Stomach and colonic microbiome of wild Japanese macaques

Short title: Gut-site-specific microbiome

Wanyi Lee[1*], Takashi Hayakawa[2,3], Yosuke Kurihara[1,4], Maho Hanzawa[5], Akiko Sawada[1,6], Akihisa Kaneko[1], Yoshiki Morimitsu[7], Takayoshi Natsume[1], Seitaro Aisu[1], Tsuyoshi Ito[1], Takeaki Honda[1], Goro Hanya[1]

[1] Primate Research Institute, Kyoto University, Inuyama, Aichi, Japan

[2] Faculty of Environmental Earth Science, Hokkaido University, Sapporo, Hokkaido, Japan

[3] Japan Monkey Centre, Inuyama, Aichi, Japan

[4] Center for Education and Research in Field Sciences, Faculty of Agriculture, Shizuoka University, Hamamatsu, Japan

[5] Department of Zoology, Graduate School of Science, Kyoto University, Kyoto, Japan

[6] Academy of Emerging Sciences, Chubu University, Kasugai, Aichi, Japan

[7] Institute of Natural and Environmental Sciences, University of Hyogo, Sanda, Hyogo, Japan

*To whom correspondence should be addressed; Correspondence to wanyi23333@gmail.com

Highlights

- Diversity, composition and function vary between stomach and colonic microbiome of Japanese macaques
- Stomach microbiome is more enriched in microbes that metabolize simple sugar, whereas the colonic microbiome has more fiber-degrading microbes.

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.

Keywords: primates, gut microbiome, Japanese macaques, gut sites

Introduction

Along the gastrointestinal (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 et al., 2016; Clayton et al., 2019), black and white colobus monkeys, and langurs (Amato 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, 2004; Hanya, Noma, & Agetsuma, 2003; Hill, 1997; Kurihara, Kinoshita, Shiroishi, & Hanya, 2020). They spend approximately 35% of their annual feeding time on

fibrous leaves and shoots (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 (Supplementary Table 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 us (A. K.), 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 (Supplementary Table 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 (Supplementary Figure 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 (74.78 ± SD 9.90 %), Bacteroidetes (12.04 ± SD 10.12 %), and Proteobacteria (4.61 ± SD 3.24 %) (Figure 1; Supplementary Table 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 1; Supplementary Table 2). The 240 ASVs uncovered in the stomach were from 6 known phyla, 12 classes, 17 orders, and 21 families.

Alpha diversity 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 2a; Shannon index: V=28, P = 0.0156, Figure 2b). 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.

PCoA plots based on unweighted (Figure 3a) and weighted UniFrac distance (Figure 3b) revealed that the 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 colon of Japanese macaques

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 4; Supplementary Table 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) (Supplementary Figure 2). In particular, the top 15 enriched genera were mostly from families Ruminococcaceae and Lachnospiraceae of the order Clostridiales.

Predicted functional difference between stomach and colon

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, $P < 0.05$). Among these, 36 pathways were enriched in the colon and 39 were enriched in the stomach (Figure 5; Supplementary Table 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 of Japanese macaques

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 1). 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 et al., 2015; Clayton 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.

Accession number. The raw data is available in the DDBJ database with accession number DRA009571.

Competing interests: The authors declare no competing interests.

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Table 1. 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

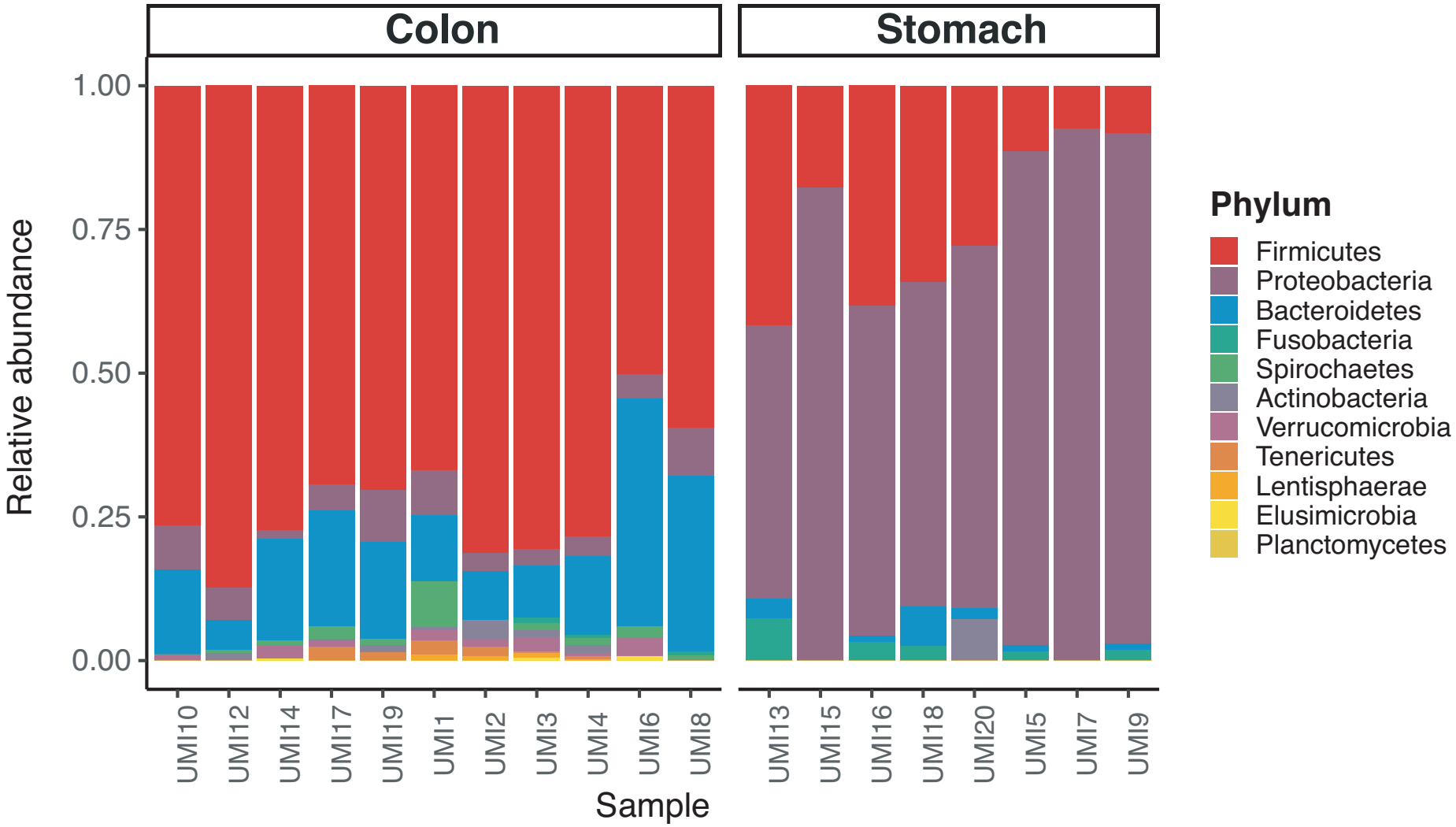


Figure 1. Relative abundance of gut bacterial taxa at phylum level (% of total sequences per sample)

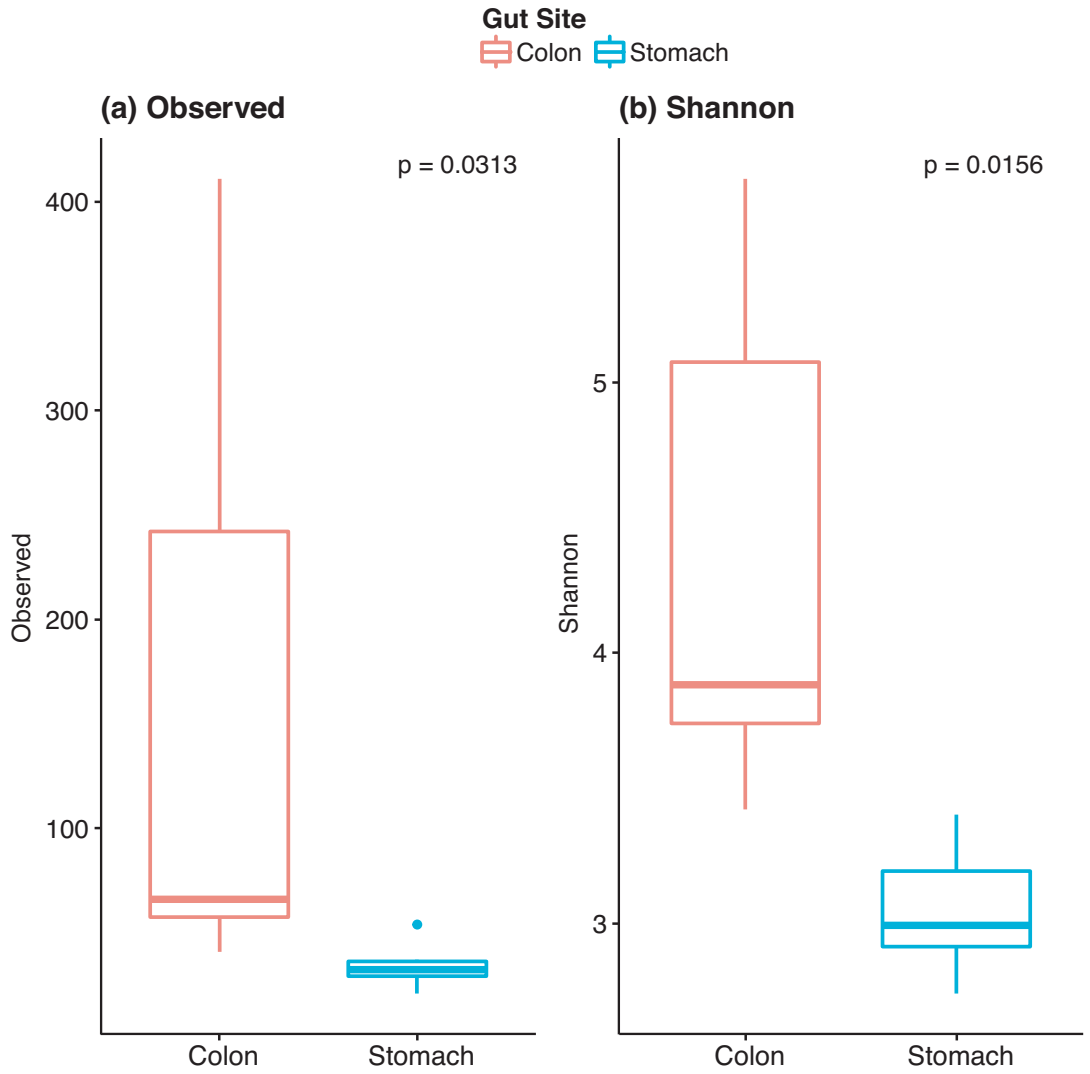


Figure 2. (a) Observed richness and (b) Shannon diversity index of stomach and colonic microbiomes of Japanese macaques

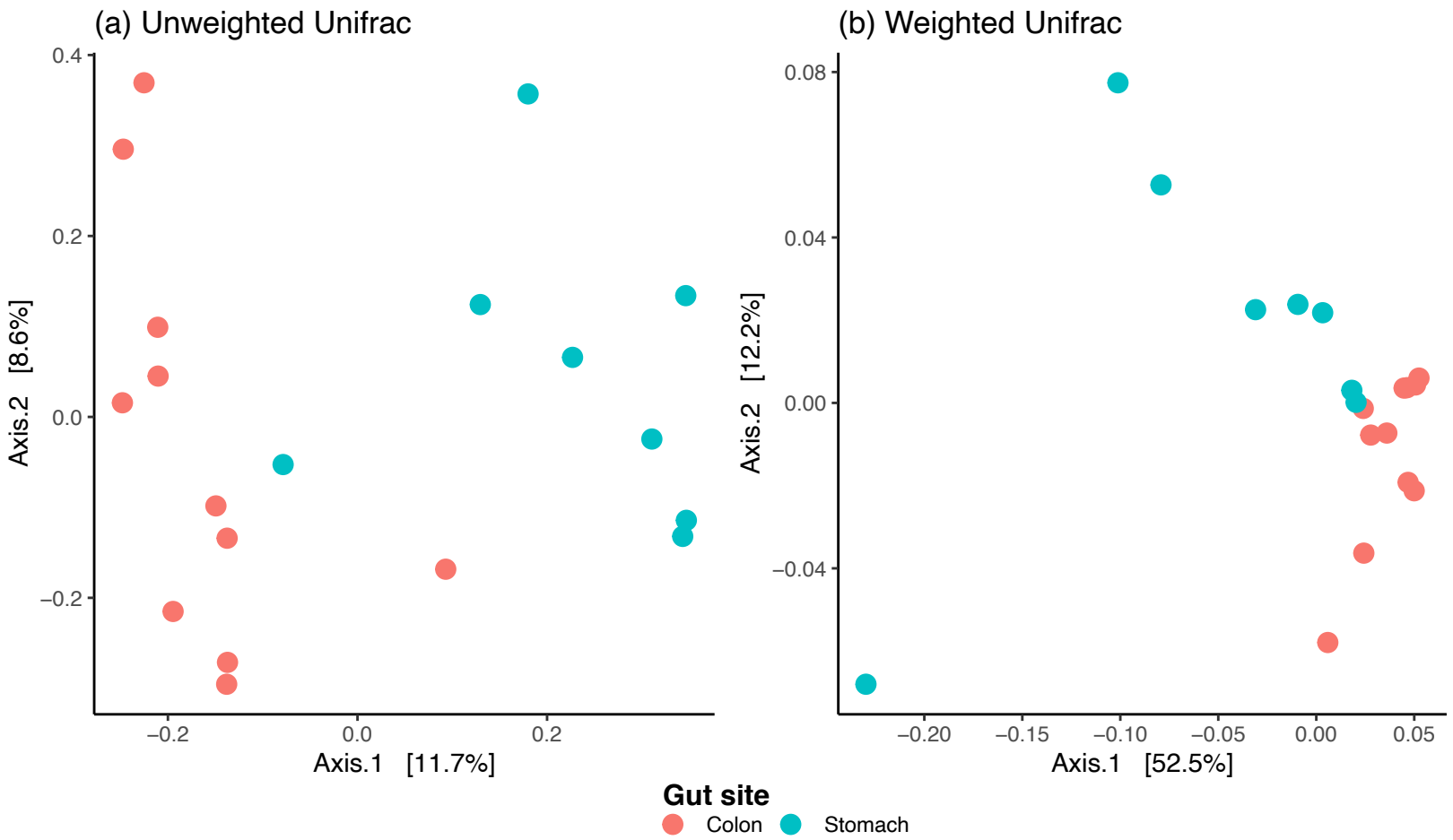


Figure 3. Principal coordinate analysis plots based on (a) unweighted and (b) weighted UniFrac distance for macaques' gut bacterial communities

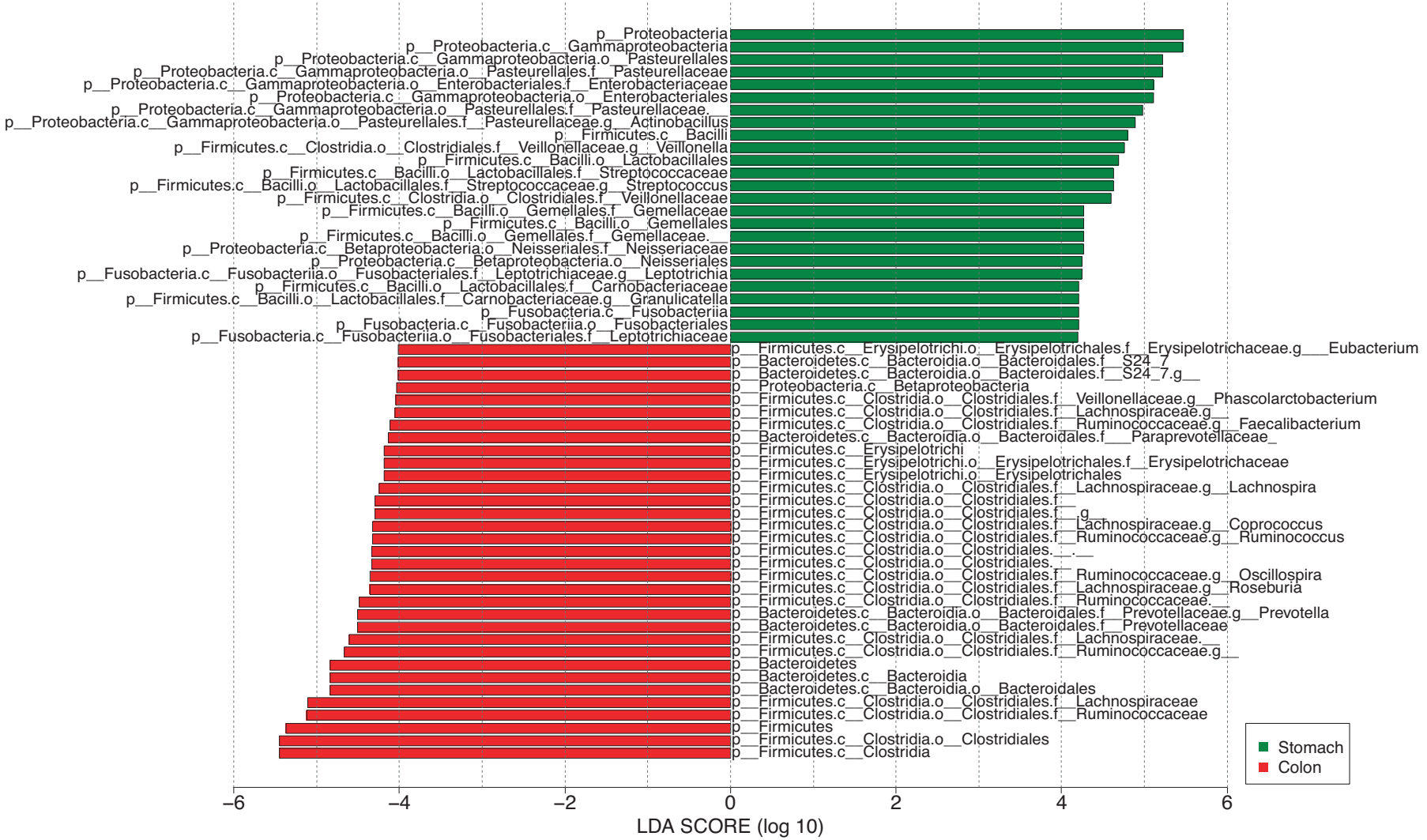
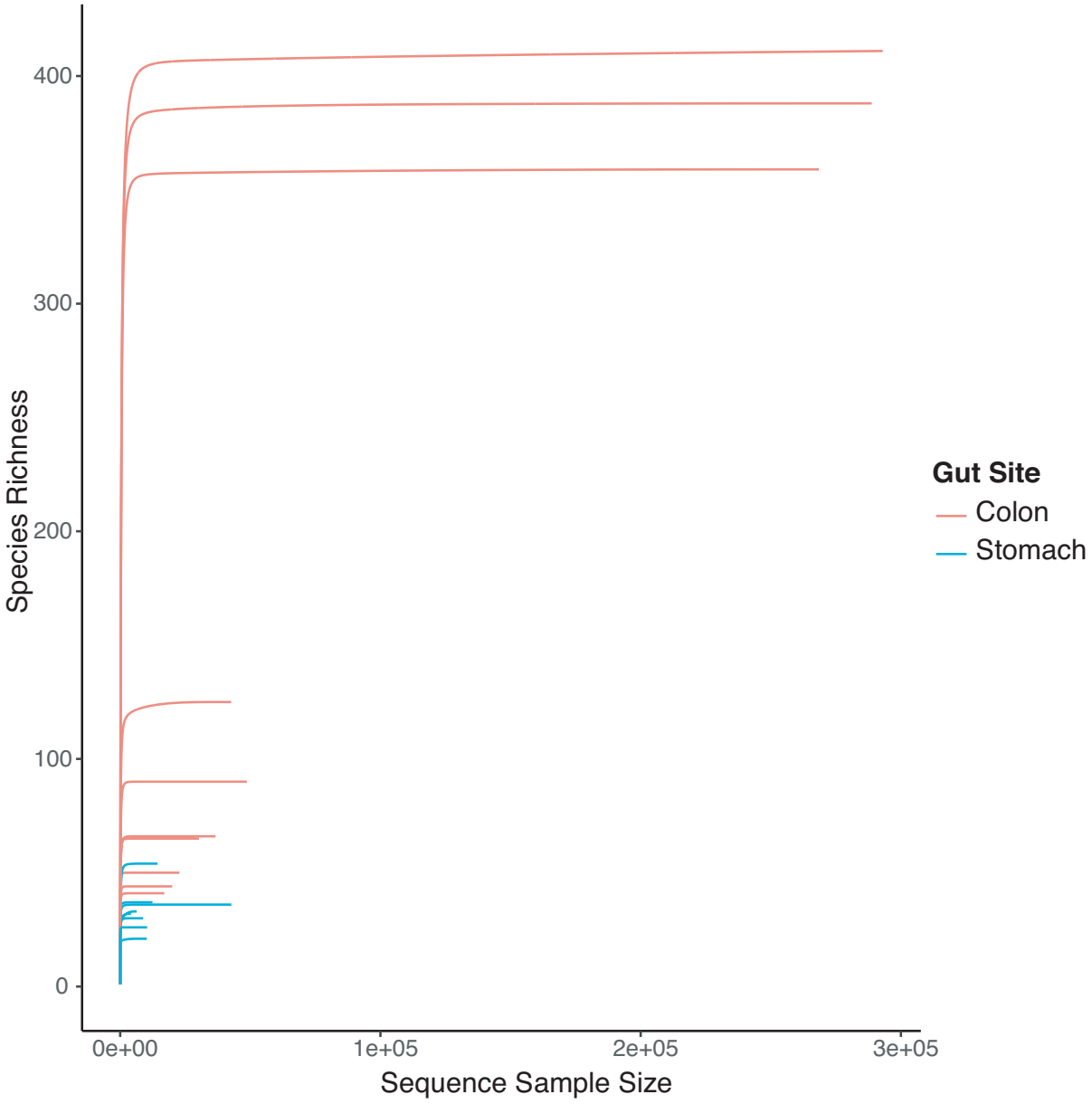
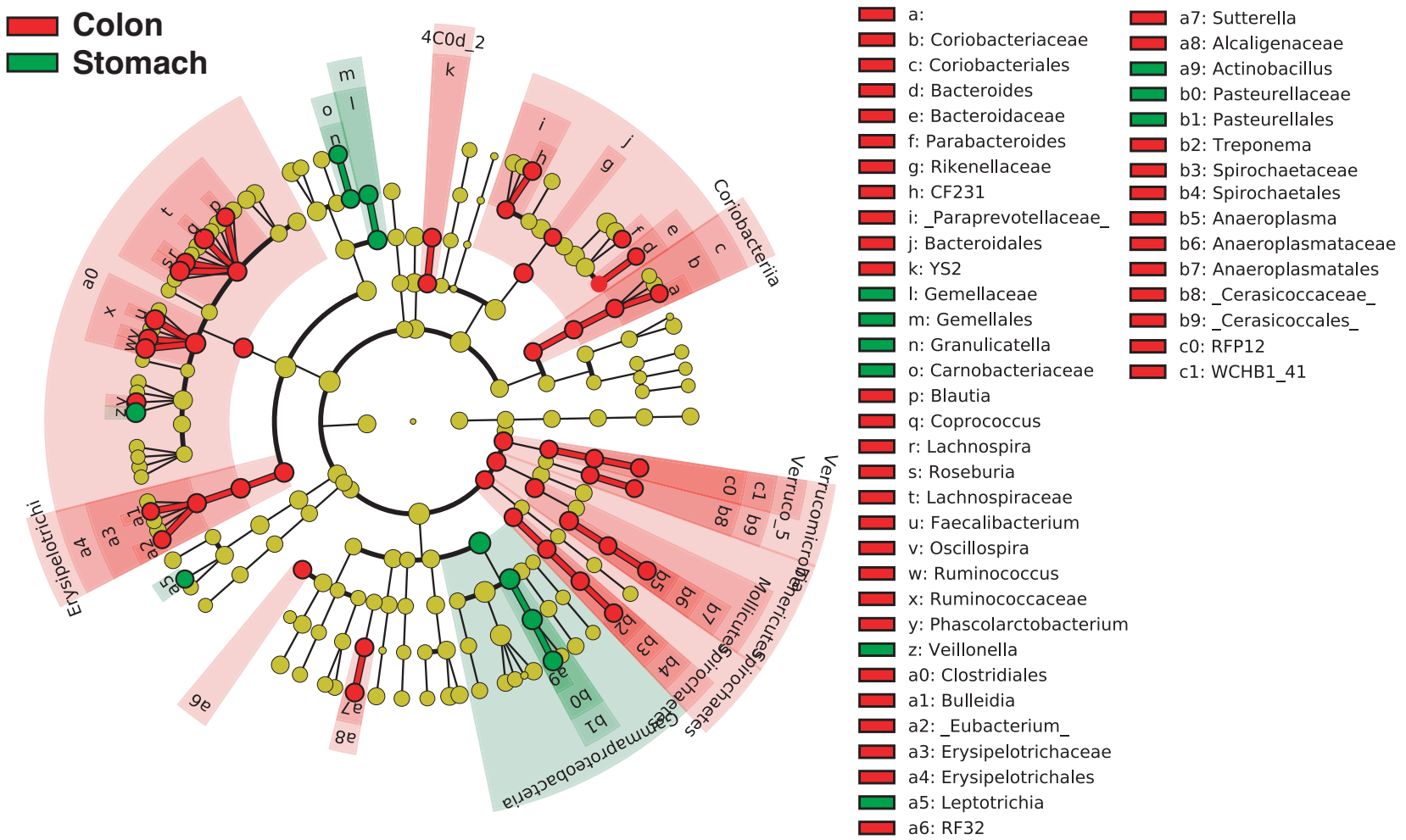


Figure 4. Gut microbial genera differentially abundant in the stomach and colonic microbiome. Plot showing the histogram of linear discriminant analysis (LDA) scores computed for differentially abundant bacterial genera (log LDA score > 5.0, p <0.05)

Figure 5. 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, $p < 0.05$)



Supplementary Figure 1. Rarefaction curve of stomach and colonic samples



Supplementary Figure 2. 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)

Supplementary Table 1. Sample information

Sample ID	Group	Sex	Collected year & month	Gut Site	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

Supplementary Table 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%

Supplementary Table 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

Supplementary Table 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