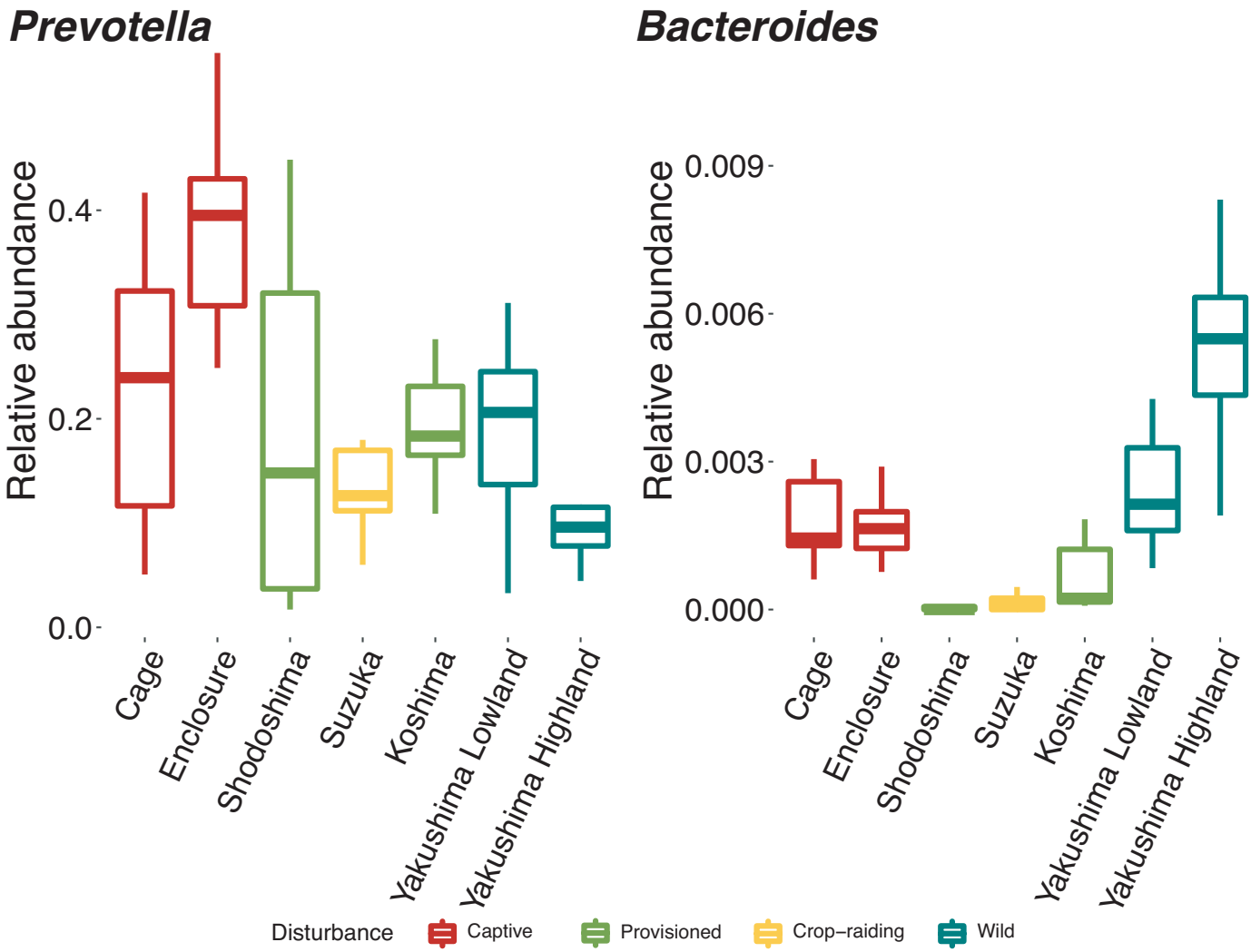

Figure 3.7 Relative abundance of the dominant bacterial genera in human, *Prevotella* and *Bacteroides*

Tables

Table 2.1 Sample information

SampleID	Group	Sex	Collected year&month	GutSite	PairedID	Seuqncing depth
UMI1	umia	male	2017 July	colon	2017AM	42628
UMI2	umia	female	2017 July	colon	2017AF	269882
UMI3	umib	female	2017 July	colon	2017BF	289151
UMI4	umia	female	2018 May	colon	2018AF	294209
UMI6	umia	male	2018 May	colon	2018AM	17427
UMI8	umib	female	2018 May	colon	2018BF	48753
UMI10	umib	male	2018 May	colon	2018BM	19976
UMI12	umic	female	2018 May	colon	2018CF	23050
UMI14	umic	male	2018 May	colon	2018CM	30265
UMI17	umia	male	2019 Septmenber	colon	2019AM	36631
UMI19	umia	female	2019 Septmenber	colon	2019AF	28918
UMI5	umia	female	2018 May	stomach	2018AF	46398
UMI7	umia	male	2018 May	stomach	2018AM	4221
UMI9	umib	female	2018 May	stomach	2018BF	10529
UMI13	umic	female	2018 May	stomach	2018CF	15547
UMI15	umic	male	2018 May	stomach	2018CM	6658
UMI16	umia	male	2019 Septmenber	stomach	2019AM	11950
UMI18	umia	female	2019 Septmenber	stomach	2019AF	15528
UMI20	umic	male	2019 Septmenber	stomach	2019CM	12760

Table 2.2 Relative abundance of microbial phyla

Phylum	Colon												
	UMI1	UMI10	UMI12	UMI14	UMI17	UMI19	UMI2	UMI3	UMI4	UMI6	UMI8	Average	SD
Actinobacteria	0.55%	0.00%	1.10%	0.00%	0.36%	1.04%	2.37%	1.03%	1.05%	0.00%	0.00%	1.21%	0.73%
Bacteroidetes	11.30%	15.50%	4.69%	22.07%	21.96%	16.05%	7.03%	9.27%	12.70%	38.17%	29.69%	12.04%	10.12%
Cyanobacteria	4.20%	0.00%	2.61%	1.04%	0.00%	0.00%	1.25%	0.85%	3.22%	1.31%	0.00%	1.65%	1.44%
Elusimicrobia	0.00%	0.00%	0.00%	0.26%	0.00%	0.00%	0.00%	0.31%	0.00%	0.72%	0.00%	0.10%	0.23%
Euryarchaeota	0.13%	0.00%	0.00%	0.37%	0.00%	0.00%	0.90%	0.00%	0.64%	0.00%	0.00%	0.40%	0.31%
Firmicutes	61.06%	71.73%	83.91%	70.21%	67.90%	71.79%	80.15%	75.98%	75.02%	50.98%	56.89%	74.48%	9.90%
Fusobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.73%	0.41%	0.00%	0.67%	0.33%	0.29%
Lentisphaerae	0.89%	0.00%	0.00%	0.00%	0.00%	0.00%	0.52%	0.71%	0.20%	0.00%	0.00%	0.40%	0.33%
OD1	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.01%	0.01%
Planctomycetes	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Proteobacteria	7.29%	11.80%	7.20%	3.38%	4.36%	8.67%	3.55%	4.18%	3.54%	3.96%	11.79%	4.61%	3.24%
Spirochaetes	6.53%	0.00%	0.49%	0.72%	2.12%	1.07%	0.06%	0.84%	0.97%	1.98%	0.65%	0.94%	1.83%
Tenericutes	4.34%	0.00%	0.00%	0.11%	2.34%	1.38%	2.19%	1.84%	0.60%	0.00%	0.18%	1.47%	1.40%
Verrucomicrobia	1.47%	0.97%	0.00%	1.86%	0.96%	0.00%	0.87%	2.02%	0.62%	2.88%	0.14%	1.12%	0.92%
WPS-2	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.66%	0.00%	0.00%	0.18%	0.20%
NA	2.24%	0.00%	0.00%	0.00%	0.00%	0.00%	1.13%	2.22%	0.36%	0.00%	0.00%	1.04%	0.90%

Phylum	Stomach									
	UMI13	UMI15	UMI16	UMI18	UMI20	UMI5	UMI7	UMI9	Average	SD
Actinobacteria	0.00%	0.00%	0.00%	0.00%	7.22%	0.00%	0.00%	0.00%	0.67%	2.55%
Bacteroidetes	3.13%	0.00%	0.93%	6.91%	1.86%	1.12%	0.00%	0.78%	1.96%	2.29%
Cyanobacteria	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Elusimicrobia	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Euryarchaeota	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Firmicutes	39.47%	18.77%	33.84%	34.18%	27.77%	11.40%	7.46%	6.13%	20.79%	13.16%
Fusobacteria	6.99%	0.00%	2.86%	2.47%	0.00%	1.53%	0.00%	1.38%	2.16%	2.34%
Lentisphaerae	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
OD1	0.00%	0.00%	0.00%	0.00%	0.00%	0.52%	0.00%	0.00%	0.21%	0.19%
Planctomycetes	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Proteobacteria	45.12%	81.23%	50.85%	56.44%	63.10%	85.10%	92.54%	66.41%	70.07%	17.09%
Spirochaetes	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Tenericutes	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Verrucomicrobia	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
WPS-2	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
NA	5.29%	0.00%	11.51%	0.00%	0.05%	0.33%	0.00%	25.31%	4.14%	9.07%

Table 2.3 Bacterial genera identified by LEfSe analysis different between stomach and colonic microbiota (log LDA score >2.0, p <0.05)

Differentially abundant taxa	Class	log_{10}(LDA score)	*p*-value
p__Firmicutes.c__Clostridia	colon	5.4511	0.0003
p__Firmicutes.c__Clostridia.o__Clostridiales	colon	5.4511	0.0003
p__Firmicutes	colon	5.3684	0.0003
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae	colon	5.1202	0.0002
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae	colon	5.1121	0.0002
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales	colon	4.8410	0.0004
p__Bacteroidetes.c__Bacteroidia	colon	4.8410	0.0004
p__Bacteroidetes	colon	4.8357	0.0004
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__	colon	4.6670	0.0002
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.__	colon	4.6074	0.0002
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Prevotellaceae	colon	4.5093	0.0015
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Prevotellaceae.g__Prevotella	colon	4.5073	0.0015
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.__	colon	4.4849	0.0002
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Roseburia	colon	4.3561	0.0005
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__Oscillospira	colon	4.3527	0.0013
p__Firmicutes.c__Clostridia.o__Clostridiales.__	colon	4.3365	0.0002
p__Firmicutes.c__Clostridia.o__Clostridiales.__.__	colon	4.3365	0.0002
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__Ruminococcus	colon	4.3207	0.0005
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Coprococcus	colon	4.3207	0.0013
p__Firmicutes.c__Clostridia.o__Clostridiales.f__.g__	colon	4.2950	0.0005
p__Firmicutes.c__Clostridia.o__Clostridiales.f__	colon	4.2950	0.0005
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Lachnospira	colon	4.2466	0.0002
p__Firmicutes.c__Erysipelotrichi.o__Erysipelotrichales	colon	4.1820	0.0002
p__Firmicutes.c__Erysipelotrichi.o__Erysipelotrichales.f__Erysipelotrichaceae	colon	4.1820	0.0002
p__Firmicutes.c__Erysipelotrichi	colon	4.1820	0.0002
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f___Paraprevotellaceae_	colon	4.1328	0.0005
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Ruminococcaceae.g__Faecalibacterium	colon	4.1131	0.0005
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__	colon	4.0554	0.0013
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Veillonellaceae.g__Phascolarctobacterium	colon	4.0432	0.0002

p__Proteobacteria.c__Betaproteobacteria	colon	4.0296	0.0277
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__S24_7.g__	colon	4.0184	0.0002
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__S24_7	colon	4.0184	0.0002
p__Firmicutes.c__Erysipelotrichi.o__Erysipelotrichales.f__Erysipelotrichaceae.g___Eubacterium_	colon	4.0108	0.0331
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f___Paraprevotellaceae_.g___Prevotella_	colon	3.9777	0.0033
p__Cyanobacteria.c__4C0d_2.o__YS2.f__	colon	3.9256	0.0076
p__Cyanobacteria	colon	3.9256	0.0076
p__Cyanobacteria.c__4C0d_2.o__YS2.f__.g__	colon	3.9256	0.0076
p__Cyanobacteria.c__4C0d_2	colon	3.9256	0.0076
p__Cyanobacteria.c__4C0d_2.o__YS2	colon	3.9256	0.0076
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f___Paraprevotellaceae_.g__	colon	3.9231	0.0163
p__Spirochaetes	colon	3.9203	0.0005
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Christensenellaceae.g__	colon	3.9092	0.0076
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Christensenellaceae	colon	3.9092	0.0076
p__Firmicutes.c__Erysipelotrichi.o__Erysipelotrichales.f__Erysipelotrichaceae.g__	colon	3.9026	0.0013
p__Tenericutes	colon	3.8969	0.0033
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.__.__	colon	3.8807	0.0013
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.__	colon	3.8807	0.0013
p__Spirochaetes.c__Spirochaetes	colon	3.8774	0.0033
p__Spirochaetes.c__Spirochaetes.o__Spirochaetales.f__Spirochaetaceae.g__Treponema	colon	3.8774	0.0033
p__Spirochaetes.c__Spirochaetes.o__Spirochaetales	colon	3.8774	0.0033
p__Spirochaetes.c__Spirochaetes.o__Spirochaetales.f__Spirochaetaceae	colon	3.8774	0.0033
p__Proteobacteria.c__Betaproteobacteria.o__Burkholderiales	colon	3.8751	0.0013
p__Proteobacteria.c__Betaproteobacteria.o__Burkholderiales.__.__	colon	3.8604	0.0331
p__Proteobacteria.c__Betaproteobacteria.o__Burkholderiales.__	colon	3.8603	0.0331
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Dorea	colon	3.8424	0.0076
p__Verrucomicrobia	colon	3.8352	0.0013
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__	colon	3.8300	0.0033
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__.g__	colon	3.8300	0.0033
p__Proteobacteria.c__Betaproteobacteria.o__Burkholderiales.f__Alcaligenaceae.g__Sutterella	colon	3.8294	0.0013
p__Proteobacteria.c__Betaproteobacteria.o__Burkholderiales.f__Alcaligenaceae	colon	3.8294	0.0013

p__Firmicutes.c__Clostridia.o__Clostridiales.f__Lachnospiraceae.g__Blautia	colon	3.8272	0.0033
p__Proteobacteria.c__Gammaproteobacteria.o__Aeromonadales.f__Succinivibrionaceae	colon	3.8051	0.0033
p__Proteobacteria.c__Gammaproteobacteria.o__Aeromonadales.f__Succinivibrionaceae.g__Succinivibrio	colon	3.8051	0.0033
p__Proteobacteria.c__Gammaproteobacteria.o__Aeromonadales	colon	3.8051	0.0033
p__Proteobacteria.c__Epsilonproteobacteria.o__Campylobacterales.f__Helicobacteraceae.g__Flexispira	colon	3.7996	0.0163
p__Proteobacteria.c__Alphaproteobacteria.o__Rickettsiales.f__.g__	colon	3.7749	0.0331
p__Proteobacteria.c__Alphaproteobacteria.o__Rickettsiales.f__	colon	3.7749	0.0331
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae.g__Bacteroides	colon	3.7680	0.0331
p__Verrucomicrobia.c__Opitutae	colon	3.7645	0.0331
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Clostridiaceae	colon	3.7611	0.0076
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Bacteroidaceae	colon	3.7577	0.0331
p__Actinobacteria.c__Coriobacteriia.o__Coriobacteriales.f__Coriobacteriaceae.g__	colon	3.7569	0.0163
p__Firmicutes.c__Erysipelotrichi.o__Erysipelotrichales.f__Erysipelotrichaceae.g__RFN20	colon	3.7558	0.0331
p__Verrucomicrobia.c__Opitutae.o___Cerasicoccales_	colon	3.7534	0.0331
p__Verrucomicrobia.c__Opitutae.o___Cerasicoccales_.f___Cerasicoccaceae_.g__	colon	3.7534	0.0331
p__Verrucomicrobia.c__Opitutae.o___Cerasicoccales_.f___Cerasicoccaceae_	colon	3.7534	0.0331
p__Actinobacteria.c__Coriobacteriia.o__Coriobacteriales.f__Coriobacteriaceae	colon	3.7450	0.0163
p__Actinobacteria.c__Coriobacteriia	colon	3.7450	0.0163
p__Actinobacteria.c__Coriobacteriia.o__Coriobacteriales	colon	3.7450	0.0163
p__Proteobacteria.c__Alphaproteobacteria.__.__.__	colon	3.7305	0.0163
p__Proteobacteria.c__Alphaproteobacteria.__.__	colon	3.7305	0.0163
p__Proteobacteria.c__Alphaproteobacteria.__	colon	3.7305	0.0163
p__Tenericutes.c__Mollicutes	colon	3.7230	0.0076
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f___Paraprevotellaceae_.g__CF231	colon	3.7057	0.0076
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Rikenellaceae	colon	3.7040	0.0033
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Rikenellaceae.g__	colon	3.7040	0.0033
p__Firmicutes.c__Clostridia.o__Clostridiales.f___Mogibacteriaceae_	colon	3.6991	0.0076
p__Firmicutes.c__Clostridia.o__Clostridiales.f___Mogibacteriaceae_.g__	colon	3.6991	0.0076
p__Tenericutes.c__Mollicutes.o__Anaeroplasmatales	colon	3.6984	0.0076
p__Tenericutes.c__Mollicutes.o__Anaeroplasmatales.f__Anaeroplasmataceae	colon	3.6984	0.0076
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__Porphyromonadaceae.g__Parabacteroides	colon	3.6944	0.0076

p__Tenericutes.c__RF3.o__ML615J_28.f__	colon	3.6869	0.0331
p__Tenericutes.c__RF3	colon	3.6869	0.0331
p__Tenericutes.c__RF3.o__ML615J_28.f__.g__	colon	3.6869	0.0331
p__Tenericutes.c__RF3.o__ML615J_28	colon	3.6869	0.0331
p__Verrucomicrobia.c__Verruco_5	colon	3.6757	0.0033
p__Verrucomicrobia.c__Verruco_5.o__WCHB1_41.f__RFP12	colon	3.6757	0.0033
p__Verrucomicrobia.c__Verruco_5.o__WCHB1_41.f__RFP12.g__	colon	3.6757	0.0033
p__Verrucomicrobia.c__Verruco_5.o__WCHB1_41	colon	3.6757	0.0033
p__Proteobacteria.c__Alphaproteobacteria.o__RF32	colon	3.6503	0.0033
p__Proteobacteria.c__Alphaproteobacteria.o__RF32.f__.g__	colon	3.6503	0.0033
p__Proteobacteria.c__Alphaproteobacteria.o__RF32.f__	colon	3.6503	0.0033
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Veillonellaceae.g__Dialister	colon	3.6481	0.0331
p__Tenericutes.c__Mollicutes.o__Anaeroplasmatales.f__Anaeroplasmataceae.g__	colon	3.6393	0.0163
p__Firmicutes.c__Erysipelotrichi.o__Erysipelotrichales.f__Erysipelotrichaceae.g__Bulleidia	colon	3.6107	0.0163
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__RF16.g__	colon	3.6099	0.0331
p__Bacteroidetes.c__Bacteroidia.o__Bacteroidales.f__RF16	colon	3.6099	0.0331
p__Proteobacteria	stomach	5.4670	0.0003
p__Proteobacteria.c__Gammaproteobacteria	stomach	5.4632	0.0003
p__Proteobacteria.c__Gammaproteobacteria.o__Pasteurellales	stomach	5.2180	0.0012
p__Proteobacteria.c__Gammaproteobacteria.o__Pasteurellales.f__Pasteurellaceae	stomach	5.2179	0.0012
p__Proteobacteria.c__Gammaproteobacteria.o__Enterobacteriales.f__Enterobacteriaceae	stomach	5.1109	0.0074
p__Proteobacteria.c__Gammaproteobacteria.o__Enterobacteriales	stomach	5.1092	0.0074
p__Proteobacteria.c__Gammaproteobacteria.o__Pasteurellales.f__Pasteurellaceae.__	stomach	4.9740	0.0074
p__Proteobacteria.c__Gammaproteobacteria.o__Pasteurellales.f__Pasteurellaceae.g__Actinobacillus	stomach	4.8884	0.0008
p__Firmicutes.c__Bacilli	stomach	4.8013	0.0003
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Veillonellaceae.g__Veillonella	stomach	4.7553	0.0012
p__Firmicutes.c__Bacilli.o__Lactobacillales	stomach	4.6845	0.0035
p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Streptococcaceae	stomach	4.6254	0.0023
p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Streptococcaceae.g__Streptococcus	stomach	4.6225	0.0023
p__Firmicutes.c__Clostridia.o__Clostridiales.f__Veillonellaceae	stomach	4.5965	0.0475
p__Firmicutes.c__Bacilli.o__Gemellales.f__Gemellaceae	stomach	4.2689	0.0010

p__Firmicutes.c__Bacilli.o__Gemellales	stomach	4.2689	0.0010
p__Firmicutes.c__Bacilli.o__Gemellales.f__Gemellaceae.__	stomach	4.2676	0.0034
p__Proteobacteria.c__Betaproteobacteria.o__Neisseriales.f__Neisseriaceae	stomach	4.2652	0.0320
p__Proteobacteria.c__Betaproteobacteria.o__Neisseriales	stomach	4.2471	0.0320
p__Fusobacteria.c__Fusobacteriia.o__Fusobacteriales.f__Leptotrichiaceae.g__Leptotrichia	stomach	4.2449	0.0109
p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Carnobacteriaceae	stomach	4.2049	0.0109
p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Carnobacteriaceae.g__Granulicatella	stomach	4.2049	0.0109
p__Fusobacteria.c__Fusobacteriia	stomach	4.2045	0.0345
p__Fusobacteria.c__Fusobacteriia.o__Fusobacteriales	stomach	4.2023	0.0345
p__Fusobacteria	stomach	4.2012	0.0345
p__Fusobacteria.c__Fusobacteriia.o__Fusobacteriales.f__Leptotrichiaceae	stomach	4.1920	0.0194

Table 2.4 KO pathways identified by LEfSe analysis different between stomach and colonic microbiota(log LDA score >2.0, p <0.05)

KO pathway	Level 3	Gut site	log_{10}(LDA score)	p-value	Level1	Level 2
ko01051	Biosynthesis of ansamycins	colon	3.917	0.001	Metabolism	Metabolism of terpenoids and polyketides
ko02030	Bacterial chemotaxis	colon	3.604	0.008	Cellular Processes	Cell motility
ko03020	RNA polymerase	colon	3.561	0.000	Genetic Information Processing	Transcription
ko00521	Streptomycin biosynthesis	colon	3.479	0.000	Metabolism	Biosynthesis of other secondary metabolites
ko00121	Secondary bile acid biosynthesis	colon	3.461	0.000	Metabolism	Lipid metabolism
ko02040	Flagellar assembly	colon	3.433	0.026	Cellular Processes	Cell motility
ko00250	Alanine, aspartate and glutamate metabolism	colon	3.380	0.000	Metabolism	Amino acid metabolism
ko00730	Thiamine metabolism	colon	3.339	0.000	Metabolism	Metabolism of cofactors and vitamins
ko01055	Biosynthesis of vancomycin group antibiotics	colon	3.333	0.001	Metabolism	Metabolism of terpenoids and polyketides
ko00300	Lysine biosynthesis	colon	3.238	0.000	Metabolism	Amino acid metabolism

ko00511	Other glycan degradation	colon	3.212	0.001	Metabolism	Glycan biosynthesis and metabolism
ko00900	Terpenoid backbone biosynthesis	colon	3.122	0.005	Metabolism	Metabolism of terpenoids and polyketides
ko00340	Histidine metabolism	colon	3.091	0.000	Metabolism	Amino acid metabolism
ko00625	Chloroalkane and chloroalkene degradation	colon	3.064	0.033	Metabolism	Xenobiotics biodegradation and metabolism
ko00770	Pantothenate and CoA biosynthesis	colon	3.046	0.008	Metabolism	Metabolism of cofactors and vitamins
ko00312	beta-Lactam resistance	colon	3.044	0.000	Human Diseases	Drug resistance: antimicrobial
ko00550	Peptidoglycan biosynthesis	colon	2.977	0.005	Metabolism	Glycan biosynthesis and metabolism
ko00500	Starch and sucrose metabolism	colon	2.905	0.048	Metabolism	Carbohydrate metabolism
ko03420	Nucleotide excision repair	colon	2.903	0.001	Genetic Information Processing	Replication and repair
ko00052	Galactose metabolism	colon	2.861	0.006	Metabolism	Carbohydrate metabolism

ko00908	Zeatin biosynthesis	colon	2.838	0.005	Metabolism	Metabolism of terpenoids and polyketides
ko00030	Pentose phosphate pathway	colon	2.752	0.021	Metabolism	Carbohydrate metabolism
ko00760	Nicotinate and nicotinamide metabolism	colon	2.722	0.003	Metabolism	Metabolism of cofactors and vitamins
ko00270	Cysteine and methionine metabolism	colon	2.712	0.002	Metabolism	Amino acid metabolism
ko00120	Primary bile acid biosynthesis	colon	2.703	0.000	Metabolism	Lipid metabolism
ko00531	Glycosaminoglycan degradation	colon	2.681	0.013	Metabolism	Glycan biosynthesis and metabolism
ko04141	Protein processing in endoplasmic reticulum	colon	2.605	0.000	Genetic Information Processing	Folding, sorting and degradation
ko00791	Atrazine degradation	colon	2.589	0.008	Metabolism	Xenobiotics biodegradation and metabolism
ko00830	Retinol metabolism	colon	2.477	0.033	Metabolism	Metabolism of cofactors and vitamins
ko04626	Plant-pathogen interaction	colon	2.460	0.008	Organismal Systems	Environmental adaptation
ko03450	Non-homologous end-joining	colon	2.436	0.001	Genetic Information Processing	Replication and repair

ko04974	Protein digestion and absorption	colon	2.390	0.001	Organismal Systems	Digestive system
ko05146	Amoebiasis	colon	2.365	0.004	Human Diseases	Infectious disease: parasitic
ko00510	N-Glycan biosynthesis	colon	2.321	0.001	Metabolism	Glycan biosynthesis and metabolism
ko04210	Apoptosis	colon	2.317	0.005	Cellular Processes	Cell growth and death
ko05120	Epithelial cell signaling in Helicobacter pylori infection	colon	2.285	0.039	Human Diseases	Infectious disease: bacterial
ko00540	Lipopolysaccharide biosynthesis	stomach	3.769	0.000	Metabolism	Glycan biosynthesis and metabolism
ko00130	Ubiquinone and other terpenoid-quinone biosynthesis	stomach	3.585	0.000	Metabolism	Metabolism of cofactors and vitamins
ko00785	Lipoic acid metabolism	stomach	3.559	0.000	Metabolism	Metabolism of cofactors and vitamins
ko00480	Glutathione metabolism	stomach	3.528	0.000	Metabolism	Metabolism of other amino acids
ko02060	Phosphotransferase system (PTS)	stomach	3.512	0.000	Environmental Information Processing	Membrane transport

ko00790	Folate biosynthesis	stomach	3.462	0.000	Metabolism	Metabolism of cofactors and vitamins
ko00780	Biotin metabolism	stomach	3.449	0.000	Metabolism	Metabolism of cofactors and vitamins
ko02010	ABC transporters	stomach	3.292	0.000	Environmental Information Processing	Membrane transport
ko00053	Ascorbate and aldarate metabolism	stomach	3.249	0.001	Metabolism	Carbohydrate metabolism
ko00660	C5-Branched dibasic acid metabolism	stomach	3.221	0.002	Metabolism	Carbohydrate metabolism
ko04122	Sulfur relay system	stomach	3.209	0.000	Genetic Information Processing	Folding, sorting and degradation
ko00910	Nitrogen metabolism	stomach	3.208	0.000	Metabolism	Energy metabolism
ko00020	Citrate cycle (TCA cycle)	stomach	3.137	0.000	Metabolism	Carbohydrate metabolism
ko00920	Sulfur metabolism	stomach	3.132	0.000	Metabolism	Energy metabolism
ko00450	Selenocompound metabolism	stomach	3.120	0.000	Metabolism	Metabolism of other amino acids
ko00473	D-Alanine metabolism	stomach	3.108	0.002	Metabolism	Metabolism of other amino acids

ko00650	Butanoate metabolism	stomach	3.003	0.000	Metabolism	Carbohydrate metabolism
ko00564	Glycerophospholipid metabolism	stomach	2.982	0.000	Metabolism	Lipid metabolism
ko00630	Glyoxylate and dicarboxylate metabolism	stomach	2.960	0.021	Metabolism	Carbohydrate metabolism
ko05322	Systemic lupus erythematosus	stomach	2.948	0.016	Human Diseases	Immune disease
ko01053	Biosynthesis of siderophore group nonribosomal peptides	stomach	2.942	0.037	Metabolism	Metabolism of terpenoids and polyketides
ko00620	Pyruvate metabolism	stomach	2.905	0.003	Metabolism	Carbohydrate metabolism
ko00633	Nitrotoluene degradation	stomach	2.890	0.010	Metabolism	Xenobiotics biodegradation and metabolism
ko00010	Glycolysis / Gluconeogenesis	stomach	2.880	0.001	Metabolism	Carbohydrate metabolism
ko00380	Tryptophan metabolism	stomach	2.879	0.008	Metabolism	Amino acid metabolism
ko00051	Fructose and mannose metabolism	stomach	2.867	0.017	Metabolism	Carbohydrate metabolism
ko03070	Bacterial secretion system	stomach	2.862	0.006	Environmental Information Processing	Membrane transport
ko00520	Amino sugar and nucleotide sugar metabolism	stomach	2.791	0.001	Metabolism	Carbohydrate metabolism

ko00440	Phosphonate and phosphinate metabolism	stomach	2.787	0.001	Metabolism	Metabolism of other amino acids
ko00350	Tyrosine metabolism	stomach	2.749	0.008	Metabolism	Amino acid metabolism
ko04146	Peroxisome	stomach	2.728	0.000	Cellular Processes	Transport and catabolism
ko00562	Inositol phosphate metabolism	stomach	2.723	0.017	Metabolism	Carbohydrate metabolism
ko00561	Glycerolipid metabolism	stomach	2.717	0.005	Metabolism	Lipid metabolism
ko00627	Aminobenzoate degradation	stomach	2.638	0.005	Metabolism	Xenobiotics biodegradation and metabolism
ko00196	Photosynthesis - antenna proteins	stomach	2.628	0.028	Metabolism	Energy metabolism
ko00361	Chlorocyclohexane and chlorobenzene degradation	stomach	2.609	0.013	Metabolism	Xenobiotics biodegradation and metabolism
ko03008	Ribosome biogenesis in eukaryotes	stomach	2.486	0.000	Genetic Information Processing	Translation
ko05150	Staphylococcus aureus infection	stomach	2.378	0.001	Human Diseases	Infectious disease: bacterial
ko05142	Chagas disease (American trypanosomiasis)	stomach	2.096	0.001	Human Diseases	Infectious disease: parasitic

Table 2.5 Foregut/stomach microbiota of the colobines and Japanese macaques

Common name	Scientific name	Captive/Wild	Observed richness (Stomach)	Observed richness (Colon)	Reference	Most abundant phylum in stomach		
						1	2	3
Japanse macaque	*Macaca fuscata*	Wild	30.0 ± SD 9.23	119.55 ± SD 109.88	Present study	Proteobacteria	Firmicutes	Bacteoidetes
Red-shanked douc	*Pygathrix nemaeus*	Captive	606.5 ± 166.52	1239.5 ± 146.57	Clayton et al., 2019	Firmicutes	Proteobacteria	Bacteroidetes
Proboscis monkey	*Nasalis larvatus*	Captive, Provisioned and Wild	501 - 962	N.A.	Hayakawa et al., 2018	Bacteroidetes	Firmicutes	Proteobacteria

Table 3.1 Basic information of sample collection sites

Disturbance type	Site	Diet	Conspecific contact	Living environment	Close interaction with humans
Captive	PRI cage	Simple diet; monkey chow	×	Artificial	High
Captive	PRI enclosure	Simple diet; monkey chow	◯	Artificial	High
Provisioned	Shodoshima	Intensive provisioning (3-4/day)	◯	Partly artificial	Medium
Crop-raiding	Suzuka-shi, Mie prefecture	No record; natural & agricultural food source	◯	Generally wild	Medium
Provisioned	Koshima	Controlled provisioning (2/week)	◯	Wild	Medium
Wild	Yakushima lowland	Frugivory based	◯	Wild	Rare
Wild	Yakushima highland	Folivory based	◯	Wild	Rare

Table 3.2 Observed richness of Japanese macaque samples

Sample ID	Collection Site	Disturbance type	Collection date	Observed richness	Shannon diversity
CA31	PRI Cage	Captive	2017.7.21	392	4.7394
CA32	PRI Cage	Captive	2017.7.21	345	4.2539
CA33	PRI Cage	Captive	2017.7.21	380	4.2564
CA34	PRI Cage	Captive	2017.7.21	438	4.8238
CA35	PRI Cage	Captive	2017.7.21	365	4.4144
CA36	PRI Cage	Captive	2017.7.21	306	4.2014
CA38	PRI Cage	Captive	2017.7.21	372	4.5146
CA39	PRI Cage	Captive	2017.7.21	396	4.4995
CA40	PRI Cage	Captive	2017.7.21	266	3.8096
EC41	PRI Enclosure	Captive	2017.7.27	399	3.9582
EC42	PRI Enclosure	Captive	2017.7.27	424	4.5641
EC43	PRI Enclosure	Captive	2017.7.27	423	4.6276
EC44	PRI Enclosure	Captive	2017.7.27	361	3.9833
EC45	PRI Enclosure	Captive	2017.7.27	409	4.2287
EC46	PRI Enclosure	Captive	2017.7.27	209	3.2750
EC47	PRI Enclosure	Captive	2017.7.27	406	4.2604
EC48	PRI Enclosure	Captive	2017.7.27	196	3.2510
EC49	PRI Enclosure	Captive	2017.7.27	343	3.9424
EC50	PRI Enclosure	Captive	2017.7.27	369	4.1574
SH51	Shodoshima	Provisioned	2017.7.5	396	4.5801
SH52	Shodoshima	Provisioned	2017.7.5	395	4.5539
SH53	Shodoshima	Provisioned	2017.7.6	437	4.7227

SH54	Shodoshima	Provisioned	2017.7.6	454	4.8484
SH55	Shodoshima	Provisioned	2017.7.6	426	4.7007
SH56	Shodoshima	Provisioned	2017.7.6	257	3.9383
SH57	Shodoshima	Provisioned	2017.7.6	423	4.6960
SH58	Shodoshima	Provisioned	2017.7.6	303	3.9445
SH59	Shodoshima	Provisioned	2017.7.6	423	4.6028
SH60	Shodoshima	Provisioned	2017.7.6	367	4.3919
SU61	Suzuka	Crop-raiding	2017.7.9	378	4.5604
SU62	Suzuka	Crop-raiding	2017.7.9	377	4.5818
SU63	Suzuka	Crop-raiding	2017.7.9	305	4.0735
SU64	Suzuka	Crop-raiding	2017.7.9	357	4.2420
SU65	Suzuka	Crop-raiding	2017.7.9	349	4.3660
SU66	Suzuka	Crop-raiding	2017.7.9	385	4.3644
SU67	Suzuka	Crop-raiding	2017.7.9	266	3.7208
SU68	Suzuka	Crop-raiding	2017.7.9	391	4.5045
SU69	Suzuka	Crop-raiding	2017.7.9	386	4.4891
SU70	Suzuka	Crop-raiding	2017.7.9	398	4.7247
KO22	Koshima	Provisioned	2017.4.27	382	4.5247
KO23	Koshima	Provisioned	2017.4.25	357	4.5926
KO24	Koshima	Provisioned	2017.4.25	240	4.1858
KO25	Koshima	Provisioned	2017.4.27	329	4.4883
KO26	Koshima	Provisioned	2017.4.25	247	4.3941
KO28	Koshima	Provisioned	2017.4.27	313	4.6082
KO29	Koshima	Provisioned	2017.4.27	399	4.6836

KO30	Koshima	Provisioned	2017.4.27	347	4.5090
YL11	Yakushima Lowland	Wild	2017.5.19	352	4.3294
YL12	Yakushima Lowland	Wild	2017.5.19	193	3.3412
YL13	Yakushima Lowland	Wild	2017.5.19	412	4.9149
YL14	Yakushima Lowland	Wild	2017.5.19	372	4.3716
YL15	Yakushima Lowland	Wild	2017.5.19	331	3.8770
YL17	Yakushima Lowland	Wild	2017.5.19	308	4.1305
YL18	Yakushima Lowland	Wild	2017.5.19	309	4.4676
YL19	Yakushima Lowland	Wild	2017.5.19	307	4.1891
YL20	Yakushima Lowland	Wild	2017.5.19	282	4.5277
YH1	Yakushima Highland	Wild	2013.8.13	440	4.6770
YH3	Yakushima Highland	Wild	2013.8.13	385	4.6580
YH4	Yakushima Highland	Wild	2013.8.13	431	4.8290
YH5	Yakushima Highland	Wild	2013.8.14	436	4.7196
YH6	Yakushima Highland	Wild	2013.8.27	434	4.6381
YH7	Yakushima Highland	Wild	2013.8.27	358	4.6746
YH8	Yakushima Highland	Wild	2013.8.27	461	4.9466
YH9	Yakushima Highland	Wild	2013.8.29	454	4.8794
YH10	Yakushima Highland	Wild	2013.9.1	394	4.8165

Table 3.3 Relative abundance of dominant gut microbial phyla in Japanese macaques experiencing different human disturbances (Order from most abundant to least abundant)

Phylum	Captive	Crop-raiding	Provisioned	Wild	Average
Firmicutes	50.25%	63.30%	58.95%	67.31%	59.95%
Bacteroidetes	40.46%	27.91%	28.15%	21.46%	29.50%
Proteobacteria	5.39%	4.35%	3.79%	4.61%	4.53%
Spirochaetes	1.31%	1.28%	3.94%	2.23%	2.19%
Verrucomicrobia	0.23%	1.05%	1.68%	0.96%	0.98%
Cyanobacteria	0.23%	0.38%	1.14%	1.27%	0.76%
Tenericutes	0.13%	0.22%	1.09%	0.65%	0.52%
Lentisphaerae	0.29%	0.18%	0.20%	0.89%	0.39%
WPS-2	0.07%	0.95%	0.32%	0.00%	0.34%
Actinobacteria	0.29%	0.26%	0.43%	0.35%	0.33%
Fusobacteria	0.92%	0.00%	0.00%	0.00%	0.23%
Fibrobacteres	0.10%	0.04%	0.21%	0.06%	0.10%
Elusimicrobia	0.30%	0.00%	0.00%	0.05%	0.09%
Unassigned	0.01%	0.05%	0.10%	0.14%	0.08%

Table 3.4. Bacterial taxa whose relative abundance correlates with human disturbance level ($p<0.05$), absolute value of Spearman correlation coefficient greater than 0.5

Phylum	Mean Decrease Accuracy	Mean Decrease Gini	Class	Mean Decrease Accuracy	Mean Decrease Gini
Tenericutes	0.0213	2.1775	Bacilli	0.0525	3.7609
Firmicutes	0.0151	1.5991	Chloroplast	0.0434	3.2617
Fibrobacteres	0.0149	1.1407	Deltaproteobacteria	0.0365	2.6073
Lentisphaerae	0.0111	1.3334	Mollicutes	0.0332	3.1345
Verrucomicrobia	0.0108	1.2505	Fibrobacteria	0.0212	1.9059
Actinobacteria	0.0081	1.1817	Verruco-5	0.0201	2.1321
Proteobacteria	0.0065	1.1792	Opitutae	0.0197	1.6246
Bacteroidetes	0.0056	0.9442	4C0d-2	0.0193	1.9955
Spirochaetes	0.0044	0.7762	Clostridia	0.0192	2.1734
Elusimicrobia	0.0022	0.3448	Coriobacteriia	0.0164	2.2905

Order	Mean Decrease Accuracy	Mean Decrease Gini	Family	Mean Decrease Accuracy	Mean Decrease Gini
Lactobacillales	0.0409	3.3585	Bacteroidaceae	0.0464	2.8068
Streptophyta	0.0390	3.5246	Streptococcaceae	0.0370	2.4373
Anaeroplasmatales	0.0382	2.9337	Lactobacillaceae	0.0302	2.414
GMD14H09	0.0347	2.4186	Rikenellaceae	0.0277	2.276
Fibrobacterales	0.0304	2.1464	Anaeroplasmataceae	0.0275	2.4845
[Cerasicoccales]	0.0250	1.8791	[Odoribacteraceae]	0.0222	1.5921
WCHB1-41	0.0233	2.3574	Veillonellaceae	0.0211	2.1263
Clostridiales	0.0189	2.1989	p-2534-18B5	0.0210	1.3708
Victivallales	0.0177	1.7147	[Cerasicoccaceae]	0.0204	1.3487
Pasteurellales	0.0158	1.7542	Porphyromonadaceae	0.0188	1.6108