Comparative analysis of gut microbiota between wild and captive Guizhou snub-nosed monkey (Rhinopithecus brelichi)

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July 16, 2024

Abstract

Maintaining a healthy status is crucial for the successful captive breeding of critically endangered Rhinopithecus brelichi, it is conducive to ex situ conservation of this species and rejuvenation of its population. However, changes in the feeding environment and food can affect the composition and function of the gut microbiota in R. brelichi, ultimately impacting its health and adaptation. Herein, 16S rRNA gene sequencing was employed to determine the gut microbiota composition and functional variations between wild and captive R. brelichi populations. The results showed that the captive group had higher alpha diversity than the wild group, and significant differences were observed in their beta diversity. Captive and wild R. brelichi showed similar microbiota at the phylum level, which mainly comprised Firmicutes, Bacteroidota, and Spirochaetota, but captivity reduced the Firmicutes/Bacteroides ratio. LEfSe analysis revealed that the relative abundance of microbiota related to cellulose degradation, such as Prevotellaceae_UCG_001, Christensenellaceae_R_7_group, Ruminococcus, and Fibrobacter, differed significantly between the two groups. Furthermore, the potential pathogens Acinetobacter and Treponema were significantly abundant in wild and captive groups, respectively. Functional predictions demonstrated that the most significant functional pathways at the second level between captive and wild monkeys were carbohydrate, amino acid, and lipid metabolisms. The captive monkeys exhibited higher digestive capacity and endocrine regulation as well as a higher risk of infectious diseases than wild monkeys. These findings can serve as a valuable theoretical basis for promoting the healthy breeding of R. brelichi and as a guide for future evaluation of the health of wild and captive monkeys.

Comparative analysis of gut microbiota between wild and captive Guizhou snub-nosed monkey (*Rhinopithecus brelichi*)

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Abstract: Maintaining a healthy status is crucial for the successful captive breeding of critically endangered *Rhinopithecus brelichi* , it is conducive to ex situ conservation of this species and rejuvenation of its population. However, changes in the feeding environment and food can affect the composition and function of the gut microbiota in *R. brelichi* , ultimately impacting its health and adaptation. Herein, 16S rRNA gene sequencing was employed to determine the gut microbiota composition and functional variations between wild and captive *R. brelichi* populations. The results showed that the captive group had higher alpha diversity than the wild group, and significant differences were observed in their beta diversity. Captive and wild *R. brelichi* showed similar microbiota at the phylum level, which mainly comprised Firmicutes, Bacteroidota, and Spirochaetota, but captivity reduced the Firmicutes/Bacteroides ratio. LEfSe analysis revealed that the relative abundance of microbiota related to cellulose degradation, such as Prevotellaceae_UCG_001, Christensenellaceae_R_7_group, *Ruminococcus* , and*Fibrobacter* , differed significantly between the two groups. Furthermore, the potential pathogens *Acinetobacter* and*Treponema* were significantly abundant in wild and captive groups, respectively. Functional predictions demonstrated that the most significant functional pathways at the second level between captive and wild monkeys were carbohydrate, amino acid, and lipid metabolisms. The captive monkeys exhibited higher digestive capacity and endocrine regulation as well as a higher risk of infectious diseases than wild monkeys. These findings can serve as a valuable theoretical basis for promoting the healthy breeding of *R. brelichi* and as a guide for future evaluation of the health of wild and captive monkeys.

Keywords: *Rhinopithecus brelichi* ; Wild and captive; Gut microbiota; 16S rRNA gene sequencing

1. Introduction

The gut microbiota is composed of bacteria, archaea, viruses, and eukaryotic microbes, which have great potential to influence host physiology in both healthy and diseased states (Wang et al., 2023). Over the course of evolution, a stable relationship of mutual adaptation and cooperation has developed between animals and their gut microbes, and co-evolution has been achieved (Ley et al., 2008). The structure of the gut microbial community in animals is the result of the co-evolution of animals and their environment. Although the gut microbial community affects physiological functions of animals, it is highly susceptible to changes in various endogenous and exogenous factors (Bennett et al., 2016). Multiple studies have shown that the high plasticity of gut microbes makes their structure and function susceptible to changes in dietary structure, phylogenetic relationships, and the geographical environment (Zhao & Wang, 2024), and phylogenetic development exerts a greater impact on the host gut microbial community than diet and geographical environment (Amato et al., 2016). However, some studies have suggested that diet plays a dominant role in determining the composition of the host gut microbiota (Hale et al., 2018; Frankel et al., 2017). A previous study reported that the environment plays a crucial role in shaping the composition of gut microbiota (Gani et al., 2024), and animals living in different environments often exhibit distinct microbial signatures (Alberdi et al., 2021). However, only a few studies have addressed the impact of environmental changes on gut microbes, and most of them have focused on relatively few target species (McKenzie et al., 2017).

The heterogeneity of the living environment can directly influence the composition and acquisition of food resources by animals, thereby impacting the diversity of their gut microbiota (Gomez et al., 2015). The heterogeneity of primate habitats is manifested in two main ways: (1) variations between different geographical areas and (2) variations between wild and non-wild populations. A study by Zhao et al. (2018) revealed significant differences in the intestinal microbial composition of *Macaca mulatta* populations in different geographical environments. In addition, many studies have shown that the microbial composition of captive primates, such as *Macaca mulatta* (Jia et al., 2022), *Macaca thibetana* (Xia et al., 2022), *Pygathrix nemaeus* (Clayton et al., 2018), *Rhinopithecus roxellanae* (Zhao et al., 2023), and *Alouatta pigra* (Nakamura et al., 2011), is significantly different from that of wild individuals, with the former showing significantly lower microbial diversity. Feeding on a single set diet may be the main cause of reduced gut microbial diversity among captive individuals (Guo et al., 2023). In addition to food-related factors, captive individuals spend less time socializing and moving due to changes in their lifestyle (Guo et al., 2023), which may reduce the

host's exposure to microbes and reduce their gut microbial diversity. However, studies have also shown the opposite trend; for instance, gorillas in captivity in zoos show higher gut microbial diversity than those living in the wild (Narat et al., 2020). The environment in which primates are held in captivity may also alter the composition of their gut microbiota; for example, the loss of the host's native microbiota due to reduced dietary fiber consumption under captive conditions (Clayton et al., 2016). It has been suggested that differences in the gut microbiome between wild and captive animals can significantly affect their overall health, particularly in terms of digestive and immune functions (Gani et al., 2024), and the loss of microbial diversity may underlie the increased disease prevalence in captive animals by resulting in microbial communities that are more susceptible to invasion or by altering host immune function (Kohl et al., 2014).

The Guizhou snub-nosed monkey (*Rhinopithecus brelichi*) is a primate belonging to the Cercopithecidae (Colobinae), and is one of the 25 most endangered primates globally (Yang et al., 2023; Huang et al., 2024). It is only found in the Fanjing Mountain National Nature Reserve (FNNR) in Guizhou Province, China, and its wild population is small in number and distribution range (Jia et al., 2022). Owing to habitat loss and fragmentation, the population has been isolated, making *R. brelichi* into a species with only a single continuous population, showing very low genetic diversity and slow population growth, along with a high risk of extinction. In this context, captive breeding is crucial to improve its reproductive success and help its population to recover (Yang et al., 2023). However, *R. brelichi* fare poorly in captivity and exhibit chronic diarrhea, poor hair coat, pale skin tone, low reproductive success, and a general failure to thrive (Hale et al., 2019). Gut microbial disorders or changes in the composition of the gut microbiome are closely related to host health. As captivity increases the contact of primates with humans, it may lead to an increase in potential pathogens in the primates' gut microbiota, thereby increasing the risk of disease (Clayton et al., 2016). Dietary differences are also an important cause of differences in gut microbes between wild and captive primates (Sun et al., 2023). *R. brelichi* is a typical leaf-eating primate. In the wild, its diet mainly consists of a large amount of leaves (Hale et al., 2019; Zhang et al., 2024), while in captivity, in addition to the leaves of various plants, its diet also includes a variety of fruits (grapes, bananas, dates, citrus, sweet potato, apple, pear, kiwi, mango, cantaloupe, peach), vegetables (lettuce, romaine, carrot, pumpkin, and eggplant), sources of protein (eggs and peanuts), and coarse grains, which are not available to its counterparts in the wild. Therefore, for the ex situ conservation of these wild animals and scientific management of their captive counterparts, it is essential to understand how the lifestyle alters the gut microbial composition, revealing interactions between the gut microbiota and host.

In this study, the gut microbiota of wild and captive *R. brelichi* was evaluated using noninvasive sampling of feces and high-throughput sequencing based on the 16S rRNA gene. Extensive studies confirmed that there are differences in the gut microbial community structure between wild and captive primates, which may be related to their diet, lifestyle, and other factors. This study aimed to provide an understanding of the composition and function of the gut microbiota in wild and captive *R. brelichi* . This study was conducted with the aim of answering the following research questions: Are there differences in gut microbial composition, diversity, and function between wild and captive *R. brelichi* ? What accounts for these differences? Are the changes in the gut microbiota of captive monkeys relevant to their health?

2. Materials and methods

2.1 Study area and sample collection

FNNR is located in the transition from Yunnan Guizhou Plateau to the hills of western Hunan and includes the main peak of Wuling Mountains (27°49'50'' to 28deg1'30''N, 108deg45'55'' to 108deg48'30''E). It is located at an altitude of 500–2,572 m, with an area of 419 km^2. In terms of vegetation, it is composed of evergreen broad-leaved, deciduous broad-leaved, evergreen deciduous broad-leaved mixed, and needle- and broad-leaved mixed forests (Xiang et al., 2009). The FNNR is one of the areas with the highest forest biodiversity protection priority in the upper reaches of the Yangtze River. The natural vegetation in the protected area is relatively well preserved, providing a good habitat for various rare and endangered animals and plants, and this area is the only habitat for the wild population of *R. brelichi* (Wu et al., 2006).

From December 2021 to November 2022, fresh fecal samples were collected from all suspected *R. brelichi* in the northern part of FNNR. During this sampling, the surface soil was removed manually using disposable medical gloves. Furthermore, plant branches, leaves, and other parts attached to the samples were removed with disposable sterile tweezers and scalpels, and the middle uncontaminated part of the sample was selected and placed in 50-mL sterile centrifuge tubes, which were then marked and packed into a sealed bag. The geographical information about the site was recorded on the sealed bags (Figure 1). To prevent DNA degradation and microbial reproduction in the fecal samples, they were immediately placed in liquid nitrogen tanks after collection and were brought back to the laboratory for storage at -80 until use. Fecal samples from captive animals were collected simultaneously using the same method. These samples were collected from five *R. brelichi* in captivity at the rescue station of FNNR Administration. A total of 42 and 15 fecal samples were collected from animals in the wild and those in captivity, respectively.

2.2 DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing

Mitochondrial CO I-based DNA barcoding was used to identify 42 fecal samples collected in the wild, of which 31 belonged to *R. brelichi* and 11 belonged to other monkey species. Total genomic DNA samples were extracted using OMEGA DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, USA), following the manufacturer's instructions, and stored at -20degC prior to further analysis. The quantity and quality of extracted DNA were measured using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and 0.8% agarose gel electrophoresis, respectively.

The V3–V4 highly variable region of the bacterial 16S rRNA gene, with a length of approximately 468 bp, was selected for PCR amplification. The primer sequences were as follows: forward primer 338F (5'-barcode+ACTCCTACGGGAGGCAGCA-3', the barcode is a seven-base oligonucleotide sequence used to distinguish different samples in the same library) and reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). PCR reactions were performed in triplicate 25 μL mixtures containing 0.25 μL of Q5 high-fidelity DNA polymerase, 5 μL of 5× Reaction Buffer, 5 μL of 5× High GC Buffer, 2 μL of 10 mmol L^{-1} dNTP, 1 μL of forward primer (10 μmol L^{-1}), 1 μL of reverse primer (10 μmol L^{-1}), and 2 μL of template DNA, with ddH_2O added up to a final volume of 25 μL. Thermal cycling consisted of initial denaturation at 98°C for 5 min, followed by 25 cycles consisting of denaturation at 98°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 45 s, with final extension for 5 min at 72°C (PCR instrument: ABIGeneAmp® 2720, USA). PCR amplicons were purified with Vazyme VAHTS^{TM} DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After the individual quantification step, amplicons were pooled in equal amounts, and paired-end 2×250 bp sequencing was performed using the Illumina NovaSeq platform with NovaSeq 6000 SP Reagent Kit (500 cycles) at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China).

2.3 Data analysis

Microbiome bioinformatics was performed with QIIME2 (https://docs.qiime2.org/2019.4/tutorials/). Specific analysis was conducted as follows: (i) Raw sequence data were demultiplexed using the demux plugin followed by primer cutting with the cutadapt plugin (Martin, 2011). (ii) Sequences were then quality-filtered, denoised, and merged, and chimeras were removed using the DADA2 plugin (Callahan et al., 2016). The obtained sequences with 100% similarity were merged, and amplicon sequence variants (ASVs) and abundance data tables were generated. (iii) The QIIME2 classify-sklearn algorithm was used (https://github.com/QIIME2/q2-feature-classifier). Specifically, for the feature sequence of each ASV, QIIME2 software was used with the default parameters and species annotation was performed using a naive Bayes classifier that had been pre-trained to obtain taxonomic information corresponding to each ASV (Bokulich et al., 2018).

We evaluated the alpha diversity of the microbial communities using Chao1, ACE, Shannon, and Simpson indexes, as calculated with QIIME2 and visualized as box plots. Additionally, we performed Kruskal–Wallis tests to examine differences in alpha diversity between the two groups (Xi et al., 2023). Principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) were used to analyze beta diversity

between the different groups to compare the intestinal microbial composition between wild and captive *R. brelichi* . Based on the Bray–Curtis similarity distance algorithm, analysis of similarities was used to test the difference between the two groups (Anderson et al., 2006).

Linear discriminant analysis effect size (LEfSe) analysis was performed to identify the taxa that differed between the wild and captive groups (Segata et al., 2011). For LEfSe, Kruskal–Wallis tests were first performed among all groups of samples, followed by comparison of the selected species that differed between the two groups via Wilcoxon rank sum tests. Linear discriminant analysis (LDA) was used to sort the selected differences to obtain an LDA histogram (LDA score of > 3, $p < 0.05$), after which the evolutionary branching diagram was obtained by mapping the differences to the classification tree with a known hierarchical structure. Clustering ASV information was compared with the sequenced microbial genome database using PICRUSt2 software to obtain the functional types and abundance of the corresponding species in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Douglas et al., 2019) (https://www.kegg.jp/). Differences in KEGG pathways between wild and captive *R. brelichi* were analyzed using Wilcoxon rank sum tests ($p < 0.05$).

The rarefaction ASV curves, Venn diagrams, stacked column charts, LEfSe difference analysis diagrams, and KEGG function annotation diagrams were drawn using Parsenno Genomics Cloud Platform (https://www.genescloud.cn/home). The clustering heatmaps at the genus level, and PCoA and NMDS analysis charts were drawn using OmicStudio Cloud Platform (https://www.omicstudio.cn/tool). Box plots were drawn using Origin software.

3. Results

3.1 Sequence statistics and diversity analysis

A total of 4,723,744 original sequences of the target fragment, with a band size of 415.26 bp, were obtained in 46 fecal samples from wild and captive *R. brelichi*. Overall, 3,762,171 valid sequences were obtained through primer removal, splicing, mass filtration, deduplication, chimera removal, and clustering of the reads, of which 2,339,533 were from wild *R. brelichi* and 1,422,638 were from their captive counterparts. The obtained sequences with 100% similarity were merged, and a total of 8,202 ASVs were obtained, with the number of ASVs for each sample ranging from 316 to 734. The rarefaction curves based on the ASVs gradually leveled off with increasing sequencing depth, indicating that most sample information had been obtained from the sequencing data, and no more ASVs were generated with additional sequencing data (Figure 2a). Of the 8,202 ASVs, 427 ASVs were shared by captive and wild *R. brelichi* , whereas 3,233 and 4,542 ASVs were unique to captive and wild *R. brelichi* , respectively (Figure 2b).

Alpha diversity analysis showed that the average alpha diversity index of the gut microbial community of captive *R. brelichi* was higher than that of wild *R. brelichi* , and there were significant differences in Chao1, ACE, and Shannon indexes between the two groups ($p < 0.05$). This indicated that the gut microbial diversity and richness of captive *R. brelichi* were significantly higher than those of wild *R. brelichi* (Figure 3a). The beta diversity analysis based on the Bray–Curtis distance matrix showed that there were significant differences in gut microbial composition between wild and captive *R. brelichi* ($p < 0.01$). In addition, PCoA analysis revealed that wild and captive sample points were significantly separated (Figure 3b). Moreover, NMDS analysis showed that the stress value was 0.09 (less than 0.2), which could better reflect the true arrangement of the data and accurately reflect the extent of difference in the intestinal microbial composition between wild and captive *R. brelichi* (Figure 3c).

3.2 Gut microbial composition of wild and captive *R. brelichi*

We identified 8,202 unique ASVs from the 46 fecal samples based on taxonomic annotation, distributed across 28 phyla, 60 classes, 126 orders, 213 families, and 497 genera. At the phylum level, Firmicutes, Bacteroidota, Verrucomicrobiota, Actinobacteriota, Spirochaetota, and Proteobacteria were the core dominant bacterial phyla in all samples, with relative abundances exceeding 1%. Campylobacterota was absent only in samples C14 and C15, and its average relative abundance was 0.23%. Meanwhile, Cyanobacteria was absent only in

sample C7, and its average relative abundance was 0.45%. Moreover, Fibrobacterota was absent in samples W1, W5, W12, and C15, with an average relative abundance of 1.87%. Firmicutes, Proteobacteria, and Bacteroidota were the predominant microbes in the gut of wild *R. brelichi* , accounting for more than 92% of the total relative abundance. Firmicutes, Bacteroidota, Spirochaetota, and Fibrobacterota were the clearly dominant groups of intestinal microorganisms in captive *R. brelichi* , accounting for more than 94% of the total relative abundance. However, the relative abundance of other bacterial groups was wild *R. brelichi* was higher than that of their captive counterparts.

At the genus level, the bacteria with [?]1% abundance were selected for analysis, and the bacteria with unclassified abundance or relative abundance of <1% were classified as others. *UCG-005* ,*Clostridia* _UCG-014, Christensenellaceae_*R-7* _group,*[Eubacterium]_coprostanoligenes_group* , Muribaculaceae (unidentified genus), *NK4A214_group* , *Treponema* ,*UCG-002* , *UCG-010* , *Monoglobus* , *RF39* ,*Ruminococcus* , and *Enterorhabdus* were bacterial genera that were common to all samples. The composition of gut microbial dominant groups differed between wild and captive *R. brelichi* . In the gut microbiota of wild *R. brelichi* , the Christensenellaceae_*R-7* _group showed the highest abundance, followed by *Acinetobacter* and *Clostridia_UCG-014* . Meanwhile, *UCG-005* was the most dominant genus of captive*R. brelichi* , followed by Christensenellaceae_*R-7* _group, Muribaculaceae (unidentified genus), and *Fibrobacter* . Other dominant genera identified in captive individuals included *Treponema* and *Sphaerochaeta,* both belonging to the phylum Spirochaetota. The clustering results indicated that wild and captive individuals clustered into a single group (Figure 4b).

3.3 Analysis of gut microbial differences between wild and captive

R. brelichi

To investigate the potential differences in composition of the microbial community between wild and captive populations, LEfSe was used to analyze the gut microbes of samples from both groups (LDA score of differences (Figure 5). At the phylum level, the relative abundances of Bacteroidota, Spirochaetota, Fibrobacterota, and Desulfobacterota were significantly higher in the captive *R. brelichi* than in their wild counterparts. In contrast, the relative abundance of Actinobacteriota was significantly higher in wild *R. brelichi* than in their captive counterparts. At the genus level, the relative abundances of Prevotellaceae_UCG_001, *UCG_005* ,*Fibrobacter, Sphaerochaeta* , *Treponema* ,*Ruminococcus* , Bradymonadales (unidentified genus),*Faecalibacterium* , and *Bacteroides* were significantly higher in the captive *R. brelichi* than in their wild counterparts. At the same time, Christensenellaceae_*R_7* _group,*Clostridia_UCG_014* , *dgA_11_gut_group* ,*Pseudomonas* , *Acinetobacter* , *RF39* , and*Akkermansia* were more abundant in wild *R. brelichi* .

3.4 Predicted functional differences of the gut microbiota between wild and captive *R. brelichi*

We performed analysis of KEGG metabolic pathways in both captive and wild *R. brelichi* . In KEGG Level l categories, the gut microbial genes of captive and wild *R. brelichi* were associated with six types of metabolic pathways (Figure 6a), three of which exhibited significant differences. Specifically, the enrichment of environmental information processing genes was significantly higher in wild *R. brelichi* than in captive *R. brelichi* , while the opposite pattern was observed for genes involved in genetic information processing and organismal systems (Figure 6b). In KEGG Level 2 categories, the gut microbial genes of captive and wild *R. brelichi* were associated with 32 types of metabolic pathways (Figure 6a), among which 15 differed significantly ($p < 0.05$). Specifically, the captive group showed significant enrichment of pathways such as cell growth and death, replication and repair, translation, amino acid metabolism, biosynthesis of other secondary metabolites, carbohydrate metabolism, glycan biosynthesis and metabolism, infectious diseases, immune system, digestive system, and endocrine system. Meanwhile, the wild group showed significant enrichment of pathways such as membrane transport, signal transduction, xenobiotics biodegradation and metabolism, and lipid metabolism (Figure 6c).

4. Discussion

4.1 Diversity of the gut microbiota of wild and captive *R. brelichi*

The living environment of wild animals is mainly caused by environmental changes (captive breeding or semi-release, etc.) caused by the protection of species characteristics, which affect the gut microbial composition of wild animals to a certain extent, especially endangered wild animals (Zeng, 2020). Multiple studies have shown that the richness and diversity of the gut microbiota were reduced to varying degrees after captivity in *Macaca fascicularis* (Sawaswong et al., 2021),*Moschus berezovskii* (Li et al., 2017), *R. roxellanae* (Zhao et al., 2023), and *Ailuropoda melanoleuca* (Cheng et al., 2020), with some of the natural flora potentially being lost. In this study, the gut microbial richness and diversity of captive *R. brelichi* were significantly higher than those of wild *R. brelichi* , which was consistent with the results of a previous study of captive and wild*R. roxellanae* by Wang et al. (2023). As a typical leaf-eating primate, *R. brelichi* has access to a more uniform diet in the wild but a richer range of food in captivity, including leaves from a variety of trees, as well as a range of fruits, vegetables, and sources of protein. We believe that this factor may be an important explanation for the richer and more diverse gut microbiota of captive *R. brelichi* (Campbell et al., 2020). In addition, captive breeding limits the roaming and foraging behaviors of *R. brelichi* , and increases their close contact with each other and with humans. It is speculated that captivity increases the likelihood of the transmission of intestinal microbiota through oral and fecal routes, and potentially pathogenic microorganisms may be more abundant in the gut microbiota of*R. brelichi* in captivity.

4.2 Composition and difference of gut microbiota in wild and captive

R. brelichi

Wild and captive *R. brelichi* feed on different foods, and the ingestion of microorganisms via food may be one of the main sources of gut microbial colonization. In addition, these monkeys are exposed to different microorganisms through the environmental conditions in their habitat, including diet, water, soil, and social activities, which are potential sources of microbes in their gut (Diaz & Reese, 2021; Sun et al., 2023). Our study revealed strong similarities in the main gut microbiota between wild and captive *R. brelichi* . Regarding the top 10 most dominant taxa in the fecal microbiota of the two groups, Firmicutes, Bacteroides, and Spirochaetota were the dominant phyla common to the two groups. This is consistent with the results of Bornbusch et al. (2022) regarding the core dominant groups of*Lemur catta* in wild and captive populations. These results suggest that wild and captive *R. brelichi* share a potential core gut microbiota despite their different dietary habits and living environments. This core microbiota may be essential for maintaining body function and can be retained even in the face of environmental changes (Tian et al., 2020). In this study, Christensenellaceae_R_7_group, followed by *Acinetobacter* and *Clostridia_UCG_014* , had the highest relative abundance in the gut microbiota of wild *R. brelichi* . In addition, LEfSe analysis showed that the abundance of these genera of wild *R. brelichi* was significantly higher than that of captive *R. brelichi* . Christensenellaceae_R_7 group and Clostridia_UCG_014 belong to the Firmicutes. The former primarily participates in cellulose and hemicellulose degradation (Wang et al., 2023), facilitating the host's digestion of cellulosic substances and nutrient absorption, while the latter plays a crucial role in amino acid metabolism (Yang et al., 2021). As leaves rich in fibrous compounds dominate the diet of wild *R. brelichi* (Xiang et al., 2012), the significant increases in the abundance of both genera were also considered a response to changes in their diet. LEfSe analysis showed that the abundance of *Fibrobacter* , *Ruminococcus* ,*UCG_-005* , and Prevotellaceae_UCG_001 significantly increased in the gut microbiota of captive *R. brelichi* . *Ruminococcus* belongs to the Ruminococcaceae. When the energy intake is low, the increased abundance of Ruminococcaceae bacteria fermenting nonstructural carbohydrates can provide additional energy and nutrients to supplement the insufficient intake of energy from the diet (Amato et al., 2015). Meanwhile, Prevotellaceae_UCG_001 belongs to the genus*Prevotella* . Its enzymes can degrade cellulose and xylan, and its significant enrichment plays an important role in alleviating disordered glucose and lipid metabolism (Tang et al., 2020). The substantial increase in the abundance of these flora in the captive group may partially compensate for the significant decrease in the abundance of Christensenellaceae_R_7_group and Clostridia_UCG_014, thereby contributing to functional balance. Previous studies have also shown that *R. brelichi* has a greater capacity to use plant fiber as an energy source than *R. bieti* and *R. roxellanae* (Xi

et al., 2023). This may be related to the wide distribution of cellulose-degrading bacteria such as *UCG-005* ,*Ruminococcus* , Christensenellaceae_R-7_group, and*Fibrobacter* in the intestine.

LEfSe analysis also showed that the abundance of *Acinetobacter* (belonging to Proteobacteria) was significantly increased in the gut of wild *R. brelichi* . This is consistent with the findings of Cabana et al. (2019), who showed that *Acinetobacter* was significantly enriched in the gut of wild *Nycticebus javanicus* , but contrasts with the findings of Sun et al. (2020), who revealed significant enrichment of Proteobacteria in the gut microbiota of captive*Moschus chrysogaster* . Studies have shown that environment, soil, and animals are the natural habitats of *Acinetobacter* for its growth and reproduction (Zhai et al., 2020). In humans and animals, this is associated with diseases such as septicemia, pulmonary infections, meningitis, and diarrhea, with susceptibility to infection being associated with low host resistance, resulting in disease risk (Xu et al., 2014; He et al., 2023). The abundance of this bacterial genus in the gut of wild *R. brelichi* was significantly higher than that in captivity, with such infection being suggested to occur through contaminated food and water sources, and wild *R. brelichi* also have more chances to ingest soil. There is thus a need for further research on the imbalance of *Acinetobacter* in the gut microbiota of wild *R. brelichi* , in combination with analyses of the environment and feeding. LEfSe analysis showed that the potential pathogen *Treponema* showed significantly increased abundance in the gut of captive *R. brelichi* . *Treponema* is a spirochetal bacterium that can infect a wide range of hosts and tissues (Mamuad et al., 2020); for example, it is associated with porcine colonic spirochetosis, a diarrheal disease that can lead to reduced productivity. (Nguyen et al., 2023). A study by Zeng (2020) showed that an increase in the abundance of *Treponema* was conducive to the fermentation of cellulose and starch in *R. roxellanae* , but the role of *Treponema* in the gut of snub-nosed monkeys remains unclear. Therefore, further studies based on the physiological characteristics of *R. brelichi* should be conducted.

4.3 Functional differences of the gut microbiota between wild and captive *R. brelichi*

Under different environmental conditions, an increase or decrease of certain gut microbiota can be an adaptation to the changing environment. In this study, the potential functions of gut microbiota in *R. brelichi* were predicted using PICRUSt2. KEGG database analysis showed that functional genes of the gut microbiota in *R. brelichi* were mainly associated with metabolic pathways such as metabolism, genetic information processing, and cellular processes, which is consistent with the results of previous studies on this species (Huang et al., 2024). KEGG functions were also influenced by food provisioning, with wild foraging monkeys showing higher functions of metabolism and environment information processing, while captive food fed monkeys exhibited increases in genetic information processing, cellular processes, and organismal systems, which is contrary to the results of Li et al. (2024) on Yunnan snub-nosed monkey. At the KEGG Level 2 categories, gut microbial functions of *R. brelichi* were found to be mainly enriched in carbohydrate metabolism, amino acid metabolism, and lipid metabolism, which is consistent with the findings of most researchers on the metabolic functions of gut microbiota in non-human primates (Sun et al., 2016; Cheng et al., 2020; Wang et al., 2023). As a typical leaf-eating primate, *R. brelichi* in the wild mainly eat large leaves, while in captivity they ingest less cellulose and increase their intake of carbohydrates and fats. This is consistent with a significant increase in the abundance of carbohydrate metabolism pathways in the captive group; the ability to digest cellulose was decreased in captive*R. brelichi* due to changes in the diet, which in turn increased its ability to digest simple carbohydrates (Wang et al., 2023); this also reflected the significant increases of Prevotellaceae_UCG_001 and*Ruminococcus* in the captive group. Meanwhile, captive *R. brelichi* exhibited enhanced digestive and endocrine regulation capabilities. Additionally, a significant increase in the enrichment of metabolic pathways related to infectious diseases was identified, suggesting a heightened risk of infectious diseases in these monkeys. Wild *R. brelichi* exhibited greater ability of the xenobiotics biodegradation and metabolism pathway, xenobiotics are manmade refractory organic pollutants that are harmful to the health of living organisms. Most of them can readily be found in various components of the environment, such as soil, sediment, and water bodies (Zhang et al., 2021). Wild *R. brelichi* live in more complex environments and are more affected by exogenous pollutants than their captive counterparts; therefore, the enhancement of this function is understandable given the greater need to maintain gut health. In addition, the enrichment of the metabolic pathway of lipid metabolism was significantly increased in the

gut of wild *R. brelichi* . This may be explained by the significant increase in the bacterial genus Christensenellaceae_R-7_group in the wild group, as a taxon mainly involved in host amino acid metabolism and lipid metabolism (Jiang et al., 2023). Overall, captivity altered the gut microbiota of *R. brelichi* , which in turn affected their functions, but these changes may have helped the host to adapt to captivity.

Data availability statement

The dataset generated and analysed during the current study is available in the NCBI repository: https://dataview.ncbi.nlm.nih.gov/object/PRJNA1130139?reviewer=o9alf6vgg7uubbebp8dpj7imvn

Conflict of interest statement

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Author contributions

Xiaolong Huang : Conceptualization, investigation, writing – original draft, writing – review and editing, funding acquisition.**Haibo Li** : Methodology, sample collection, writing – original draft, writing – review and editing. **Lan Zhang** : Data analysis, writing – original draft, writing – revise. **Xu Zhang and Shaochuan Cheng** : Funding acquisition, formal analysis, and visualization. **Wei Yang** : Methodology, sample collection.**Yuying Yan** : Data curation, funding acquisition.**Bingshun Meng** : Sample collection, sampling distribution map is drawn, resources, visualization. **Juanjuan Zhao and Zuobo Wang** : Formal analysis, visualization, writing – original draft.**Jingcheng Ran** : Conceptualization, funding acquisition, writing – review and editing, formal analysis. All authors contributed to the study conception and design as well as to material preparation, data collection, discussion, and commented on the manuscript as well as read and approved the final version.

Acknowledgements

This research was supported by the following projects:

1. Study on field monitoring and habitat protection techniques of Guizhou snub-nosed monkey, China (No. Qiankehe zhicheng [2024] Yiban 129).

2. This research was supported by the Forestry Research Foundation of Guizhou Province, China (No. Qianlin Kehe [2022]10), study on gut microbial community structure of Guizhou snub-nosed monkey.

3. The Forestry Research Foundation of Guizhou Province, China (No. Qianlin Kehe [2022]13), the diet habits of Guizhou golden monkey using DNA sequencing technology.

4. Research on the risk factors of the Guizhou snub-nosed monkey population and the key techniques of manual intervention and control of breeding in the wild, China (No. Qiankehe zhicheng [2023] Yiban 188).

5. This research was supported by the Forestry Research Project of Guizhou Province, China (No. Qiankehe [2024]14).

6. Capacity building of ecosystem optimization and innovation in key ecological areas of Guizhou, China (No. Qiankehe fuqi [2023]009)

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Figure legends

Figure 1. Fecal sampling points for the study of *R. brelichi* in Fanjing Mountain National Nature Reserve. Identification was based on the mitochondrial COI technique: 31 fecal samples belonged to *R. brelichi* and 11 to other monkey species.

Figure 2. Rarefaction ASV curves (a) and Venn diagram (b) of gut microbiota of wild and captive *R. brelichi* . W stands for wild, C stands for captive, here and in similar places below.

Figure 3. Analysis of gut microbial alpha and beta diversity in wild and captive *R. brelichi* . (a) The alpha diversity differences between groups as reflected in the Chao1, ACE, Shannon, and Simpson indexes were analyzed. (b) PCoA (b) and NMDS (c) analyses based on Bray–Curtis distance matrix. $*p < 0.05$, $**p < 0.01$, here and in similar places below.

Figure 4. Gut microbial community composition in wild and captive*R. brelichi* . (a) Histogram analysis of the relative abundance of bacterial phyla. (b) Clustering heat map of bacterial genera with relative abundance of >1%. The color scale ranges from blue (low abundance) to red (high abundance). The blue, pink, black, green, orange, red, purple, and yellow characters represent the dominant bacterial phyla of Firmicutes, Bacteroidota, Proteobacteria, Desulfobacterota, Spirochaetota, Fibrobacterota, Actinobacteriota, and Verrucomicrobiota, respectively.

Figure. 5 LEfSe analysis of the gut microbiota in wild and captive*R. brelichi* . (a) Evolutionary branching diagram: the diagram shows phylum, class, order, family, and genus in this order from the inside to the outside. The size of the small circle indicates the relative abundance of species at the taxonomic level, with species with no significant differences marked in white and species with significant differences marked in red and blue. (b) Histogram length represents the LDA score.

Figure 6 The prediction of the enrichment of KEGG pathways for all samples. (a) Annotated statistical chart of KEGG Level 2 metabolic pathways in the gut microbiota of wild and captive *R. brelichi* . The x-axis represents the relative abundance of annotations to the corresponding metabolic pathway, while the y-axis corresponds to the KEGG Level 2 metabolic pathways, with the Level 1 category to which each metabolic pathway belongs being listed on the right. (b), (c) Analysis of the differences of metabolic functions between groups at the first and second levels. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.