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Letter to the editor Open Access

Unique characteristics of gut microbiota in black snub-nosed monkeys (*Rhinopithecus strykeri*) reveal an enzymatic mechanism of adaptation to dietary vegetation

DEAR EDITOR,

The Myanmar or black snub-nosed monkey (*Rhinopithecus strykeri*) is a recently discovered and critically endangered colobus primate with an unknown gut microbiota. Here, we characterized and compared the gut microbiota of *R. strykeri* with those of two closely related snub-nosed monkey species, *R. roxellana* and *R. bieti*. Results showed that *R. strykeri* exhibits a unique gut microbiota composition, with higher bacterial and fungal diversity and greater abundance of carbohydrate-active enzymes (CAZymes) related to pectate and glucose metabolism. In addition, we identified core microbial taxa shared among the three snub-nosed monkey species, involved in the digestion of carbohydrates that cannot be digested by the host, such as cellulose, hemicellulose, and lignin. Our findings provide insights into the important role of the gut microbiota in facilitating adaptation to dietary vegetation in snub-nosed monkeys, and enabling the expansion of their dietary niches.

Rhinopithecus strykeri is a recently discovered snub-nosed monkey species located in the Gaoligong Mountains on the Sino-Myanmar border (Geissmann et al., 2011). All snub-nosed monkeys are highly folivorous, exploiting diets composed predominantly of leaves, seeds, bark, and lichen (Zhou et al., 2014). Despite exhibiting a similar dietary structure to other snub-nosed monkeys, *R. strykeri* consumes more fruits, seeds, buds, and flowers than *R. roxellana* and *R. bieti* (Yang, 2019). The gut microbiota plays a crucial role in foregut fermentation during leaf consumption in *R. roxellana* (Zhou et al., 2014). Coker (2022) recently reported that the fungal and viral communities are also critical components of the animal gut microbiota, essential for maintaining intestinal homeostasis and promoting animal survival. To date, however, these microbial communities have not been characterized in any snub-nosed monkey species, and there are no reports on the gut microbiota of *R. strykeri*.

In the current study, to clarify the potential uniqueness of the *R. strykeri* gut microbiota and elucidate the dietary adaptive mechanisms of the three species, we collected fecal samples of 42 individuals in the wild, including 13 *R. strykeri* (Rst), 15 *R. roxellana* (Rro), and 14 *R. bieti* (Rbi) monkeys, from seven different locations (Supplementary Table S1). We adopted shotgun metagenomic sequencing to explore the similarities and differences in the bacterial, fungal, and viral communities and to detect the carbohydrate hydrolases among the three species.

Sequence taxonomy was resolved using Kraken2 to estimate microbial abundance at each taxonomic level. Alpha and beta diversities were calculated based on the abundance of each genus. Bacterial, fungal, and viral genera that generally existed in at least one monkey group were filtered to identify coexisting microbial clusters, and then the unique characteristics of each group were detected. Single-sample metagenomic assembly and carbohydrate-active enzyme (CAZyme) annotation were used to compare differences in carbohydrate metabolism capacity among the three groups. Core microbes and CAZymes from the three groups were then selected to explore the general characteristics of the gut microbiota in the three snub-nosed monkey species. Further details on methods are available in the Supplementary Materials.

Our results indicated that the gut microbiota structure at the phylum level was similar among the three species, primarily composed of bacteria, fungi, and viruses (Supplementary Figure S1A). The most abundant bacterial phyla detected in the three groups were Firmicutes (mean 56.17%), Proteobacteria (mean 16.19%), and Bacteroidetes (mean 14.32%). The most abundant fungal and viral phyla were Ascomycota (mean 88.91%) and Uroviricota (mean 77.04%), respectively. The Venn diagram showed that most microbial genera and carbohydrate hydrolases were shared among the three monkey species (Supplementary Figures S1B, S2A). However, beta and alpha diversities showed significant differences between Rst and the other two groups. Beta diversity analyses, including non-metric multidimensional scaling (NMDS) and principal coordinate analysis (PCoA), demonstrated that Rbi and Rro were clustered together and significantly distant from Rst (Figure 1A; Supplementary Figures S3A, S2B; $P<0.005$). Alpha diversity was calculated using the Chao1 and Shannon indices at the genus level across the three monkey groups. Results showed that the gut microbiota diversity of Rst was significantly lower than that of Rbi and Rro (Figure 1B; Supplementary Figure S3B; $P<0.0001$), with no significant differences detected between the latter two species.

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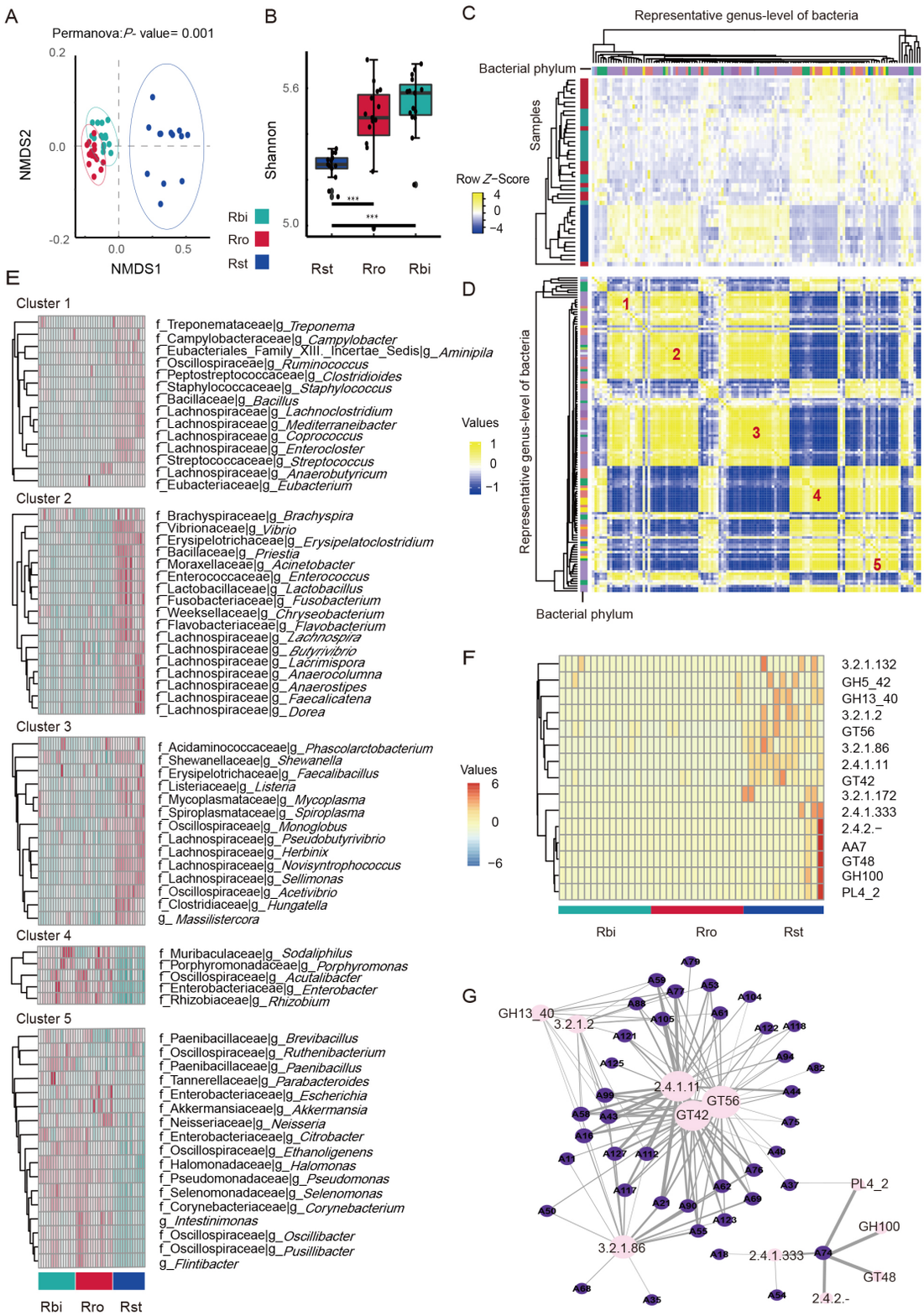

Figure 1 Gut microbial diversity and uniqueness in three snub-nosed monkey species

A: Representative NMDA plots of gut microbiota community composition of three snub-nosed monkey species at the genus level. B: Representative alpha diversity of gut microbiota of three snub-nosed monkey species at the genus level. C: Heatmap representing relative abundance of each bacterial genus in each sample. Heatmap was hierarchically clustered using average linkage based on Euclidean distance and showed the coexistence of several groups of bacterial genera within specific groups. D: Correlation matrix calculated among vectors of abundances of bacterial genera across all samples. Bacterial genera were hierarchically clustered using average linkage based on Euclidean distance. E: Five clusters of coexisting bacterial genera were visually selected, and their abundances in corresponding hosts are depicted in Figure 1D. Clusters 1, 2, and 3 coexisted in Rst; whereas Clusters 4 and 5 coexisted in Rro and Rbi. F: Heatmap of CAZymes that were significantly more abundant in Rst compared with Rbi and Rro. G: Correlation network diagram of coexisting bacteria and significantly more abundant CAZymes detected in Rst.

Based on the above results, although the gut microbiota of the three species shared many of the identified microbial genera and enzymes, gut microbiota structure and function were more similar in Rro and Rbi than in Rst. Furthermore, gut microbial diversity was significantly higher in Rbi and Rro than in Rst. Both Rbi and Rro inhabit mixed deciduous broad-leaved and coniferous forests, where lichens are an important dietary component (Zhou et al., 2014). In contrast, Rst inhabits humid mixed evergreen broad-leaved, coniferous, and bamboo forests (Chen et al., 2022; Ma et al., 2014), where fruits, flowers, and buds are more abundant (Yang, 2019). In addition, the Rst samples were only collected from one location (Gaoligong Mountains), whereas the Rro and Rbi samples were collected from three different habitat locations, which may explain their greater gut microbial diversity. Previous research has suggested that host lifestyle can influence gut microbiota more than geographic location in shaping the microbiomes of primates, including humans (Campbell et al., 2020). Thus, we speculate that the similar lifestyles of Rro and Rbi contribute to the similarities in their gut microbiota.

Based on the distinctive structure of the Rst gut microbiota, we analyzed the relationship between the gut microbiome and phylogeny in the three species. We identified 130 bacterial, 43 fungal, and 121 viral genera in more than 20% of individuals in each monkey group (Supplementary Table S2). Based on cluster and correlation analyses of the microbial genera, we identified several clusters of coexisting gut microbes within each group (Figure 1C, D; Supplementary Figures S4, S5). Notably, bacterial Clusters 1, 2, and 3 coexisted in Rst, whereas Clusters 4 and 5 coexisted in Rbi and Rro (Figure 1C, D). Most of the 45 bacterial species coexisting in Rst are related to pectate and glucose degradation, including the Lachnospiraceae (a typical pectin-decomposing family) and Oscillospiraceae families, as well as the *Massilistercora*, *Faecalibacillus*, and *Vibrio* genera (Figure 1E). In contrast, among the 22 bacterial species coexisting in Rro and Rbi (Figure 1E), *Oscillibacter* and *Parabacteroides* are involved in fiber digestion. Similar results were obtained following analysis of coexisting fungal genera among all monkeys. Yeast fungal clusters associated with pectate and glucose fermentation were more abundant in the Rst gut microbiota (Supplementary Figure S4), with most yeast genera belonging to the Saccharomycetaceae family, including *Lachancea*, *Saccharomyces*, and *Kazachstania*. No obvious viral genera clusters were detected for the different monkey groups (Supplementary Figure S5). We also compared CAZymes among the three species and identified 15 CAZymes significantly enriched in Rst (Figure 1F; Supplementary Table S3), with most involved in starch and glucose metabolism, such as glycogen (starch) synthase (2.4.1.11), phospho-beta-glucosidase A (3.2.1.86), and glycosyltransferase (GT42). Furthermore, the CAZymes and coexisting bacteria were correlated in Rst (Figure 1G; correlation coefficient>0.6; P>0.005). Notably, several families of Firmicutes, including Lachnospiraceae; Bacillaceae, Enterococcaceae, Oscillospiraceae, and Clostridiaceae, were highly correlated with the CAZymes. These findings demonstrated a strong correlation between the bacteria and CAZymes clustered and enriched in Rst, indicating consistent distribution and functional trends of the bacterial and functional genes.

A greater number of microbes and carbohydrate-digesting enzymes related to pectin and glucose metabolism were detected in the gut microbiota of Rst (Figure 1E–G; Supplementary Figure S4). Compared with Rro and Rbi, the Rst diet contains a higher proportion of flowers (Rst: ~15.2%; Rbi: 1.1%–1.9%; Rro: 1.13%–1.3%), buds (Rst: ~13.8%; Rbi: ~3%; Rro: 5.36%–5.8%), and fruits/seeds (Rst: ~15.7%; Rbi: 1.1%–10.5%; Rro: ~9.5%) (Yang, 2019). These plant parts contain a higher proportion of pectin and glucose compared to leaves. Thus, we suggest that the enhanced ability of the gut microbiota in *R. strykeri* to digest pectin and glucose may be an adaptation to the higher proportion of these nutrients in the diet, enabling the monkeys to stabilize in their unique habitat (Chen et al., 2022; Ma et al., 2014). Diet can rapidly and reproducibly alter the human gut microbiome, thus reflecting high variability in the gut microbiota in feeding adaptation (David et al., 2014). Multiple studies have also revealed the important role of the gut microbiota in maintaining adaptation to different habitats and in expanding diets (Xiang et al., 2012; Zhou et al., 2014). Similar dietary patterns have also been observed in the Tonkin snub-nosed monkey (*R. avunculus*) and gray snub-nosed monkey (*R. brelichi*), which consume relatively higher proportions of flowers, buds, and fruits in their diets (Xiang et al., 2012; Yang, 2019). We deduce that their gut microbes may play a similar role in feeding adaptation; however, the characteristics of their gut microbiota remain to be explored.

We extracted the core microbes and CAZymes from 42 individuals to explore common gut microbiota functions among the three species of snub-nosed monkeys (Supplementary Table S4). The core bacteria mainly belonged to the phyla Firmicutes, Bacteroidetes, and Proteobacteria, with *Paenibacillus* (5.22%), *Clostridium* (3.68%), and *Faecalibacterium* (3.25%) being the most abundant genera (Supplementary Table S4A). The core fungi mainly belonged to the phylum Ascomycota, with *Fusarium* (9.59%), *Aspergillus* (8.06%), and *Eremothecium* (7.38%) being the most abundant genera (Supplementary Table S4B). The core viruses mainly belonged to the phyla Nucleocytoviricota and Uroviricota, with *Mimivirus* (9.6%) being the most abundant genus (Supplementary Table S4C). Core CAZymes primarily belonged to 10 glycoside hydrolase families and seven glycosyl transferase families (Supplementary Table S4D). Glycoside hydrolases are a widespread group of enzymes that hydrolyze the glycosidic bond between two or more carbohydrates, or between carbohydrate and noncarbohydrate moieties. Glycosyltransferases enzymes are involved in the biosynthesis of disaccharides, oligosaccharides, and polysaccharides, as well as the metabolism of various carbohydrates, such as xylan, starch, mannose, and galactose.

Although the gut microbiota of *R. strykeri* reflects advantages in pectin and glucose digestion, as a typical leaf-eating nonhuman primate, it also shares similar dietary adaptations to those of other snub-nosed monkeys. At our sampling sites, *R. roxellana* and *R. bieti* predominantly consume lichens (Rro: 38.4%–43.28%; Rbi: 50.6%–82.1%) and leaves (Rro: 22.4%–32.22%; Rbi: 9.1%–24.7%) (Yang, 2019). Similarly, *R. strykeri* consumes a large proportion of leaves (37.9%) and twigs (7.8%), including total nonstructural carbohydrates of 34.9% and neutral detergent fiber of 34.68% (Yang, 2019). Our results related to core microbes and CAZymes indicated the three snub-nosed monkey species have a strong ability to digest complex carbohydrates, including cellulose, hemicellulose, starches, and pectin, in

addition to simple carbohydrates, such as glucose and oligosaccharides. Consistent with our results, Xu et al. (2015) detected numerous glycoside hydrolases enriched in the gut microbiota of *R. bieti*. Glycoside hydrolases have also been found in abundance in the rumen microbiome of bovines and in the gut of termites (*Trinervitermes trinervoides*) (Brulc et al., 2009), with abundant glycosyltransferases also detected in the cow rumen. Thus, these dietary adaptations are not only widely observed in the gut microbiota of snub-nosed monkeys, but also in other herbivores, reflecting potential convergent evolution of gut microbiota during dietary adaptation (Muegge et al., 2011).

SCIENTIFIC FIELD SURVEY PERMISSION INFORMATION

Before conducting sampling in the wild, we obtained approval from the State Forestry and Nature Reserves and the Institutional Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences.

DATA AVAILABILITY

Sequencing data can be found in the Sequence Read Archive (NCBI, nlm.nih.gov/sra) under BioProjectID PRJNA916620, GSA (https://ngdc.cncb.ac.cn/gsa/) under BioProjectID PRJCA014890, and Science Data Bank (https://www.scidb.cn/c/zoores) databases DOI:10.57760/sciencedb.07331.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

M.L. and Z.F.X. designed the project. X.C.W., J.L.Z., and H.J.P. compiled the data and conducted the analyses. Y.X.C. and Z.F.X. conducted the sampling. X.C.W., J.L.Z., H.J.P., and M.L. wrote the paper. S.X.M., J.W.Q., Y.S., and M.Y.Z. provided help in analysis. All authors read and approved the final version of the manuscript.

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Supplementary Materials

Unique characteristics of gut microbiota in black snub-nosed monkeys (*Rhinopithecus strykeri*) reveal an enzymatic mechanism of adaptation to dietary vegetation

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SUPPLEMENTARY MATERIALS AND METHODS

Sample collection

We collected 42 fecal samples from wild population, including 13 *R. strykeri* (Rst) individuals from the Gaoligong Mountain National Nature Reserve in Nujiang Prefecture, Yunnan Province, 15 *R. roxellana* (Rro) individuals from the Jiuzhaigou population in Sichuan Province (three samples), Yuhuangmiao population in Shaanxi Province (five samples), and Shennongjia population in Hubei Province (seven samples), and 14 *R. bieti* (Rbi) individuals from the Xianggujing population in Yunnan Province (five samples), Lasha Mountain population in Yunnan Province (five samples), and Xiaochangdu population in Tibet (four samples) (Supplementary Table S1). As the fecal samples were collected from wild populations, we were unable to identify individual monkeys, except for the Shennongjia population. We did not obtain information on age or sex but could infer that the samples were from adult monkeys based on the size of the feces. No duplicate individuals were sampled (with simultaneous collection at overnight trees or gathering sites) and samples were collected in different seasons. The samples were used to explore potential similarities in the gut microbiota of snub-nosed monkeys from the seven different habitat sites, and the unique adaptive characteristics of black snub-nosed monkeys. Fresh fecal samples were immediately collected in 3 mL of RNAlater (Blekhman et al., 2016; Vlckova et al., 2012), then transported on dry ice within one week and stored at −80 °C until DNA extraction at the Institute of Zoology, Chinese Academy of Sciences (CAS), Beijing, China.

DNA extraction and sequencing

Microbial DNA was extracted from the fecal samples using a QIAamp DNA stool mini kit (Qiagen, Valencia, CA, USA) following standard protocols. DNA quality and quantity were determined using Nanodrop (ND-1000) spectrophotometry (Nanodrop Technologies, Wilmington, DE, USA) and agarose gel electrophoresis, respectively. DNA samples were stored at −20 °C until use. Shotgun sequencing was performed using an Illumina NovaSeq 6000, with at least 10 Gb per sample. Raw data were filtered using Trimmomatic v0.36 (Bolger et al., 2014) to trim low-quality reads: 3' tailing sequences were removed when the average quality over a 4 bp sliding window was less than 20 and reads less than 70 bp were discarded. The genomes of *R. strykeri* (assembly ASM2376470v1), *R. bieti* (assembly ASM169854v2), *R. roxellana* (assembly ASM756505v1), and *Homo sapiens* (assembly GRCh38.p13) were used to remove contamination with bowtie2 v2.3.5 (Langmead & Salzberg, 2012) to obtain clean data.

After removal of low-quality and contaminated reads, an average of 12.26 Gb of high-quality nonhost sequences were obtained per sample in the Rst, Rbi, and Rro groups (Supplementary Table S1). We assessed 4 623 bacterial terms (38 phyla, 10 classes, 14 orders, 25 families, 43 genera, and 73 species), 169 fungal terms (three phyla, 78 classes, 180 orders, 398 families, 1 197 genera, and 2 732 species), 527 viral terms (12 phyla, 16 classes, 20 orders, 39 families, 169 genera, and 271 species), and 575 CAZymes, which formed the gut microbiomes of the three snub-nosed monkey species (Supplementary Table S5).

Determination of relative abundance of taxonomic and functional terms

Sequence taxonomy was resolved using Kraken2 and the PlusPF database (Standard plus protozoa & fungi database) with default parameters (Wood et al., 2019). The Kraken2-mapped sequence counts were subsequently re-estimated using Bracken, a rapid read-level classifier, along with information about the genomes themselves to estimate abundance at the species, genus, or higher level of bacteria, fungi, and viruses (Lu et al., 2016). We further calculated the relative abundance of each taxonomic term for bacteria, fungi, and viruses using an in-house script. Taxonomic terms that did not exceed a maximum relative abundance of 1×10^{-4} were excluded from further analysis, together with taxonomic terms accounting for less than 20% of the samples in each cohort (Wirbel et al., 2019).

We used single-sample metagenomic assembly and functional annotation to analyze the gut

microbiota of all three species. Briefly, assemblies were produced with MEGAHIT (v1.2.6) (Li et al., 2015), and gene identification was performed on contigs longer than 300 bp using MetaGeneMark (Zhu et al., 2010). The contigs were then annotated with carbohydrate-active enzymes (CAZymes) (Lombard et al., 2014) to obtain a complete picture of the carbohydrate digestibility capabilities of gut microbiota using DIAMOND (v0.9.24) (Buchfink et al., 2015) with parameters -d -q -e 1e-5 -k 1. We further calculated the relative abundance of each functional term, then removed terms detected (i) in less than 20% of the samples in each group or (ii) in no sample in any group. Specifically, functional terms that did not exceed a maximum relative abundance of 1×10^{-5} were excluded from further analysis (Wirbel et al., 2019).

Analysis of bacterial coexistence in three snub-nosed monkey species

Bacterial, fungal, and viral genera present in at least 20% of individuals within at least one host group and with a relative abundance greater than 0.005 were screened for coexistence analysis (Doron et al., 2021). First, we constructed a heatmap representing relative abundance of each genus (columns) in each sample (rows). The heatmap was hierarchically clustered using average linkage based on Euclidean distance, showing coexistence of bacterial genera within host groups. Second, a correlation matrix was calculated between vectors of abundances of bacterial genera across all samples. Genera were hierarchically clustered with average linkage based on Euclidean distance. Third, clusters of coexisting genera were visually selected and their abundance in the corresponding hosts was visualized using a heatmap. To relate the clusters back to the groups that contained them, heatmaps using average relative abundance of genera for each snub-nosed monkey group were visualized for each cluster.

Analysis of gut microbiota diversity of three snub-nosed monkey species

Alpha diversity (Chao1 and Shannon indices) was calculated based on genus abundance for the three groups using the R package "vegan" (Oksanen et al., 2019). The Shannon diversity index accounts for genus richness and evenness, whereas the Chao1 index extrapolates the number of rare taxa that may have been detected with deeper sampling. Each metric was further compared using the Wilcoxon rank sum test with the R package "coin" (Hothorn et al., 2006), and *P*-values were adjusted using the Benjamini-Hochberg false discovery rate (FDR) (Benjamini & Hochberg, 1995). Principal component analysis (PCoA) and non-metric multidimensional scaling (NMDS) based on PERMANOVA were performed using the R package "vegan" to analyze the gut microbiota of the three groups (Oksanen et al., 2019), with the results visualized using the "ggplot2" package. The *P*- and R-values were calculated based on "ANOSIM" using the R package "vegan" (Oksanen et al., 2019).

Analysis of functional differences among three snub-nosed monkey species

Diversity analysis indicated that the gut microbiota structures of Rbi and Rro were highly similar but differed significantly from that of Rst. To explore the unique or significantly enriched functional terms in Rst, we compared the relative abundances of CAZymes across all individuals in the three host groups. We used the fold-change method to conduct differential analysis and selected functional terms with Log2 fold-change >2 in both Rst/Rbi and Rst/Rro, as well as functional terms with zero relative abundance in Rbi and Rro but not in Rst, as significantly more abundant terms in the Rst group (Supplementary Table S3). To explore the correlation between coexisting bacteria in Rst and significantly abundant functional terms, we constructed a correlation network diagram between the genera and functional terms. Pearson correlation analysis was performed between genera and functional terms, then a matrix of correlation coefficients was visualized using a correlation network diagram (https://www.omicstudio.cn/tool/).

Analysis of core microbes and CAZymes

We selected core microbes and CAZymes from Rst, Rro, and Rbi to explore the general characteristics of the gut microbiota in snub-nosed monkeys. Specifically, we choose microbes

present in all individuals, with average relative abundance in each population greater than 0.1%. The same approach and thresholds were used for the selection of core CAZymes. In total, 95 bacterial genera, 24 fungal genera, 12 viral genera, and 21 CAZymes were screened among all individuals of the three snub-nosed monkey species (Supplementary Table S4).

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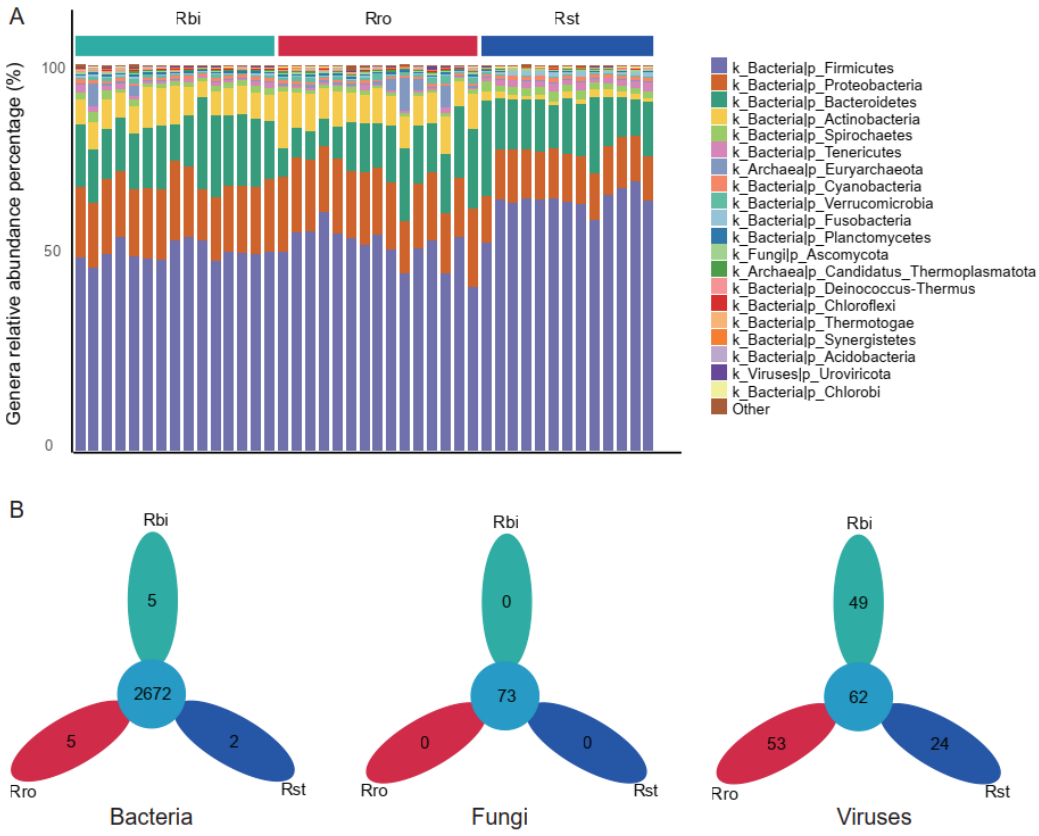
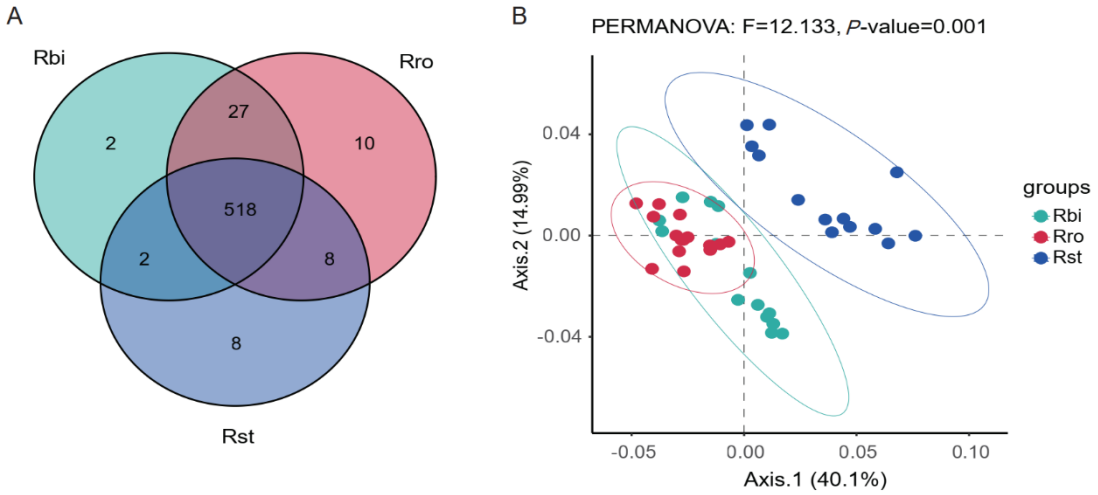


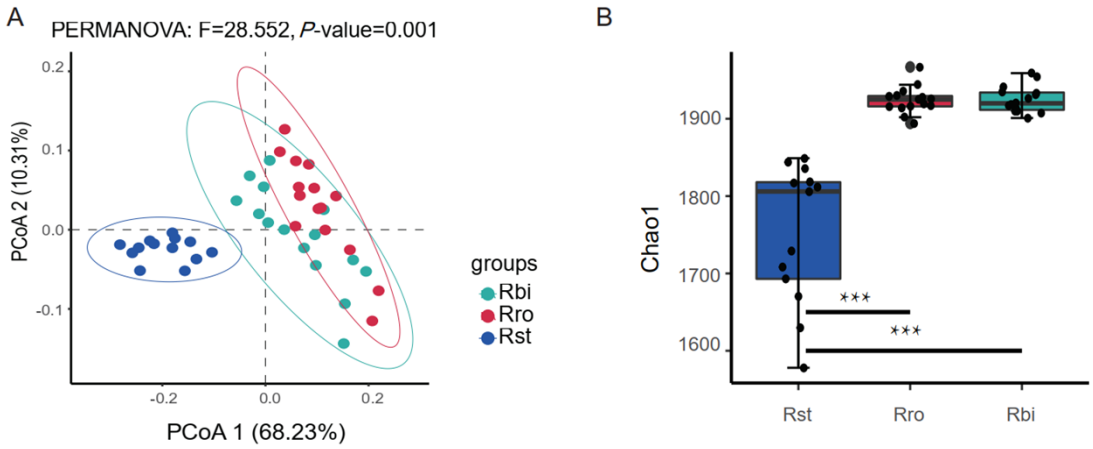
Supplementary Figure S1 Community components of gut microbiota in three snub-nosed monkey species

A: Relative abundances of gut microbial communities, including bacteria, fungi, and viruses, at the phylum level in three snub-nosed monkey groups. B: Venn diagram of bacteria, fungi, and viruses at the genus level in three snub-nosed monkey groups.



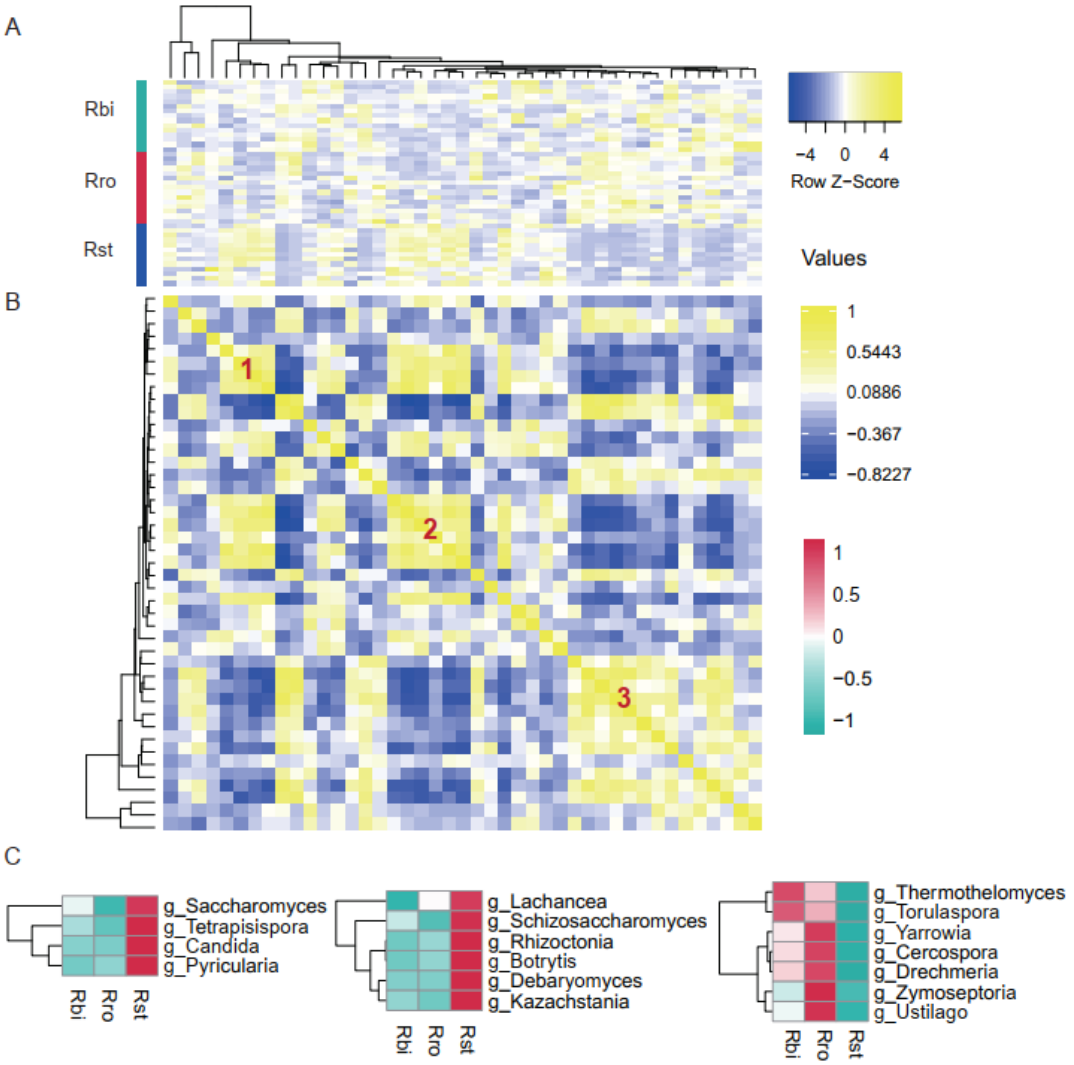
Supplementary Figure S2 Differential analysis of taxa and CAZymes among three snub-nosed monkey species

A: Venn diagram of microbial abundances in three snub-nosed monkey species. B: Bray-Curtis PCoA based on CAZy database functional terms.



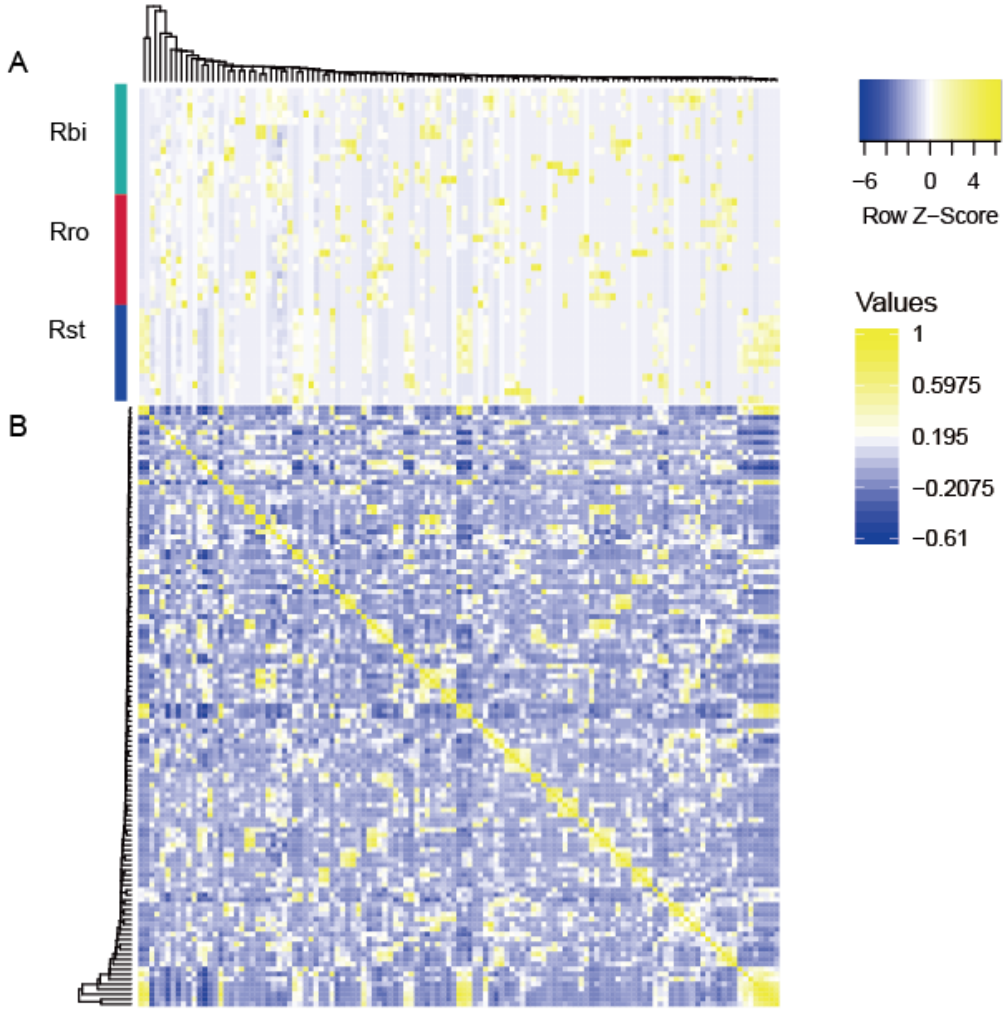
Supplementary Figure S3 Community structure, richness, and diversity of gut microbiota of three snub-nosed monkey species

A: Representative PCoA plots of gut microbiota community composition of three snub-nosed monkey species at the genus level. B: Representative alpha diversity of gut microbiota of three snub-nosed monkey species at the genus level.



Supplementary Figure S4 Association between microbiota composition and host class, expressed as coexisting fungal clusters

A: Heatmap representing relative abundance of each fungal genus in each sample. Heatmap was hierarchically clustered using average linkage based on Euclidean distance, showing coexistence of several groups of fungal genera within specific groups. B and C: Correlation matrix calculated among vectors of abundances of fungal genera across all samples. Fungal genera were hierarchically clustered using average linkage based on Euclidean distance. Three clusters of coexisting fungal genera were visually selected, with their abundances in corresponding hosts depicted in (C).



Supplementary Figure S5 Association between microbiota composition and host class, expressed as coexisting viral clusters

A: Heatmap representing relative abundance of each viral genus in each sample. Heatmap was hierarchically clustered using average linkage based on Euclidean distance, showing coexistence of several groups of viral genera within specific groups. B: Correlation matrix calculated among vectors of abundances of viral genera across all samples. Viral genera were hierarchically clustered using average linkage based on Euclidean distance. There were no significant clusters of coexisting viral genera.

Supplementary Tables

Supplementary Table S1 Sample information of three snub-nosed monkey species

Supplementary Table S2 Coexisting bacterial and fungal clusters in three snub-nosed monkey species

Supplementary Table S3 CAZymes with high abundance in Rst

Supplementary Table S4 Core bacteria, fungi, viruses, and CAZymes in three snub-nosed monkey species

Supplementary Table S5 Relative abundance of bacteria, fungi, viruses, and CAZymes in three snub-nosed monkey species

Supplementary Tables S1–**S5** are listed separate excel files due to their large size.