

Comparison of gastrointestinal microbiota in golden snub-nosed monkey (*Rhinopithecus roxellanae*), green monkey (*Chlorocebus aethiops sabaeus*), and ring-tailed lemur (*Lemur catta*) by high throughput sequencing

Y. Zeng^{a,1}, Y. Pu^{b,1}, L.L. Niu^{b,1}, J.B. Deng^b, D. Zeng^a, K.R. Amato^c, Y. Li^a, Y. Zhou^a, Y.C. Lin^a, J. Wang^a, L.Q. Wu^a, B.H. Chen^a, K.C. Pan^a, B. Jing^a, X.Q. Ni^{a,*}

^a College of Veterinary Medicine, Sichuan Agricultural University, Chengdu 611130, China

^b Chengdu Wildlife Institute, Chengdu Zoo, Chengdu 610081, China

^c Department of Anthropology, Northwestern University, Evanston 60208, USA

ARTICLE INFO

Keywords:

Non-human primates
Gut microbiome
Microbial function
16S rRNA V4 gene sequencing
Metagenomics

ABSTRACT

High throughput sequencing of gut microbiota helps to understand the nutrition and health of the host. In this sense, it is an important tool for improving wildlife conservation and management. Little is known about the microbial communities occupying the gastrointestinal tract and the differences of microbiota among primate species. We conducted a microbiome study of the gastrointestinal tract in golden snub-nosed monkey (*Rhinopithecus roxellanae*), green monkey (*Chlorocebus aethiops sabaeus*), and ring-tailed lemur (*Lemur catta*), using the 16S rRNA gene amplicon sequencing and shotgun metagenomic sequencing. The gastrointestinal tract of all three primates were characterized by Firmicutes, Bacteroidetes, and Proteobacteria, although the relative abundance of these taxa differed across non-human primate species and gastrointestinal segments. In addition, the stomach of the golden monkey demonstrated a strong signal of microbial metabolic activity, especially in metabolizing carbohydrate. *Prevotella multisaccharivorax* and *Selenomonas bovis* were the main contributors to the function, and the enzymes involved in these metabolic activities were mainly derived from the glycoside hydrolase (GH) family. Microbial composition and function vary widely across non-human primate species and gastrointestinal segments. Therefore, while faecal samples are necessary for studying many mammals, particularly in the wild, data from the gastrointestinal tract have the potential to greatly improve our knowledge of host-gut microbe interactions. This information can contribute to the development of conservation interventions and animal care protocols in the wild and captivity to more fully describe a species' nutritional needs and advance health and survival.

* Correspondence to: College of Veterinary Medicine, Sichuan Agricultural University, Huimin Road, Gongping District, Chengdu, Sichuan 611130, China.

E-mail address: xueqinni@foxmail.com (X.Q. Ni).

¹ These authors contributed equally to this work.

1. Introduction

The gastrointestinal microbiota plays a crucial role in host health and disease. Currently, gastrointestinal microbiota research is a potentially important means for improving our understanding of wildlife ecology, evolution, and conservation, especially important for the threatened species conservation (West et al., 2019). Nevertheless, the studies of wild animals account for only a small proportion (14.3%) of mammalian gut microbiome research (Pascoe et al., 2017). A majority of these studies focus on non-human primates, linking the microbiome to their ecology, behavior, and responses to captivity. This is mainly due to that most non-human primates are endangered or have a higher level of protection and are not allowed to be dissected *in vivo*. However, most studies of gastrointestinal microbiota in the non-human primates rely on faecal samples since they are relatively easy to collect non-invasively. Faecal samples are not representative of the gastrointestinal microbiota (Clayton et al., 2018), and given that different gastrointestinal segments often have different microbiota (Tropini et al., 2017). Therefore, the gastrointestinal microbiota of different mammalian species, including non-human primates, need further research to improve our understanding of wildlife species.

Non-human primates use a range of terrestrial and arboreal habitats globally, with a variety of associated diets (Strier, 2016). As a result, different non-human primate species have evolved different gastrointestinal tract structures (Langer and Clauss, 2018). For example, members of the subfamily *Colobinae* consume a diet high in hard-to-digest mature leaves, unripe fruits, and seeds, and have developed foregut fermentation to help process it (Matsuda et al., 2019). These diets contain a lot of carbohydrates, such as cellulose, hemicellulose, lignin and pectin. In contrast, other Non-human primates such as *Hapalemur* generally rely on hindgut fermentation (Campbell et al., 2000). These gastrointestinal compartments likely expose food to distinct microbial communities. In addition, captivity also greatly affects the intestinal flora of wild animals of the same species (McKenzie et al., 2017). In fact, phylogeny and associated physiology has been shown to be an important driver of gut microbiota in non-human primates (Amato et al., 2019).

Currently, studies of the microbiome of the partial or full gastrointestinal tract have been published for only a few captive non-human primate species, including the red-shanked douc (*Pygathrix nemaeus*) (Clayton et al., 2019), olive baboon (*Papio anubis*) (Yuan et al., 2020), aye-aye (*Daubentonia madagascariensis*) (Greene and McKenney, 2018), and macaque (*Macaca mulatta*) (Lee et al., 2021). All of these studies targeted a single host species and described only the microbiome taxonomic composition. Another study examined the gastrointestinal tract of several captive colobine species comparatively (Amato et al., 2016). However, these species were closely related, and again, no microbiome functional data were generated, limiting the scope of the results. To date, no study has compared the microbiome of the gastrointestinal tract of distantly related primates or has examined variation in both microbiome taxonomy composition and function. Microbiome functional data are particularly important for understanding microbial contributions to host health.

Here, we address these gaps by comparing the gastrointestinal tract of three distantly related primates: a captive green monkey (*Chlorocebus aethiops sabaeus*), a captive ring-tailed lemur (*Lemur catta*), and a wild golden monkey (*Rhinopithecus roxellanae*). The golden monkey is an endangered colobine (subfamily Colobinae) (Zhang et al., 2016) with a sacculated foregut (Matsuda et al., 2019). The green monkey is a closely related Old World monkey from the subfamily Cercopithecinae. However, it consumes an omnivorous diet and uses hindgut fermentation (Yasuda et al., 2015). The lemur is a distantly related primate from the suborder Strepsirrhini that consumes an omnivorous diet and uses hindgut fermentation (Campbell et al., 2000). Previous studies have demonstrated the microbiota in the fecal of these three non-human primates is dominated by the phyla Bacteroidetes, Firmicutes, and Proteobacteria, with variations according to captivity, diet, age, sex, and health status (McKenzie et al., 2017). However, the microbiota of the gastrointestinal tract is still unexplored. We hypothesized that the microbiota composition and abundance would differ across non-human primate species and gastrointestinal segments. Specifically, we expected that the microbiota of the golden monkey foregut would be more specialized (e.g. more carbohydrate metabolism) than of the green monkey and lemur.

2. Materials and methods

2.1. Sample collection

We collected gastrointestinal contents and faecal samples from three primates that died naturally (Supplementary Fig. S1): a wild golden monkey, a captive green monkey, and a captive lemur. The golden monkey was an 18-year-old female rescued by the Sichuan Pingwu Animal Rescue Station. The habitat of this primate includes deciduous broad-leaved mixed forest, deciduous broad-leaved forest and coniferous broad-leaved mixed forest. The golden monkey was sent to the Chengdu Zoo for routine clinical examination and received rehydration therapy in March 2016. Clinical anatomy and pathological data indicated that cause of death may be heart disease. The unpreserved gastrointestinal tract was stored in a – 80 °C freezer for two months. The green monkey was an old female primate raised in the Chengdu Zoo for which no necropsy report was provided. The lemur was also raised at the Chengdu Zoo. This small primate had clinical symptoms such as abdominal pain and flatulence before death. The gastrointestinal samples of the lemur were collected after death within one day of death. The diets of the green monkey and lemur included fruits, vegetables and the steamed cornbread. Gastrointestinal segments sampled included the stomach, duodenum, jejunum, ileum, cecum, appendix (lemur), colon, rectum, and faecal (golden monkey). At the same time, faecal samples from green monkeys and lemurs were collected from living individuals occupying the same building. All samples were placed in sterile tubes, frozen immediately within 20 min, and stored at – 80 °C until further analysis.

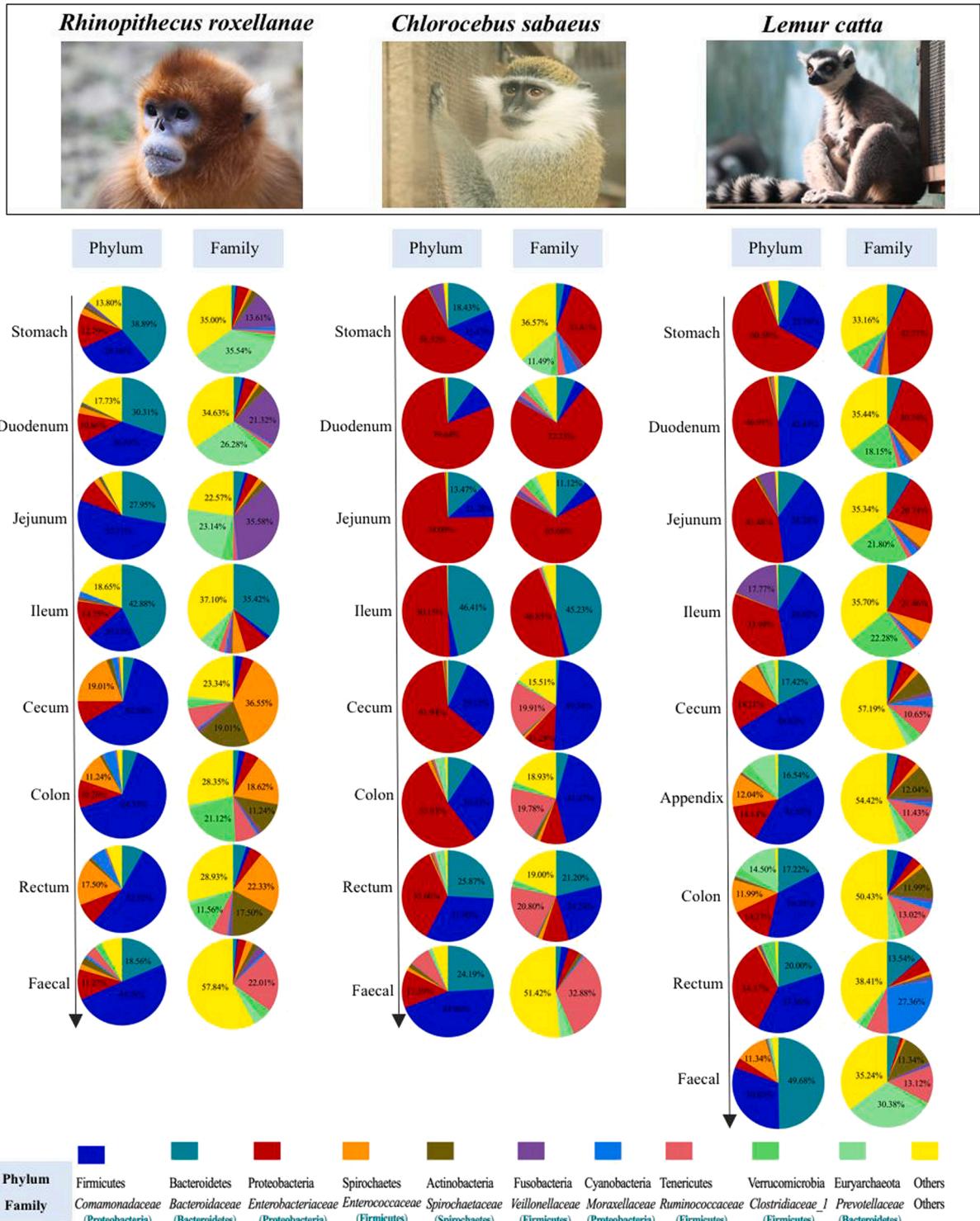


Fig. 1. Distribution of bacterial taxa in the three primates. The pie diagrams show the top ten bacterial composition at phylum and family levels. The heatmaps show the top three genera, the number of graphic annotations represent the number of bacterial sequences. RR, CS, and LC represent the golden monkey, green monkey, and lemur, respectively. Sto, Duo, Jej, Ile, Col, Cec, App, Rec, and Fae represent samples from stomach, duodenum, jejunum, ileum, colon, cecum, appendix, rectum, and faecal, respectively. The following is the same. The portrait of the three primates was provided by Pu Yang from Chengdu zoo.

2.2. DNA extraction and sequencing

We extracted the total genomic DNA from gastrointestinal samples using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's protocol. The genomic DNA concentrations were measured by a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). According to Caporaso et al., the 16S rRNA gene (V4 region) of the DNA was amplified using 515F (GTGCCAGCMGCCGCGTAA) and 806R (GGACTACHVGGGTWTCTAAT) primers with a 6 bp error-correcting barcodes (Caporaso et al., 2011). The PCR reaction was performed using Phusion® High Fidelity PCR Master Mix (New England Biolabs). The PCR products were mixed and purified using a Qiagen gel extraction kit (Qiagen, Germany). A sequencing library was obtained using a PCR-free system (TruSeq® DNA PCR-Free sample preparation kit) (Jones et al., 2015). Library quality was assessed using a Qubit@ 2.0 fluorometer (Thermo Scientific) and an Agilent Bioanalyzer 2100 system. Finally, bacterial DNA paired end sequencing was performed using an Illumina HiSeq 2500 platform (insert size 250 bp, read length 150 bp).

Six samples were subjected to metagenomic sequencing, including the stomach and ileum of golden monkey, the stomach and cecum of green monkey, the stomach and duodenum of lemur. NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) and Qubit® dsDNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA) were used for the quality control. A sequencing library of sample DNA (2 µg per sample) was constructed according to the instructions of the NEB Next® Ultra™ DNA Library Preparation Kit (NEB, USA). The DNA sample was fragmented to a size of 350 bp. The PCR product was purified using an AMPure XP system. Library preparation was sequenced using the Illumina HiSeq 4000 platform (150-bp paired end reads). All bacterial sequences were performed by Novogene (Beijing, China).

2.3. Processing of microbiome data

For 16S rRNA sequence data, the raw sequence reads were obtained using FLASH V1.2.7 (Magoc and Salzberg, 2011) after removal of the barcode and primer sequences. The clean reads were then obtained according to the QIIME 1.7.0 (Caporaso et al., 2010) quality control procedures (quality threshold <=19, default length 3, continuous high-quality base length > 75% tags). The Gold database and the UCHIME algorithm (Edgar et al., 2011) were used to obtain valid reads and remove chimeric sequences. Bacterial operational taxonomic units (OTUs) with an identity threshold of 97% similarity was constructed using UPARSE V7.0.1001 (Edgar, 2013). Species annotations (threshold 0.8⁻¹) were performed by the SSUrRNA database in SILVA (Quast et al., 2012) with no chloroplast and mitochondrial reads. Analysis of good's coverage, rarefaction curves, UPGMA sample clustering tree, and PCoA were analyzed using QIIME1.7.0 and R software 2.15.3 (McMurdie and Holmes, 2013). The ternary diagram was analyzed to compare the difference between the three groups of the centroid plot of three variables, and the sum of the three variables was constant (ggplot2) (Bulgarelli et al., 2015). Linear discriminant analysis coupled with effect size (LEfSe) was performed by the LEfSe software with the filter value of LDA score was set as 4 by default (Segata et al., 2011).

The metagenomic data set was filtered through a Readfq V8 with low quality bases (the default read length is 40 bp, the default length of the N base is 10 bp, and the default overlap length of a portion of the adapter is 15 bp). After removing host gene sequence contamination by using SoapAligner (identity≥90%, -l30, -v 7, -M 4, -m 200, -x 400) (Qin et al., 2014), SOAPdenovo V 2.04 was used to assemble the valid sequence (Luo et al., 2012) (-d 1, -M 3, -R, -u, -F, -K 55). ScafTigs (\geq 500 bp) gene prediction was performed using MetaGeneMark V2.10 (Zhu et al., 2010). Gene catalogue (Unigenes) was obtained using SoapAligner 2.21 (Li et al., 2014) (-m 200, -x 400, identity \geq 95%) and gene was removed (reading \leq 2) (Li et al., 2014). Gene annotation and functional gene blasting, and DIAMOND V0.7.9 (Buchfink et al., 2014) in KEGG database V201609 (<https://www.kegg.jp/kegg/>) (Kanehisa et al., 2013) and CAZy database V20150704 (<http://www.cazy.org/>) (Cantarel et al., 2008).

3. Results

3.1. Microbial biogeography along the gastrointestinal tract of golden monkey, green monkey, and lemur

All gastrointestinal samples had good sequencing quality and were well characterized by rarefaction curves and box-plots of species accumulation (Supplementary Fig. S2). A total of 1, 533, 170 high quality paired-end reads and 24,044 OTUs were obtained from the 16S rRNA gene sequencing data after removal of the lower quality sequences (Supplementary Table S1). Differences in microbial composition abundance between each intestinal segment were observed of each non-human primate (Fig. 1). For example, at the family level, the stomach, duodenum, and jejunum of the golden monkey were dominated by Veillonellaceae and Prevotellaceae. However, the ileum of this primate was dominated by Bacteroidaceae. In addition, Enterococcaceae and Ruminococcaceae were the main bacteria in its large intestine and faecal, respectively. Similarly, such differences in the microbiota along the gastrointestinal segments were observed in the microbiome data of the green monkey and lemur. Nevertheless, gastrointestinal bacteria of the golden monkey, green monkey, and lemur were major characterized by Firmicutes, Bacteroidetes, and Proteobacteria (Fig. 1).

3.2. Microbial composition differences among primate species

The microbiota demonstrated predictable differences among non-human primate species. The principal coordinate analysis (PCoA) of the microflora showed that the distribution of the gastrointestinal samples was relatively scattered among different primate species (Supplementary Fig. S2c). In addition, the differences of microbiota among different primate species were obtained from the phylogenetic tree with weighted UniFrac (Supplementary Fig. S2d). Among the major bacteria, Firmicutes, Bacteroidetes, and

Proteobacteria, the former two were abundant in the gastrointestinal tract of the golden monkey, and the latter one was dominated in the gastrointestinal tract of the green monkey. Similar differences were revealed by the Ternary Phase Diagram (Supplementary Fig. S2e-2g). In the Ternary Phase Diagram at the family and genus level, we observed the golden monkey were dominated by Prevotellaceae, *Enterococcus*, *Prevotella_7*, and *Sphaerochaeta*. The difference of flora among the three primates was typical observed in the comparison of microbiota in the stomach and colon at the phylum, family, and genus level (Fig. 2). *Prevotella_7* belonged to Prevotellaceae (belonged to Bacteroidetes) and *Clostridium_sensu_stricto_1* from the Clostridiaceae_1 (belonged to Firmicutes) were the major genera in stomach and colon of golden monkey, respectively. However, *Escherichia-Shigella* and *Citrobacter* belonged to Enterobacteriaceae (belonged to Proteobacteria) were the major bacteria in stomach of green monkey and lemur, respectively. The colon of these two primates was both dominated by *Comamonas* belonged to Comamonadaceae (belonged to Proteobacteria).

LEFSe analysis showed the differential flora among the three primates with higher LDA score (Fig. 3a). *Prevotella_7*, *Escherichia-Shigella*, and *Sphaerochaeta* were the differential genera of the golden monkey. *Clostridium_sensu_stricto_1*, *Treponema_2*, *Acinetobacter*, *Arcobacter*, *Pseudomonas*, and *Erysipelotrichaceae_UCG-004* were the differential genera of the lemur. In addition, *Escherichia-Shigella* and *Comamonas* were observed the different flora in the gastrointestinal tract of the green monkey. Finally, we used the line chart to show the differential flora at genus level along each gastrointestinal segment of the three primates (Fig. 3b-d). We found the main differential flora (*Prevotella_7*) in the stomach and ileum of golden monkey fluctuates greatly. This genus was found at high abundance level in the stomach, duodenum and jejunum, but the levels drop to very low in and beyond the ileum. The reverse pattern was seen for the abundance levels of *Enterococcus* and *Sphaerochaeta*. Similarly, large fluctuations in main differential flora (*Escherichia-Shigella*) were observed in the stomach and cecum of the green monkey. In addition, the main differential flora (*Clostridium_sensu_stricto_1*) showed a large fluctuation in the stomach and duodenum of the lemur. Therefore, these intestinal samples with significant bacterial differences need further metagenomic sequencing to explore main flora and metabolism.

3.3. KEGG and carbohydrate metabolism profiling

Using the KEGG database, we obtained 109 differential metabolic pathways from the gastrointestinal microbiota of the three primates. These metabolic pathways contributed the most to carbohydrate metabolism (4093 sequences, 16.05%), amino acid

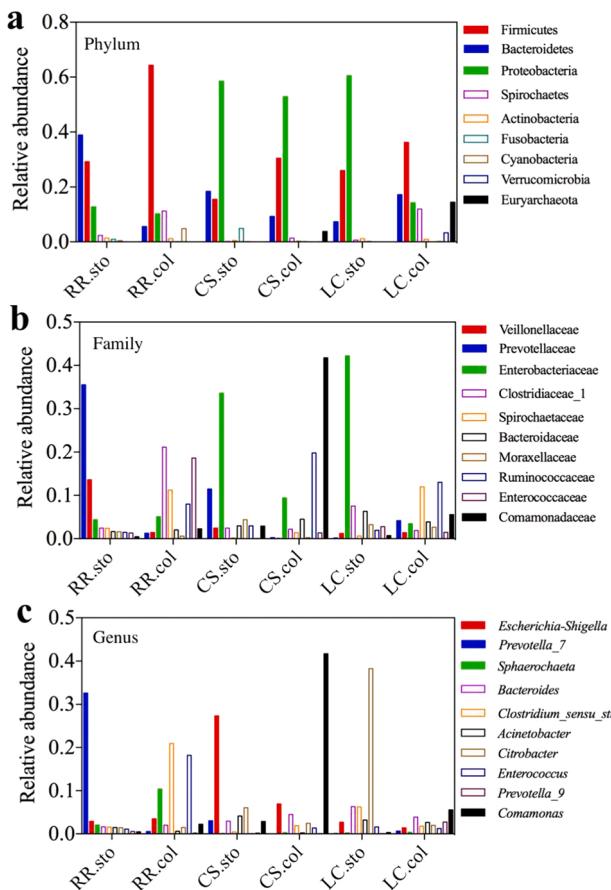


Fig. 2. Differences in the bacterial composition of stomach and colon of the three primates. Relative abundance of flora at phylum (a), family(b), and genus (c) level.

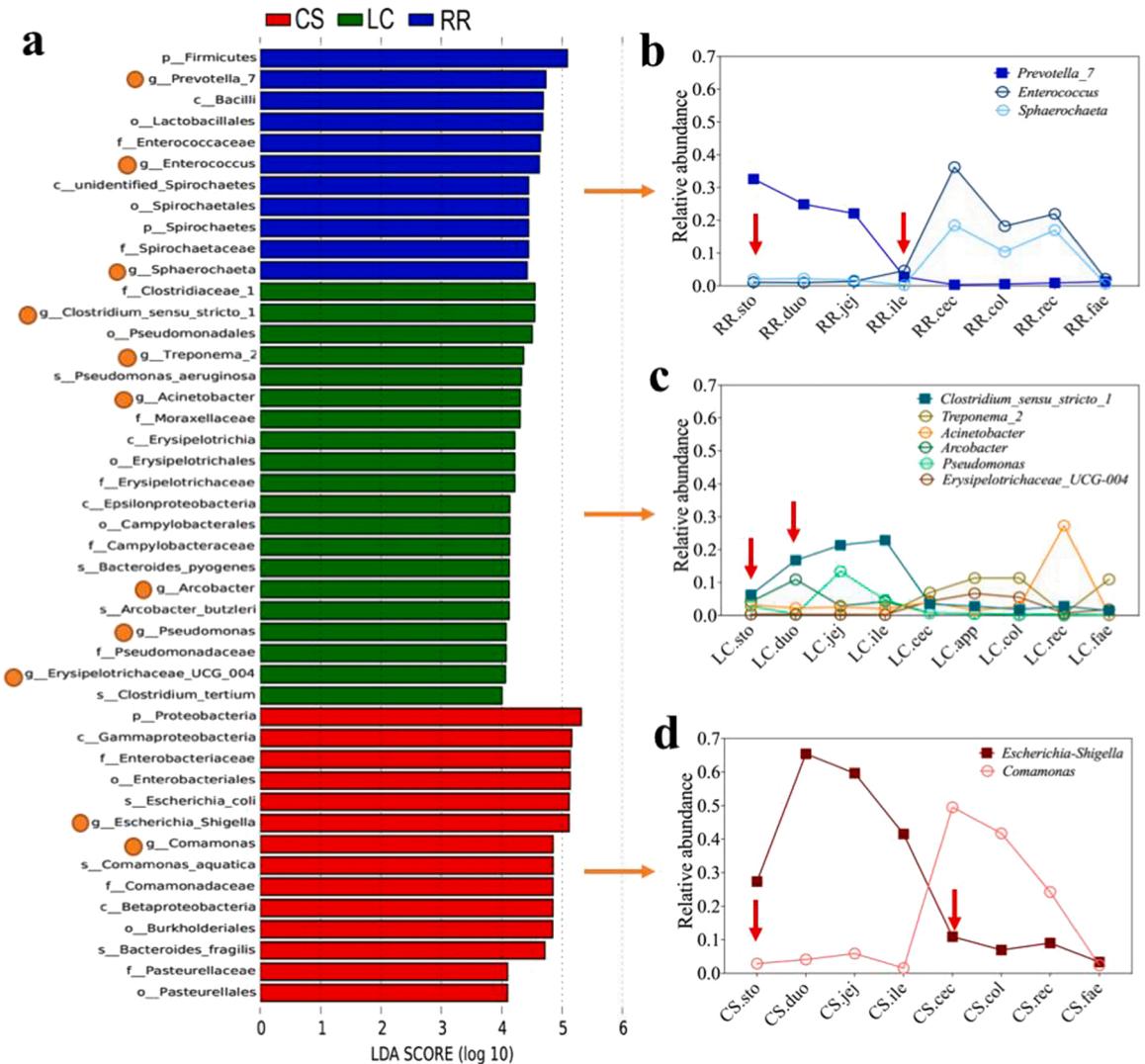


Fig. 3. Difference in bacterial composition between the three primates. Taxa identification of potential biomarkers by LEfSe of RR, CS, and LC (a). Line chart with significantly bacteria at genus level (b-d). The solid orange circle indicates the genus heteromycetes. The red arrow indicates the sample for metagenomic analysis. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

metabolism (4085 sequences, 16.02%), energy metabolism (3292 sequences, 12.91%), cofactor and vitamin metabolism (2683 sequences, 10.52%), and nucleotide metabolism (2281 sequences, 8.95%) (Fig. 4a).

For putative genes involved in carbohydrate metabolism, we annotated bacterial sequences in the CAZy database. A total of 177 putative CAZymes were identified for the three primates' gastrointestinal tracts (Fig. 4b). CAZymes were primarily of the glycoside hydrolases (GHs) family, followed by the glycosyltransferase (GT) family (Supplementary Fig. S3). As shown in Figs. 4b and 5b, the CAZyme families were abundant in the stomach of golden monkey. In this primate, families of GT2, GH2, GH3, GH43, GT4, GH13, GH28, and four modules (CBM50–32–48–20) contributed most of the carbohydrate metabolism. Notably, the top 35 abundant enzymes from the CAZy families showed that a total of 18 enzymes belonged to the GH13 family, following by the GT2 family (11 enzymes) (Fig. 5c). Among these enzymes, five enzymes from the GH13 family (e.g. EC 3.2.1.93, EC 3.2.1.10, EC 2.4.1.7, EC 2.4.1.18, and EC 2.4.1.25) and an enzyme from the GT2 family (e.g. EC 2.4.1.12) participated in the metabolism of starch and sucrose. The cluster heatmap of CAZyme showed that families GH13, GH3, GT5, GH31, GT4, GH73, GT51, GT28, and module CBM48 clustered together (Fig. 5b). In addition, families GT2, GH2, GH78, GH92, GT83, CE1, GH97, GH51, GH43, GH105, GH28, PL1, GH77, and CE8 gathered together.

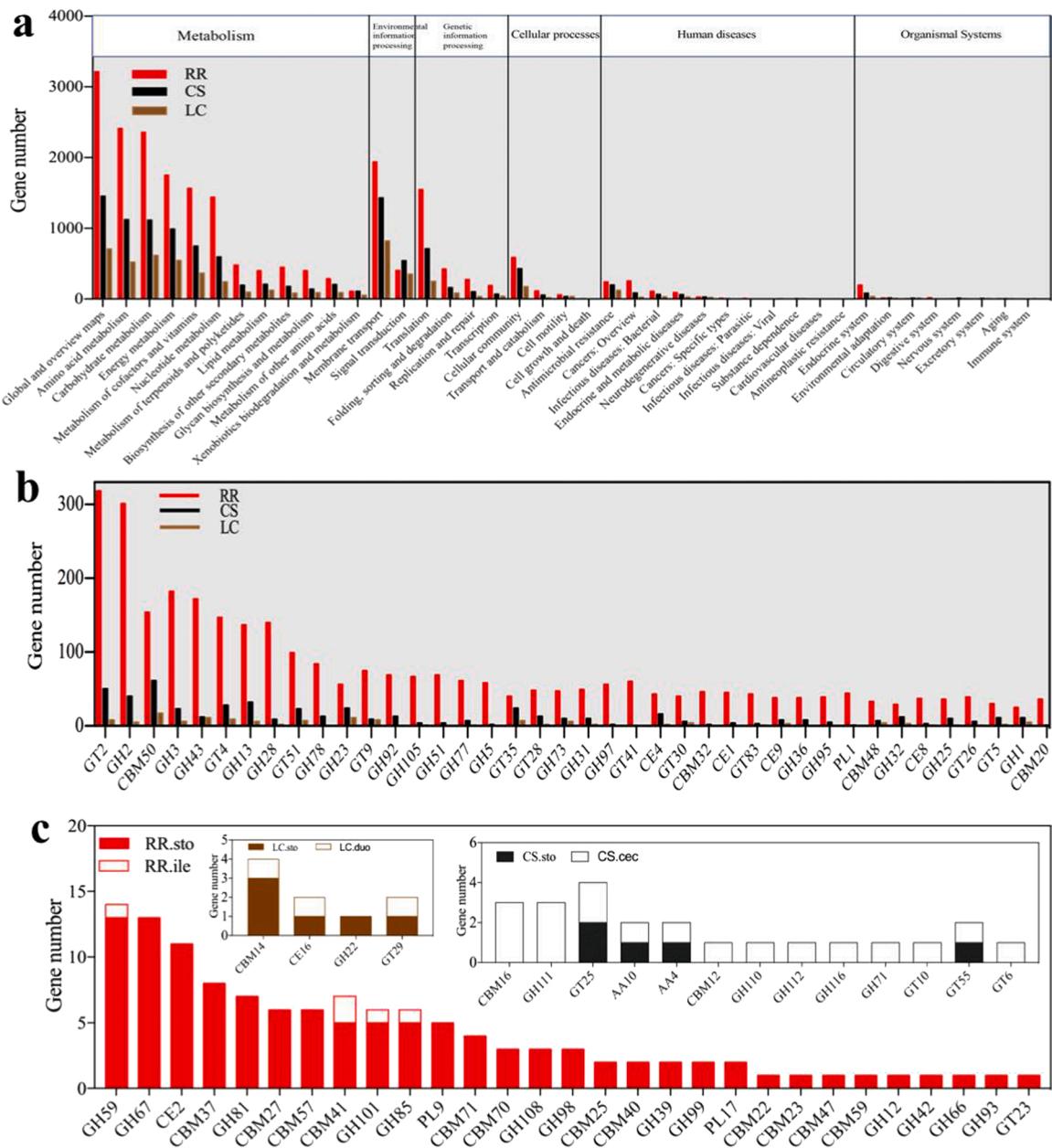


Fig. 4. Functional categories. The gene numbers of functional categories from KEGG subcategories (a). Top 40 CAZy families and carbohydrate-binding modules, respectively (b). Unique CAZy families and carbohydrate-binding modules in the GIT of RR, CS, and LC, respectively (c).

3.4. Major microbiota and contributors of the carbohydrate metabolism

Bacteria from Bacteroidetes (*Prevotella multisaccharivorax* and *Prevotella* sp. AGR2160) and Firmicutes (*Selenomonas bovis*, *Selenomonas ruminantium*, *Clostridium* sp. CAG:288, *Ruminococcus* sp. CAG:177, and *Ruminococcus* sp. CAG:177) were major contributors to the carbohydrate metabolism (Fig. 5a). Among these bacteria, the main five bacteria (*Prevotella multisaccharivorax*, *Selenomonas bovis*, *Prevotella* sp. AGR2160, *Selenomonas ruminantium*, *Clostridium* sp. CAG:288) were clustered together and were mainly found in the stomach of the golden monkey (Fig. 5a, d). The families GT4, GH43, GH2, GH3, GH5, and GH1 from these five bacteria contributed most to carbohydrate metabolism. *Prevotella multisaccharivorax* had the second number of bacterial sequences (5507 sequences) and the highest gene number in the families GT2 (504 genes), GH43 (442 sequences), GT4 (316 sequences), GH43 (261 sequences), and GH2 (102 sequences) (Fig. 5b, c). However, the main enzyme of *Selenomonas bovis* with the highest bacterial sequence (6087 sequences) in carbohydrate metabolism was from the family GH1 (138 sequences). The major five bacteria were clustered with eight bacteria, including *Prevotella albensis*, *Mitsuokella jalaludinii*, *Prevotella histicola*, *Sphaerochaeta pleomorpha*, *Prevotella multacidica*,

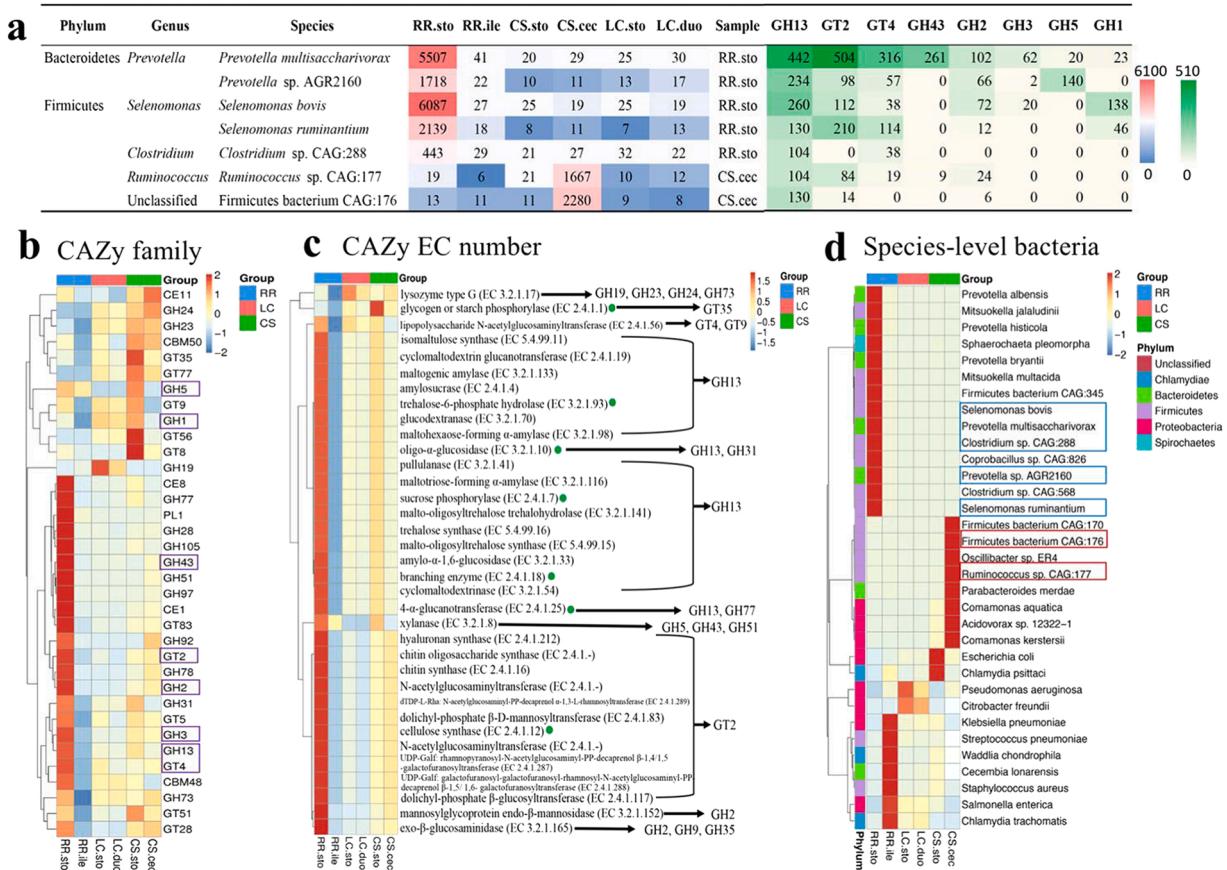


Fig. 5. Major microflora, enzyme genes related to the carbohydrate metabolism. Major microflora related to carbohydrate metabolism, the numbers under sample name represent the bacteria absolute unigenes number (a). The number under CAZy families represented the absolute unigenes number. The clustering heatmap of CAZy family (b), eight major CAZy families are emphasized by the purple box. The clustering heatmap of enzymes in CAZy family (c), green solid circle represented the enzymes involved in the metabolism of starch and sucrose. The clustering heatmap of the microbiota at species level (d). The bacteria with the blue and red box represent the major bacteria mainly observed from RR stomach and CS cecum, respectively.

Firmicutes bacterium CAG:345, *Coprobacillus* sp. CAG:826, and *Clostridium* sp. CAG:568 (Fig. 5d). In the green monkey cecum, Firmicutes bacterium CAG:176 (2280 sequences) and *Ruminococcus* sp. CAG:177 (1667 sequences) showed strong carbohydrate metabolism, particularly associated with the GH13 family (Fig. 5a). Importantly, 13 unique CAZymes were identified in the cecum of green monkey, with the main contributors being CBM16 and the family GH111 (Fig. 4c).

4. Discussion

4.1. Gastrointestinal segment determines the geographical microbiota within the species

The structure and function of each gastrointestinal segment of mammals are different and adapt to distinct nutritional functions (Vasapolli et al., 2019). Stomach is the first large container to receive and initially digest food, other gastrointestinal segments have an important role in food delivery and nutrient absorption. In addition, great differences were observed in the stomachs of different species. For example, herbivores (*Colobus abyssinicus* and *Loxodonta africana*) have a higher stomach pH than omnivores (*Homo sapiens*) (Beasley et al., 2015). Carnivores require pepsin to break down proteins, the acidic environment created by pepsin inhibits pathogen growth (Stevens and Hume, 2004). Herbivores need to approach a neutral stomach environment to promote more microbial growth and fiber material digestion. For example, at the 5th hour after eating, the cow's food digestion metabolism (e.g., starch and sucrose metabolism and energy metabolism) reaches its highest value, while the rumen's pH remains weakly acidic (Shaani et al., 2018).

Significant differences in microbial populations have been observed in different gastrointestinal segments of primates, including red-shanked doucs (Clayton et al., 2019), olive baboons (Yuan et al., 2020), aye-ayes (Greene and McKenney, 2018), proboscis monkeys (Hayakawa et al., 2018), and macaques (Matsuda et al., 2019). Some of these patterns are conserved across species. Because host phylogeny largely determines the differences of intestinal microbiota (Nishida and Ochman, 2018; Amato et al., 2019). The major two bacteria *Prevotella multisaccharivorax* and *Selenomonas bovis* showed higher abundance in the stomach of the golden monkey

(Fig. 5). Although the physiological structure of the gastrointestinal tract is different, the stomach is the first important gastrointestinal segment for receiving and digesting food. It is speculated that many microbial communities in the stomach have the same function (Martinez-Guryn et al., 2019). The metabolic pathways of the microbes of golden monkey, green monkey, and the lemur can be slightly illustrative, but this is not enough (Figs. 4, 5). In addition, the colon is another important intestinal tract for host digestion (e.g., hindgut animals), immunity and health (Litvak et al., 2018), especially in hindgut fermentation animals (e.g., omnivorous and frugivores). This promotes the colonization of more bacterial species in the colon, including the *Clostridium*, *Ruminococcus*, *Prevotella*, *Bacteroides*, and *Akkermansia* (Lee et al., 2021).

4.2. Primate species have specialized gastrointestinal microbiota

Currently, many studies have confirmed that host phylogeny is the main factor affecting the mammalian gut microbiota (Amato et al., 2019; Contijoch et al., 2019). Consistent with these findings, we found significant differences gastrointestinal bacteria of the golden monkey, green monkey, and lemur, with these bacteria major dominated by Firmicutes, Bacteroidetes, and Proteobacteria. In addition, the gastrointestinal microbiota of the green monkey and lemur clustered together (Supplementary Fig. S2d). This pattern mainly could be the result of gastrointestinal physiology. In the first scenario, although the phylogenetic relationship between the golden monkey and the green monkey is closer than that of the lemur (Rowe, 2016), the former is an herbivorous primate relying on fermentation in the foregut (stomach), and the latter two primates, as described above, are the omnivorous primates that rely on fermentation in the hindgut (green monkey: colon; lemur: cecum).

The intestinal characteristics of the two hindgut-fermented primates, green monkey and lemur can be characterized by an increased in the Ruminococcaceae in the hindgut (Figs. 1, 2b). Ruminococcaceae in the faecal mainly degrade dietary fiber in herbivores, including folivores primates (e.g., *Rhinopithecus brelichi* (Hale et al., 2019) and *Colobus guereza* (Amato et al., 2019) and ruminants (e.g., sheep, camel, and goat (Al-Masaudi et al., 2019). In addition, we observed that the stomach of the golden monkey was dominated by Bacteroidetes (Fig. 1). Similarly, Bacteroidetes were also found in the stomach of other foregut fermented herbivores (e.g., bison, yak, goat, and water deer) (Bergmann, 2017; Xie et al., 2021). Bacteroidetes mainly degrades complex polysaccharide carbohydrates (e.g., Cellulose, and resistant starch) (Lapébie et al., 2019). This is due to the colobines monkey (e.g. *Rhinopithecus roxellanae* and *Nasalis larvatus*) with four chambers of the stomach have a higher digestibility of fiber. In addition, this inter-individual variation may be due to the fact that the golden monkey in our study was from the wild and required more complex carbohydrate metabolism.

4.3. Carbohydrate metabolism is vital in folivore primates

Folivore primates eat a large amount of fiber diets, including leaves, branches and bark, especially in the wild where high-protein food is scarce (Frankel et al., 2019). Colobinae monkeys, including *Rhinopithecus roxellanae* (stomach) (Zhou et al., 2014) and *Rhinopithecus bieti* (faecal) (Xu et al., 2015), have been described for their good carbohydrate metabolism activity with intestinal microbes to digest complex carbohydrates in their diet (Hou et al., 2018). In our study, the lemur and green monkey received sufficient food (e.g., apple, orange, banana, fresh enough leaves of the *Ligustrum quihoui* Carr., and fiber-rich zoo-made nest) all year round but have a relatively narrow range of activities and were more in contact with humans. In contrast, the golden monkeys live in a wild environment with little contact with humans and their food is more limited in availability, particularly in the winter (Hou et al., 2018). This may greatly contribute to the abundant carbohydrate metabolism in the stomach in the golden monkey than that of the green monkey and lemur in our study.

The carbohydrate metabolism in the stomach of the golden monkey mainly from the glycoside hydrolases (GHs, e.g., mainly GH13 and GH43) and glycosyltransferase (GTs, mainly GT2 and GT4) in our study. The GH13 family containing α -glucoside linkages has been identified as having a common function of the digestive process, primarily degrading starch from the oral cavity to the colon (El Kaoutari et al., 2013). In addition, the enzymes involved in cellulolytic degradation in the GH2, GH3, GH5, GH28, and GH43 families were very abundant in the stomach of golden monkey. Similar results were observed in the gut microbiota study of the Yunnan snub-nosed monkey (Xu et al., 2015), North American beaver (*Castor canadensis*) (Armstrong et al., 2018), bovine (Brulc et al., 2009), and pygmy slow loris (*Nycticebus pygmaeus*) (Xu et al., 2013). In our study, the major species *Prevotella multisaccharivorax* and *Selenomonas bovis* were found contributed most of the GH13. *Prevotella multisaccharivorax* is anaerobic Gram-negative bacteria known to digest various sugars (Sakamoto et al., 2005; Stewart et al., 2018). Additionally, it has been found to be one of the contributors associated with oral diseases, such as the root caries (Chen et al., 2015) and acute apical abscesses (George et al., 2016), and is positively correlated with the expression of IgA (Zhang et al., 2018). *Selenomonas bovis* is believed to be effective in degrading cellulose in the rumen of yak (Zhang and Dong, 2009). Therefore, *Prevotella multisaccharivorax* and *Selenomonas bovis* were the major two contributors of the carbohydrate metabolism in the stomach of golden monkey, their identified metabolic pathways for the carbohydrate metabolism need further study.

5. Conclusions

In summary, we have revealed for the first time the microbial differences between golden monkey, green monkey, and lemur, using the gastrointestinal tract. Microbial composition and function vary widely across non-human primate species and gastrointestinal segments. The stomach of the golden monkey has a strong signal of microbial metabolic activity in carbohydrate metabolism compared to green monkey and lemur. However, our study of the primate gastrointestinal microbiota has some limitations. For example, the primates in our study were not replicated biologically, and gastrointestinal samples were collected from dead animals. While we

attempted to fully characterize the metabolic activity of the microbiome using two different databases (e.g., the KEGG and CAZY databases), future studies of host intestinal digestion and absorption also need to consider the effects of the enzyme and other microorganisms (e.g., parasites, fungi, bacteriophages and viruses). It will be important to isolate and culture key bacteria and determine their associated enzymes and functions directly. Additionally, captivity can affect the composition of intestinal microbiota, which was not considered in this study. Together, this information will ultimately help improve our understanding of microbiota and non-human primate species ecology and health, with important applications for wildlife management and species conservation.

Ethics statement

Collection of the full gut content sample of the dead golden monkey, green monkey, and lemur were approved by the Ethical Committee of Animal Care and Use Commission of Chengdu Institute of Wildlife, Chengdu Zoo. Laboratory experiments approved by the Animal Microecology Institute of Veterinary Medicine, Sichuan Agricultural University (approval number: SYXKchuan2019-187).

Funding

This study was supported by the Funded Project of Chengdu Giant Panda Breeding Research Foundation (CPF2017-04) and Sichuan Science and Technology Program (2019YFH0060).

Author contributions

Yan Zeng, Yang Pu, Lili Niu, and Xueqin Ni designed the experiment and wrote the first draft. Jiabo Deng, Yi Zhou, and Yiceng Lin collected the samples. Yang Li, Jie Wang, Liqian Wu, and Benhao Chen performed the experiments. Yan Zeng and Yang Pu analyzed the data. Katherine Ryan Amato, Xueqin Ni, Kangcheng Pan, Bo Jing, and Dong Zeng revised the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data accessibility

The raw sequence data of 16S rRNA (PRJNA545140) and metagenomic data (PRJNA578354) without host genes for this study are available at the Sequence Read Archive of NCBI.

Acknowledgments

We would like to thank Chengdu Wildlife Institute, Chengdu Zoo for their supporting of the collection of primate's gastrointestinal samples.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.gecco.2021.e01946](https://doi.org/10.1016/j.gecco.2021.e01946).

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