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Community series in the wildlife gut microbiome and its implication for conservation biology, volume II

Edited by
Lifeng Zhu and Jianjun Wang

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Community series in the wildlife gut microbiome and its implication for conservation biology, volume II

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Editorial: Community series in the wildlife gut microbiome and its implication for conservation biology, volume II

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Editorial on the Research Topic
Community series in the wildlife gut microbiome and its implication for conservation biology, volume II

Over the past decade, mounting evidence has highlighted the pivotal role of the gut microbiome in the physiological development, nutrient absorption, and immune health of wildlife (Wei et al., 2019). The integration and application of non-invasive sampling methods, genomics, and metagenomics has provided valuable insights into important aspects such as population status, genetic diversity, and health conditions of wild animal species (Wei et al., 2019; Huang et al., 2021). These approaches offer invaluable perspectives and tools for investigating the connections between the gut microbiome and wildlife health, while concurrently enhancing our understanding of the ramifications of human-induced disruptions, habitat alterations, and environmental pollution on the host symbiotic microbiome (Menke et al., 2017; Lavrinienko et al., 2018; Degregori et al., 2021). In addition, employing comprehensive research methods that delve deeply into the role and influencing factors of the gut microbiota enable a more comprehensive understanding and protection of the health and habitat of wildlife, facilitating effective monitoring and improvement of conservation efforts for endangered wildlife (Yao et al., 2019; Zhu et al., 2021a). Moreover, by elucidating the correlation between gut microbiota and conservation, it becomes feasible to further assess the efficacy of conservation strategies and furnish scientific substantiation for the formulation of adaptive management strategies (Wei et al., 2019; Huang et al., 2023).

Building on Volume I (Zhu et al., 2021b), the second volume of the Research Topic titled *Community Series in the Wildlife Gut Microbiome and Its Implication for Conservation Biology* in *Frontiers in Microbiology* comprises a comprehensive collection of 20 original research articles that provide valuable insights for further study of the relationship between wildlife gut microbes and conservation biology. Below is a summary of the articles included in the volume II of this topic.

The specificity of gut microbiome in wildlife in response to varying conservation conditions

Over the years, captive breeding, translocation, and reintroduction have proven to be effective strategies in safeguarding endangered wildlife, rendering substantial contributions to the realm of wildlife conservation endeavors (Harding et al., 2016; Gross et al., 2023; Smith et al., 2023). Nonetheless, as summarized in our editorial in the first volume at 2021, the environmental changes resulting from different conservation strategies pose challenges to the ecological adaptability of the host species (Zhu et al., 2021b). In this regard, the gut microbiome exhibits specificity as a potential indicator reflecting the health status and adaptability of wild animals (de Jonge et al.; Zhou et al., 2022). In the second volume, we have made efforts to acquire additional relevant articles, which further expand our understanding of the specific gut microbiota responses in different wild animal species under variable conservation strategies. On one hand, in comparison with wild populations, captive conservation can exert a beneficial influence on the gut microbiota structures and functions of wild animals. In this topic, it has been showed that captive alpine musk deer (*Moschus chrysogaster*) individuals exhibit higher alpha diversity (alpha and beta diversity) and stronger functions of chemoheterotrophy and fermentation, which could potentially enhance the stability and complexity of the gut microbial community (Zhang et al.). This reinforcement contributes to the host's ecological adaptation mechanisms in the habitat environment. On the other hand, captive breeding may have detrimental effects on the host's health by increasing the abundance of potential pathogenic bacteria in the gut. For instance, Tang et al. discovered a higher presence of potentially pathogenic bacteria in the gut microbiota of captive tokay gecko (*Gekko gecko*). Similarly, Xia et al. ascertained reduced α-diversity, heightened abundance of potential pathogenic bacteria (e.g. *Streptococcus* and *Sarcina*), and the enrichment of antibiotic resistance (KEGG pathway) of gut microbiome within captive populations of tibetan macaques (*Macaca thibetana*). In addition, in a successful translocation case involving koalas (*Phascolarctos cinereus*), Blyton et al. found that the formation of the koala gut microbiota is primarily determined by the acquisition and development of microbial communities during early life stages, while dietary variations resulting from the translocation process did not significantly affect the host gut microbiota. In summary the gut microbiota of different wildlife species exhibits remarkable specificity in their responsiveness to alterations in the habitat environment. Consequently, fully elucidating the host gut microbiota's reaction under varying conservation conditions assumes pivotal practical significance for safeguarding and managing endangered wildlife.

The negative response of wildlife gut microbiome to the ecological pressures of the Anthropocene

In the Anthropocene epoch, the escalation of human activities, encompassing urbanization, agricultural expansion, hunting, and logging, etc., has wrought severe devastation upon global biodiversity and the pristine habitats of wildlife (Hockings et al., 2015; Otto, 2018; Schmidt et al., 2020). Wildlife encounters grave perils arising from habitat fragmentation and numerous anthropogenic disturbances, constituting one of the pivotal concerns impinging upon wildlife conservation endeavors (Sánchez-Barreiro et al., 2021). These disturbances engender not only direct biodiversity loss but also exert deleterious ramifications on wildlife well-being through modifications to the symbiotic microbiome, concomitantly amplifying the risk of localized extinction on a population scale (Barelli et al., 2015; Hockings et al., 2015; Xi et al., 2023). Hence, delving into the response of wildlife gut microbiome to the ecological stressors imposed by human interference furnishes indispensable insights for the pursuit of animal conservation initiatives (Cortés-Avizanda et al., 2022). In this topic, it has been reported that the effects of the human environmental factors on the host microbiome. For example, Wasimuddin et al. studied the gut microbiome of gray-brown mouse lemurs (*Microcebus griseorufus*) in south-western Madagascar. They revealed the impact of anthropogenic disturbance on the gut microbiome of wild lemurs, highlighting its disruptive nature and negative health consequences, with a decline in gut microbial diversity and beneficial bacterial species, accompanied by a slight rise in potentially pathogenic bacteria (Wasimuddin et al.). Similarly, Zhou et al. assessed the impact of urbanization on the symbiotic microbial community of wild amphibians (*Pelophylax nigromaculatus*, *Fejervarya multistriata*, and *Bufo gargarizans*) and found that although urbanization increased the diversity of symbiotic microbiota and the number of keystone species taxa, it came at the cost of a depletion of the stability and complexity of the microbial community.

Spatiotemporal dynamics of environmental variables changes affecting the host-microbiome

The macroscopic spatiotemporal dynamics of environmental variables exert a substantial influence on the structure, function, and adaptive evolution of the host-microbiome (Ma et al., 2019; Li et al., 2023). The changes in dietary resources due to seasonal fluctuations can induce alterations in the community structure of the host microbiome, thereby significantly impacting the physiological functions of the host (Huang et al., 2022; Li et al., 2023). The issue was investigated by Qin et al. and Li et al. in this topic. It was found by Qin et al. that seasonal dietary changes significantly impact the gut microbiota diversity (α and β diversity) and metabolic function of goitered gazelles (*Gazella subgutturosa*). Specifically, during winter, both the alpha diversity and metabolic function of gut microbiome were higher compared to summer. Li et al. revealed that seasonal changes in the gut microbiota of golden snub-nosed monkeys (*Rhinopithecus roxellana*) are primarily caused by variations in macronutrient intake, and the metabolic adaptation of gut microbiota helps the host compensate for inadequate macronutrient intake. Furthermore, at global geographic scales, gradient variation, including altitude and latitude, may also influence the functional adaptation and

convergent evolution of the host-microbiome through stringent ecological selection pressures (Karl et al., 2018; Ma et al., 2019; Zuo et al., 2022). Wang et al. studied the adaptation of ungulates gut microbiome to the environmental extremes of the Tibetan Plateau, and their results showed that while high altitude may not surpass the phylogenetic factors in driving the convergent evolution of ungulate gut microbiome compositions, it does play a significant role in in promoting the convergent evolution of alpha diversity and indicator microbiota within the gut microbiome of ungulates. Another study presented a latitudinal pattern of gut microbial diversity in frogs (*Fejervarya limnocharis*) along the eastern coast of mainland China, showing a significant negative correlation between alpha diversity and the latitudinal gradient (Zhao et al.).

Conclusion and perspectives

Building upon the investigations of the gut microbiome structure and functional adaptations in wild animals explored in Volume I (Zhu et al., 2021b), we further delve into examining the link between their gut microbiome and conservation biology within this topic. Overall, the current efforts in this field contribute to the reconstruction and maintenance of healthy microbial ecosystems in wildlife, enabling effective responses to external disturbances and threats originating from human or natural environments. However, as emphasized in our previous editorial in Volume I regarding the significance of sequencing depth, bioinformatics analysis, and database quality in host microbiome research, there will be an increased focus on whole-genome and multi-omics technologies (Wei et al., 2019; Skarzyńska et al., 2020; Zhu et al., 2021b; Li et al., 2022). In this topic, Zhu et al. employed a combination of metabolomics and 16S rDNA sequencing techniques to elucidate the association of host metabolism and gut microbiome with phylogeny and environmental adaptation in mountain dragons. This further highlights the value and potential of multi-omics technologies in future research, providing new insights into the study and conservation of the gut microbiome in wildlife. The theoretical research on gut microbiome has provided important guidance for the conservation and management of endangered animals. Gut microbiome of different wild animals showed specific responses to environmental changes (e.g. captivity or translocation), which subsequently influence the ecological adaptability of their hosts (Tang et al.; Xia et al.; Zhang et al.). Furthermore, gut microbiome holds the potential to serve as biomarkers for captive or reintroduced hosts at different stages of life, thus enabling more effective monitoring and management of the health status of endangered wildlife (Huang et al., 2023; Zhou et al.). Simultaneously, this has also fostered the development of new approaches for assessing the adaptive capacity and survival status of endangered animals, providing a basis for implementing appropriate conservation measures and management strategies. Undoubtedly, future research will continue to expand our understanding of the role and value of gut microbiota in endangered wildlife, making significant contributions to the conservation of biodiversity and ecosystem health.

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Comparative Analysis of Gut Microbial Composition and Functions in Przewalski's Gazelle (*Procapra przewalskii*) From Various Habitats

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Gut microbiota of mammals participates in host nutrient metabolism and plays an important role in host adaptation to the environment. Herein, to understand the relationship between environment differences and the composition and abundance of the gut microbiota of Przewalski's gazelle (*Procapra przewalskii*) in almost all its habitats, high throughput sequencing of the 16S rRNA gene was used to compared the characteristics of the gut microbiota based on total 120 fecal samples. The results showed that Przewalski's gazelle exhibited different characteristics of microbiota diversity in different habitats. The Jiangxigou Rescue Station (JX), Nongchang (NC), and Ganzihe and Haergai townships (GH) groups had a relatively high microbiota diversity, while the Niaodao scenic area (ND) group had the lowest diversity. This finding seemed to follow a similar pattern of change in the population of Przewalski's gazelle. *Bacteroidetes* and *Actinobacteria* were the phyla with significant differences, especially between the Wayu township (WY) and the other groups. The difference in the microbiota mainly included the *Ruminococcaceae UCG-005*, *Christensenellaceae R-7 group*, and *Bacteroidaceae* and was enriched in the ND, WY, and other regions. We speculated that the difference in the gut microbiota was due to a difference in environmental characteristics, particularly the food resources that the host can obtain. We speculated that a similar microbiome has important functions for species survival and represents the evolutionary commonality of Przewalski's gazelle, while a different microbiome plays an important role in the adaptation of Przewalski's gazelle to a different environment. The results of this study illustrate how the same species adapts to different environments from the perspective of gut microbiota plasticity and therefore are of great significance for the protection and restoration of the population of this species.

Keywords: Przewalski's gazelle, gut microbiome, environmental adaptability, 16S rRNA gene sequencing, pathogenic bacteria, co-occurrence network

INTRODUCTION

There are many strategies for species to adapt to the environment, such as adjusting physiology and behavior and changing morphology to enable organisms to respond to changes in the living environment (Rosalino et al., 2014). Gut microbiota has strong plasticity and is considered to play an important regulatory role in the host's environmental adaptability (Candela et al., 2010, 2012). The gut microbiota has evolved with the host and has formed a complex microecosystem with the animal gastrointestinal tract. It participates in important physiological activities such as food digestion, nutrient absorption, and immune regulation of the host and is crucial for the health of the host (Dethlefsen et al., 2007; Sampson and Mazmanian, 2015). In addition, the disturbance of the gut microbiota will also affect the health of the host.

The composition and function of gut microbiota are influenced by genetic background, age, different parts of the digestive tract, and external factors such as food composition, geographical environment, and season. Although the host genotype is considered important for shaping the gut microbiota, the external environment is also a crucial factor in controlling the gut microbiota and maintaining homeostasis in the intestine through interaction with the host (Spor et al., 2011). There is a great deal of evidence that environmental factors can change the bacteria and the community structure of the gut microbiota. The gut microbial diversity, functional gene diversity, metabolic pathway, and cellulolytic enzyme activity of captive giant pandas (*Ailuropoda melanoleuca*) were lower than those of wild pandas, while antibiotic resistance genes, heavy metal tolerance genes, and disease risk showed an increasing trend (Guo et al., 2019). Comparative studies of the gut microbiota of free-ranging and captive Namibian cheetahs (*Acinonyx jubatus*) showed no difference in microbial α diversity in captive individuals, but showed higher abundance of OTUs and transfer of disease-related functional pathways associated with potential pathogens (Wasimuddin et al., 2017). As Chinese Rhesus Macaques (*Macaca mulatta*) adapt to different geographical environments, new unique bacteria have been established to help macaques improve their adaptability under corresponding environmental conditions (Zhao et al., 2018).

Przewalski's gazelle (*Procapra przewalskii*) is currently one of the most endangered ungulates in the world, which is also endemic to the Qinghai-Tibet Plateau. At present, almost all the individuals are believed to be distributed near the Qinghai Lake Basin in China. This species was once widely distributed in Western China. However, the population of this species has greatly declined and its habitat has shrunk due to habitat fragmentation and severe human disturbances. The current distribution range and activity area of this species are relatively small, and several independent small populations are scattered (Jiang et al., 2000; Li et al., 2012). The general environmental conditions of Przewalski's gazelle are very similar. For instance, their habitat in the Qinghai-Tibet Plateau exhibits the characteristics of low temperature, low oxygen, and high radiation. There are certain differences of habitats for Przewalski's gazelle. In particular, the vegetation types would directly lead to differences in the available food resources. It is commonly known that the diet of the majority of herbivores is compatible with plant diversity and composition found in their environment. Overwhelming evidence has shown that diet plays a key role in determining the composition of gut microbiota (Muegge et al., 2011; Scott et al., 2013; Flint et al., 2015; Graf et al., 2015). Therefore, we collected fecal samples of Przewalski's gazelle from eight small geographical areas in the same season and performed the 16S rRNA gene sequencing. The chosen areas cover almost all the current ranges of the Przewalski's gazelle. Our objectives are to (1) understand the gut microbial composition and the dominant bacteria in Przewalski's gazelle in various regions, (2) analyze the differences in the gut microbiota of Przewalski's gazelle between different regions as well as the possible reasons for the differences, and (3) explore the adaptation strategies of Przewalski's gazelle to different environments from the perspective of gut microbiota.

MATERIALS AND METHODS

Sample Collection

Based on the ecological characteristics and current spatial distribution of the Przewalski's gazelle, our previous results have divided the distribution of this species into 8 relatively independent habitat patches (Zhang et al., 2021). Therefore, during the winter of late 2018-early 2019, 15 fresh fecal samples of different Przewalski's gazelle were collected from the Shengge township (SG), Wayu township (WY), Niaodao scenic area (ND), Ganzihe and Haergai townships (GH), Nongchang (NC), Shadao scenic area (SD), and Ketu township (KT), respectively, in Qinghai Province (**Figure 1**), covering almost all distribution areas of Przewalski's gazelle with different vegetation types (**Table 1**). Additionally, we randomly collected 15 semi-captive fecal samples from the Jiangxigou Rescue Station (JX). Each fecal sample was taken using sterile disposable PE gloves and sterile sampling bags to avoid cross-contamination between the samples.

All samples were temporarily preserved in liquid nitrogen and then stored at −80°C in an ultra-low temperature freezer in the laboratory. A total of 120 fecal samples were collected for DNA extraction, amplification, and sequencing. Furthermore, the vegetation types of Przewalski's gazelle in different distribution areas were obtained by using 1:1 million vegetation map of China (Hou, 2019).[1,2] There were distinct vegetation types in different distribution areas of Przewalski's gazelle (**Table 1**).

DNA Extraction, Amplification, and Sequencing

Genomic DNA from each fecal sample was extracted using an EZNA® soil DNA kit (Omega Bio-tek, Norcross, GA, United States). The concentration and purity of DNA were

[1]http://data.tpdc.ac.cn/zh-hans/data/eac4f2cf-d527-4140-a35d-79992957f043
[2]https://www.plantplus.cn/cn/datas/11

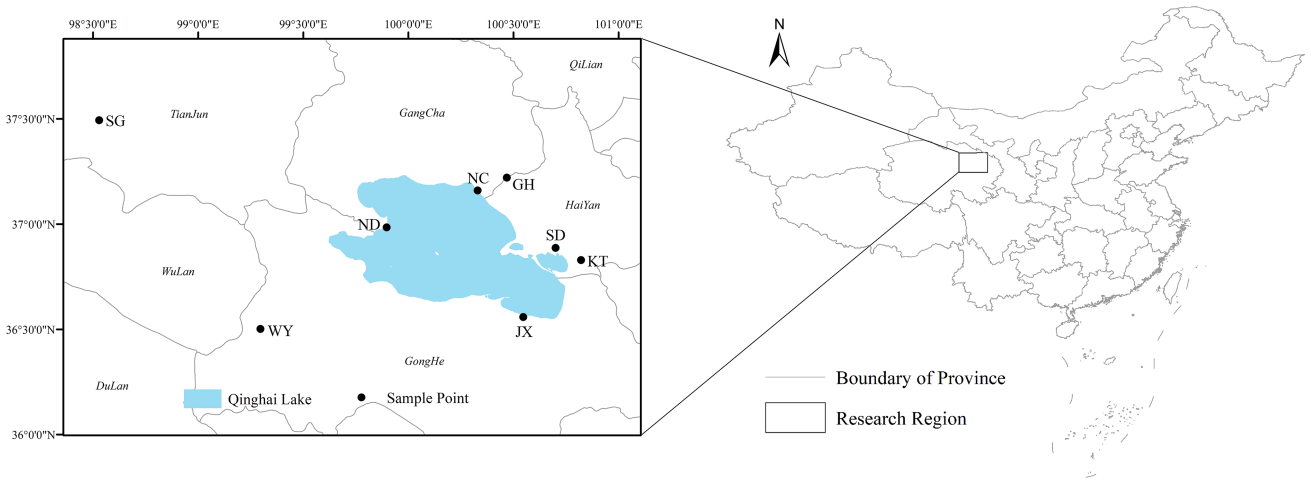


FIGURE 1 | The eight geographical regions of the fecal samples of Przewalski's gazelle.

then determined using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, United States). Gel electrophoresis in a 1% agarose gel was used to assess the DNA quality. The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified by the microbial universal primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′).

PCR reactions were carried out in triplicate in a final volume of 20 μl consisting of 4 μl TransStart FastPfu buffer (5×), 2 μl dNTPs (2.5 mm), 0.8 μl upstream primer (5 μm) and 0.8 μl downstream primer (5 μm), 0.4 μl TransStart FastPfu DNA polymerase, 10 ng template DNA, and ddH_2O to complete the 20 μl volume. Amplification reactions were performed in an ABI GeneAmp® 9,700 PCR instrument (Applied Biosystems, Foster City, CA, United States) under the following conditions: 95°C for 3 min (initial denaturation); 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s; and 10 min at 72 C. Gel electrophoresis in a 2% agarose was used to evaluate PCR product yields. A genomic DNA library was constructed using the NEXTFLEX Rapid DNA-Seq Kit (Bioo Scientific, Austin, TX, United States). Sequencing was conducted on the Illumina Miseq PE300 platform (Illumina, San Diego, CA, United States). Sequences of approximately 410 bp were obtained.

Genetic and Statistical Analyses

Quality control of the raw sequences was performed with Trimmomatic (version 0.39). The paired reads were merged into a sequence with a minimum overlap length of 10 bp using FLASH (version 1.2.7). The sequences were clustered into operational taxonomic units (OTUs) at 97% similarity using UPARSE (Edgar, 2013;[3] version 7.1), and chimera were removed. The sequence with the highest frequency in each OTU was selected as the representative sequence for further annotation. Subsequently, to obtain the species classification information corresponding to each OTU, the Silva database (silva 132/16S) was adopted and the Ribosomal Database Project (RDP; version 2.11) classifier was used for taxonomic analysis of OTU representative sequences at 97% similar level. The confidence threshold was set to 0.8.

To estimate alpha diversity, the Shannon, sobs (the observed richness), ACE, and Chao1 indices were calculated using Qiime software at the OTU level (Caporaso et al., 2010). The principal coordinates analysis (PCoA) plot based on the Bray Curtis dissimilarity and unweighted and weighted UniFrac distance matrices were used to calculate the differences in beta diversity between each group, as well as to test the differences between the groups at the phylum and genus levels (Jiang et al., 2021). The community of the samples was clustered with a distance matrix and a hierarchical clustering tree of the samples was built. All the differences between the groups were analyzed using ANOSIM and Adonis. The potential pathogens were collected at phylum and genus level by literature search, and the differences between groups were analyzed.

A Wilcoxon signed-rank test and a Kruskal–Wallis rank-sum test was used with the STAMP software to test the species differences between the groups at a 95% confidence interval. FDR-based multiple test corrections were performed and Welch's (uncorrected) 0.95 was used for post-hoc testing. To better understand the functions of the gut microbiota and their differences or similarities in various habitats of Przewalski's gazelle, the Tax4Fun software package was used to transform the 16S taxonomic lineage based on the Silva database into the lineage of prokaryotes in the KEGG (Kyoto Encyclopedia of Genes and Genomes)[4] and EggNOG databases. In addition, the correlation of the top 30 bacterial genera was calculated and the co-occurrence network of the bacteria was analyzed with Gephi v0.9.2 (Jacomy et al., 2014).

[3]http://drive5.com/uparse/

[4]http://www.genome.jp/kegg/

TABLE 1 | Vegetation types in different study areas.

Study area	Vegetation formation and sub-formation	Vegetation type
Shengge township (SG)	Kobresia, Kobresia pygmaea, Stipa purpurea meadow, Stipa	Alpine grass and carex grassland, meadow of kobresia and miscellaneous grass
Jiangxigou Rescue Station (JX)	Stipa breviflora Griseb, Achnatherum splendens	Temperate tufted dwarf grass, dwarf semi-shrub desert steppe
Wayu township (WY)	Ceratoides	Typical grassland of temperate tufted grasses, desert of ceratoides, and desert of salt claw
Niaodao scenic area (ND)	Myricaria	Meadow of kobresia and miscellaneous grass, temperate deciduous thickets
Ganzihe and Haergai townships (GH)	Achnatherum, Artemisia, Blysmus sinocompressus, Carex spp. Meadow, Sabina, Spring barley, spring wheat, navew, rape field, Stipa	Typical grassland of temperate tufted grasses, temperate half shrub, low half shrub desert, meadow of kobresia and miscellaneous grass, typical grassland of temperate tufted grasses, one crop a year short growth period hardy crops
Nongchang (NC)	Spring barley, spring wheat, navew, rape field, Achnatherum	Typical grassland of temperate tufted grasses, one crop a year short growth period hardy crops
Shadao scenic area (SD)	Achnatherum	Typical grassland of temperate tufted grasses, temperate half shrub, low half shrub desert
Ketu township (KT)	Achnatherum, Artemisia, Kobresia	Temperate half shrub, low half shrub desert, typical grassland of temperate tufted grasses

RESULTS

16S rRNA Sequencing Data

The total number of effective sequences obtained from 120 samples was 13,650,739, and the average length of the obtained sequences was 410 bp. According to the statistical results of the OTU species classification, 3,867 effective OTUs were extracted, screened and classified into 23 phyla, 43 classes, 110 orders, 208 families, and 473 genera.

A rarefaction curve was obtained by plotting the number of OTUs (*y*-axis) against the number of read (*x*-axis) per sample, showing that the curve gradually rises as the amount of sequencing increases (**Supplementary Figure S1**). When the amount of sequencing reached 48,100, the curve tended to be flat, indicating that the sequencing data were reliable and that the size of the samples from this sequencing was sufficient to reflect the diversity of the gut microbiota of Przewalski's gazelle in the eight different areas.

The Composition of the Gut Microbiota of Each Group

The composition of the dominant phyla (relative abundance >1%) and the top 30 bacterial genera were analyzed. At the phylum level, *Firmicutes* and *Bacteroidetes* were the dominant phyla in each group (**Figure 2A**). Their cumulative relative abundance was higher than 88%. In the eight study locations JX, KT, NC, GH, SD, ND, SG, and WY, the relative abundances of *Firmicutes* were 69.46, 71.54, 68.25, 70.40, 69.89, 71.26, 71.20, and 72.61%, respectively, whereas the relative abundances of *Bacteroidetes* were 26.09, 22.71, 27.33, 22.89, 26.00, 24.21, 24.48, and 16.19%, respectively.

At the genus level, the dominant bacteria (relative abundance >1%) in Przewalski's gazelle in all the study areas were *Ruminococcaceae UCG-005*, *Christensenellaceae R-7 group*, *Rikenellaceae RC9 gut group*, and *Bacteroides* (**Figure 2B**). The cluster heat map analysis showed that the gut microbiota of Przewalski's gazelle in the ND and WY groups were far from the other six groups based on the top 30 bacterial genera. A relative abundance bar chart was plotted to visually show the distribution of the top four phyla and genera in each sample (**Figure 2**). Species composition and abundance are also illustrated by the graph.

Analysis of Alpha and Beta Diversity

The alpha diversity of the eight different groups showed that at the OTU level, the values of the Shannon and ACE indices in the JX, NC, and GH groups were higher than those of the other five groups, suggesting that the gut microbial richness and uniformity in the above-mentioned three groups were higher than in the other groups (**Figures 3A–D**). Nevertheless, the values of the Shannon and ACE indices in the ND group were the lowest (**Figure 3**).

Beta diversity analysis was used to compare the differences in the gut microbiota of Przewalski's gazelle in the different study areas based on three distance matrices (Bray Curtis, unweighted UniFrac, and weighted UniFrac) with ANOSIM and Adonis (**Figure 4**; **Supplementary Table S1**). The PCoA analysis showed that the differences in the gut microbiota of Przewalski's gazelle between the eight groups were significantly greater than the differences within the groups at the OTU level.

The relative abundances of *Firmicutes* and *Verrucomicrobia* in the gut microbiota of Przewalski's gazelle from all the regions showed no significant intergroup differences, while that of *Bacteroides* and *Actinobacteria* displayed significant intergroup differences (**Figure 5A**). Almost all the dominant genera in Przewalski's gazelle from the different regions presented significant intergroup differences except for *Prevotellaceae UCG-004* (**Figure 5B**).

Functional Prediction of the Gut Microbiota

Based on the KEGG database, the gene function annotation analysis of the gut microbiome showed that the most important

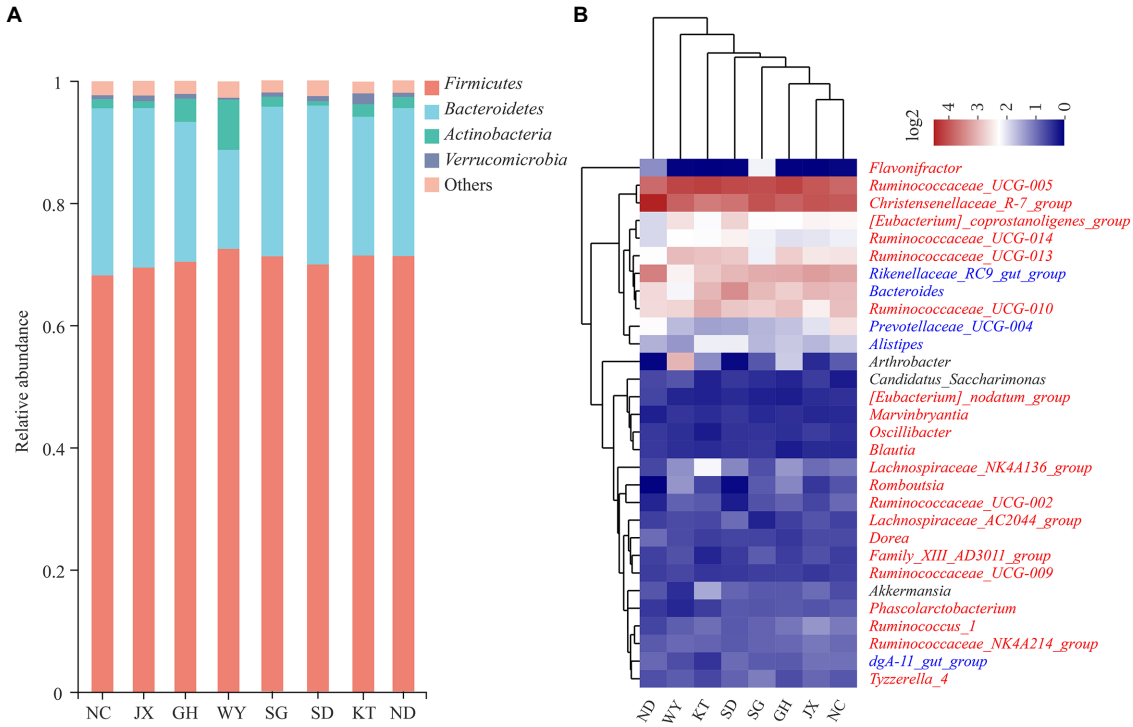


FIGURE 2 | The composition of the gut microbiota of Przewalski's gazelle. **(A)** Relative abundance of the dominant bacteria among the groups of Przewalski's gazelle at the phylum level. **(B)** Cluster heat maps of the top 30 bacterial genera in terms of abundance.

functions at level 1 were metabolism with a relative abundance (RA) value of 60.51% ± 0.2872, followed by environmental information processing (RA, 18.61% ± 0.2151) and genetic information processing (RA, 13.27% ± 0.0807). The functions exhibited significant differences, particularly the metabolic function of various substances of the gut microbiome among the eight groups at level 1 and level 2 (**Figure 6**). Moreover, the eight groups had different advantage functions. For example, the ND group had a higher abundance in the functions of organismal systems (i.e., environmental adaptation, digestive system, and immune system) and cellular processes (i.e., cell motility and eukaryotic community). The gut microbiota in the WY group in particular was significantly different from the other regions in the function of metabolism and organismal systems. For instance, metabolism of cofactors and vitamins, metabolism of terpenoids and polyketides, and biodegradation of xenobiotics had the highest abundance ($p < 0.05$), while glycan biosynthesis and metabolism, digestive system ($p < 0.05$), and immune system showed the opposite result ($p < 0.05$; **Supplementary Table S2**).

Based on the EggNOG database, the metabolic functions were selected for analysis. The results showed that amino acid transport and metabolism (8.34% ± 0.0729), carbohydrate transport and metabolism (7.86% ± 0.0829), and inorganic ion transport and metabolism (5.31% ± 0.0547) were the dominant metabolic functions of the gut microbiome in Przewalski's gazelle. Analysis of the differences between the groups showed that all the metabolic functions of various substances differed significantly based on the KEGG and EggNOG databases (**Figure 6**).

Analysis of Enterotypes and Pathogenic Bacteria

The Jensen–Shannon divergence (JSD) distance matrices was selected to calculate the distance between the fecal samples of Przewalski's gazelle in the different study areas based on the OTU relative abundance matrix. The larger the Calinski–Harabasz (CH) index, the closer the samples are within each cluster. In addition, the more dispersed the types, the better are the clustering results. Analysis of the enterotype diagram showed that the optimal number of clusters was 4 (**Figure 7A**). Consequently, the fecal samples of Przewalski's gazelle from the eight areas could be divided into four enterotypes. In this study, 30, 55, 19, and 16 of the 120 samples of Przewalski's gazelle belonged to enterotype 1, enterotype 2, enterotype 3, and enterotype 4, accounting for 25, 45.8, 15.8, and 13.3%, respectively. The SD and KT groups were mainly of enterotype 1, whereas the NC, SG, JX, and GH groups were mainly of enterotype 2, the WY group was mainly of enterotype 3, and the ND was mainly of enterotype 4 (**Figure 7B**). Marker bacteria of each enterotype were *Ruminococcaceae UCG-005*, *norank f p-251-o5*, *Arthrobacter,* and *Christensenellaceae R-7 group*, respectively (**Figure 7C**).

Furthermore, a total of 15 potentially pathogenic bacterial genera were obtained, 13 of which showed significant differences

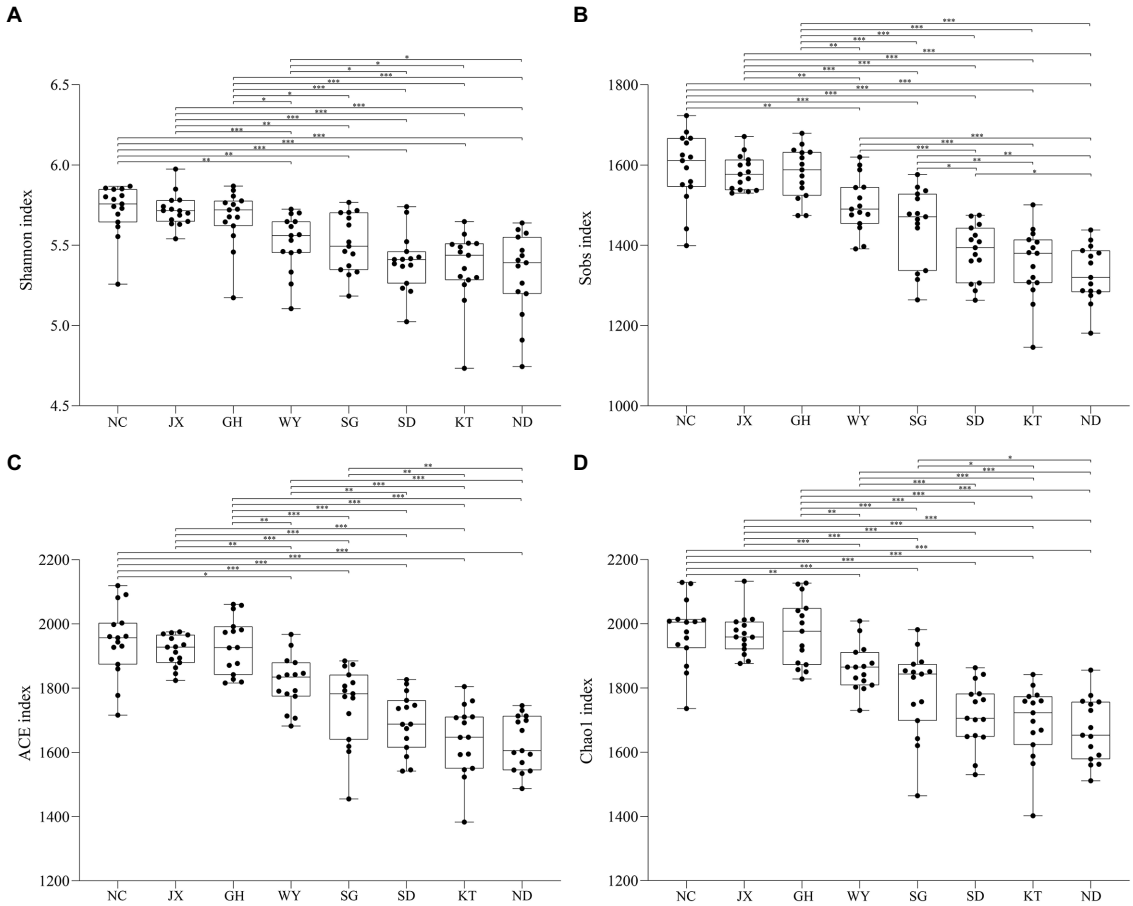


FIGURE 3 | Alpha diversity indices of the gut microbiota of Przewalski's gazelle found in the eight study areas. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.

between the groups. We noted that *Oscillibacter* and *Treponema* 2 were the dominant potentially pathogenic bacteria. The SD group had the highest cumulative relative abundance of potential pathogens, while the KT group had the lowest cumulative relative abundance of potential pathogens (**Figure 7D**).

Co-occurrence Network of the Core Bacteria

The co-occurrence network of the 30 most abundant genera revealed the relationships among the gut bacteria in the eight distribution areas of Przewalski's gazelle. The results showed that the complexity of the gut microbial network structure of Przewalski's gazelle was significantly different in the different distribution areas. The bacterial group in the SG group was the most complex since the bacteria in this group had more links (65 links), followed by the NC group (47 links) and the KT group (47 links).

The bacterial genera with the greatest number of edges were *Ruminococcaceae UCG-010*, *Christensenellaceae R-7 group*, *Marvinbryantia*, *Ruminococcaceae UCG-005*, *Family XIII AD3011 group*, *Ruminococcaceae NK4A214 group,* and *Ruminococcaceae UCG-010* (**Figure 8**).

DISCUSSION

With the rapid development of sequencing technology, studies on gut microbial composition and diversity based on high-throughput sequencing technology can provide novel strategies for the protection of endangered wildlife. Due to the difficulty in collecting wildlife samples, there are very few studies on the gut microbiota of Przewalski's gazelle. In this study, we applied high-throughput sequencing technology of the V3-V4 hypervariable regions of 16S rRNA to investigate the composition of the gut microbiota of Przewalski's gazelle in its different distribution areas, as well as to establish a theoretical basis for implementing conservation measures of Przewalski's gazelle backed by scientific research.

The study showed that Przewalski's gazelle had similar dominant bacteria in all its distribution areas. This result indicated the presence of a commonality in the gut microbiota of Przewalski's gazelle in different areas, which was also an essential outcome demonstrating the co-evolution of mammalian gut microbiota and their host. At the phylum level, both *Firmicutes* and *Bacteroidetes* were the dominant bacterial phyla, with a total proportion of over 88%, which is consistent with the conclusions of most studies (Samsudin et al., 2011; Ishaq and Wright, 2014;

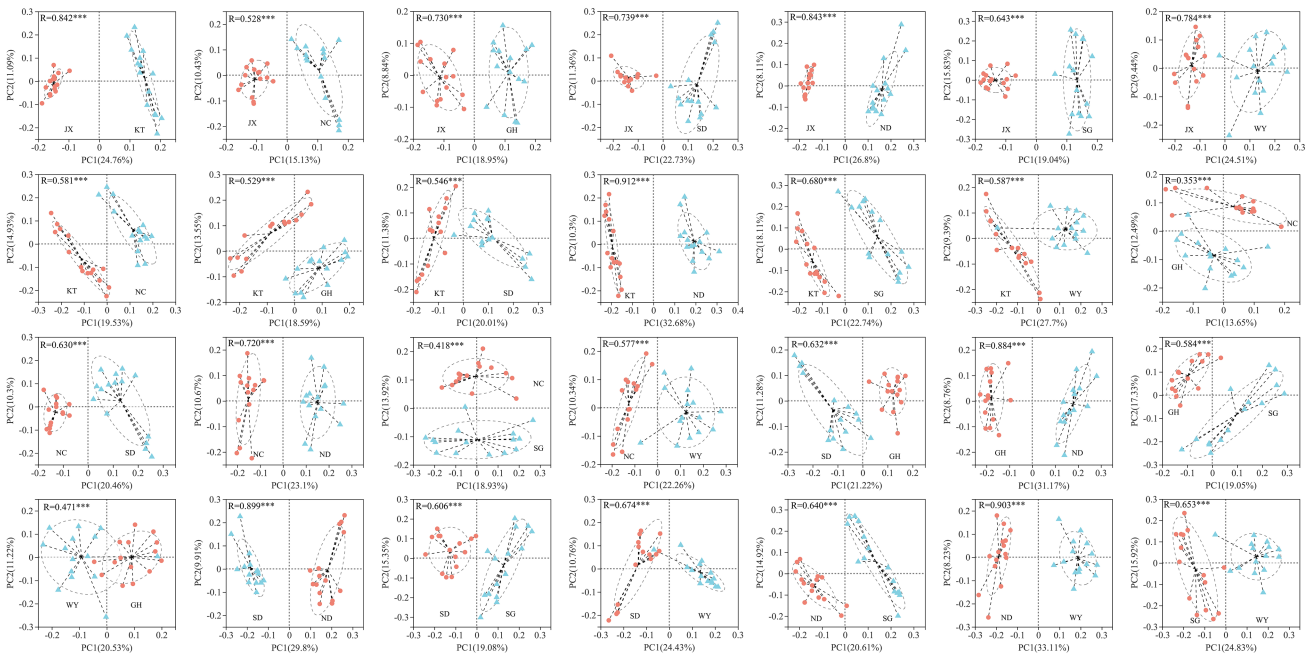
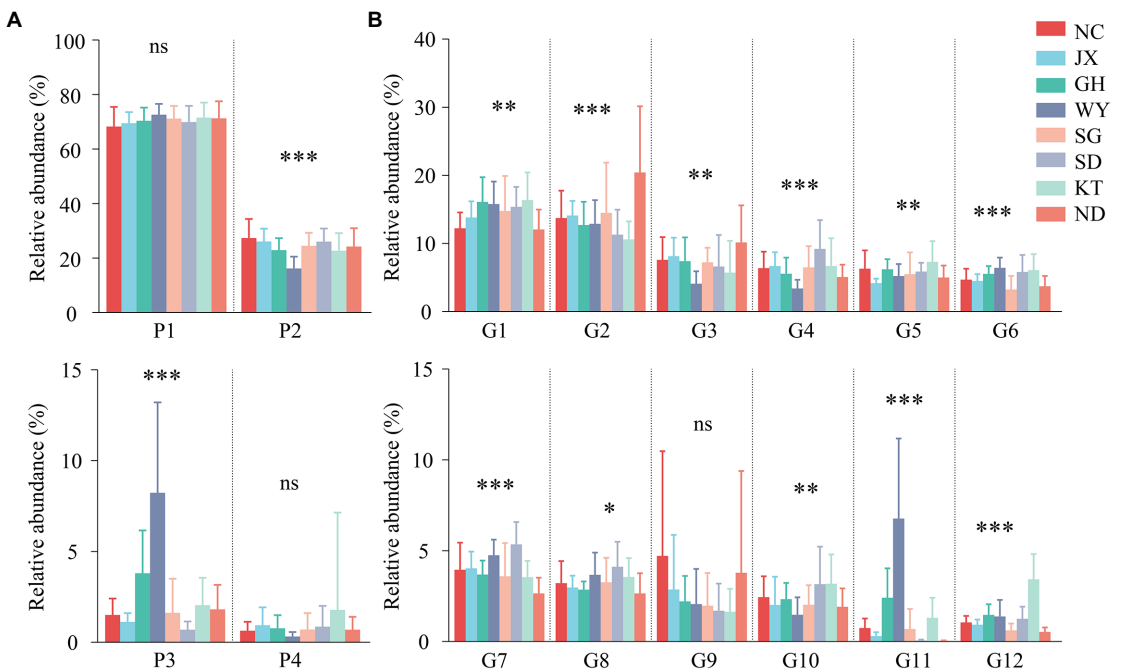


FIGURE 4 | PCoA analysis of gut microbiome in Przewalski's gazelle based on Bray-Curtis distance matrices. ***$p < 0.001$.



P1: *Firmicutes* G1: *Ruminococcaceae UCG-005* G5: *Ruminococcaceae UCG-010* G9: *Prevotellaceae UCG-004*
P2: *Bacteroidetes* G2: *Christensenellaceae R-7 group* G6: *Ruminococcaceae UCG-013* G10: *Alistipes*
P3: *Actinobacteria* G3: *Rikenellaceae RC9 gut group* G7: *[Eubacterium] coprostanoligenes group* G11: *Arthrobacter*
P4: *Verrucomicrobia* G4: *Bacteroides* G8: *Ruminococcaceae UCG-014* G12: *Lachnospiraceae NK4A136 group*

FIGURE 5 | Analysis of the differences between the dominant bacteria in Przewalski's gazelle at the phylum **(A)** and the genus **(B)** levels. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$. ns, no significance.

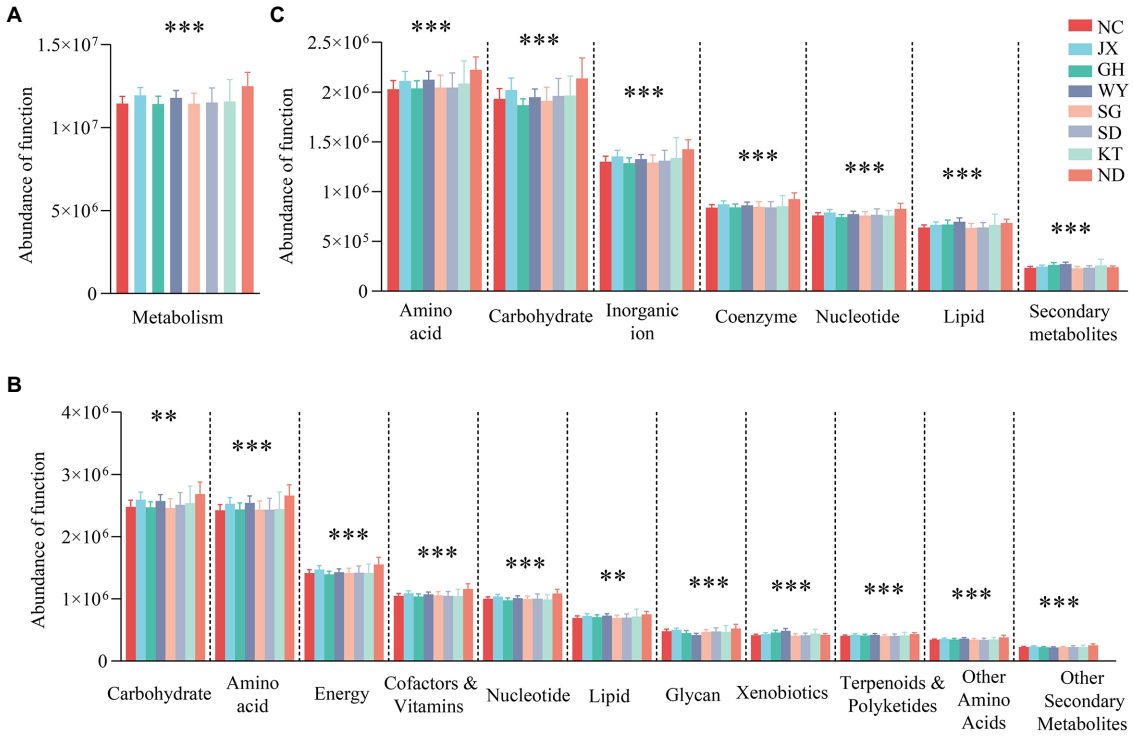


FIGURE 6 | Analysis of the differences in metabolic functions between the groups based on the KEGG database at level 1 **(A)**, level 2 **(B)** and EggNOG database **(C)**. $^{**}p < 0.01$; and $^{***}p < 0.001$.

Li et al., 2017). The phylum *Firmicutes* can degrade fiber and cellulose into volatile fatty acids (Thoetkiattikul et al., 2013; Hu et al., 2017), and the phylum *Bacteroidetes* contributes to the breakdown of carbohydrates and proteins and improve nutrient utilization in the host (Waite and Taylor, 2014; Nuriel-Ohayon et al., 2016). In addition, both of them play an important role in enhancing host immunity and maintaining the balance of gut microbiota (Sears, 2005; Shang et al., 2016). The phylum *Actinobacteria* is also an essential component in each group. The members of this phylum play important roles in the degradation of cellulose and lignin (Heurich et al., 2015; Lewin et al., 2016). At the genus level, the co-occurrence network analysis showed that most of the bacteria at the core of the network belong to the family *Ruminococcaceae* in the rumen, which is closely related to food digestion. The genera *Ruminococcaceae UCG-005* and *Christensenellaceae R-7 group* were the most abundant genera. *Ruminococcaceae UCG-005* belongs to the family *Ruminococcaceae*, which are important bacteria involved in the degradation of cellulose and hemicellulose in the rumen (Matulova et al., 2008; La Reau et al., 2016). The bacteria can produce a large amount of cellulase and hemicellulase and convert dietary fiber in foods into various nutrients required by the host (La Reau and Suen, 2018), which plays a key role in food digestion and carbohydrate metabolism of ruminants. *Christensenellaceae R-7 group* is mainly involved in host amino acid and lipid metabolism (Waters and Ley, 2019). Moreover, these bacteria were considered to be potentially beneficial since they are involved in the positive regulation of the intestinal environment and are associated with immune regulation and healthy homeostasis (Kong et al., 2016; Kashtanova et al., 2020; Li et al., 2020). Dominant gut microbes are considered as the result of strong selection and long-term co-evolution with the host (Groussin et al., 2020). Furthermore, they play an effective role in ensuring the health of the species by coping with long-term environmental changes (Asnicar et al., 2017; Sun et al., 2019a). We speculated that these dominant bacteria can help Przewalski's gazelle decompose cellulose, obtain nutrients and improve immunity more effectively, and adapt better to the various environment.

Diversity results revealed that the diversity and uniformity of the gut microbiota in Przewalski's gazelle in the JX, GH, and NC regions were higher than that in other regions. In addition to feeding on local vegetation, Przewalski's gazelle may receive additional supplementary feeding in winter in these three regions, such as oats. The GH area is currently the largest distribution area of Przewalski's gazelle. The vegetation types and food resources in this area are relatively diverse, which are probably the main reason for the high microbial diversity (Tinker and Ottesen, 2016; Chi et al., 2019). The NC area has also sufficient food resources. The JX area belonged to a semi-captive environment where Przewalski's gazelle can obtain the most food resources, as there is no competition for resources from domestic animals. In our opinion, a better living environment will increase the diversity of the gut

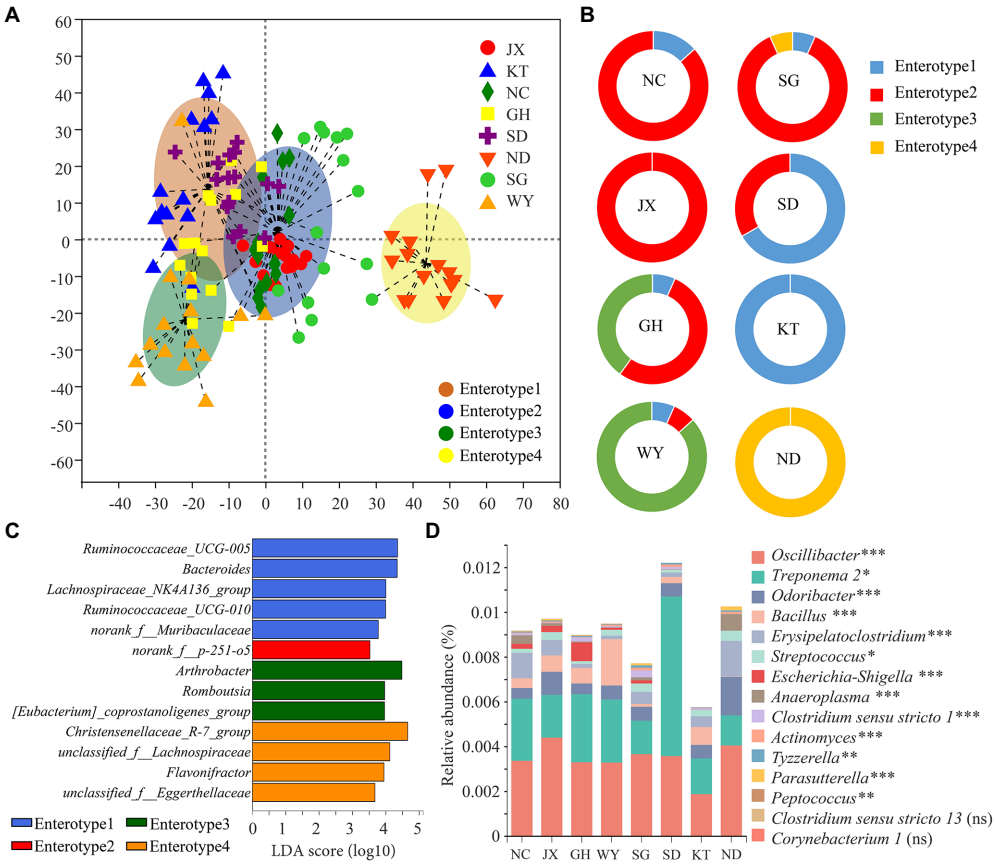
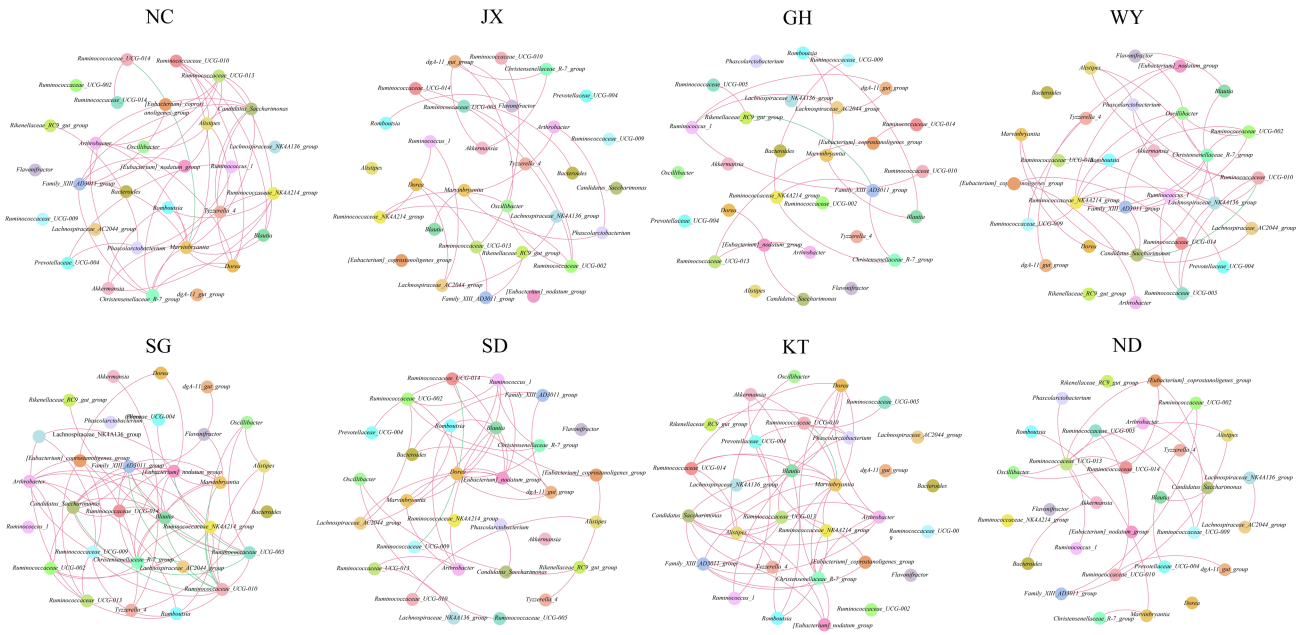


FIGURE 7 | Analysis of the enterotype and the pathogenic bacteria of Przewalski's gazelle from the different study areas. **(A)** Analysis of the diagram of the enterotype. **(B)** Analysis of the enterotype distribution of Przewalski's gazelle in the different study areas. **(C)** Marker bacteria of each enterotype. **(D)** Analysis of the differences between the groups of the potentially pathogenic bacteria. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$. ns, no significance.

microbiome of Przewalski's gazelle, while the α diversity of gut microbiota in Przewalski's gazelle in the ND area was the lowest compared to the other regions. We speculated that the limitations of these environmental factors would affect the species' gut microbial diversity because the ND area is currently the smallest distribution area of Przewalski's gazelle, with a single vegetation type and limited food resources. Studies have shown that a high diversity of gut microbiota is beneficial to the host's health and is also a sign of maturity of the gut microbiome (Turnbaugh et al., 2007; Le Chatelier et al., 2013). We also found that the wild population of Przewalski's gazelle is relatively higher in the GH and NC areas; therefore, we speculated that there is a certain connection between the diversity of the gut microbiome and the population number.

Przewalski's gazelle is currently a species that occurs only in a narrow range around Qinghai Lake, with extremely small distribution area. However, there are still certain differences between the habitats of several independent small populations, which are mainly represented in the vegetation types. Vegetation in different regions determines in part the type of food available to Przewalski's gazelle. The study revealed that in different habitats, the composition of gut microbiota in Przewalski's gazelle was also different. This variation is most likely due to the species' adaptation to diverse habitats. The relative abundance of *Bacteroidetes* was the lowest in the WY, while that of the *Actinobacteria* was the highest in the WY, and these values are significantly different from those found in the other regions. We noticed that there was an obvious difference between the vegetation types in the WY and the other areas. The habitat type of Przewalski's gazelle in the WY area included *Ceratoides arborescens* and salt claw desert, a vegetation type in the area that are halophytes with high fiber content, but low carbohydrate and protein content. We speculated that the difference in abundance may be used to improve the adaptability of these populations in special environments (Gao et al., 2020). *Proteobacteria* were most abundant in the SG group, followed by the GH, WY, and JX groups, but less abundant in the SD, KT, and ND groups. Previous studies have indicated that the surge of *Proteobacteria* in the gut was a manifestation of the imbalance and instability of the gut microbial community structure (Shin et al., 2015). As a result, we speculated that gut microbiota experiences minor variations in a single habitat.

Fibrobacteres are an important phylum of cellulose-degrading bacteria (Ransom-Jones et al., 2012, 2014), yet they were not



Network indices	NC	JX	GH	WY	SG	SD	KT	ND
Total nodes	30	30	30	30	30	30	30	30
Total edges	47	24	18	37	65	35	47	21
Positive edges	46	24	17	36	55	34	46	21
negative edges	1	0	1	1	10	1	1	0
Average degree	3.133	1.6	1.2	2.467	4.333	2.333	3.133	1.4
Average weighted degree	1.94	0.968	0.703	1.532	1.901	1.396	1.94	0.865
Network diameter	5	9	4	6	5	7	5	6
Average path length	2.085	3.706	1.636	2.665	2.369	2.959	2.085	2.61
Density	0.108	0.055	0.041	0.085	0.149	0.08	0.108	0.048
Average clustering coefficient	0.591	0.427	0.538	0.553	0.537	0.505	0.591	0.115

FIGURE 8 | Co-occurrence networks of the 30 most abundant genera in Przewalski's gazelle from eight different areas. Each line represents Pearson correlation coefficient, whose absolute value was higher than 0.5 and *p* value was less than 0.05. The red and green lines represent positive and negative correlations, respectively.

present in the SG and ND groups, which was a significant result. The absence of these bacteria in these areas may be related to the environment, probably because these areas lack specific plants that the bacteria require to degrade, or another bacterial structure evolved in the intestine and effectively replaced the function of the bacteria to decompose and utilize certain cellulose. *Rikenellaceae RC9* had the lowest abundance in the WY group, but the highest abundance in the ND group. There is evidence that *Rikenellaceae RC9* can not only play a potential role in the degradation of plant-derived polysaccharides but also have a function in lipid metabolism (He et al., 2015; Sun et al., 2019b; Tavella et al., 2021). Because Przewalski's gazelle confronts more survival obstacles in the NC than in the other locations, a larger abundance of *Rikenellaceae RC9* may assist Przewalski's gazelle to receive more nutrients from limited food. The diet of the species in the WY group, on the other hand, was predominantly halophyte, with more cellulose but less sugar or lipids, which may explain the low abundance of this bacteria in the species of this region.

The functional prediction analysis revealed that Przewalski's gazelle in the WY area and the other regions exhibited unique characteristics in a variety of functions, including glycan biosynthesis and metabolism, metabolism of secondary metabolites, and xenobiotics biodegradation and metabolism. It is abundant in the aforementioned functions and has generated major differences in the other regions. In addition, Przewalski's gazelle presented in the WY obvious low abundance and differences in organismal systems, such as environmental adaptation, digestive system, and immune system, confirming the particularity of Przewalski's gazelle in the WY's food resources. We speculated that these functional changes play an important role in the adaptation of Przewalski's gazelle to a special environment. We intend to conduct a metagenomic study in the future to better understand the role and adaptability of the gut microbiota of Przewalski's gazelle. We found that the enterotype and the geographic distance of Przewalski's gazelle were related, with the SD and KT being very close and having a similar habitat type.

The WY and ND have relatively independent geographical locations, which may be the main reason of the significant difference of the enterotypes from the other areas. The four enterotypes had different marker bacteria, and their relative abundance may be closely related to food composition. We also speculated that the difference in wild animal habitats is responsible for the composition and function of their gut microbiota.

Bacteroides have been shown to have a beneficial impact on the mucosal immune system (Malmuthuge et al., 2015) and have been proven to have an important influence on the development of immunological tolerance to commensal microbiota (Mazmanian et al., 2008). Nonetheless, *Bacteroides* are pathogenic bacteria that can cause endogenous infections when the immune system or gut microbiota malfunctions (Patrick, 2002; Wexler, 2007). *Oscillibacter* and *Treponema* 2 were found to account for a high proportion of potentially pathogenic bacteria. *Oscillatoria* is an anaerobic pathogenic bacterial genus that may induce intestinal metabolic dysfunction and metabolic diseases in the host (Hae-Jin et al., 2015). *Treponema* 2 can cause inflammation of the colon and is related to dysentery (Kelly et al., 2016). Research on the composition and abundance of the potentially pathogenic bacteria in Przewalski's gazelle will help understand the physiology and the pathological conditions of the population in different environments, which is crucial for population recovery.

CONCLUSION

In conclusion, this study compared the gut microbiota of almost all distribution areas of Przewalski's gazelle. The results revealed that environmental differences affect the gut microbiota of the species. The dominant gut microflora of Przewalski's gazelle distributed in various geographical areas had great similarities. These bacteria are likely to play an essential role in the long-term evolution of the host and gut microbiota. However, the gut microbiome of Przewalski's gazelle in multiple regions differed also in species, abundance, and function. These differences are a result of adapting to different environments.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI GenBank, accession number PRJNA722780 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA722780?reviewer=65b4tumbfel4l7nv7r96tlpoqa).

AUTHOR CONTRIBUTIONS

JZ and TZ conceived and designed the experiments. JZ wrote the first draft of the manuscript. JZ, HG, FJ, DL, YH, XC, WQ, PS, and ZC contributed to sampling and laboratory work. JZ, FJ, and HG conducted data analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Effects of Habitat River Microbiome on the Symbiotic Microbiota and Multi-Organ Gene Expression of Captive-Bred Chinese Giant Salamander

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The reintroduction of captive-bred individuals is a primary approach to rebuild the wild populations of the Chinese giant salamander (*Andrias davidianus*), the largest extant amphibian species. However, the complexity of the wild habitat (e.g., diverse microorganisms and potential pathogens) potentially threatens the survival of reintroduced individuals. In this study, fresh (i.e., containing environmental microbiota) or sterilized river sediments (120°C sterilized treatment) were added to the artificial habitats to treat the larvae of the Chinese giant salamander (control group—Cnt: 20 individuals, treatment group 1 with fresh river sediments—T1: 20 individuals, and treatment group 2 with sterilized river sediments—T2: 20 individuals). The main objective of this study was to test whether this procedure could provoke their wild adaptability from the perspective of commensal microbiotas (skin, oral cavity, stomach, and gut) and larvae transcriptomes (skin, spleen, liver, and brain). Our results indicated that the presence of habitat sediments (whether fresh or sterilized) reshaped the oral bacterial community composition. Specifically, Firmicutes decreased dramatically from ∼70% to ∼20–25% (mainly contributed by *Lactobacillaceae*), while Proteobacteria increased from ∼6% to ∼31–36% (mainly contributed by Gammaproteobacteria). Consequently, the proportion of antifungal operational taxonomic units (OTUs) increased, and the function of oral microbiota likely shifted from growth-promoting to pathogen defense. Interestingly, the skin microbiota, rather than the colonization of habitat microbiota, was the major source of the pre-treated oral microbiota. From the host perspective, the transcriptomes of all four organs were changed for treated individuals. Specifically, the proteolysis and apoptosis in the skin were promoted, and the transcription of immune genes was activated in the skin, spleen, and liver. Importantly, more robust immune activation was detected in individuals treated with sterilized sediments. These results suggested that the pathogen defense of captive-bred individuals was improved after being treated, which may benefit their survival in the wild. Taken together, our results suggested that the pre-exposure

of captive-bred Chinese giant salamander individuals to habitat sediments could be considered and added into the reintroduction processes to help them better adapt to wild conditions.

Keywords: amphibian, conservation, immunity, microbiome, pathogen, reintroduction

INTRODUCTION

Amphibian is one of the most threatened taxa on Earth today, with approximately one-third (32%) of the wild populations declining worldwide due to global changes caused by human activities (Stuart et al., 2004; González-Del-Pliego et al., 2019). Therefore, amphibian conservation has been advocated and suggested to be more urgent by ecologists and biologists (WWF, 2020). The reintroduction of captive-bred individuals to the wild is one of the most important measures for the rebuilding of natural amphibian populations. Typically, the artificial habitats of the captive individuals are designed to be suitable environments for them to grow (e.g., stable habitat structure, clean food, and water). Therefore, once being released to the wild, the complicated environmental condition may induce a low survival rate of captive-bred animals. Accordingly, determining how to increase the released individuals' survival rate and fitness should be the priority for species conservation.

Prerelease training is a common approach to teach captive-bred animals to prey and live in the wild (e.g., giant panda *Ailuropoda melanoleuca*; Wei et al., 2015, and Crested Ibis *Nipponia nippon*; Nagata and Yamagishi, 2016). Regarding amphibians, due to their thin, moist, and permeable skins, they are more vulnerable to the effects of desiccation, toxic chemicals (Mcmenamin et al., 2008; Wang X. et al., 2019; Zhu et al., 2022), and in particular, environmental pathogens (O'hanlon et al., 2018; Fisher and Garner, 2020). Previous studies have demonstrated that symbiotic microbes (i.e., skin, oral cavity, stomach, and gut microbes) played important roles in regulating amphibian capacity for environmental adaptation (Chang et al., 2016; Guo et al., 2021). Moreover, the composition of symbiotic microbiota is associated with host susceptibility and the composition of environmental pathogens (Rebollar et al., 2016; Bletz et al., 2017). Therefore, the reorganization of symbiotic microbiota is a potential strategy for amphibian to cope with the colonization of the host by pathogens (Muletz et al., 2012; Longo and Zamudio, 2017). Accordingly, symbiotic microbes were important for amphibian disease immunity (Hopkins, 2007; Harris et al., 2009). Different microbiota community structures were correlated with the specific immune status of the host, maintaining the mutualistic nature of the host-microbial relationship (Hooper et al., 2012). Consequently, we argued that the diversity and community structure of the symbiotic microbiota may be an important factor influencing the fitness and survival of released captive-bred individuals.

Previous studies have indicated that symbiotic microbes can shift with the change in their natural habitat (Bletz et al., 2016; Bird et al., 2018; Muletz et al., 2018), and increasing attention has been paid to understand how the symbiotic microbiota responds to these environmental changes and the implications to the host fitness (Ellison et al., 2019; Barnes et al., 2021). For example, reduced microbiome stability stemming from habitat alterations could compromise the population health of amphibians, even in the absence of pathogen infection (Neely et al., 2021). Constant exposure to the environment with complex microbiota would shape the physiological development and evolution of animals. However, since captive-bred animals usually exhibited low diversity of symbiotic microbiota (Kueneman et al., 2016; San et al., 2021), it is not clear how their health can be affected by the wild habitat with complex environmental microbiota (Hyde et al., 2016). Adding soil collected from their natural environment into the captive environment seems to be an applicable approach to rescue this situation (Loudon et al., 2014). It has been reported that amphibians could recruit putatively beneficial bacteria to defend themselves from fungal infection (Longo and Zamudio, 2017). Pre-exposure to the natural microbiota might enable an opportunity for amphibian to reorganize their microbiota and thus reinforce their immunity to potential pathogens.

The Chinese giant salamander (*Andrias davidianus*) is a flagship species for amphibian conservation. It is the largest extant amphibian species in the world, and its evolutionary history can be traced back to 16 Ma (Fei et al., 2009; Yan et al., 2018). Recent studies showed that this species was comprised of at least seven lineages, thus it should be considered as *Andrias* species in China (Yan et al., 2018; Liang et al., 2019; Turvey et al., 2019). Historically, *Andrias* species were widely distributed in central and southern China throughout the Yangtze, Yellow, and Pearl River drainages (Pierson et al., 2014; Wang et al., 2017). However, due to the dramatic decline of the wild populations (over 80%) in the past 60 years (Wang et al., 2017; Turvey et al., 2019), the distribution of *Andrias* species was concentrated in 12 patches in China currently (IUCN, 2016). These declines are largely due to habitat degradation, water pollution, and in particular, overexploitation (Liang et al., 2004; Zhao et al., 2020). Consequently, *Andrias* species were evaluated as critically endangered in the International Union for Conservation of Nature Red List (IUCN, 2016), and also in China (Jiang et al., 2016). Accordingly, increasing attention has been paid to *Andrias* species conservation by the Chinese government and national and international organizations (Pierson et al., 2014). Specifically, 47 natural reserves have been established in China to protect *Andrias* species since 1980, covering more than 240,000 km^2 of the total area (Zhao et al., 2017). Another important protective measure for *Andrias* species conservation is the reintroduction of captive-bred individuals, and the first activity was conducted in Zhangjiajie National Nature Reserve in 2002 (China Aquatic Wildlife Conservation, 2015). Based on the records, a total of more than 287,000 captive-bred *Andrias davidianus* individuals were released into natural reserves by the end of 2019 (Shu et al., 2021). Despite a large number of individuals have been

released, it is still rare to observe *Andrias* species in the field. The physiological status of captive-bred salamander may be maladaptive to the wild environment. With the increasing attentions on the potential role of symbiotic microbiota in conservation biology, the Chinese giant salamander is a great model to examine the potential effects of habitat microbiota exposure on the symbiotic microbiota and host status.

In this study, we used experimental approaches to (1) test whether pre-treatment (i.e., the pre-exposure to the field habitat sediments) altered captive-bred *Andrias* individuals in the skin, oral cavity, stomach, and gut microbe composition and (2) verify whether the change in the composition of the microbiota was related to the host physiological response (e.g., upregulation of the expression of the immune-related genes). We predicted that differentiation in the skin, oral cavity, stomach, and gut microbiota, as well as the physiological traits, can be detected between treated and controlled *Andrias* individuals. We hope our study can provide a potential approach that can activate the innate immune system of captive-bred *Andrias* individuals before releasing them back to the field. More importantly, we also hope it can increase the fitness-related traits or indices of the released individuals and thus improve the conservation strategies of *Andrias* species.

MATERIALS AND METHODS

Animal Culture and Treatment

Sixty *A. davidianus* larvae from the same clutch were collected ∼350 days after hatching (d.a.h) from a hatchery in Hongya County of Sichuan Province, China. These larvae were then transferred to Chengdu Institute of Biology, Chinese Academy of Sciences on 20 September 2019. They were acclimated in an artificial climate chest (BIC-250, Shanghai Boxun, China), raised in two plastic containers (29 × 20 × 9.7 cm), with each of them containing 30 individuals and 4 L aerated water. The artificial climate chest was kept under controlled temperature (15 ± 0.5°C) and a 12-h light/dark condition. Larvae were overfed with red worms (larvae of *Chironomus* sp.) every day at 9:00 a.m. Water in the containers was renewed entirely at 18:00 every day. The acclimation lasted for 20 days, until the start of the treatment (10 October 2019). Sterilized tap water was used as a water source in our experiment. The animal use protocol in this study (permit: 2016-AR-JJP-02) was reviewed and approved by the Animal Ethical and Welfare Committee of Chengdu Institute of Biology, Chinese Academy of Science, China. Chengdu 610041, China.

Given the demersal habit of *Andrias* individuals, we intended to create an environment with field microbiota by introducing river bottom sediments from reintroduction sites into containers containing aerated water. These sediments were convenient to be stored in large quantities in the laboratory. Therefore, sediments of Tongma River (Dujiangyan City, Sichuan Province, China; 103°38′-103°39′E, 31°02′N) were collected. We selected this river as it was an empirical habitat for the reintroduction of captive-bred *A. davidianus* since 2017. Specifically, six sites (i.e., two sites each at upstream, midstream, and downstream locations, respectively, **Supplementary Figure 1**) were randomly selected to collect river sediments using a mud dredger (TC-600BD-1/40, China), spanning 1.5 km of the river segment. In total, we collected ∼30 kg of sediments (i.e., 5 kg per site) in the field. Approximately 50 g of the sediments from each site were separately stored in poly zipper bags for environmental microbiome analyses, and the rest sediments were mixed. All of them were preserved in a cool box in the field and were subsequently brought back to the laboratory. Sediments in the zipper bags were −80°C preserved in the freezer, which were used for analyzing microbial diversity. The mixed sediments were divided into two parts, with one part being sterilized at 120°C for 20 min in a steam sterilizer. Later, sterilized and fresh sediments were divided into 40 small packets preserved in gauzes (∼300 g for each), separately, which were stored at 4°C in the fridge before utilization. For unsterilized sediments, the storage duration should not exceed 7 days, otherwise, fresh sediments were collected.

Before treatment, 60 *A. davidianus* larvae were measured for the body length to the nearest 0.01 mm by a digital caliper, weighted to the nearest 0.01 g by a scale, and were then randomly divided into three groups (20 individuals per group). For each group, the 20 individuals were randomly delivered into four plastic containers (29 × 20 × 9.7 cm), with five individuals for each. Specifically, the control group (Cnt) contained only 3,000 ml of aerated water in the plastic containers, the T1 group contained 3,000 ml of aerated water and 300 g of sterilized sediments (weight ratio of sediments to water is 1:10) in the plastic containers, and the T2 group contained 3,000 ml of aerated water and 300 g of fresh sediment (weight ratio of sediments to water is 1:10) in the plastic containers (**Figure 1A**). Although the gauze packets did not allow burrowing or other behaviors in the sediment, the animals are accessible to the sediment in the packets due to the good permeability of the gauze. Treatments were conducted in the artificial climate chest with the same condition as those in acclimation. Experiments were started on 11 October 2019, and the treatments lasted for 20 days until 30 October 2019. Larvae were fed with sufficient red worm once a day (at 9:00 a.m.), while the sediments packets and water were replaced every 2 days (at 18:00).

Sample Collection

After 20 days of treatment (Bletz et al., 2016), ten individuals from each treatment group were randomly selected. For each treatment group, the ten individuals were from four plastic containers, with 2–3 individuals being sampled from each plastic container to avoid the cage effect (animals from the same cage may share more symbiotic microbes due to exchange). The body weight of sampled individuals was measured to the nearest 0.01 g using a scale, and their body length was measured to the nearest 1 mm using a digital caliper. The skin, oral, stomach, and gut microbiota, as well as their brain, liver, spleen, and skin tissues, were collected. Details of the approaches are as follows: ultra-pure water was first used to rinse the larvae three times to remove potential transient bacteria. Sterile swabs were then used to collect the skin microbes by wiping the dorsal, ventral, and lateral sides of the larvae (Xu et al., 2020). For oral microbial sampling, sterile swabs were used by swabbing the oral cavity of

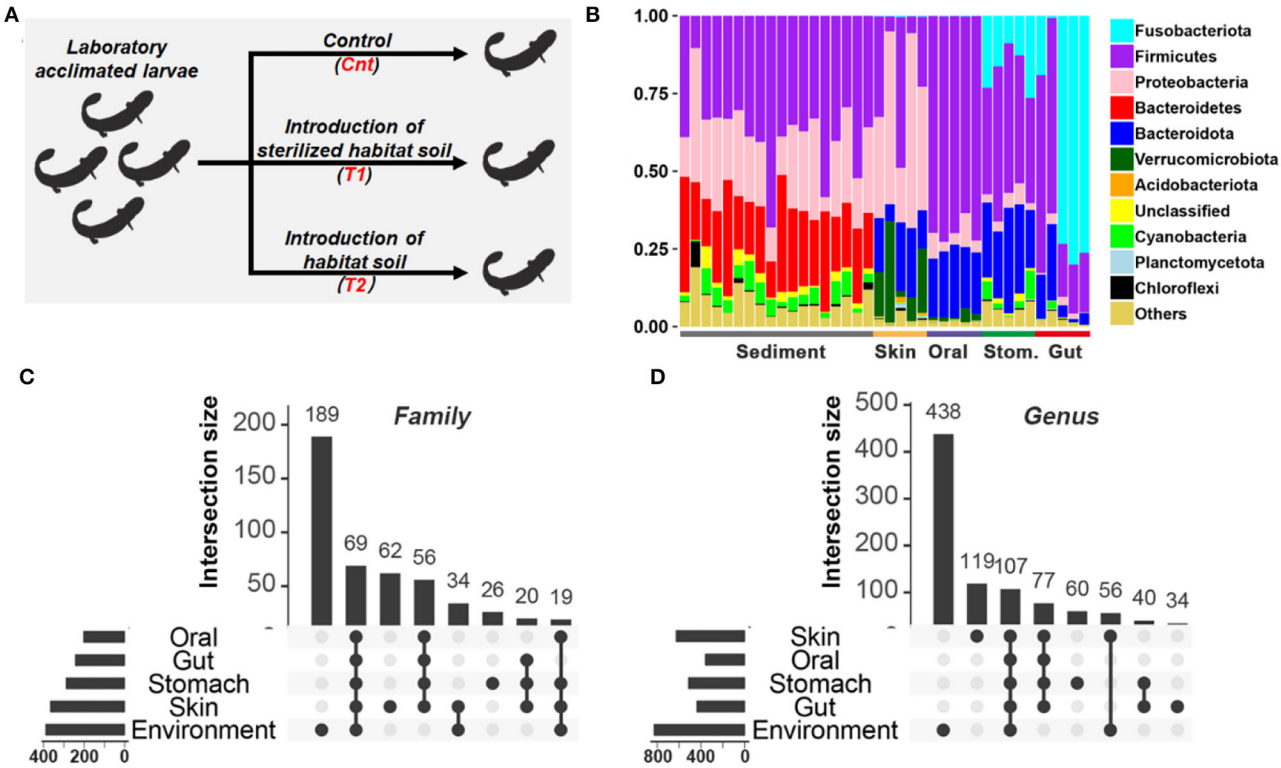


FIGURE 1 | Comparison of microbiota between habitat river sediments and captive giant salamander. **(A)** Experimental design. **(B)** The relative abundance of the bacterial taxon in the habitat river sediments and captive giant salamander at the phylum level. **(C,D)** The number of shared and unique bacterial families **(C)** or genera **(D)** between river sediments and commensal microbiota. The horizontal bars represent the family or genus numbers of each source, while the vertical bars denote the numbers of their shared or unique bacterial families/genera.

the larvae gently and repeatedly. Later, larvae were euthanized with 0.1% MS-222 and dissected to collect the gut and stomach contents, which were preserved in 2 ml of the aseptic centrifuge tubes, separately. These samples were further used to do the gut and stomach microbial analyses, respectively.

16S rRNA Gene-Based Microbiome Analyses

The skin, oral, stomach, and gut microbes of two random individuals were pooled as one sample, and thus, we obtained five samples or biological replicates for each tissue and each group. The two individuals for pooled samples may be either from the same cage or not, as they were randomly selected. Additionally, 18 sediment samples were also prepared for analyses of bacterial diversity (n = 6 replicates for upstream, midstream, and downstream locations, respectively). The QIAamp DNA Stool Minikit (Qiagen, Valencia, CA, USA) was used to extract DNA from the samples at room temperature. The V4 region of the 16S rRNA gene was amplified with 515F (5 = -GTGCCAGCMGCCGCGGTAA-3=) and 806R (5 = -GACTACHVGGGTWTCTAAT-3=) primers (Caporaso et al., 2012). We used the following PCR thermocycling conditions: 95°C for 5 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, with a final extension step at 72°C for 10 min. High-throughput sequencing of amplicons was performed using the Illumina MiSeq platform. Sequencing was performed by Mingke Biotechnology Co., Ltd. (Hangzhou, China).

QIIME 1.9 was used to process the raw sequences and to obtain clean sequences (Caporaso et al., 2010). In the trimming analysis, *Usearch* was used for chimerism check in order to remove low-quality sequences (Edgar, 2010), *flash* program was used for splicing (Magoc and Salzberg, 2011), and *trimmomatic* program was used for quality control with default parameters (e.g., Window Size: 20 base pair; Minimum Read Length: 50 base pair) (Bolger et al., 2014). Operational taxonomic units (OTUs) were defined as sharing >97% sequence identity by annotating clean sequences to the SILVA132 database (Quast et al., 2013). The taxon summary was conducted using the OTU table in QIIME 1.9.

The alpha diversity (e.g., OTU richness and Shannon index) was calculated using QIIME 1.9 (Caporaso et al., 2010). A one-way ANOVA was used to investigate the differences in the microbial alpha diversity in each type of symbiotic microbiome between groups. The dissimilarity matrices (Bray-Curtis distances and unweighted and weighted UniFrac distances) were produced by the QIIME pipeline. The Bray-Curtis distances only considered the abundances of each

OTU but not the phylogenetic relationships between bacterial taxa. The unweighted Unifrac and weighted Unifrac distances took the phylogenetic relationships into consideration. The difference is that the weighted Unifrac distances considered the abundance of each OTU, while the unweighted Unifrac distances only considered the presence or absence of each OTU in samples. In this study, the weighted Unifrac distances were preferentially used for comparative analyses, but analyses based on the other two types of distances were also provided in **Supplementary Materials**. Principal coordinates analysis (PCoA, based on dissimilarity matrices) and hierarchical clustering were used to visualize the dissimilarity of beta diversity, and PERMANOVA (Adonis function in the Vegan package) was performed to test the composition differences at the OTU level (Dixon, 2003). Benjamini–Hochberg (BH) correction was conducted to obtain the corrected *p*-values. The differences in microbial compositions between groups were compared using the linear discriminant analysis (LDA) effect size (LEfSe) method (Segata et al., 2011).

Source-Tracker 0.9.5 was used to assess the contribution (microbiome transmission) of the sediment and initial symbiotic microbiota (before treatment) to the building of the symbiotic microbiotas in treated larvae (Knights et al., 2011). Since the control group faced no variation in environmental conditions, we speculated that they kept the initial state of the symbiotic microbiota. For example, to explore the source of the oral microbiota of T1, the microbiotas of T1 individuals were set as sinks, while the sediment microbiotas and the skin, oral, stomach, and gut microbiotas of Cnt individuals were set as sources.

To evaluate the potential antifungal capacity of the microbiota, the 16S rRNA sequence of the OTUs was queried against an antifungal bacterial 16S rRNA database (Woodhams et al., 2015). Comparison hits with sequence similarity >95% were considered as valid one, and these OTUs were screened as antifungal strains and calculated for relative abundance (Xu et al., 2020).

Transcriptomic Analyses

For each treatment group, the tissue samples of three individuals were randomly selected for transcriptomic analyses, and thus, there were three biological replications for each tissue and each group. The protocols of RNA extraction, purification, and cDNA library construction were used following the description from Chang et al. (2021). After cluster generation, the library preparations were sequenced on an Illumina HiSeq 4000 platform by Annoroad (Beijing), and paired-end reads were generated. The read quality was verified using the FastQC (version 0.10.0) software. Poly-N and low-quality reads, as well as those containing adapters, were removed. The total clean reads from all libraries were assembled *de novo* using Trinity as a reference transcriptome (refer to the assembly summary in **Supplementary Table 1**). The resulting unigenes were annotated by querying databases NCBI Non-Redundant Protein and Nucleotide Sequence databases (NR and NT databases), and Swiss-Prot with an *E*-value threshold of 1.0E-5, gene ontology (GO) with an *E*-value threshold of 1.0E-6, and Kyoto Encyclopedia of Genes and Genomes (KEGG) with an *E*-value threshold of 1.0E-10. The sequencing data from this study have been submitted to the Genome Sequence Archive (GSA; http://bigd.big.ac.cn/gsa/s/B7y0wob0) under accession number PRJCA004234.

Differently expressed genes (DEGs) were identified by DESeq (based on R) based on pairwise comparison (corrected $p < 0.05$, BH correction), or by one-way ANOVA across three groups ($p < 0.01$ or 0.05). Functional enrichment analyses were conducted by querying DEGs against the KEGG database (based on KOBAS 3.0, with default parameters) (Xie et al., 2011).

Statistical Analyses

Basic statistical analyses were conducted using IBM SPSS 22.0 (SPSS Inc., Chicago, USA). Heatmaps were created by heatmap.2 in "gplot" package based on R software. The columns (samples) of the heatmap were not reordered, while the rows (genes) were reordered in default parameters. Graphs were drawn using Graphpad prism 5 or ggplot2, an R package (Wickham, 2009).

RESULTS

The sediment and host microflora are different in the bacterial community structure (**Figure 1B**, **Supplementary Figure 2**). There are 189/390 families and 438/830 genera exclusively existing in the sediment microflora (**Figures 1C,D**). Before treatment, the body length of the salamanders was 17.13 ± 0.25 cm (mean ± SE), and their body weight was 23.79 ± 0.87 g (mean ± SE). After 20 days of treatment, the body lengths of Cnt, T1, and T2 were mean = 18.12 cm ± 0.45 (SD), 17.68 cm ± 0.53 (SD), and 18.53 cm ± 0.48 (SD), respectively. Individual body weights of Cnt, T1, and T2 were mean = 31.23 g ± 1.66 (SD), 30.51 g ± 2.83 (SD), and 33.68 g ± 2.54 (SD), respectively. No significant difference in salamander growth rate was observed between groups ($p > 0.05$, one-way ANOVA; **Supplementary Figure 3**).

Variations in Symbiotic Microbiota After Pre-exposure

The OTU richness in the gut microbiota was higher in T1 and T2 than in the Cnt group, while there were no intergroup differences in Shannon diversity of the gut microbiota (at corrected $p < 0.05$, one-way ANOVA, and BH correction; **Figure 2A**). No intergroup variations in alpha-diversity were observed for oral, skin, and stomach microbiota (at corrected $p < 0.05$, one-way ANOVA, and BH correction; **Figure 2A**).

There was a significant difference in the larvae oral bacterial composition between Cnt and T1 groups ($R^2 = 0.60$, corrected $p = 0.039$ for unweighted UniFrac distance; $R^2 = 0.56$, corrected $p = 0.015$ for weighted UniFrac distance), as well as that between Cnt and T2 groups ($R^2 = 0.60$, corrected $p = 0.024$ for unweighted UniFrac distance; $R^2 = 0.43$, corrected $p = 0.015$ for weighted UniFrac distance) (PEMANOVA and BH correction). The oral samples could be divided into control and treatment groups (T1 & T2) by PCoA and Hcluster based on both weighted and unweighted UniFrac distances (**Figures 2B–D**). No significant differences in microbiota composition in the skin, stomach, or gut were observed between larvae from the control

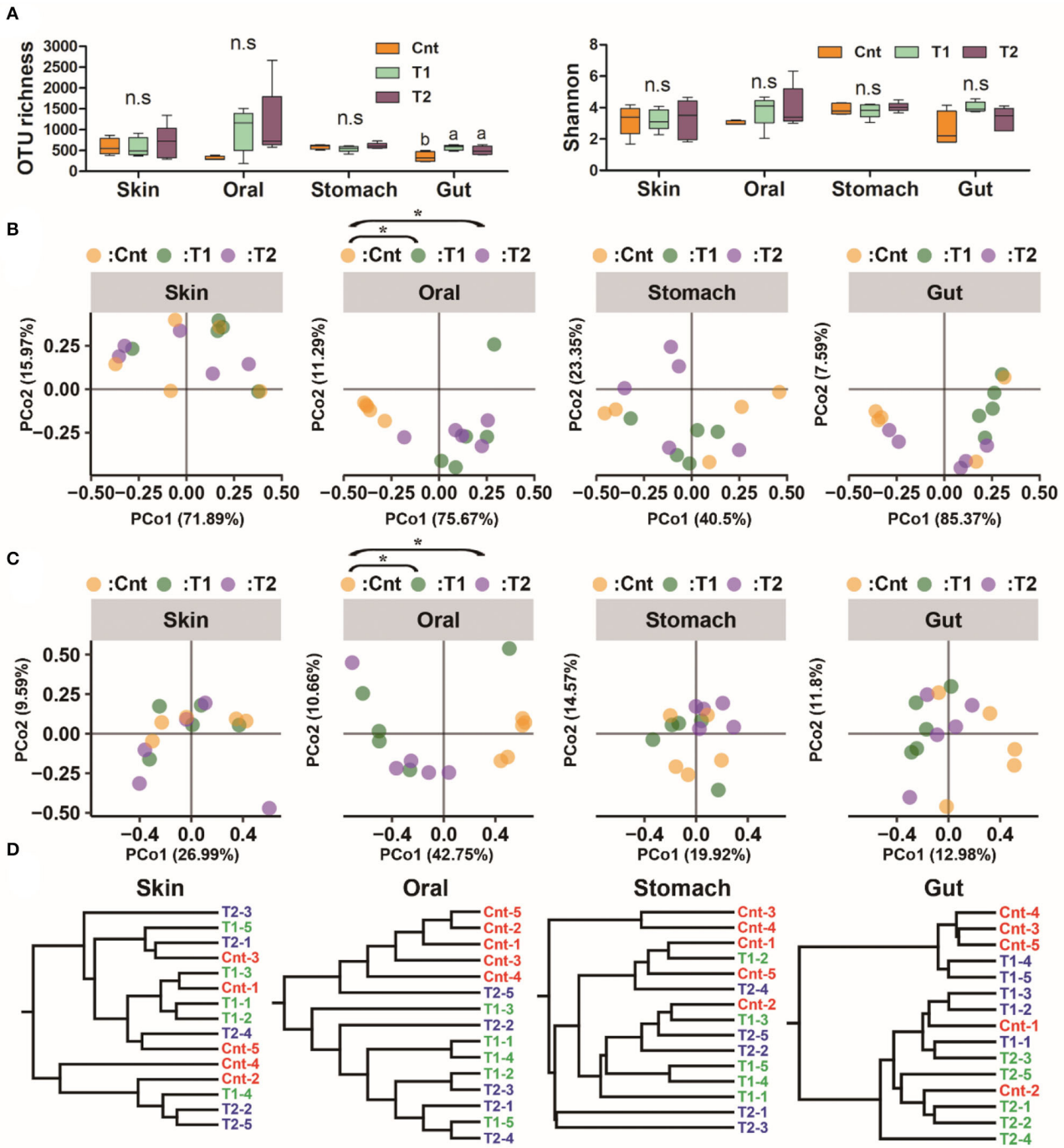


FIGURE 2 | Influence of pre-exposure on the symbiotic microbiota diversity of giant salamander. **(A)** Variation of microbiome alpha diversity (left: operational taxonomic units (OTU) number; right: Shannon index). Data are presented as means ± SE ($n = 5$). The influences of pre-exposure on alpha diversity were tested using a one-way ANOVA and the Benjamini–Hochberg (BH) correction at the level of corrected $p < 0.05$. Different superscripts denote significant differences between groups (LSD *post-hoc* tests at $p < 0.05$) if there is a significant influence. **(B,C)** PCoA scatter plots present the dissimilarity of microbiomes at the OTU level [**(B)**, based on weighted UniFrac distance; **(C)**, based on unweighted UniFrac distance]. *Corrected $p < 0.05$, PERMANOVA followed by BH correction. **(D)** Hierarchical clustering based on weighted UniFrac distance.

and treated groups (Cnt vs. T1 and Cnt vs. T2) (corrected $p >$ 0.05, PERMANOVA and BH correction). "Source-track" analyses indicated that the original skin and oral microbiomes were the major sources of the oral microbiomes from both T1 and T2 larvae, while the habitat sediment microbiota contributed less (**Figure 3A**). Correspondingly, the individual oral microbiome

(at phylum level) was more similar to the skin microbiome in the presence of sediment (**Figure 3B**). The weighted UniFrac distance between oral and gut microflora decreased significantly, while that between oral and stomach increased significantly ($p < 0.05$, one-way ANOVA, **Figure 3C**). PCoA and PERMANOVA of weighted UniFrac distance further indicated that the oral and skin microbiota showed increased similarity in the presence of wild sediment (**Figures 3D,E**).

The oral bacterial community of T1 and T2 groups was characterized by the increase of Proteobacteria while the decrease of Firmicutes (**Figures 4A,B**). The decrease of Firmicutes was mainly due to the reduced abundance of Bacilli at the class level, Lactobacillales at the order level, Lactobacillaceae at the family level, and *Lactobacillus* at the genus level (**Figures 4C,D**). The increase of Proteobacteria was mainly contributed by gamma-Proteobacteria at the class level, Burkholderiales and Chitinophagales at the order level, and Comamonadaceae, Chitinophagaceae, and Oxalobacteraceae at the family level (**Figures 4C,D**). These variations were accompanied by a significant increase in antifungal OTU proportions in the oral microbiota (corrected $p < 0.05$, one-way ANOVA and BH correction; **Figure 5**).

Transcriptional Changes of the Animal After Pre-exposure

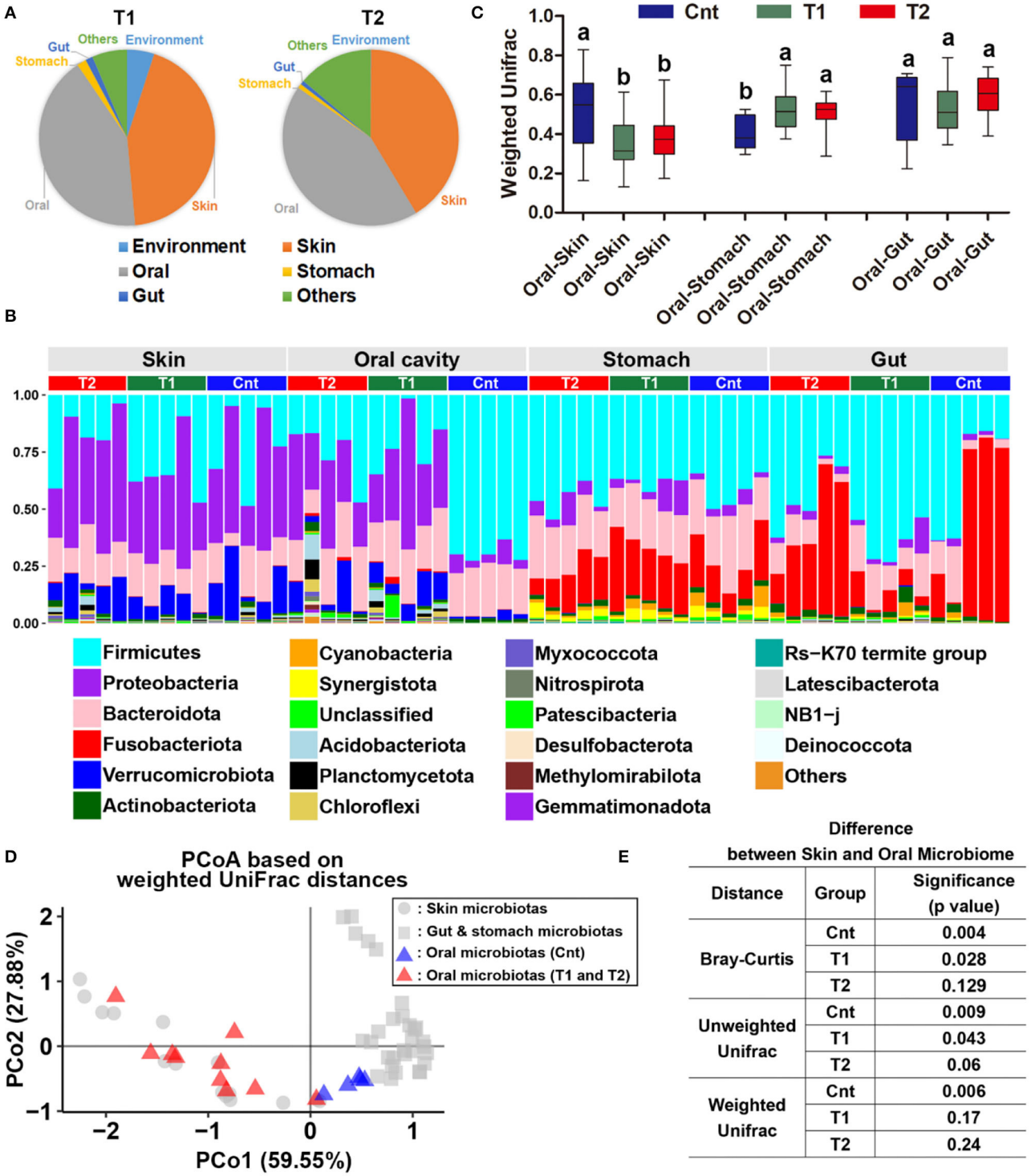
For the pairwise comparison between groups, there were more DEGs (corrected $p < 0.05$, *T*-test, and BH correction) between the control and treatment groups than those between the T1 and T2 groups for all four organs (**Supplementary Figure 4A**). The four organs shared few DEGs with each other, and the spleen showed the most dramatic variations in their transcriptomes (**Supplementary Figures 4B,C**). The cnt individuals could be distinguished from sediment-treated ones by the skin transcriptomes, and the Cnt, T1, and T2 individuals could be divided into their respective groups by the spleen, liver, or brain transcriptomes [principal component analysis (PCA) analyses, **Figure 6A**]. Functional enrichment analyses were conducted for DEGs ($p < 0.01$, one-way ANOVA) across Cnt, T1, and T2 groups. The top 10 enriched pathways (sorted by corrected *p*-value, KOBAS 3.0) of each organ were presented to show the primary transcriptional variations (**Figure 6B**). In the spleen, these pathways were mainly involved in immune response and infectious diseases (e.g., herpes simplex virus 1 infection, human T-cell leukemia virus 1 infection, and endocytosis). In the skin, most of these pathways were also related to immune responses and infectious diseases, including amoebiasis and the NOD-like receptor signaling pathway. Moreover, protein metabolic pathways were also highlighted, such as protein processing in ER and lysosome. In the liver, the top 10 enriched pathways were diverse in function, covering cellular proliferation (e.g., cell cycle and oocyte meiosis) and numerous signaling transduction (e.g., FoxO signaling pathway and Apelin signaling pathways). However, immune-related pathways (i.e., tuberculosis and human immunodeficiency virus 1 infection) were still involved. In the brain, oxidative phosphorylation (OPP) was highlighted. In addition, OPP enzymes were also responsible for the enrichment of many other pathways (e.g., thermogenesis, Alzheimer's disease, Huntington's disease, and cardiac muscle contraction). Moreover, functional enrichment analyses also highlighted many immune-and disease-related pathways (e.g., human papillomavirus infection, chemokine signaling, and Fc epsilon RI signaling pathway) (**Supplementary Figure 5**). Further analyses classified the significantly enriched pathways (corrected $p < 0.05$) into functional categories defined in the KEGG database. At the primary classification level, the "Human diseases" category accounted for the largest number of enriched pathways in the spleen, liver, and skin (**Figure 6C**). At the secondary classification level, "Immune system" and "Infectious diseases" occupied large proportions in these three organs (**Figure 6D**).

The variation trends were presented for DEGs ($p < 0.01$, one-way ANOVA) with closely related functions. In the spleen and liver, DEGs enriched in immune-related pathways showed an overall upregulation in treated larvae, and the T1 group showed more prominent upregulation than the T2 group (**Figures 7A,B**). This variation pattern was still presented when focused on DEGs with definite immune functions (e.g., interleukin-18, cathelicidin, and immunoglobulin; screened manually) (**Figures 7A,B**). In the skin, the treatment caused an overall upregulation of immune (most identified inflammatory cytokines) and proteolytic and apoptotic components (e.g., most of the identified ubiquitination components and apoptosis-inducing factors) (**Figures 7C,D**). No significant difference existed in the transcriptional level of these genes between the T1 and T2 groups. In the brain, the OPP enzymes showed prominent upregulation in the T1 group, but not in the T2 group (**Figure 7E**). In addition, the differently expressed immune-related genes (e.g., MHC, pattern recognition proteins, and pro-inflammatory factors) were screened, and most of these genes in the liver, spleen, and skin showed increased transcription after pre-treatment (**Supplementary Figure 6**).

DISCUSSION

Compositional and Functional Shift of Symbiotic Microbiome

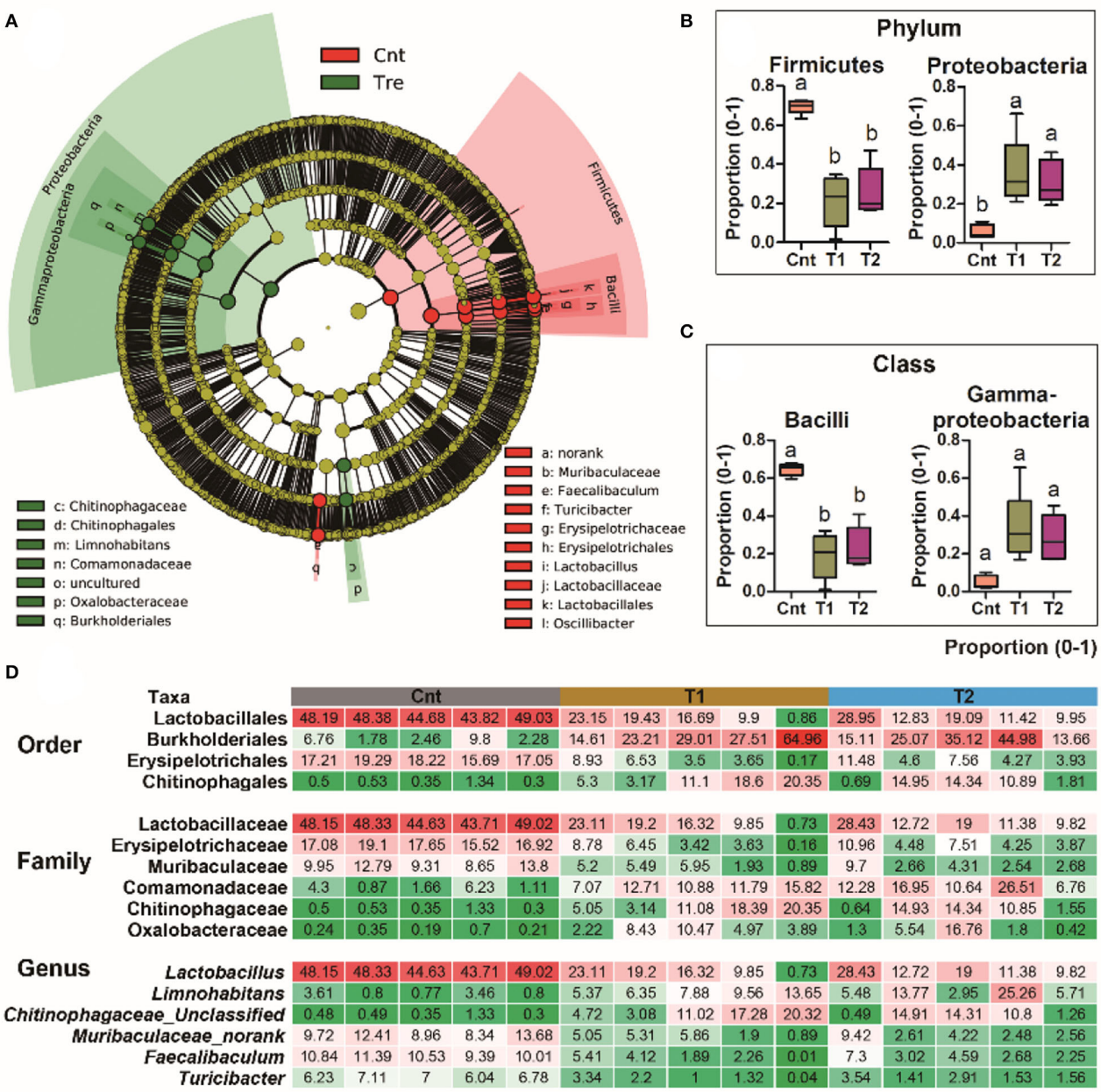
Our results provided a detailed description of the community structure of multiple symbiotic microbiotas in captive *A. davidianus*. Consistent with our previous studies (Zhang et al., 2019; Zhu et al., 2021), Firmicutes, Bacteroidetes, Fusobacteria, and Proteobacteria were the dominating bacteria in the stomach and gut microbiome of *A. davidianus*. During pre-exposure, a large amount of new bacterial taxa can be brought to the living environments of captive individuals (**Figure 1**), providing opportunities for these new microbes to colonize the skin and digestive tract of the host (i.e., captive *A. davidianus* larvae). However, no significant change in the bacterial community was observed in the skin, stomach, and gut after the treatment (**Figure 2**). It suggested that the symbiotic microflora in the skin, stomach, and gut of captive individuals is relatively stable. The skin microbiome of amphibians has been proved to play an important role in their innate immunity (**Figure 5**; Woodhams et al., 2007; Colombo et al., 2015; Varga et al., 2019; Douglas et al.,



Difference between Skin and Oral Microbiome

Distance	Group	Significance (p value)
Bray-Curtis	Cnt	0.004
	T1	0.028
	T2	0.129
Unweighted Unifrac	Cnt	0.009
	T1	0.043
	T2	0.06
Weighted Unifrac	Cnt	0.006
	T1	0.17
	T2	0.24

FIGURE 3 | Reorganization of the oral bacterial community. **(A)** Source-tracking analyses for the oral microbiota of larvae in treatment groups. The habitat/environment and original commensal microbiota were considered as the bacteria sources. Since the control group faced no variation of environmental conditions during treatment duration, their commensal microbiota was considered the original commensal microbiota of captive *Andrias davidianus*. **(B)** Variation of weighted UniFrac distances between oral and other commensal microbiota after treatment. Data are presented as means ± SE. Different superscripts denote significant differences between groups ($p < 0.05$, one-way ANOVA and LSD *post-hoc* tests). **(C)** Bar plots represent the variation of microbiome similarity (at phylum level) between control, T1, and T2 groups. **(D)** A PCoA scatter plot presenting the variations of oral microbiota in the similarity to the skin, gut, and stomach microbiotas (Weighted UniFrac distance). **(E)** Results of PERMANOVA.



D

	Taxa	Cnt					T1					T2				
Order	Lactobacillales	48.19	48.38	44.68	43.82	49.03	23.15	19.43	16.69	9.9	0.86	28.95	12.83	19.09	11.42	9.95
	Burkholderiales	6.76	1.78	2.46	9.8	2.28	14.61	23.21	29.01	27.51	64.96	15.11	25.07	35.12	44.98	13.66
	Erysipelotrichales	17.21	19.29	18.22	15.69	17.05	8.93	6.53	3.5	3.65	0.17	11.48	4.6	7.56	4.27	3.93
	Chitinophagales	0.5	0.53	0.35	1.34	0.3	5.3	3.17	11.1	18.6	20.35	0.69	14.95	14.34	10.89	1.81
Family	Lactobacillaceae	48.15	48.33	44.63	43.71	49.02	23.11	19.2	16.32	9.85	0.73	28.43	12.72	19	11.38	9.82
	Erysipelotrichaceae	17.08	19.1	17.65	15.52	16.92	8.78	6.45	3.42	3.63	0.16	10.96	4.48	7.51	4.25	3.87
	Muribaculaceae	9.95	12.79	9.31	8.65	13.8	5.2	5.49	5.95	1.93	0.89	9.7	2.66	4.31	2.54	2.68
	Comamonadaceae	4.3	0.87	1.66	6.23	1.11	7.07	12.71	10.88	11.79	15.82	12.28	16.95	10.64	26.51	6.76
	Chitinophagaceae	0.5	0.53	0.35	1.33	0.3	5.05	3.14	11.08	18.39	20.35	0.64	14.93	14.34	10.85	1.55
	Oxalobacteraceae	0.24	0.35	0.19	0.7	0.21	2.22	8.43	10.47	4.97	3.89	1.3	5.54	16.76	1.8	0.42
Genus	*Lactobacillus*	48.15	48.33	44.63	43.71	49.02	23.11	19.2	16.32	9.85	0.73	28.43	12.72	19	11.38	9.82
	Limnohabitans	3.61	0.8	0.77	3.46	0.8	5.37	6.35	7.88	9.56	13.65	5.48	13.77	2.95	25.26	5.71
	Chitinophagaceae_Unclassified	0.48	0.49	0.35	1.33	0.3	4.72	3.08	11.02	17.28	20.32	0.49	14.91	14.31	10.8	1.26
	Muribaculaceae_norank	9.72	12.41	8.96	8.34	13.68	5.05	5.31	5.86	1.9	0.89	9.42	2.61	4.22	2.48	2.56
	Faecalibaculum	10.84	11.39	10.53	9.39	10.01	5.41	4.12	1.89	2.26	0.01	7.3	3.02	4.59	2.68	2.25
	Turicibacter	6.23	7.11	7	6.04	6.78	3.34	2.2	1	1.32	0.04	3.54	1.41	2.91	1.53	1.56

FIGURE 4 | Differential analyses of the oral microbiota between control and treatment groups. **(A)** A LEfSe analysis on the oral bacteria composition between control and treatment groups. **(B,C)** The primary variations at phylum and class levels. Data are presented as boxplots ($n = 5$). Different superscripts denote significant differences between groups ($p < 0.05$, one-way ANOVA and LSD *post-hoc* tests). **(D)** Heatmap presents the major variations of microbiota at order, family, and genus levels. The values denote the abundances in percentages.

2020), and the gut microbiome is responsible for maintaining gut metabolic homeostasis and coordinating immune responses (Kau et al., 2011; Shapira, 2016). Their stability may benefit the host to prevent the colonization of pathogens (Lauer et al., 2008; Harris et al., 2009; Kung et al., 2014).

Unlike the situation for the skin, stomach, and gut, the oral microflora showed prominent reorganization in treated individuals. It seemed that the oral microbiome was more flexible in composition than that of the skin, stomach, and gut. The oral cavity is a transitional region linking the external environment and the intestinal space. Oral microbiota is affected by diet, age, and environmental factors, and synergy and interaction of variable oral microorganisms can help the host against the invasion of undesirable stimulation outside (Gao et al., 2018). In humans, the variation of oral microbiota is associated with oral diseases (e.g., caries and mucosal diseases) and systemic

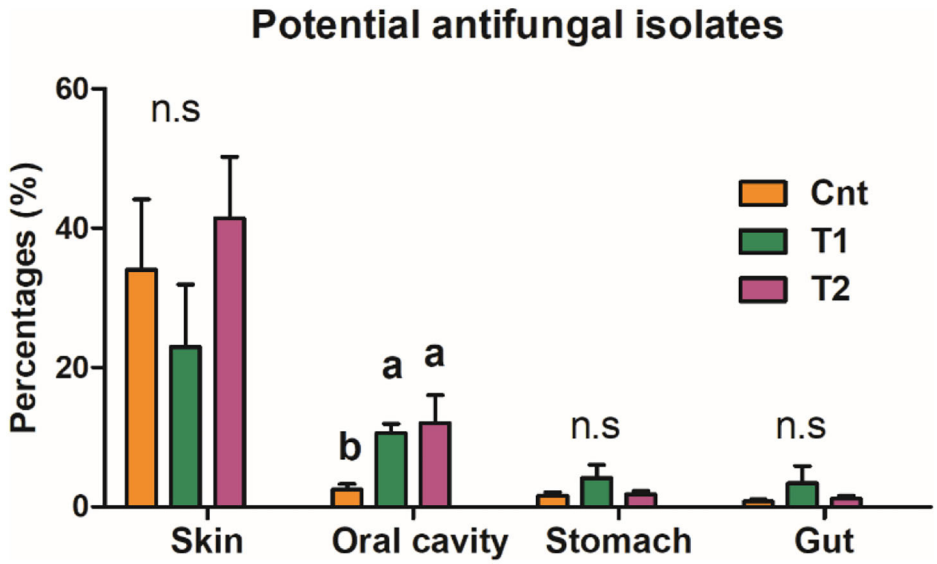


FIGURE 5 | Proportion of antifungal OTUs in commensal microbiota. The influences of pre-exposure on the abundance of antifungal isolates were tested by a one-way ANOVA and the BH correction at the level of corrected $p < 0.05$. Different superscripts denote significant differences between groups (LSD *post-hoc* tests at $p < 0.05$) if there is a significant influence.

diseases (e.g., diabetes and obesity) (Gao et al., 2018; Mameli et al., 2019; Frias-Lopez and Duran-Pinedo, 2020). This is suggestive of the immune and metabolism-regulatory functions of the oral microbiota. Pre-exposure to the soil sediment caused the dominating oral bacteria to change from Firmicutes to Proteobacteria (**Figure 2**). A high proportion of Firmicutes is associated with increased nutrient absorption and energy storage in mammals (Turnbaugh et al., 2006; Krajmalnik-Brown et al., 2015; Dugas et al., 2016). In addition, it has been reported that there is an association between obesity and increased abundance of Firmicutes in oral biofilm in humans (Zeigler et al., 2012). Specifically, the reduction of Firmicutes and Bacilli in the oral cavity was largely contributed by the loss of Lactobacillus, which has been used as probiotics to promote growth and survival in the breeding industry (Venkat et al., 2004). Taken together, the oral microbiota of the captive individuals seemed to be in favor of host nutrient absorption and growth. This might be a result of long-term adaptation to an artificial environment, where animal growth rate and body weight were pursued. In contrast, Proteobacteria is a typical dominating phylum in the skin microbiota of amphibians (Catenazzi et al., 2018), which is related to amphibian defense and immunity (Woodhams et al., 2007; Colombo et al., 2015; Varga et al., 2019; Douglas et al., 2020). The increased proportion of Proteobacteria in the oral microbiome was largely contributed by the raise of gamma-Proteobacteria. This class contains higher proportions of anti-fungi strains than others (Catenazzi et al., 2018; Rebollar et al., 2019, 2020). In fact, the abundance of anti-fungus strains indeed increased after pre-exposure. Thus, we might expect increased capacity in fungal pathogen defense of the pre-adapted oral microbiome. Such a structural shift in oral microbiota might be beneficial to coping with the complicated microbial community of the natural habitats.

Importantly, this change was not contributed by the introduction of exogenic microorganisms (**Figure 3A**). This was supported by the fact that the changes in oral microbiota were observed no matter whether the soil sediment was sterilized or not. The previous study has indicated that the oral and skin microbiomes of amphibians and captive Komodo dragons are strongly affected by the environment (Hyde et al., 2016; Xu et al., 2020). One of the explanations for our observation was that prolonged treatment was required to allow the colonization of the environmental microbiota to the captive *A. davidianus*, especially when considering the low dosage of the sediment introduced into the experimental system. Further pre-exposure studies should take the developmental stage or age into consideration, which can shape the gut microbiota of *A. davidianus* (Zhang et al., 2019), as it is possible for variation in the plasticity of symbiotic microbiota with age in response to the environment. Our results suggested a significant contribution of skin microbiota to the change of oral microbiota when exposed to the habitat sediment. This was reasonable because there is a robust exchange between skin and oral microbiota in animals (Buerger, 2020). The easy transmission of microbes from the skin to oral microbiota may be explained by the "old friends" hypothesis (Rook, 2013), which postulates that the members of the symbiotic microbiome would be identified as non-pathogenic by the immune system during an exchange event.

The Transcriptional Changes of Immune and Nervous Systems in the Host

Our data provided an insight into the transcriptional architecture of multiple organs of *A. davidianus*. Pre-exposure to habitat

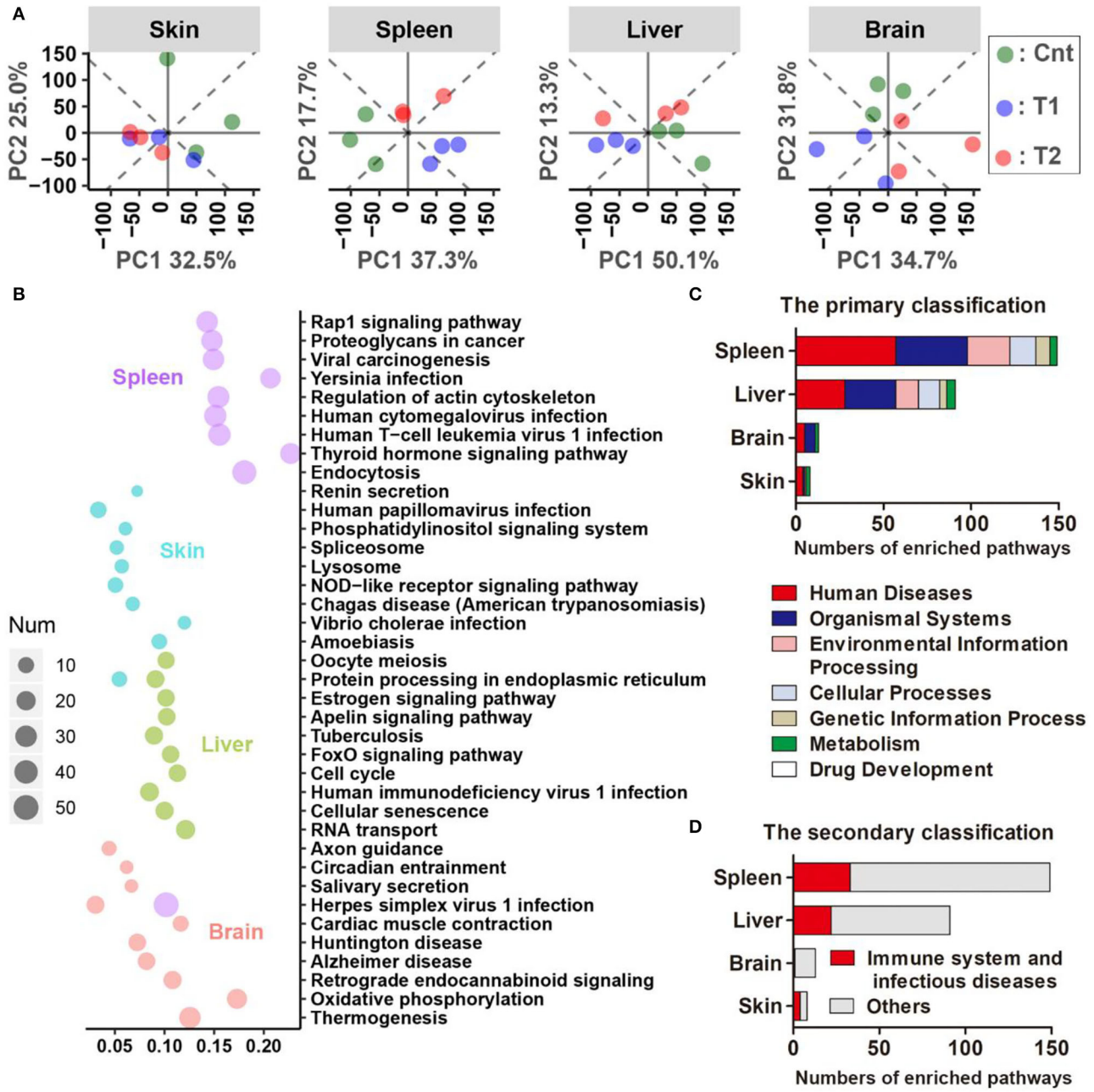


FIGURE 6 | Results of comparative transcriptomic analyses. **(A)** Principal component analysis (PCA) scatter plots present the dissimilarity of the transcriptional profile between groups. **(B)** The top 10 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (ordered by *q*-value) of each organ are enriched by the differently expressed genes (DEGs) ($p < 0.01$, one-way ANOVA). The size of each dot denotes the number of DEGs enriched in each pathway. The horizontal axis stands for the enrichment cover rate of each pathway (enriched gene number/total gene number of this pathway). It should be noted that many immune pathways in the KEGG database are named after the pathogens, but they may share considerable immune proteins in their signal transduction networks. For example, the highlight of the Herpes simplex virus 1 pathway might not mean the infection of this virus in the giant salamander, but it emphasizes that there were a lot of immune-related genes that varied after pre-exposure. **(C,D)** Statistics on the functional category of all the significantly enriched pathways (corrected $p < 0.05$). KEGG pathways are classified into seven categories at the primary classification level. Each primary category is further divided into secondary classes according to its cellular function (https://www.kegg.jp/kegg/pathway.html).

sediments caused dramatic transcriptional variations in the brain, liver, spleen, and skin of captive *A. davidianus* (**Figure 6A**). In the skin, the variations of genes were characterized by the upregulation of inflammatory systems (**Figure 7C**, **Supplementary Figure 6**), indicating the possibility of pathogenic stimulus (Akira et al., 2006; Chen et al., 2018).

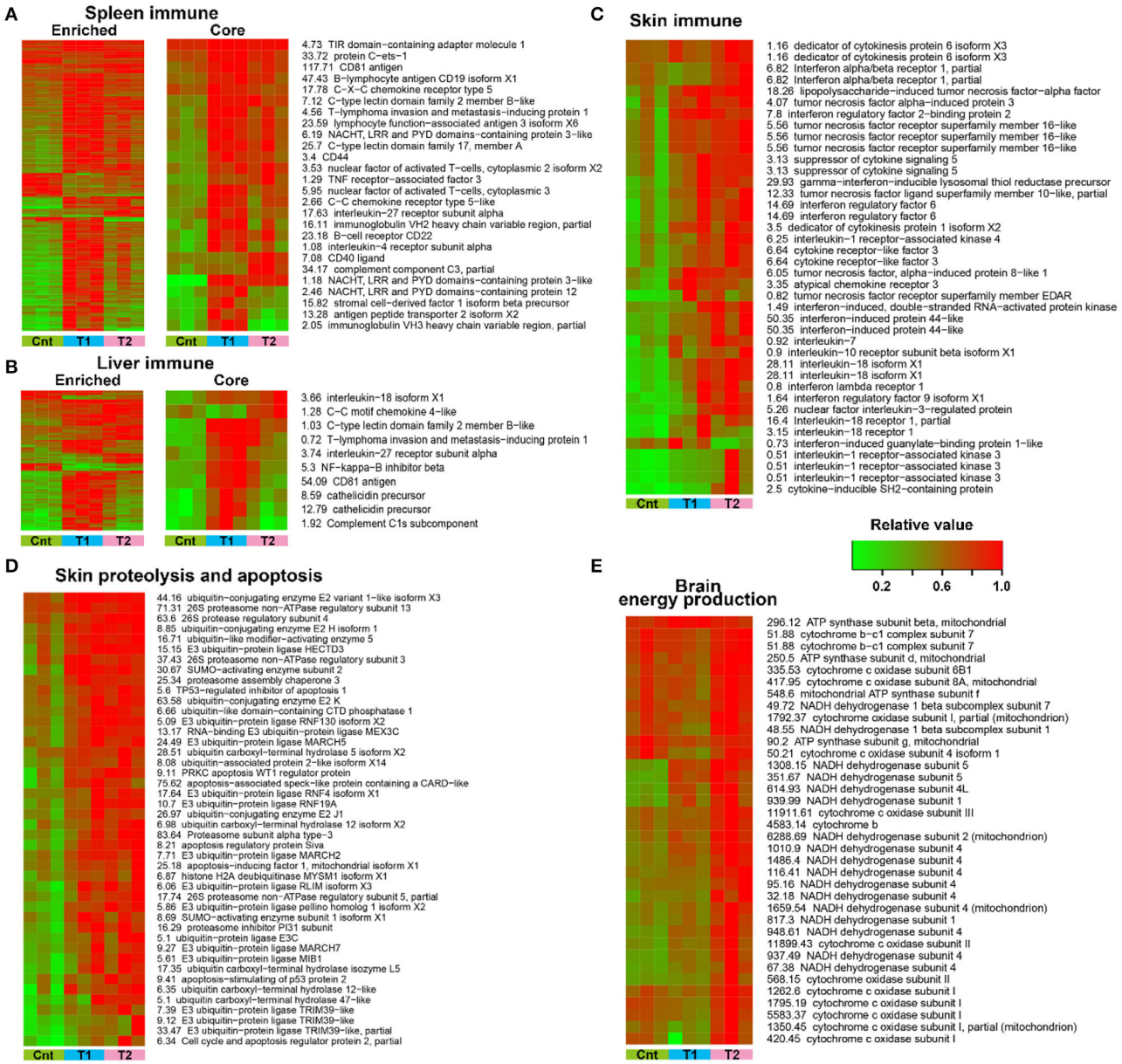


FIGURE 7 | Transcriptional variation trends of crucial and significantly enriched cellular processes. **(A,B)** Expression pattern of DEGs enriched in immune-related pathways in the spleen **(A)** and liver **(B)**. The core immune genes refer to those with defined immune functions. **(C–E)** Expression pattern of inflammation-related DEGs in the skin **(C)**, proteolysis and apoptosis-related DEGs in the skin **(D)**, and energy metabolic DEGs in the brain **(E)**.

Meanwhile, the ubiquitin-dependent proteolysis and apoptosis were transcriptionally activated (**Figure 7D**), suggesting the accelerated turnover of skin tissue. It might be an approach to void exogenous microorganism colonization by promoting antigen recognition and pathogen elimination (Goldberg and Rock, 1992; Miyairi and Byrne, 2006; Sasakawa and Hacker, 2006). Further validation was necessary to verify whether there was a causation relationship between transcriptional activation of inflammation and proteolysis. Existing studies indicate that dead pathogen stimulation can also enable a similar magnitude of acquired resistance for amphibian skin (Mcmahon et al., 2014). This might explain why similar transcriptional changes were also detected in the skin of T1 individuals, where the soil sediment was sterilized before treatment. The spleen is responsible for adaptive immune response through humoral and cell-mediated pathways in vertebrates (Mebius and Kraal, 2005). The upregulation of immune-related genes (**Supplementary Figure 6**) in the spleen likely suggested enhanced immune function in *A. davidianus* larvae after exposure to habitat sediment. It was interesting to detect a robust immune response in the spleen and the liver of

individuals from the T1 group (**Figures 7A–C**). This might be attributed to the high temperature (during sterilization) that could break down the cellular structure of microorganisms, as well as the spatial structure of macromolecules, thus causing the exposure of hidden antigen epitopes, which would induce strong local immune stimulation (Cattoretti et al., 1993). Additionally, the killed microorganisms could be broken into small peptides or oligosaccharides with antigen epitopes by digestive enzymes. Additionally, these fragments might be transported into the host to activate systematic immune responses (such as the oral vaccines) (Brayden et al., 2005). In the brain, individuals from the T2 group showed transcriptional upregulation of OPP enzymes (**Figure 7E**), suggesting the improved activity of the central nervous system. Considering the absence of this phenomenon in T1 individuals, we guessed that this transcriptional change likely resulted from the stimulation of living organisms or heat-sensitive components from the sediments. It may be explained by the microbiota–gut–brain axis, which describes the influence of gut microbiota on the host neuroendocrine system by generating metabolic products or interacting with the immune system (Foster and Mcvey Neufeld, 2013; De Vadder et al., 2014; Yano et al., 2015; Sharon et al., 2016). The specific inducement for these transcriptional variations and the underlying mechanisms require further investigation. In addition, increased energy metabolism in the brain likely improves the capacity of information processing (e.g., foraging) to adapt to complicated environments (Lefebvre et al., 1997; Dunbar and Shultz, 2007). Overall, these results suggested that

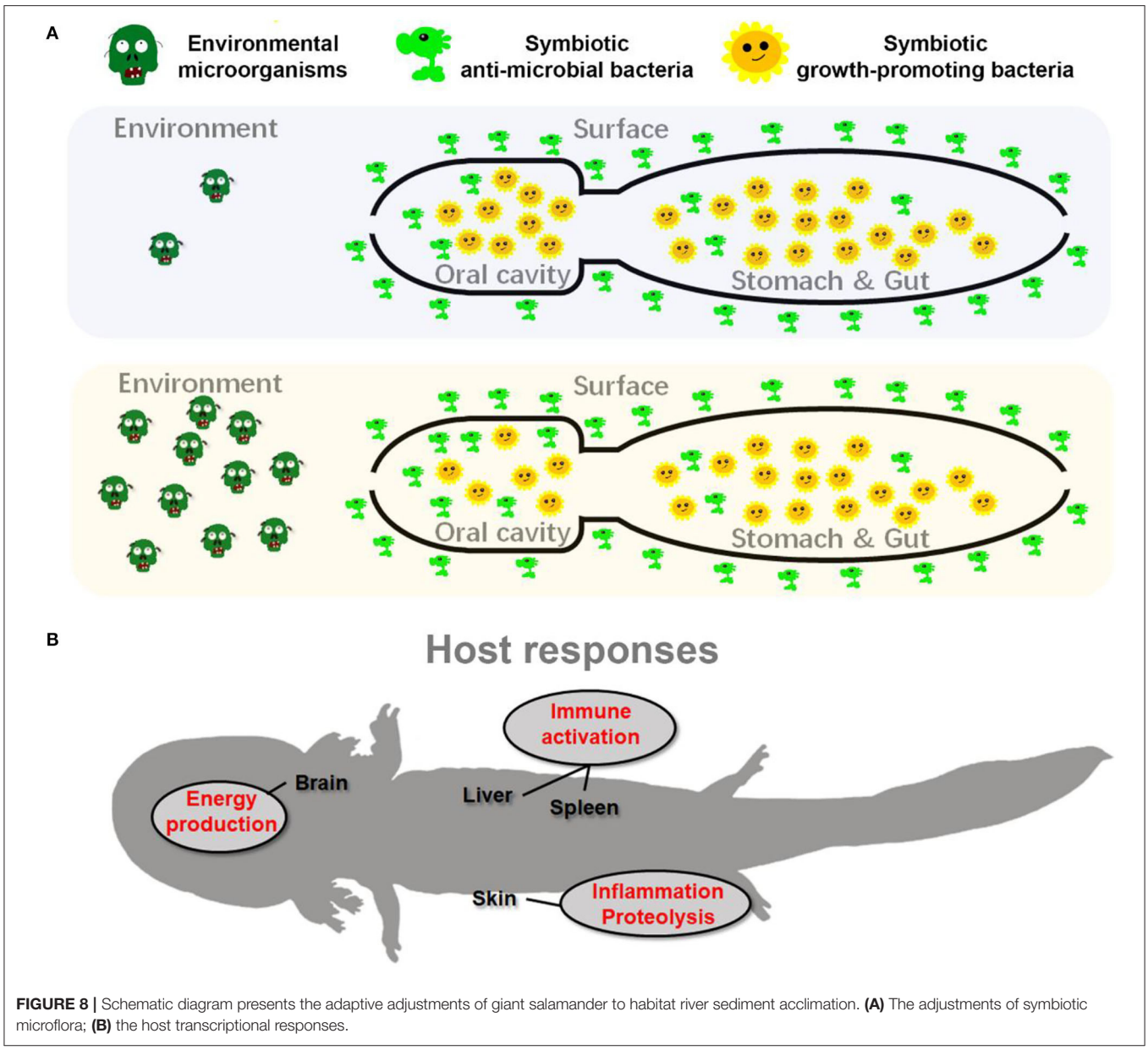


FIGURE 8 | Schematic diagram presents the adaptive adjustments of giant salamander to habitat river sediment acclimation. **(A)** The adjustments of symbiotic microflora; **(B)** the host transcriptional responses.

pre-exposure to habitat sediments might have beneficial effects on captive *A. davidianus* by pre-activating the immune systems and brain activity (**Figure 8B**).

Implications for *A. davidianus* Reintroduction

Our results indicated that pre-exposure to habitat sediments could induce a series of physiological responses in captive *A. davidianus* (**Figure 8**). Although these adjustments might also be induced in larvae released to the field without pre-exposure, the expression degree of these adaptive traits might be limited. The major limitation may lie in the nutrient acquirement and energy budget. From the perspective of symbiotic microbiota, the reorganization of oral microflora may be related to the improved pathogen defense along aside from decreased nutrient absorption of these animals (**Figure 8A**). From the perspective of the host, maintaining brain metabolism and immune responses account for a large proportion of the body's energy requirement (Rolfe et al., 1997; Wang A. et al., 2019; Cunnane et al., 2020). Upregulation of the brain and immune activities during pre-exposure would constrain the energy budget in *A. davidianus* larvae. For larvae reintroduced to the field, we might not expect good nutrient status for their unskilled foraging and hunting at the beginning. Consequently, the nutrient status of the *A. davidianus* larvae might either limit the expression of these adaptive traits or impact the individual growth. Pre-exposure in an artificial environment where food can be provided sufficiently, which likely benefits their activation of adaptive responses. It can guarantee the investment in immune function and somatic growth simultaneously and thus improve their survival rate (**Supplementary Figure 3**). Therefore, we argued that pre-exposure to the field environmental microbiota can be included in the standard procedure of captive-bred *A. davidianus* larvae reintroduction. However, despite experimental approaches supporting the effectiveness of pre-exposure to the reorganization of symbiotic microbiota and host transcriptomes in captive-bred *A. davidianus* larvae, long-time field monitoring is still needed in the future to quantify its advantages in reintroduction activities.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: http://bigd.big.ac.cn/gsa/s/B7y0wob0, PRJCA004234.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethical and Welfare Committee of Chengdu Institute of Biology, Chinese Academy of Science.

AUTHOR CONTRIBUTIONS

WeiZ, TZ, and JJ conceived the project. TZ, CZ, JC, JF, WenZ, FX, and CL collected the samples. CZ, WeiZ, JF, and WenZ performed the experiments. WeiZ, LC, and JL analyzed the data. WeiZ and TZ wrote the manuscript. All authors edited and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.884880/full#supplementary-material

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The Gut Microbiome of 54 Mammalian Species

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The gut microbiome plays a critical role in many aspects of host life, and the microbial community composition is heavily influenced by the prevailing conditions in the gut environment. Community composition has been suggested to have large implications for conservation efforts, and gut health has become of interest for optimizing animal care in captivity. In this study, we explore the gut microbiome of a wide range of animals in the context of conservation biology. The composition of the gut microbial community of 54 mammalian animal species was investigated using 16S rRNA gene amplicon sequencing. The composition of the gut microbiota clearly reflects diet and the structure of the gastrointestinal system, and it is to a certain degree more similar between closely related animals. Specific clusters of taxa were observed across animals of the same species, diet, and gut morphology. The microbiota retained regardless of captivity status is hypothesized to cover important symbiotic relationships with the host, while the remaining part reflects the artificial living conditions and can therefore be used as a future tool for conservation biologists. For five animal species (giraffes, horses, baboons, elephants, and zebras), it was possible to compare the microbiota of wild and captive individuals. Differences were observed in the proportion of microbiota detected between wild and captive specimens of the same animal species. We propose that the gut microbiota harbours important species, which can potentially serve as indicators for the well-being of the animal and the effect of living in captivity.

Keywords: gut microbiota, diet, captivity, gut physiology, mammals, conservation biology

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INTRODUCTION

The gut microbiome has an important role in relation to the health and well-being of the host, primarily for its role in regulating nutrient and energy uptake through assisting in the energy uptake by facilitating digestion of complex food items (Stevens and Hume, 1998). But the microbiome has also been linked to other processes such as the development and maintenance of the immune system, behaviour, and reproduction (Kamada et al., 2013; McFall-Ngai et al., 2013; Bahrndorff et al., 2016; Pickard et al., 2017). Furthermore, the gut microbiome also protects the host from the establishment of potential pathogens (Pickard et al., 2017; Kogut et al., 2020), thereby functioning as a barrier. This symbiotic relationship has co-evolved over numerous generations, and the composition of the microbiome is influenced by factors relating to host physiology and ecology, as well as other biotic and abiotic environmental factors (McFall-Ngai et al., 2013; Moeller and Sanders, 2020).

Conservation efforts such as breeding or rehabilitation have a significant impact on the lives of the animals concerned (Bahrndorff et al., 2016). Animals living in captivity often experience

extreme conditions, including an altered diet and social structure, compared to the environment in which they co-evolved (Redford et al., 2012). Furthermore, in human-made environments, increased population densities, contact with humans, medical treatment, and antibiotic administration in particular are also introduced (Kirkwood, 2003). All of these changes can contribute to the phenomena of selective pressure (pressure relaxation or increased pressure from altered diet composition) and can influence the composition of the gut microbiota and potentially cause negative effects on the health of animal health (Redford et al., 2012; Hauffe and Barelli, 2019). In addition, inbreeding has also been shown to have a negative impact on many aspects of animal life, including the gut microbiota (Bahrndorff et al., 2016). It is hypothesized that a better understanding of the effects of captivity on an animal's microbiome is important for providing optimal care, specifically in regards to health and general welfare (Bahrndorff et al., 2016; Hauffe and Barelli, 2019).

The gastrointestinal system and co-habiting microbiota of vertebrate hosts have co-evolved alongside the host, becoming specialized to the nutritional needs and preferences of each species (Stevens and Hume, 1998; Moeller and Sanders, 2020). The earliest evolved mammals had a simple gut system, a gut morphology that is still observed in many animals with a meat-based dietary preference such as representatives of the order Carnivora (Van Valkenburgh, 2007), as well as a number of omnivores including pigs and humans (Stevens and Hume, 1998). Animals are not able to produce the endogenous cellulolytic enzymes required to digest plant-based materials and rely on symbiosis with their gut microbiota to achieve this, as well as similar enzyme-dependent tasks, leading to a more complex gastrointestinal tract development (and microbiota) compared to carnivores (Ley et al., 2008; Bayané and Guiot, 2011). Strict herbivores can digest their food by either hindgut (e.g., horses and elephants) or foregut fermentation (e.g., ruminants such as giraffes and kangaroos) (Stevens and Hume, 1998; Bayané and Guiot, 2011). Gut morphology and dietary preferences cannot be strictly divided into specific orders of animals, as some species within the same order have developed specialized dietary strategies (and thus microbiota) over time (Stevens and Hume, 1998; McFall-Ngai et al., 2013). Prominent examples of this type of specialization are the giant pandas, which consume a strictly herbivoric diet while possessing a simple gut morphology (Van Valkenburgh, 2007; Li et al., 2015), and the koala, which lives almost exclusively on foliage from the genus *Eucalyptus* (Brice et al., 2019).

High-throughput sequencing technology has made it possible to gain insight into the gut microbiota of a diverse range of animal species, including primates (Amato et al., 2013; Clayton et al., 2016; Greene et al., 2021), carnivores (An et al., 2017), reptiles (Tang et al., 2020), and birds (Roggenbuck et al., 2014; García-Amado et al., 2018), among others. The majority of all microbiome studies have been conducted on single species, which provide excellent conditions for investigating short-term exposure effects such as diet changes. Studies across multiple species, on the other hand, allow investigations into evolutionary dependencies such as the effects of phylogenetic traits (including gut morphology) (Ley et al., 2008) and universal feeding strategies. Important parameters for shaping the gut microbiota composition in individual animal species have been shown to include dietary preferences (Muegge et al., 2011; Poulsen et al., 2017), and to a lesser degree, also factors such as sex, social interaction, biogeography, and the individual's genetic profile (Yatsunenko et al., 2012; Moeller et al., 2013; Song et al., 2013; Yuan et al., 2015; Eisenhofer et al., 2021).

A number of studies have compared the microbiota of wild and captive animals. Studies in diverse animal species, including Antarctic seals (Nelson et al., 2013), various primates (Amato et al., 2013; Hale et al., 2019; Greene et al., 2021), and horses (*Equus ferus caballus*) (Metcalf et al., 2017) showed significant differences in the composition of the gut microbiota in wild individuals compared to captive relatives. Another study showed that primates living in captivity gain a more human-like microbiota composition over time (Clayton et al., 2016), and that this is reproducible between captive populations (Houtz et al., 2021). These results support the hypothesis that transfer of animals from their natural habitat to captivity can induce rapid and extensive changes to the gut microbiota composition and thereby affect other aspects of animal welfare. However, many unanswered questions relating to the effect of habitat changes on gut microbiota in animals still exist, as a meta-analysis study has also shown that the differences between wild and captive microbiomes are heterogeneously distributed and cannot simply be generalized across species (Alberdi et al., 2021).

In studies investigating a large number of animal species, it is possible to compare microbiota between groups or habitats (Ley et al., 2008; McKenzie et al., 2017; O'Donnell et al., 2017; Youngblut et al., 2019; Alberdi et al., 2021). A frequent observation has been a reduced microbial diversity in captive individuals compared to their wild counterparts in some animal species (Kohl et al., 2014), while others have observed species-specific differences (McKenzie et al., 2017; Alberdi et al., 2021). Besides changes to the overall microbiota composition in the gut of captive animals, the functional composition of the microbiota has been suggested to be even more important (Lozupone et al., 2012), with species-specific responses potentially determining which animals adapt better to changes than others. However, this aspect of the animal gut microbiome remains largely unexplored (Hauffe and Barelli, 2019). In summary, overarching trends in the microbiota of wild animals and captive relatives have been observed, but many questions related to the specific response of animal gut microbiota to living in captivity and the effects on the host remain unanswered.

The aim of this study was to investigate the gut microbiota of mammalian animal species and across a wide cohort to explore the influence of phylogeny, gut morphology, and dietary choices on the microbial community composition. The microbiomes of 54 mammalian species from captive and wild habitats were analyzed using 16S rRNA gene amplicon sequencing, and alpha and beta diversity indices between the different animal species were explored in detail. Differences in the gut microbiomes of animals of the same species living in captivity were compared, as well as that of captive animals and their wild relatives to investigate the effects of captivity.

METHODS

Sample Collection

Samples from animals living in captivity at four different zoos in Denmark ($n = 52$) and Norway ($n = 4$) were collected in the period of October 2016 to July 2018. Fecal deposits were collected within 1 h of deposition, and where possible, only the interior of the fecal deposit was sampled to limit potential contamination. Samples were transported directly to the laboratory on ice, or when shipped cross-border, stored in RNAlater (Sigma-Aldrich), and immediately stored at −18°C upon arrival. In addition, samples from wild animals were obtained from various locations in Denmark, in Tanzania, and in Zimbabwe ($n = 22$). These samples were collected based on normal appearance (color, odor, and consistency) and the criteria that no significant evidence of exposure to the environment and dehydration were visible, and they were transported in 96% ethanol and under cold conditions where possible. An overview of all samples included in the study is shown in **Supplementary Table S1**. All samples were stored at −18°C until further processing.

Ethical approval for obtaining animal fecal samples was not required as per national guidelines. A written statement from Aalborg Zoo's Veterinary Service was obtained, declaring that no animals experienced any break from disturbances in their daily routines during sampling for this study.

DNA Extraction

All samples were gently homogenized by stirring to obtain a representative subsample of approximately 0.5 g. Total genomic DNA was extracted using the FastDNA Spin kit for soil (MP Biomedicals) following the manufacturer's instructions, with hot phenol pre-treatment as described elsewhere (Albertsen et al., 2013). The quality of the DNA extracts was assessed using a TapeStation 2200 and Genomic DNA ScreenTapes (Agilent), and their concentration was estimated using a Qubit 3.0 fluorometer and Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific).

16S rRNA Gene Amplicon Sequencing

The amplicon sequencing approach using the 16S ribosomal RNA gene as a phylogenetic marker was chosen as it provides an efficient and cost-effective approach for microbiome analysis. The hypervariable V4 region of the 16S rRNA gene was amplified using the welldescribed primer sets 515F GTGCCAGCMGCCGCGGTAA and 806R GGACTACHVGGGTWTCTAAT (Caporaso et al., 2011) fused with Illumina adapters. Genomic DNA (10 ng) was amplified in 25 μL duplicate PCR reactions, as previously described (Bahrndorff et al., 2018). Equimolar amounts of all sample libraries were sequenced on an Illumina MiSeq platform using reagent kit v3 (2 × 300 PE) and a 20 % Phi-X spike-in. Raw sequence data were treated using the AmpProc pipeline (v5.1) (https://github.com/eyashiro/AmpProc). In brief, USEARCH11 was used for read quality filtering, PhiX removal, chimeric and spurious read removal, and merging of paired-end reads (Edgar, 2010). Amplicon sequencing variants (ASVs) were generated using the UNOISE3 algorithm (Edgar, 2016b). Taxonomy was assigned using SINTAX (Edgar, 2016a) and using SILVA release S138 as the reference database (Quast et al., 2013).

Data Analysis

The microbial community data were analyzed using R version 4.0.2 (R Development Core Team, 2021), with RStudio version 1.3.959 (www.rstudio.com). Alpha and beta diversities were explored using the package ampvis2 (Andersen et al., 2018), and the clustered heatmap was generated using the packages vegan (Oksanen et al., 2016) and gplots (Warnes et al., 2019). All other visualizations were created using the package ggplot2 (Wickham, 2016). Alpha diversity was measured using the ChaoI index (Chao, 1984), and beta diversity was estimated using non-metric multi-dimensional scaling (NMDS) on Bray-Curtis distances (Bray and Curtis, 1957). Differences in alpha diversity between groups were tested using the Wilcoxon ranked sum test with Benjamini–Hochberg correction for multiple testing, and differences in beta diversity between groups were tested using PERMANOVA and ANOSIM with 999 permutations. A clustered heatmap was generated using Ward agglomerative clustering (Murtagh and Legendre, 2014) on Bray-Curtis distances of raw abundance data. All quantitative data for sequences and ASVs are presented as mean ± standard deviations.

RESULTS

Sequence Quality

Sequencing of 76 animal fecal samples generated a grand total of 3,304,384 reads. Sequencing depth was examined using rarefaction curves (**Supplementary Figure S1**), and a minimum number of 7,500 sequences per sample were determined to be sufficient for further downstream analysis. After quality filtering, 70 samples entered the microbial community analysis, of which 54 were from captive animals and 16 from wild animals, with an average of 47,096 ± 36,399 reads per sample.

Alpha Diversity of Captive Animal Species

The richness of the gut microbial communities of the captive animals was measured using the observed number of ASVs and the Chao1 index (**Figure 1**). The average ratio between the observed and estimated number of ASVs (Chao1) in each sample was 0.76 ± 0.07, which indicates that the majority of the diversity in the gut microbiota had been captured (**Supplementary Table S2**). The highest richness was seen in the sample from the order Pilosa (Giant anteater), with 6,440 ASVs (**Figure 1A**). Overall, the orders Perissodactyla ($n = 9$) and Artiodactyla ($n = 15$) contained a high gut microbial richness, compared to the order Carnivora ($n = 13$) which had a significantly lower richness per sampled animal ($p < 0.05$). Foregut fermenting animals ($n = 14$) had the overall highest gut microbiota richness within the different gut morphologies (**Figure 1B**), while in the animals with a simple gut physiology ($n = 21$), the lowest overall microbial diversity was observed. Foregut fermenting animals had a significantly higher microbiota diversity, compared to the hindgut fermenters and simple gut groups ($p < 0.0001$). Sorted by dietary choices (**Figure 1C**), the herbivores had the highest richness ($n = 32$), while the

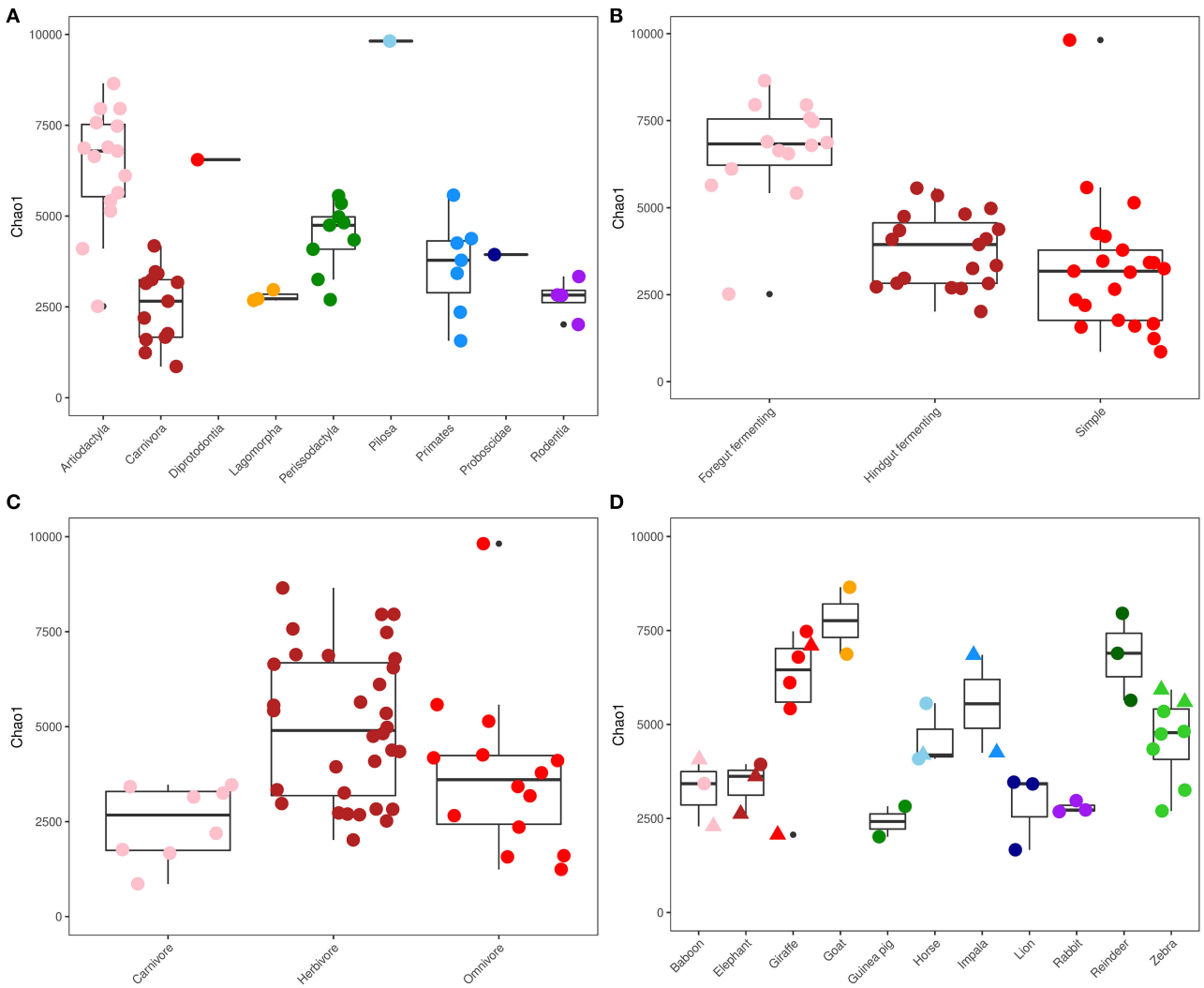

FIGURE 1 | Alpha diversity. Chao1 diversity index measurements for captive mammals, sorted and colored by phylogeny **(A)**, gut morphology **(B)**, diet **(C)**, and microbiomes of animals with multiple specimens living in captivity (circle) and in the wild (triangle) **(D)**. Data are displayed as boxplots that bound the interquartile range (IQR) divided by the median and whiskers that extend 1.5 × IQR past the box. Outliers are shown as solid black points.

carnivores had the lowest microbial community diversity ($n = 8$). Estimated microbiota richness was significantly different between the herbivores and carnivores ($p = 0.004$).

Specific Clusters of Microorganisms Associate With Animals Based on Phylogeny and Diet

The presence of distinct groups of gut microbes associated with specific groups of animals based on either phylogeny, gut morphology, or diet preference was investigated by generating a hierarchical clustered heatmap of the most abundantly observed microorganisms (the 50 most abundantly observed ASVs in herbivores, omnivores and carnivores; **Figure 2**). Overall, groupings of ruminants (cluster I), horses (and zebras) (cluster II), carnivores (cluster III), and a mixed cluster of herbivores and omnivores (cluster IV) were formed. An abundant group of microbes was observed to be ubiquitously present in a tightly clustered group of all foregut fermenting animals (cluster I, $n = 15$), including reindeer, giraffes, and goats (Artiodactyla). These included representatives of *Oscillospiraceae*, *Lachnospiraceae*, *Monoglobus*, and *Ruminococcaceae* UCG-005. Representatives of the families Clostridiaceae and Peptostreptococcaceae were primarily observed in a group of carnivores (including lions, cheetah, and African wild dog) that were clustered together (cluster III). Omnivoric bears, including the South American coati and red panda, were found on a separate branch from the other members of the mixed cluster of herbivores and omnivores (cluster IV), but directly adjacent to the order Carnivora, and were associated with a cluster of microorganisms including *Turicibacter* and a representative of the *Enterobacteriaceae*. The largest cluster consisted of omnivores such as bears and primates,

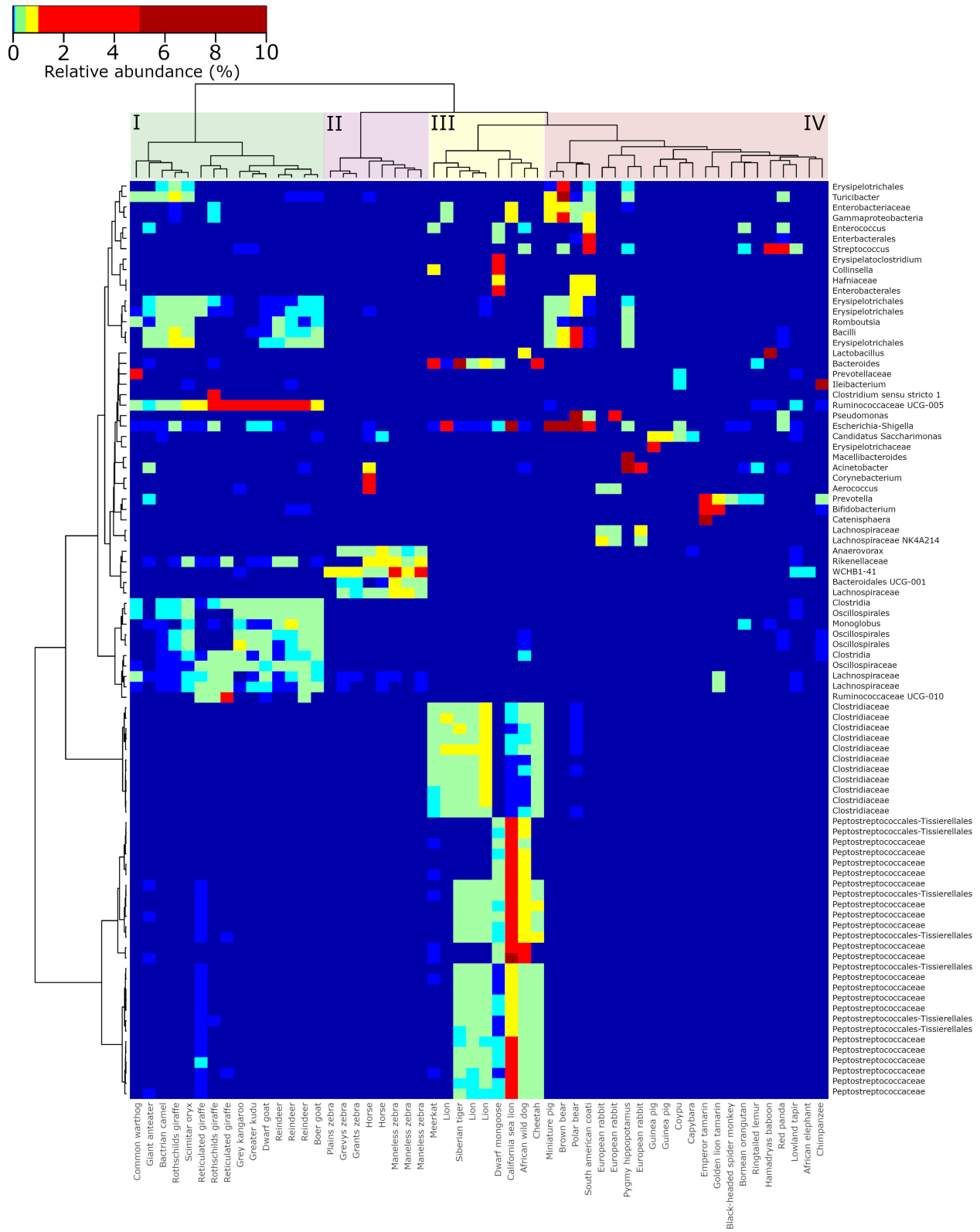

FIGURE 2 | Hierarchically clustered heatmap of the most abundant genera observed in the gut microbiota of captive animals. The 50 most abundant ASVs were selected from samples stemming from herbivores, carnivores, and omnivores, respectively. These ASVs were re-aggregated to the genus level, a distance matrix of Bray-Curtis distance was generated, and clustering was performed using Ward agglomerative clustering. Colored boxes highlight the four major clusters of ruminating herbivores (I), non-ruminating herbivores (II), carnivores (III), and omnivores (IV).

as well as herbivores such as capybara and guinea pigs (cluster IV). In these samples, *Bifidobacterium*, *Pseudomonas*, *Prevotella*, and *Escherichia-Shigella* were observed abundantly (>0.1% of total reads).

Species Relationships and Traits Shape the Microbiota Composition of Captive Animals

The similarities and differences in gut microbiota composition in 54 animal specimens across 42 species were explored using non-metric, multi-dimensional scaling analysis based on Bray-Curtis distances (**Figure 3**). Statistical analysis using PERMANOVA showed that the phylogenetic relatedness of the host was the major factor to explain the observed differences ($p < 0.001$, $R^2 = 0.83$), followed by gut morphology ($p < 0.001$, $R^2 = 0.66$) and dietary preferences ($p < 0.001$, $R^2 = 0.35$). This was also visible in the beta diversity analysis, in which all animal orders represented in the study were clearly separated from each other (ANOSIM; $p < 0.001$, $R^2 = 0.81$). The primarily herbivoric orders Perissodactyla, Proboscidae, Diprodontia, Artiodactyla, and Pilosa were relatively clustered together, while Carnivora, Rodentia, Lagomorpha, and Primates were grouped separately. Animals from the order Artiodactyla and Perissodactyla were clustered closer together within their own groups compared to other animal groups, while the greatest variation in gut microbiota composition was seen in the groups with primates and carnivores.

Gut Microbiota Differences Between Wild and Captive Animals of the Same Species

This study included 12 animal species (disregarding subspecies) where multiple individuals were sampled, either from different captive habitats or where both wild and captive specimens were included in the study. The difference in measured richness in the gut microbiome of these animals varied; the lowest variation in diversity was observed in rabbit and guinea pigs (**Figure 1D**), while a greater variation was observed between the sampled zebras and giraffes, both of which included both wild and captive specimens. Beta diversity analysis of the replicated animals (**Supplementary Figure S2**) showed that the differences between specimens of the same species were small for horses, goats, rabbits, and reindeer, while a greater variation in gut microbiota composition was observed in elephants, lions, zebras, giraffes, and baboons (ANOSIM; $p < 0.001$, $R^2 = 0.87$). Overall, the sampled captive animals clustered near their respective wild relatives in an NMDS analysis (**Supplementary Figure S3**) and within their own cluster based on evolutionary relationships, except for one giraffe and one elephant, which were observed near the bottom of the plot, grouping away from the other samples. In addition, the inclusion of the wild animals into the NMDS analysis increased the variation within the individual phylogenetic groups, and the orders Perissodactyla and Proboscidae now overlapped (ANOSIM; $p < 0.001$, $R^2 = 0.77$).

Captive animals from the same species, regardless of whether they were from the same or a different habitat, generally shared a great similarity in their overall gut microbial community structure (**Supplementary Figure S4**). This similarity also extended across certain animal species, especially ruminants and other herbivores. The differences between the gut microbial communities of animals from the same species sampled in both their captive and natural habitats (baboons, elephants, giraffes, horses, and zebras) were compared through analysis of their alpha diversity (**Figure 1D**), as well as the microbiome composition (**Supplementary Figure S5**). The smallest variation in microbial community composition between individuals of the 5 animals of interest was seen among the horses (**Supplementary Figures S2**, **S4**), while a greater variation between individuals was observed for baboons, elephants, giraffes, and zebras, but no clear separation was visible based on captivity status. The greatest similarity in microbial community composition between wild and captive specimens was observed in the horses, zebras, and giraffes (Perissodactyla and Artiodactyla) (**Supplementary Figure S5**), while a tendency toward greater variation was seen in the elephants and baboons. In both the horses ($n = 3$) and giraffes ($n = 6$), approximately 40 % of the abundantly identified ASVs (≥0.1% of total reads) were observed in both the wild and captive specimens (**Supplementary Figure S5D,E**). In elephants ($n = 3$) and baboons ($n = 3$) (**Supplementary Figures S5B,C**), this overlap was a lot smaller, with around 15 and 25% of abundant ASVs shared, respectively.

DISCUSSION

The aim of this study was to examine the gut microbiota composition of 54 different animal species stemming from wild and captive habitats. High-throughput sequencing of 70 samples yielded high-quality reads covering the majority of the diversity present in the samples, although the rarefaction curves of the analyzed samples (**Supplementary Figure S1**) did show that not all of the microbiota was captured, and it is likely that the single individuals analyzed for most species are not fully representative for the animal species. This was taken into account by performing the analysis with conservative considerations in mind. The approach used in this study was to sample different animals and compare the differences in gut microbiota among animal species rather than to focus on multiple individuals from a limited number of species. The large number of animal species sampled allowed for cross-species comparisons to gain valuable information regarding phylogeny and feeding strategies. In addition, a number of animal species included in the study were sampled with multiple replications from different captive and wild habitats to explore differences between individuals.

Microbial Community Composition of Captive Animals Is Shaped by Phylogeny and Diet

Samples representing the orders Artiodactyla and Perissodactyla ($n = 24$ collectively) contained the greatest microbial community diversity (**Figure 1**), while samples from carnivoran representatives had the lowest richness. Overall, this is in line

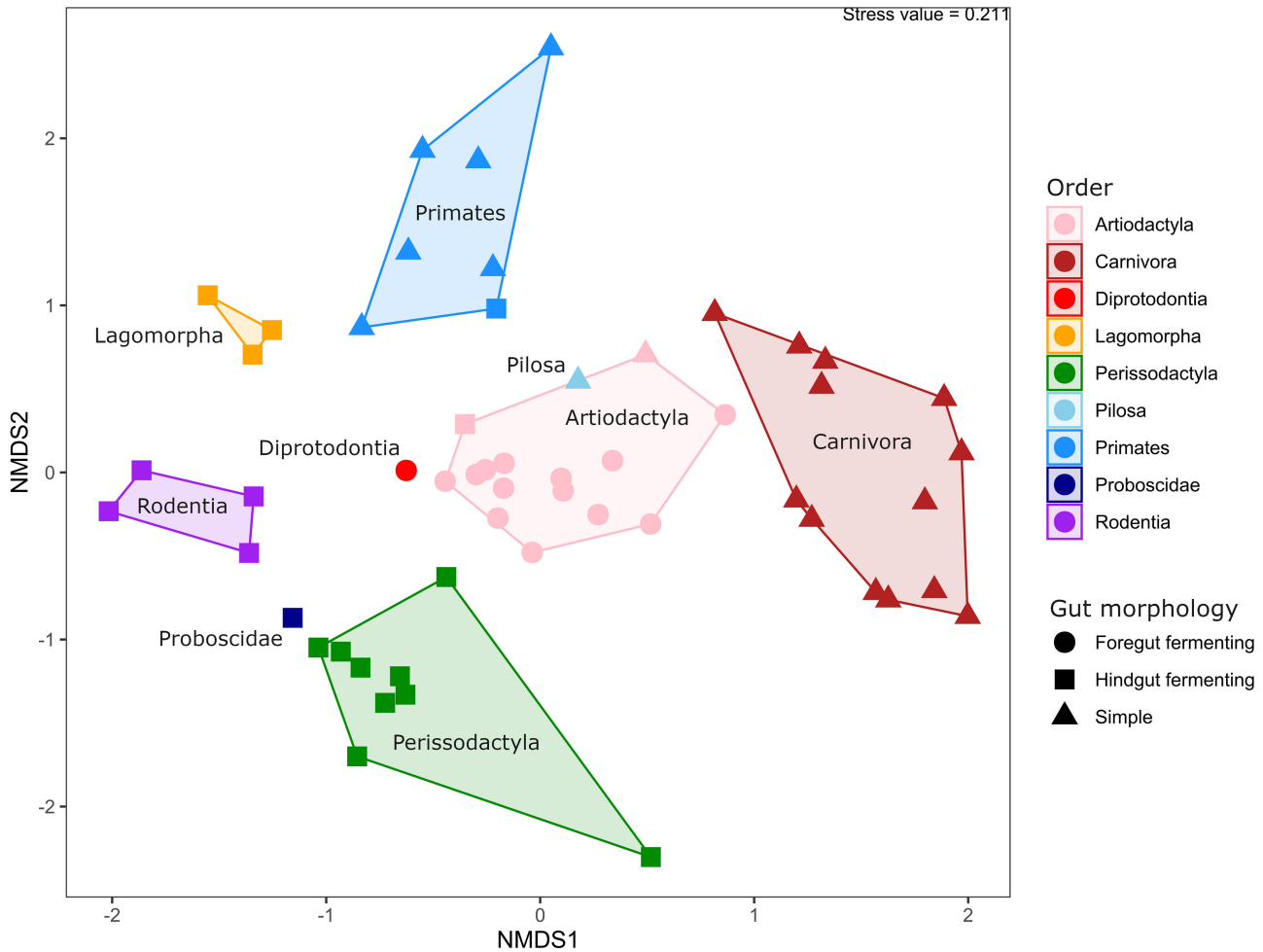

FIGURE 3 | Beta diversity of animals in captivity. Non-metric dimensional scaling analysis based on Bray-Curtis distances, with samples colored by phylogeny and shaped by gut morphology. A polygon is drawn around samples from the same phylogenetic group, and the names of the individual groups are displayed within their respective polygons.

with the results of a previous study that explored the microbial community of 41 mammals, which also observed a large difference in diversity between the same phylogenetic groups (McKenzie et al., 2017). A herbivoric diet requires a more diverse set of microorganisms to digest efficiently compared to meat-rich diets (Bayané and Guiot, 2011), which explains the increased diversity of the gut microbiome profiles observed for animals with these dietary preferences. The predominant taxa identified in fecal microbiota across all investigated animals, regardless of their diet, were *Firmicutes* and *Bacteroidetes*, which is in line with previous studies (O'Donnell et al., 2017; Youngblut et al., 2019).

Hierarchical clustering analysis revealed specific and abundant associations of *Ruminococcaceae*, *Oscillospiraceae*, and a few other microbial taxa that were ubiquitously associated with the ruminants in the dataset. These organisms (and others) were also observed abundantly in ruminants in a previous study focused on domesticated animals in Ireland (O'Donnell et al., 2017), as well as in giraffes (Schmidt et al., 2018). Ruminants have a specific gut morphology and metabolism compared to many other animal groups (Stevens and Hume, 1998; Henderson et al., 2015), and it was therefore expected to see a more uniform microbiota composition and less variation between individuals compared to non-ruminants. Adversely, the gut microbiota profiles of animals with carnivoric dietary preferences were the least diverse among the sampled animals (**Figure 1**). Previous microbiota studies in carnivores, including leopard cats (*Prionailurus bengalensis*), Eurasian otters (*Lutra lutra*), raccoon dogs (*Nyctereutes procyonoides*) (An et al., 2017), and Asiatic black bears (*Ursus thibetanus*). Song et al. (2017) observed a similar fecal microbiota diversity to those observed in this study. The families *Clostridiaceae* and *Peptostreptococcaceae* were specifically associated with sampled carnivores and have often been observed in the fecal microbiota of animals that include meat in their diet (An et al., 2017; Youngblut et al., 2019).

In this study, the gut microbiota composition was influenced by evolutionary history, gut morphology, and diet, in order of importance. This is in line with several previous studies that also examined a larger range of animal species (Ley et al., 2008; McKenzie et al., 2017), but also with studies in which the diet was reported to be a strong predictor of gut microbiota composition (Muegge et al., 2011; Youngblut et al., 2019). Collectively, these studies show that despite focusing on different aspects of gut microbiota, microbial community analyses in animals have strong predictive capabilities.

Comparison of Gut Microbiome Community of Captive and Wild Animals

The gut microbiota profiles of animal species where multiple individuals and both wild and captive species were sampled (baboons, elephants, giraffes, horses, and zebras) were examined in detail (**Figure 1D, Supplementary Figures S2–S5**). Some variation in the microbiome of sampled individuals of the same species was observed, but no significant correlational tendencies were identified based on either phylogeny or dietary preferences of the analyzed species. This is in line with a recent study comparing many different microbiota samples in different vertebrates, which found that differences between wild and captive individuals were heterogeneously distributed among species (Alberdi et al., 2021). Individual variation in the gut microbiome is determined by many factors (Pascoe et al., 2017), and the limited number of replications and samples from individual organisms investigated in this study was not sufficient to reflect on this aspect. However, retention of microbiota did show an interdependency for the animal species where both captive and wild individuals were sampled. Horses and giraffes showed a greater overlap in the microbial consortia observed in individuals from either habitat compared to zebras and elephants. Previous studies in horses and giraffes have likewise shown a high microbial community diversity in these animals (Zhao et al., 2016; Schmidt et al., 2018). However, horses have also been shown to contain lower microbial diversity in their fecal microbiota in captivity compared to wild individuals (Metcalf et al., 2017). One of the most divergently observed microorganisms was *Fibrobacter*, a microbe associated with fiber content in lignocellulosic biomass and prominently observed in animals consuming a plant-based diet (Neumann et al., 2017). The highly diverse and specialized microbiota of animals with herbivoric diets, and ruminating animals in particular, may be more resilient to changes due to their essential function in the degradation of lignocellulosic materials that cannot be achieved without microbial symbiosis (Bayané and Guiot, 2011).

The greatest divergence in gut microbiota composition between wild and captive individuals was observed in elephants. Furthermore, a large proportion of the microbiota found in the baboons was divergent between wild and captive specimens, suggesting altered microbiota. This is in agreement with recent studies where a strong correlation was found between gut microbiota and food availability, habitat and dietary composition in primates and elephants (Nakamura et al., 2011; Clayton et al., 2016; McKenzie et al., 2017; Kartzinel et al., 2019; Zhang et al., 2019). Among the most differentially abundant organisms were the genera *Collinsella* (abundant in the wild specimens) and *Lactobacillus* (abundant in captive individuals). These genera have been associated with dietary fiber content (Barrett et al., 2018) and dairy products (Azad et al., 2018), respectively, suggesting that the dietary composition between these sampled individuals may have played a large role in the observed differences.

Gut Microbiota Studies in Wild and Captive Animals

An increasing number of studies have investigated the gut microbiota of various animals in natural and captive habitats (McKenzie et al., 2017; Pascoe et al., 2017; Youngblut et al., 2019). One of the globally occurring tendencies across animal microbiome studies has been a trend toward lower gut microbial community richness among captive animals compared to their wild relatives. However, cross-examination of several studies also showed that microbiota diversity changes between wild and captive individuals are not evenly distributed across all species (Alberdi et al., 2021). Decreasing microbial richness has been linked to differences in the diversity and availability of food items in bears (Borbón-García et al., 2017), as well as a selection of African megafauna (Kartzinel et al., 2019). The observed differences could also relate to the increased contact with humans and modern human food items with little variations among captive primates that slowly cause humanization of the gut microbiota (Clayton et al., 2016), something that has also been shown to be both predictable and reproducible in non-human primates (Houtz et al., 2021). Based on these and other studies, it can be hypothesized that the overall changes to the host microbiota are generally more profound when an animal lives in a fully human-made environment, compared to a semi-controlled environment such as a nature reserve. A significantly changed microbiome could also compromise animal health, as the gut microbiota are also known to protect against pathogenic bacteria that are ingested with food items, e.g., dead prey (Roggenbuck et al., 2014; Blumstein et al., 2017). Furthermore, it has recently been shown that certain predators are able to metabolize bacterial toxins through their microbiota (Levin et al., 2021), which further highlights the contribution of host–microbe symbiosis to the functional capabilities of the microbiome in the gut.

The data obtained in this study also indicates that it is possible to identify microorganisms in animal microbiome data that are of potential importance to the host regardless of captivity status. Microbiota retained in both wild and captive individuals may provide valuable information about the symbiotic dependencies of the well-being of an animal (McKenzie et al., 2017). In horses, giraffes, and zebras, up to 50% of the identified ASVs were found in both wild and captive animals, while it was only 20% in elephants (**Supplementary Figure S5**). A distinct difference could also be seen in some of the abundant microbes found in the baboons (**Supplementary Figure S5B**), suggesting a potential shift in important microbiota. Previous studies have shown that large primates in captivity evolve a more human-like microbiota

over time, primarily due to a change in diet (Clayton et al., 2016). Identification of keystone microbes in the gut microbiota can serve as indicators of the host's adaptation to the surrounding environment. Furthermore, monitoring of keystone organisms of high importance for not only the gut microbiome composition but also its metabolic functional potential can provide important new tools in conservation efforts. However, identification of keystone organisms cannot be based on microbiota comparisons alone; information regarding temporal dynamics, function, activity, and a given microbe's interconnectedness within the microbiome are needed in order to identify true keystone organisms from a given ecosystem (Banerjee et al., 2018).

The Potential of Microbial Community Data in Conservation Efforts

The findings from this study, as well as the previous animal microbiota studies collectively, support the proposed link between animal health, responses to the environment, and gut microbiota composition (McKenzie et al., 2017; Metcalf et al., 2017; Hauffe and Barelli, 2019; Stothart et al., 2019; Levin et al., 2021), providing evidence that the gut microbiome of animals can provide information about different aspects of host welfare. This could potentially serve as an important tool for future conservation biologists.

Non-invasive fecal microbial community analysis also have the potential to become a valuable toolbox for monitoring the health and general well-being of animals living in captivity (Redford et al., 2012; Stumpf et al., 2016). It has been suggested that the multitude of studies surrounding the gut microbiota in humans and model organisms can also be extended to other animal species, to provide a framework for monitoring of animal microbiota in relation to host health and disease (Amato, 2016; Bahrndorff et al., 2016). This framework would need to be based on individual species knowledge regarding temporal variation and stability of the microbial community in order to elucidate changes in gut microbiota and discern meaningful information regarding the host. Monitoring gut composition could be used to assess environmental stress and determine whether semi- or fully man-made environments such as natural parks and zoos are adequate for the animal. Future studies might also reveal how gut microbiota monitoring can provide important information on habitat fractionation, urbanization and climate change. In addition, it has also been suggested to consider the microbiome as a source of adaptive potential instead (Hauffe and Barelli, 2019). The results of this study support the potential of applying microbiome data in animal welfare and conservation processes, but additional data regarding animal diet, behaviour, health, and other parameters are needed to support microbial community data in order to make meaningful inference in relation to conservation.

CONCLUSION

This study showed that gut microbiota composition in mammalian animals is driven by phylogenetic relatedness, gut morphology, and diet, as well as by large environmental differences in terms of living in captivity or in the wild. The presence of animal or diet-specific microbes and the retention of microbiota between captive and wild animals of the same species suggest that keystone microbes are present in the animal gut, and that these may be retained regardless of habitat status. Differences in the proportion of microbiota retention between animal species have implications for the monitoring of (gut) health in animals in captivity. Our findings support the potential and importance of gut microbiota analysis in current conservation efforts as an additional measure of animal welfare and health.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ebi.ac.uk/ena, PRJEB36809.

AUTHOR CONTRIBUTIONS

JN conceived the ideas, designed the work, and achieved the funding with contributions from NJ and CP. BC and MC did the sampling and laboratory work with guidance from NJ. NJ analyzed the data with guidance from JN. NJ and JN led the writing of the manuscript with contributions from CP, BC, and MC.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.886252/full#supplementary-material

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Comparison of Gut Microbiota Diversity Between Captive and Wild Tokay Gecko (*Gekko gecko*)

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Captive animals and wild animals may exhibit different characteristics due to the heterogeneity of their living environments. The gut microbiota play an important role in the digestion and absorption, energy metabolism, immune regulation, and physiological health of the host. However, information about the gut microbiota of captive and wild *Gekko gecko* is currently limited. To determine the difference in gut microbiota community composition, diversity, and structure between captive and wild geckos, we used the Illumina miseq platform to conduct high-throughput sequencing and bioinformatics analysis of the v3–v4 hypervariable region of 16S rRNA in 54 gecko samples. Our results showed that Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria were the dominant gut microbiota phyla of the gecko. The dominant genera comprised mainly *Pseudomonas*, *Burkholderia-caballeronia-paraburkholderia*, *Ralstonia*, *Romboutsia*, and *Bacteroides*. Captive geckos had significantly higher alpha diversity and potential pathogenic bacteria than wild populations. Moreover, significant differences in beta diversity of gut microbiota were observed between two populations. Functional prediction analysis showed that the relative abundance of functional pathways of wild geckos was more higher in metabolism, genetic information processing and organismal system function than those in captive geckos. Total length significantly affected gut microbial community (R^2 = 0.4527, p = 0.001) and explained 10.45% of the total variation for gut microbial community variance between two groups. These results may be related to differences in diet and living environment between two populations, suggesting that the management of captive populations should mimic wild environments to the greatest extent possible to reduce the impact on their gut microbiota.

Keywords: ***Gekko gecko*, captive population, 16S rRNA, gut microbiota, wildlife, conservation, microbial community diversity**

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INTRODUCTION

The gut microbiota has symbiosis and coordinated evolution with the host, forming an overall system of interaction (Nicholson et al., 2012; Tremaroli and Bäckhed, 2012; Flint et al., 2015). The gut microbiota plays important roles in host growth and development (Egert et al., 2006; Sommer and Bäckhed, 2013), impacting energy budget (Semova et al., 2012), nutritional metabolism (Cani and Everard, 2016; Greer et al., 2016), vitamin synthesis (LeBlanc et al., 2013), digestion and

absorption, colonization resistance and immune homeostasis (Hooper et al., 2012; Bengmark, 2013), and behavior and emotion (Heijtz et al., 2011; Ezenwa et al., 2012). At the same time, various factors also affect the gut microbiota, including host diet (Ley et al., 2008; De Filippo et al., 2010; Muegge et al., 2011; Wu et al., 2011), genetic and environmental history (Moeller et al., 2013), physiological status (Amato et al., 2013; McCord et al., 2014), and external environmental factors. Studies on human gut microbiota have identified the relationship between gut microbiota disorders and host infections with opportunistic pathogens (Shin et al., 2015), allergies (Bisgaard et al., 2011), and diseases (Ley et al., 2006; Sobhani et al., 2011; Vaarala, 2011).

Currently, the gut microbiota has been widely studied in invertebrates (Clark and Walker, 2018), amphibians (Bletz et al., 2016) and reptiles (Trevelline et al., 2019; Zhang et al., 2019 Eliades et al., 2021), mammals (Hu et al., 2017; Song et al., 2017; Li et al., 2021), and birds (Ryu et al., 2012; Waite et al., 2012; Grond et al., 2018). In recent years, research into the gut microbiota in captive animals, such as *Takydromus septentrionalis* (Zhou et al., 2020), *Neotoma albigula* (Martínez-Mota et al., 2020), and *Macaca mulatta* (Chen et al., 2019) has also attracted extensive attention. The gut microbiota of captive animals may differ from those of wild animals due to dietary differences, antibiotic treatment, human activities, and exposure to other species in captivity (Alfano et al., 2015; Clayton et al., 2016; Chen et al., 2019; Eliades et al., 2021). Studies on mammals and reptiles—such as *Hydrorga leptonyx* (Nelson et al., 2013), *Peromyscus maniculatus* (Schmidt et al., 2019), *Macaca mulatta* (Chen et al., 2019) and *Shinisaurus crocodilurus* (Jiang et al., 2017), *Crocodylus siamensis* (Lin et al., 2019), and *Brachylophus vitiensis* (Eliades et al., 2021)—have confirmed that captivity may change the composition and abundance of the gut microbiota. Moreover, captivity may lead to an increase in potential pathogens in the animal gut microbiota, leading to an increased incidence of disease (Berry et al., 2012; Amato et al., 2016; Kohl et al., 2016; Xie et al., 2016). Conversely, a few studies reported that captivity may improve host immune status (Montalban-Arques et al., 2015), and even have beneficial effects on the development and behavior of the host (Heijtz et al., 2011). Therefore, an evaluation of the effects of captivity on the gut microbiota of wild animals in conservation and rescue breeding is necessary (Eliades et al., 2021).

In China, Tokay geckos are distributed mainly in Guangdong province, Guangxi Zhuang autonomous region, Yunnan province, Fujian province, and Taiwan (Huang et al., 1995; Tang et al., 1997). Geckos are used in traditional Chinese medicine (Li et al., 1996; Bauer, 2009). While medical demand for geckos is increasing, the captive breeding population cannot meet the market demand (Li et al., 1996). In addition, the natural habitat of geckos has been destroyed and gradually reduced, affecting their survival (Zhang et al., 2015). These factors have caused a decline in wild gecko populations, rendering *ex-situ* conservation the effective conservation approach for this species. Due to the heterogeneity of captive and natural environments, however, captive and wild geckos may differ greatly in diet composition. Moreover, captive populations are exposed to a greater extent to human activity than wild populations. The effect of different living environments on the gut microbiota of geckos remains unclear. We therefore analyzed the gut microbiota community of captive and wild geckos by 16S rRNA gene sequencing technology, and compared the microbial community diversity, richness, structure, and function of the two groups. Considering the differences in living environment between wild animals and captive animals, we propose the following predictions: (1) wild geckos have a higher diversity of gut microbiota than captive geckos and (2) the gut microbiota of captive geckos include more opportunistic pathogens.

MATERIALS AND METHODS

Sample Collection

We collected a total of 54 samples, including 24 from wild geckos (18 males and 6 females) and 30 from captive geckos (22 males and 8 females). We captured the wild geckos in Jiangzhou District, Chongzuo City, Guangxi, and acquired the captive geckos from Nanning Junhao Wildlife Science and Technology Development Co., Ltd. Wild geckos eat mainly insects and moths from their natural environment (Liu et al., 1981; Tang et al., 1997). Captive individuals are fed on *Zophobas morio* and *Tenebrio molitor* and kept in temperature conditions of about 20°C. Average total length (TL) of wild and captive geckos is 233.92 ± 38.93 mm and 266.38 ± 32.82 mm, respectively. All Tokay geckos were healthy during the sampling period (**Supplementary Table 1**).

We used cloacal swabs—an acceptable source for non-destructive sampling of the gut microbiota of reptiles (Colston et al., 2015; Jiang et al., 2017)—to collect the gut microbiota of geckos. After sampling, we released all individuals back into the site of capture. We stored samples in a thermal insulation bucket with ice bags and transported them to a refrigerator at −20°C for storage.

DNA Extraction, PCR Amplification, and Sequencing

We extracted the total gut microbiota community genomic DNA from all samples using the FastDNA® Spin Kit for Soil (MP Biomedicals, United States) based on the manufacturer's instructions. We detected the extraction quality of DNA by 1% agarose gel electrophoresis and determined the concentration and purity of DNA with a NanoDrop 2000 UV-vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States). We amplified the hypervariable region v3–v4 of the bacterial 16S rRNA gene with primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) by an ABI GeneAmp® 9700 polymerase chain reaction (PCR) thermocycler (ABI, CA, United States) (Mori et al., 2014; Chen et al., 2019). Our PCR reaction parameters included initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s, and ended with a final extension at 72°C for 10 min. For the PCR test we used TransGen AP221-02: TransStart Fastpfu DNA Polymerase with 20 μL of reaction system containing

5 × FastPfu Buffer 4 μL, 2.5 mM deoxy-ribonucleoside triphosphates (dNTPs) 2 μL, Forward Primer (5 μM) 0.8 μL, Reverse Primer (5 μM) 0.8 μL, FastPfu Polymerase 0.4 μL, bovine serum albumin (BSA) 0.2 μL, template DNA 10 ng, and finally ddH_2O up to 20 μL. We performed PCR reactions in triplicate. We extracted the PCR product from 2% agarose gel, purified it using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) according to manufacturer's instructions, and quantified it using Quantus™ Fluorometer (Promega, United States). We pooled purified amplicons in equimolar and paired-end sequenced them (2 × 300) on an Illumina miseq platform (Illumina, San Diego, CA, United States) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

Processing of Sequencing Data and Quality Evaluation

We demultiplexed the raw 16S rRNA gene sequencing reads, quality-filtered them by Trimmomatic, and merged them by FLASH with the following criteria: (i) we truncated the 300 bp reads at any site receiving an average quality score of <20 over a 50 bp sliding window, and discarded both the truncated reads shorter than 50 bp and reads containing ambiguous characters; (ii) we assembled only overlapping sequences longer than 10 bp according to their overlapped sequence; we adopted a maximum mismatch ratio of overlap region of 0.2; we discarded reads that could not be assembled; (iii) we distinguished samples according to the barcode and primers, and adjusted the sequence direction, exact barcode matching, and 2 nucleotide mismatches in primer matching.

We obtained a total of 2,603,017 raw sequences from the v3–v4 region of the hypervariable region of 16S rRNA gene in 54 samples. After quality control, 2,504,574 sequences were effective (average ± SD = 48,204.02 ± 9,796.15 for each sample), and the average length of the sequences was 420.66 ± 7.13 bp (**Supplementary Table 2**). Our rarefaction curve tended to be flat, indicating that the amount of sequencing was reasonable, and the sequencing depth was sufficient (**Supplementary Figure 1**). The Good's coverage estimates of the 54 samples ranged from 98.89% to 99.91%, indicating that we had identified almost all bacterial communities in the samples (**Table 1**).

Bioinformatics Analysis

We clustered operational taxonomic units (OTUs) with 97% similarity cutoff (Edgar, 2013) using UPARSE (version 7.0.1090[1]) and identified and removed chimeric sequences using UCHIME. We analyzed the taxonomy of each OTU representative sequence by RDP Classifier (version 2.11[2]) against the 16S rRNA database (for instance, Silva Release138[3]) using a confidence threshold of 70%. We used rarefaction curves to reflect whether sequencing data was reasonable, and the coverage index to indicate the real situation of sequencing results. We used the Mothur program to calculate the alpha diversity index to reflect community diversity (Shannon index and Simpson index) and community richness (Abundance-based Coverage Estimator (Ace) and Chao index) (version v.1.30.2[4]). We used the Bray–Curtis distance algorithm for sample hierarchical cluster analysis of gut microbiota of captive and wild geckos and assessed beta diversity by principal coordinate analysis (PCoA) based on weighted and unweighted UniFrac distance metrics using QIIME (version 1.9.1[5]). Adonis permutational multivariate analysis (Adonis/PERMANOVA) was performed to evaluate the dissimilarity among samples with permutation set at 999. We used the Wilcoxon rank-sum test to analyze gut microbiota diversity differences between wild and captive groups and adjusted the p values under the control of FDR level at 0.05. We performed linear discriminant analysis (LDA) effect size (LEfSe) analyses to identify potential microbial biomarkers between groups.[6] For the LDA interpretation, we considered differences as significant for a $p < 0.05$ and an LDA score >4. We predicted functional profiles of microbial communities using PICRUSt2 (phylogenetic investigation of communities by reconstruction of unobserved states 2; Langille et al., 2013) (version 2.2.0[7]). We performed the Wilcoxon rank-sum test to test the differences of functional pathways between the two groups in the Kyoto Encyclopedia of Genes and Genomes (KEGG) using IBM SPSS (version 23.0), and the threshold on the p value was set at 0.05. Detrended correspondence analysis (DCA) was conducted to obtain the length of first axis (R version 3.3.1, vegan package), RDA (Redundancy analysis), or CCA (Canonical correlation analysis) was chosen based on the value of DCA 1 (>4, CCA; <3 RDA; 3–4 RDA/CCA). The DCA analysis showed that the length of DCA 1 was 6.05, indicating a unimodal-model-based CCA analysis was more suitable than RDA analysis to test the effect of geckos' total length and sex on microbial community at OTU level. A variation partitioning analysis (VPA) was conducted to examine the contribution of total length and sex factors in influencing microbial community structure as determined by CCA analysis (R version 3.3.1, vegan package). Further, the partial Mantel test was used to detect the Bray–Curtis distance matrix correlation between total length and sex and microbial community with 999 permutations [QIIME version 1.9.1 (see footnote 5)]. We used the non-parametric Spearman correlation test to analyze the correlation between total length and sex of geckos and the relative abundance of the top 50 microbial genera (R version 3.3.1, pheatmap package).

RESULTS

Differences in Alpha and Beta Diversity of Gut Microbiota

Alpha diversity analysis showed that there were significant differences in community richness and diversity ($p < 0.05$; **Table 1**); captive geckos had higher Ace, Chao, and Shannon

[1]http://www.drive5.com/uparse/

[2]http://sourceforge.net/projects/rdp-classifier/

[3]https://www.arb-silva.de/

[4]https://www.mothur.org/wiki/Download_mothur

[5]http://qiime.org/install/index.html

[6]http://huttenhower.sph.harvard.edu/galaxy/root?tool_id=lefse_upload

[7]https://github.com/picrust/picrust2/

TABLE 1 | Alpha diversity index of the gut microbiota in Tokay gecko.

Estimators	Captive	Wild	*p* value
Shannon	2.686 ± 0.603	1.930 ± 1.240	0.004
Simpson	0.207 ± 0.088	0.398 ± 0.278	0.025
Ace	494.530 ± 111.510	401.820 ± 304.350	0.001
Chao	497.870 ± 109.330	383.370 ± 301.540	<0.001
Good's coverage	0.998 ± 0.001	0.997 ± 0.002	0.577

indices and a lower Simpson index than those of wild geckos (**Table 1** and **Figure 1**).

Hierarchical cluster analysis based on Bray–Curtis distance algorithm showed that the wild and captive geckos divided into five small branches in total, without making two specific clusters according to groups (**Figure 2**). Our analysis clustered captive geckos in two branches, and wild geckos in three smaller branches. PCoA based on unweighted and weighted UniFrac distances revealed high clustering of the gut microbiota according to group, highlighting a significant separation between captive and wild geckos (Unweighted UniFrac: $R^2 = 0.2023$, $p = 0.001$; Weighted UniFrac: $R^2 = 0.2177$, $p = 0.001$; **Figure 3**).

Differences in the Composition and Abundance of Gut Microbiota

We obtained 4,851 OTUs at 97% sequence similarity and classified them into 52 phyla, 153 classes, 353 orders, 586 families, and 1,320 genera. Of the 4,851 OTUs, 1,290 OTUs were shared by captive and wild geckos, whereas 2,130 OTUs and 1,431 OTUs were unique to captive geckos and wild geckos, respectively (**Figure 4A**). At the genus level, a total of 685 genera were shared by captive and wild geckos, with 358 genera specific to the captive group and 277 genera specific to the wild group (**Figure 4B**).

At the phylum level, Proteobacteria were the predominant phylum in two groups (captive: 63.88% ± 22.99%; wild: 50.67% ± 36.90%), followed by Firmicutes (captive: 15.29% ± 11.18%; wild: 31.45% ± 33.94%), Bacteroidetes (captive: 14.55% ± 22.11%; wild: 6.90% ± 9.90%), and Actinobacteria (captive: 3.51% ± 2.96%; wild: 8.87% ± 10.41%), respectively; Wilcoxon rank-sum test showed that the relative abundance of Acidobacteriota, Chloroflexi, Deferribacterota, and Patescibacteria was higher in the captive geckos than those in wild geckos (**Figure 5A**; **Supplementary Table 3**).

At the genus level, the main gut microbiota included *Burkholderia-caballeronia-paraburkholderia* (captive: 27.77% ± 13.56%; wild: 6.73% ± 8.94%), *Ralstonia* (captive: 23.05% ± 11.21%; wild: 0.59% ± 0.47%), *Pseudomonas* (captive: 0.41% ± 0.76%; wild: 25.40% ± 38.72%), and *Bacteroides* (captive 8.59% ± 18.71%; wild: 4.16% ± 6.38%); Wilcoxon rank-sum test showed that the relative abundance of *Romboutsia* and *Rhodococcus* was higher in wild geckos, and the relative proportion of *Burkholderia-caballeronia-paraburkholderia*, *Ralstonia*, *Salmonella*, *Staphylococcus*, *Citrobacter*, and *Mycoplasma* was higher in captive geckos (**Figure 5B** and **Supplementary Table 4**).

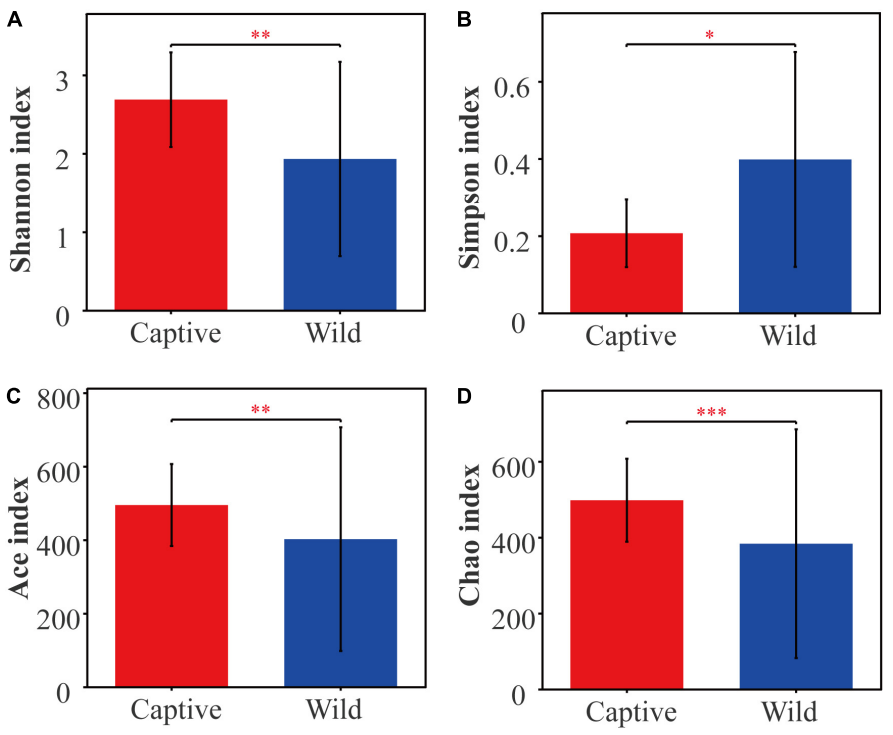

FIGURE 1 | Alpha diversity of gut microbiota between captive and wild geckos. **(A)** Shannon index; **(B)** Simpson index; **(C)** Ace index; **(D)** Chao index. Significant difference $0.01 < p \leq 0.05$ was marked as "*", $0.001 < p \leq 0.01$ was marked as "**", and $p \leq 0.001$ was marked as "***".

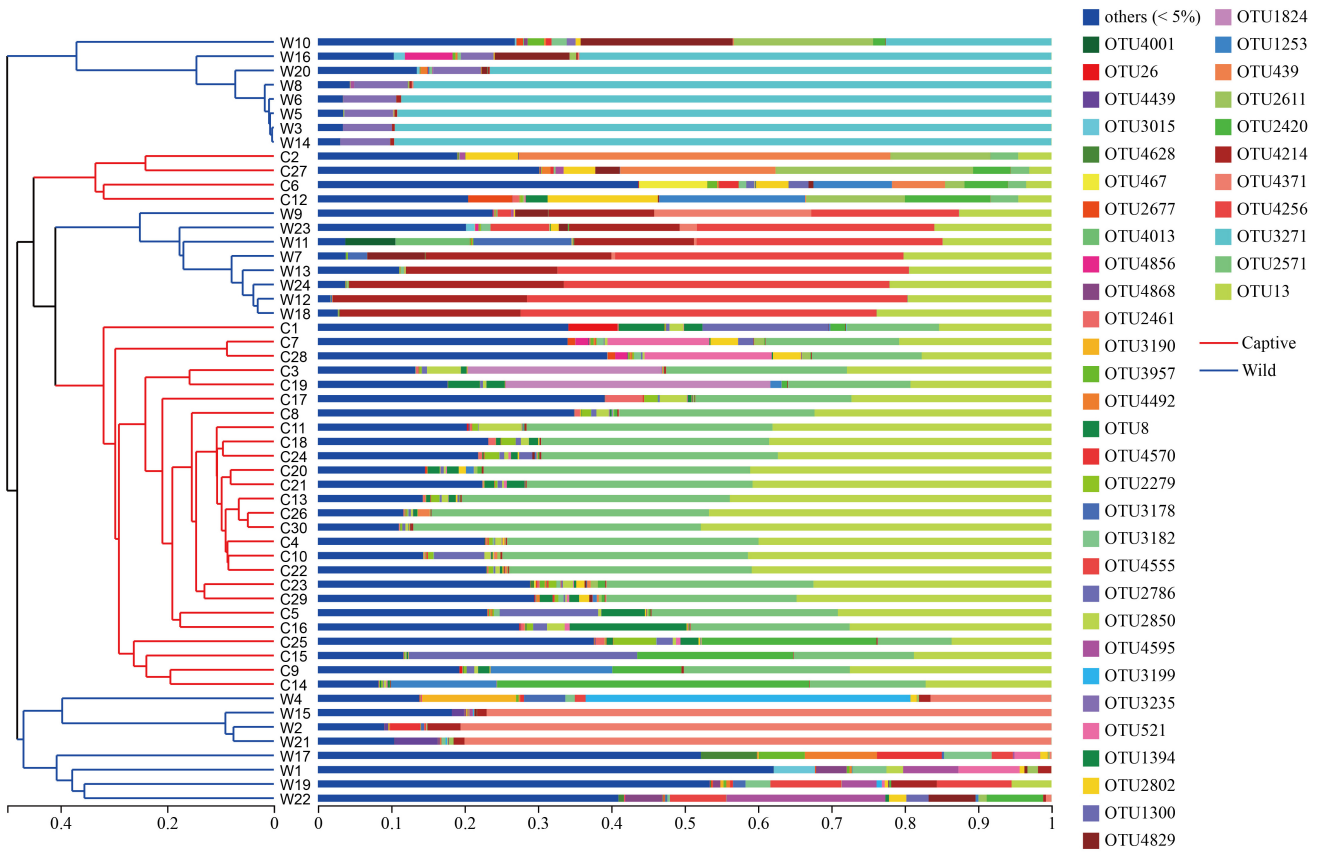

FIGURE 2 | Hierarchical cluster analysis based on Bray–Curtis distance algorithm at the OTU level.

LEfSe analysis (LDA score > 4.0) showed that the order Burkholderiales, the family Burkholderiaceae, and the class Gammaproteobacteria were the most important taxa contributing to gut microbiota differences between captive and wild geckos (**Figure 6**).

Gut Microbiota Functional Profile Prediction

Our results identified significant differences between two groups in three pathways at the KEGG pathway level 1 (Wilcoxon rank-sum test, $p < 0.05$), including those in metabolism, genetic information processing, and organismal systems, with a greater relative abundance in wild geckos than in captive geckos (**Figure 7A**). We detected 46 functional pathways at the KEGG pathway level 2, among which we identified significant differences in 27 pathways between captive and wild samples. The relative abundance of amino acid metabolism, energy metabolism and lipid metabolism in the gut microbiota of wild samples were higher than those of the captive population. However, we detected significant enrichment in global and overview maps, carbohydrate metabolism, and membrane transport in captive samples (**Figure 7B**).

Effects of Total Length and Sex on Gut Microbiota of Tokay Gecko

Canonical correlation analysis results showed the first two axes explained 5.42% of total variance (CCA1: 4.04%; CCA2: 1.38%). Total length ($R^2 = 0.4527$, $p = 0.001$) and sex ($R^2 = 0.3705$, $p = 0.001$) significantly affected gut microbial community (**Figure 8A**). VPA demonstrated that total length of Tokay geckos explained 10.45% of the total variation for gut microbial community variance (**Figure 8B**). A partial Mantel test showed that total length was weakly and positively correlated with gut microbial community ($r = 0.147$, $p = 0.019$, conditioning on sex), whereas no significant correlation was observed between sex and gut microbial community ($r = 0.004$, $p = 0.421$, conditioning on total length). Spearman correlation showed that total length was significantly positively correlated with *Burkholderia-Caballeronia-Paraburkholderia* ($r = 0.558$, $p < 0.001$), *Ralstonia* ($r = 0.478$, $p < 0.001$), and *Staphylococcus* ($r = 0.309$, $p = 0.023$) (**Supplementary Table 5**).

DISCUSSION

In terms of microbial composition, Proteobacteria, Firmicutes and Bacteroidetes were the most dominant phyla in both

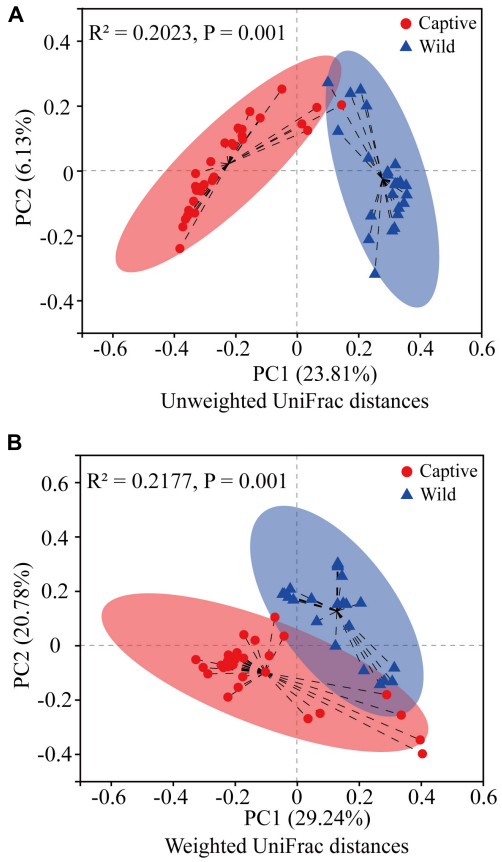

FIGURE 3 | Difference in gut microbiota community structure between captive and wild geckos using Adonis test (permutation = 999). **(A)** A principal coordinate analysis of unweighted UniFrac distances; **(B)** A principal coordinate analysis of weighted UniFrac distances.

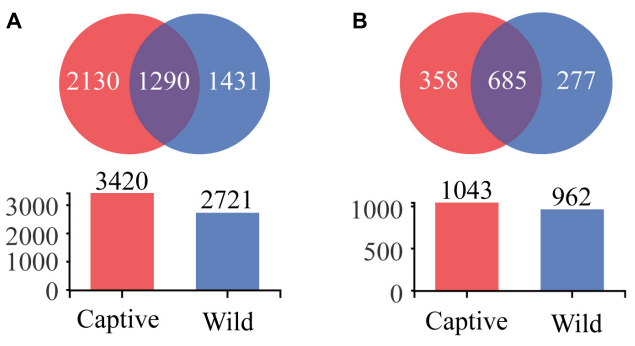

FIGURE 4 | Venn diagram of gut microbiota in Tokay gecko at the OTU **(A)** and genus **(B)** level.

captive and wild geckos, suggesting these microbes may play an important role in maintaining relative stability of gut microbiome. Other studies have also shown these microbes to be the main dominant phyla among vertebrate gut microbiota, including *Moschus berezovskii* and *Moschus chrysogaster* (Hu et al., 2017), *Ciconia boyciana* (Wu et al., 2021), *Naja atra*, *Ptyas mucosus*, *Elaphe carinata*, and *Deinagkistrodon acutus* (Zhang et al., 2019). However, we observed significant differences in microbial community structure between the captive and the wild geckos (**Figures 2**, **3**). We also discovered a higher alpha diversity of gut microbiota in captive geckos than that in wild geckos, which was inconsistent with our prediction 1. Similar results have been found in several species, including *Takifugu bimaculatus* (Lei et al., 2020), *Shinisaurus crocodilurus* (Tang et al., 2020), *Psittaciformes* (Xenoulis et al., 2010), and *Macaca mulatta* (Chen et al., 2019). Moreover, captive geckos contained a larger total number of OTUs and a greater abundance of unique OTUs. The captive environment increased human contact, individual interaction, and drug use (McKenzie et al., 2017), which might allow a greater microbial diversity and more distinct taxa to colonize the intestinal tract of captive geckos. In addition, differences in dietary composition and quantities may also play an important role (De Filippo et al., 2010; Jiang et al., 2017). Captive geckos feed mainly on locusts, woodlouse worms and barley worms, whereas wild geckos eat mainly insects and moths (Liu et al., 1981; Tang et al., 1997). Moreover, captive geckos are fed adequate food at regular intervals, but wild geckos have erratic diets because of their need to search for natural food and the fluctuations in weather or habitat conditions. Therefore, although the gut microbiota of geckos inhabiting different living environments did not differ in their most dominant phyla, the diversity and structure of the microbial community differed significantly, indicating that the captive environment had an obvious influence on gecko gut microbiota.

At the genus level, we detected a higher relative abundance of *Burkholderia-caballeronia-paraburkholderia*, *Ralstonia*, and *Staphylococcus* in captive geckos. Many of the species in these genera have been confirmed to be pathogenic in humans and animals; for instance, *Burkholderia pseudomallei* was known to cause melioidosis (Price et al., 2010); *Ralstonia* was proinflammatory during Parkinson's disease (Keshavarzian et al., 2015). The higher abundance of opportunistic pathogens in captive geckos was consistent with our prediction 2. In captivity, both dietary shift, constant cohabitation with other congeners, limited range of activity, increased exposure to human-related microbes, and medicine intervention provide pathways for transmission of opportunistic pathogens, leading to differences in the gut microbial communities of captive and wild populations (Jiang et al., 2017; McKenzie et al., 2017). Nonetheless, the causes of differences in the gut microbiota between wild and captive geckos were unknown, since environmental microbes and dietary data were not collected. In addition, our result found total length was significantly positively correlated with these taxa (**Supplementary Table 5**). Thus, it is difficult to determine whether these bacteria, which are pathogenic to other animals, have an adverse effect on the geckos. More data are needed for further study.

Firmicutes and Bacteroidetes have an impact on the host's metabolism and immune function mechanisms (Thomas et al., 2011; Zhang et al., 2019). Firmicutes can help to digest

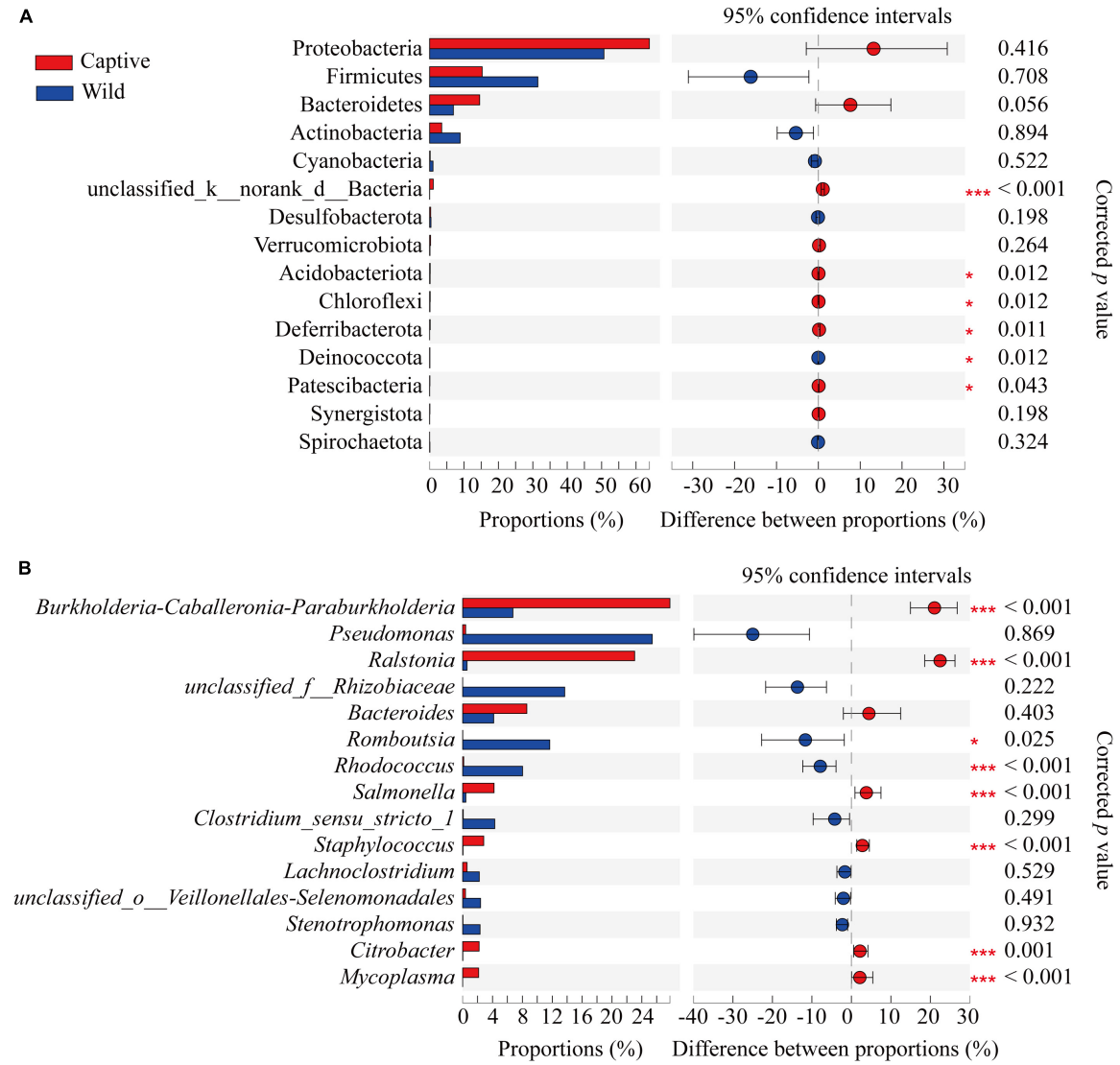

FIGURE 5 | Differences in gut microbiota composition between captive and wild geckos at the phylum level **(A)** and genus level **(B)** of the top 15 taxa. Significant difference $0.01 < p \leq 0.05$ was marked as "*", and $p \leq 0.001$ was marked as "*****".

and absorb proteins and other nutrients (Kaakoush, 2015; Bernini et al., 2016; Berry, 2016). Most species of Bacteroidetes contribute to the degradation of carbohydrates and proteins (Fernando et al., 2010; Jami et al., 2014). The Firmicutes to Bacteroidetes (F/B) ratio has been studied in both humans and animals and appears to be related to host obesity (Ley et al., 2006; Turnbaugh et al., 2006; Vebo et al., 2016). An increased F/B ratio in gut microbiota indicates a greater energy harvesting capacity for hosts (Turnbaugh et al., 2006; Murphy et al., 2010). In our study, the F/B ratio in the gut microbiota of wild geckos was higher than that of captive geckos. While the diet of captive geckos includes *Z. morio* and *T. molitor*, with high protein and fat content, the diet of wild geckos remains unclear due to limiting conditions in the field. The higher F/B ratio in the wild geckos indicates gut microbiota more efficient at digesting food to help hosts obtain energy in wild populations—a favorable adaptive strategy for wild geckos that survive in harsh natural environments (Jami et al., 2014; Wu et al., 2021). Furthermore, the KEGG pathway analysis revealed that metabolism, genetic information processing, and organismal systems pathways were significantly enriched in wild geckos, providing further evidence that wild geckos may improve adaptability through the strong metabolic potential provided by their gut microbiota.

In summary, there were significant differences between captive and wild geckos in their respective gut microbiota community structures and diversity. Captive geckos had a higher diversity of gut microbiota, but more pathogenic bacteria, while wild geckos had a higher F/B ratio and more metabolism, genetic information processing, and organismal

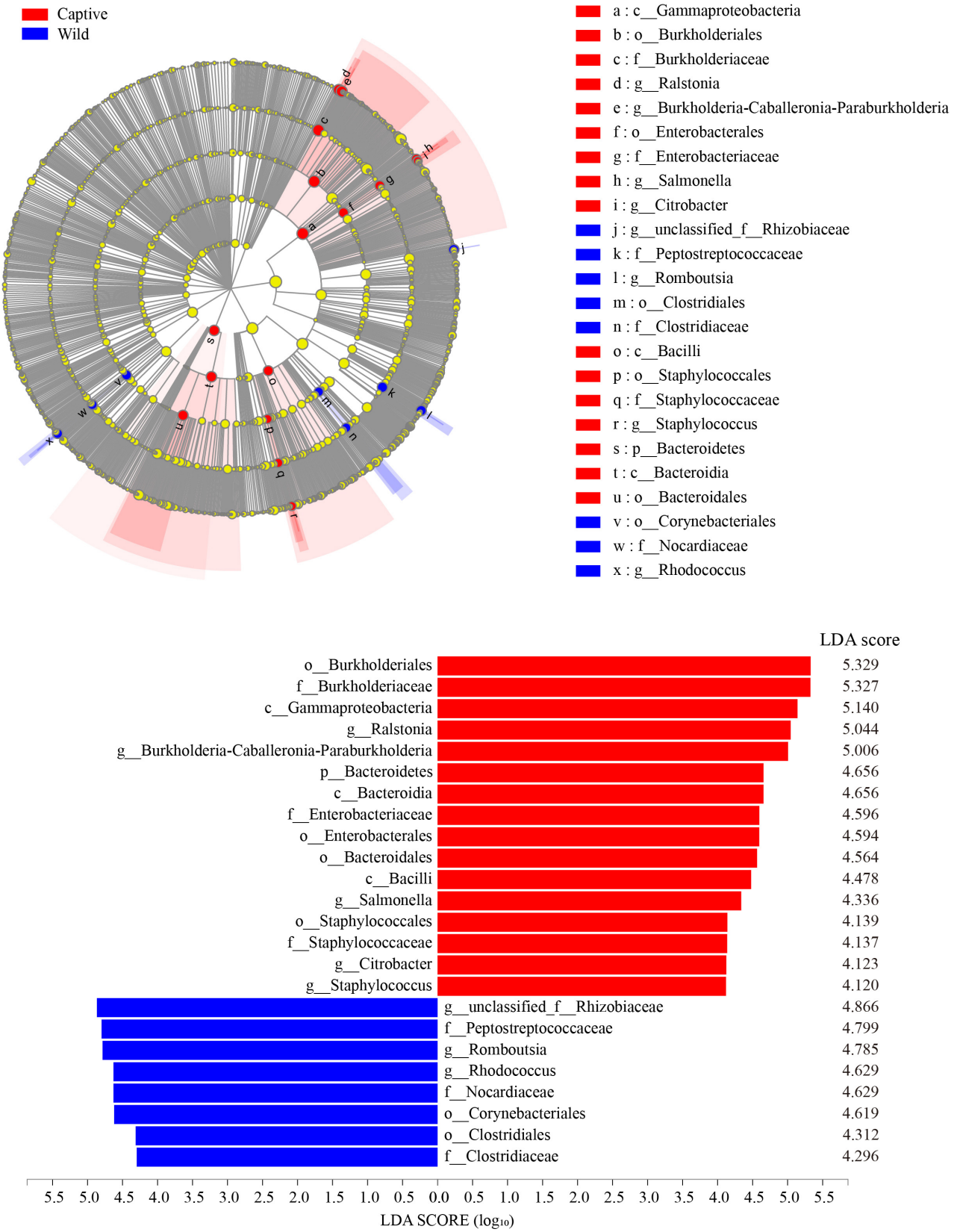

FIGURE 6 | LEfSe (LDA Effect Size) analysis of the gut microbiota in Tokay gecko (LDA score > 4.0).

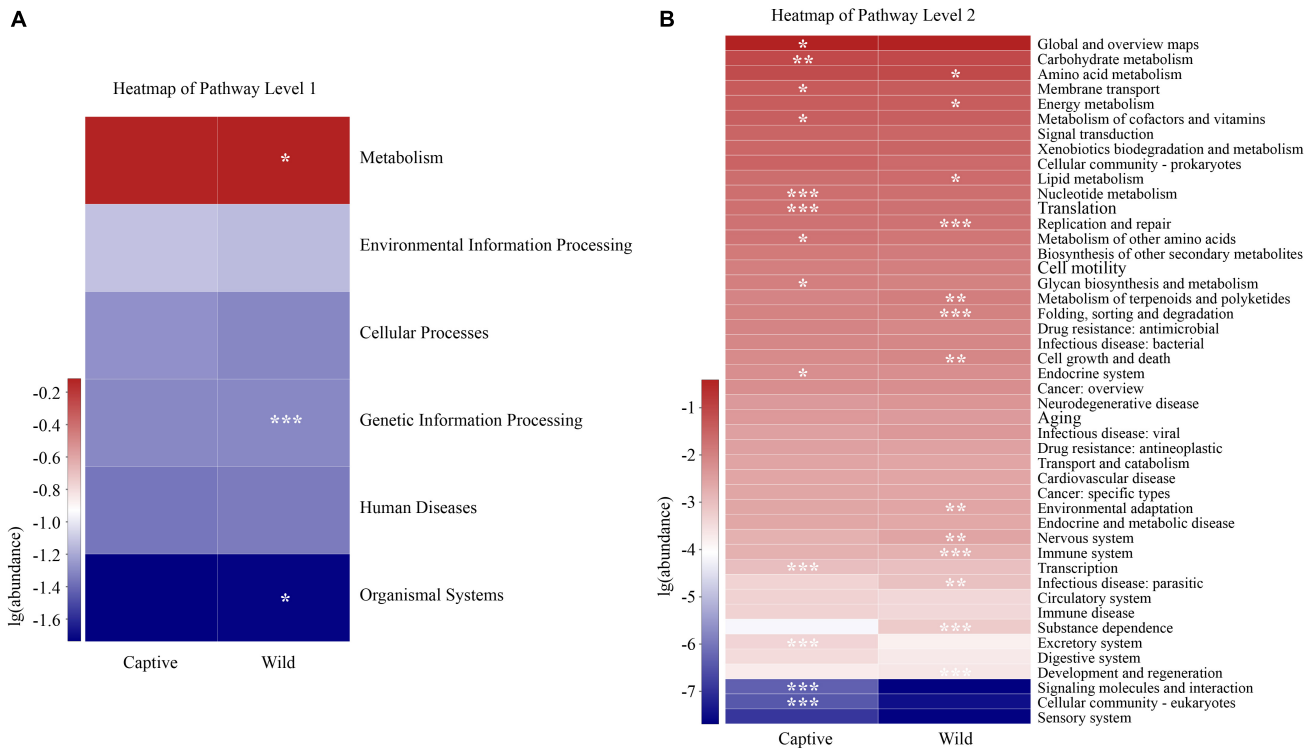

FIGURE 7 | Differences in the functional profiles in level 1 **(A)** and level 2 **(B)** pathways of the gut microbiota in Tokay gecko. The *p* value was represented by asterisks. Significant difference $0.01 < p < 0.05$ was marked as "*", $0.001 < p < 0.01$ was marked as "**", and $p < 0.001$ was marked as "***".

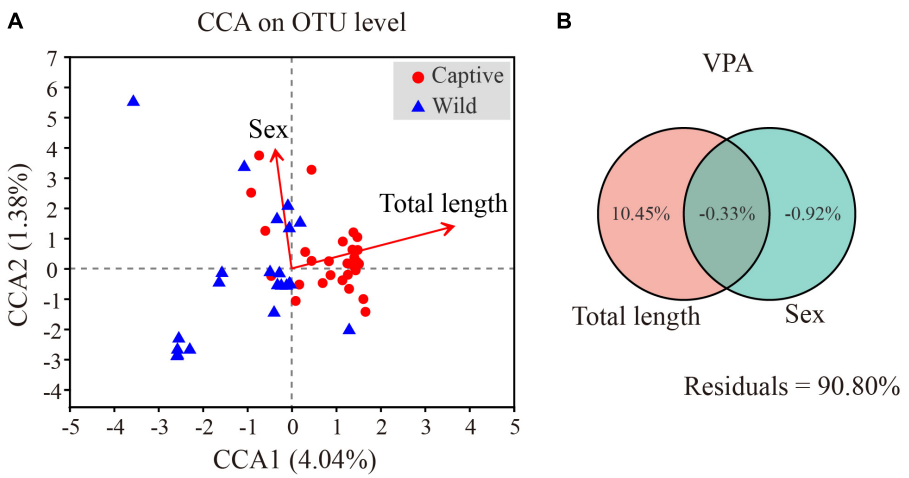

FIGURE 8 | Effects of total length and sex on gut microbiota of Tokay gecko **(A)**. Canonical correlation analysis of the microbial community on OTU level and physical parameters of Tokay gecko **(B)**.

systems pathways. These differences are probably related to differences in living environments and diets of the two gecko populations. Our results could inform researchers in their efforts to further understand the relationship between the gut microbiota of geckos and their living environments and contribute to the comprehensive protection and management of this species in the future.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioProject - PRJNA817415 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA817415).

ETHICS STATEMENT

The animal study was reviewed and approved by Laboratory Animal Care and Animal Ethics Committee of Guangxi Normal University.

AUTHOR CONTRIBUTIONS

ZC and ZW contributed to conception and design of the study. SY and YTL organized the database. CH performed the statistical analysis. ST and YHL wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.897923/full#supplementary-material

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Anthropogenic Disturbance Impacts Gut Microbiome Homeostasis in a Malagasy Primate

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Increasing anthropogenic disturbances in Madagascar are exerting constrains on endemic Malagasy lemurs and their habitats, with possible effects on their health and survival. An important component of health is the gut microbiome, which might be disrupted by various stressors associated with environmental change. We have studied the gut microbiome of gray-brown mouse lemurs (*Microcebus griseorufus*), one of the smallest Malagasy primates and an important model of the convergent evolution of diseases. We sampled two sites: one situated in a national park and the other consisting of a more disturbed site around human settlement. We found that more intense anthropogenic disturbances indeed disrupted the gut microbiome of this lemur species marked by a reduction in bacterial diversity and a shift in microbial community composition. Interestingly, we noted a decrease in beneficial bacteria (i.e., members of the Bacteroidaceae family) together with a slight increase in disease-associated bacteria (i.e., members of the Veillonellaceae family), and alterations in microbial metabolic functions. Because of the crucial services provided by the microbiome to pathogen resistance and host health, such negative alterations in the gut microbiome of mouse lemurs inhabiting anthropogenically disturbed habitats might render them susceptible to diseases and ultimately affecting their survival in the shrinking biodiversity seen in Madagascar. Gut microbiome analyses might thus serve as an early warning signal for pending threats to lemur populations.

Keywords: anthropogenic impact, gut microbiome, 16S rRNA gene, gray-brown mouse lemur (*Microcebus griseorufus*), Madagascar

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INTRODUCTION

Anthropogenic disturbances frequently have direct negative influences on wildlife distribution, reproduction, physiology, and health. However, they can also exert indirect effects by changing the abundance pattern of species communities and their interactions, ultimately influencing the population dynamics of wild-living species (Otto, 2018). Often, these disturbances act in synergy, exacerbating the decline of animal populations. Madagascar has been identified as one of the world's biodiversity hotspots that continues to be under severe anthropogenic

pressure (Myers et al., 2000; Vieilledent et al., 2018). High forest fragmentation, encroachment into wildlife areas, habitat degradation, and heavy pressures on forest resources are critical problems in Madagascar, because of the exponential rise in its human population coupled with extreme poverty (Estrada et al., 2018; Morelli et al., 2019; Bank, 2021). Rapid modifications of their habitats place immediate pressures on animal populations, with the possible extinction of species that cannot tolerate anthropogenic alterations (Otto, 2018). Primates are especially susceptible to such disturbances, as primate habitat ranges often overlap extensively with the growing human population. Indeed, out of the 107 lemur species assessed by the IUCN in Madagascar, 103 were considered to be threatened, with 33 of them being listed as critically endangered, making lemurs the most threatened vertebrate taxon in Madagascar (IUCN, 2021). The drivers causing the decline of lemurs can act synergistically, although habitat loss because of cultivation and timber harvesting is the major contributor (Irwine et al., 2010; Schwitzer et al., 2014), which is amplified by the negative impact of anthropogenic disturbance on the physiology and health of primates (Junge et al., 2011; Schwitzer et al., 2011; Bublitz et al., 2015). The response of species to habitat disturbance has shown to be mixed and species-trait dependent, e.g., small-bodied and mixed folivorous/frugivorous primates show a higher resilience compared with large bodied species and those with a more specialized diet (Eppley et al., 2020). However, more resilient species might also suffer from human encroachment, especially if this disturbs the host-gut microbiome (Fackelmann et al., 2021).

Now known as an important component of health, the gut microbiome provides several necessary services to its host, ranging from nutrition uptake and metabolism to the shaping of immunity, disease resistance, and behavior and its overall support of the host's adaptation to various environment conditions (Thomas et al., 2017; McKenney et al., 2018; Fackelmann et al., 2021; Henry et al., 2021). The gut microbiome itself is shaped by several intrinsic (i.e., genetics) and extrinsic (i.e., environment and social interactions) factors (Amato, 2013; Bahrndorff et al., 2016). Anthropogenic disturbance can induce negative changes in natural gut microbiome homeostasis, because of the loss of microbial diversity, especially a decrease in commensal bacteria and an increase of pathogens, and can result in a state of "dysbioses," with negative implications for animal health. Indeed, recent studies have emphasized the negative effects of landscape modifications and habitat fragmentation on the homeostasis of the host-gut microbiome. For example, the gut microbiome of howler monkeys living in fragmented areas or in captivity has been shown to have a lowered diversity (Amato et al., 2013), with several follow-up studies confirming these findings in various settings and for diverse non-human primate species (Barelli et al., 2015, 2020; Hayakawa et al., 2018; Trosvik et al., 2018). Habitat fragmentation is additionally associated with dietary shifts and microbiota variability in common vampire bats (Ingala et al., 2019). Recent wildlife studies have highlighted that gut homeostasis is also subject to disturbance by viral infections, which facilitate co-infections and the spread of zoonotic diseases (Wasimuddin et al., 2018; Wasimuddin et al., 2019), and is influenced by the host's immune genetic diversity (Montero et al., 2021). The associated behavioral and physiological traits of host species also play an important role in rendering resistance or susceptibility within the gut microbiome (Barelli et al., 2020).

Here, we have studied the impact of anthropogenic disturbances on the gut microbiome of the gray-brown mouse lemur (*Microcebus griseorufus*) from Southern Madagascar. Gray-brown mouse lemurs are small-bodied (40–80 g) arboreal nocturnal primates inhabiting both natural and degraded forests, exhibiting a potential range overlap with humans and other domestic species (Mittermeier et al., 2010). *Microcebus* spp. represent important model species for understanding the convergent evolution of diseases (Ezran et al., 2017; Hozer et al., 2019) and provide a good study system for disentangling the effects of anthropogenic disturbances on the homeostasis of the gut microbiome and on overall host health. The diet of the gray-brown mouse lemur consists of mostly gum and fruits, but also includes insects. In the dry season when the food supply is short, the lemurs reduce their metabolic activities and exhibit different energy saving strategies from daily torpor to a hibernation-like stage for several weeks (Kobbe et al., 2011). Small-bodied primates and nocturnal primates have been suggested to be less sensitive to anthropogenic disturbances (Eppley et al., 2020; Hending, 2021). Nevertheless, anthropogenically modified habitats with substantial contact zones between lemurs and humans increase disturbance frequencies and modify habitat and microclimatic characteristics, including food resources. In order to understand the influence of anthropogenic disturbances on gut microbiome community structure and function, we have compared the gut microbiome of mouse lemurs from two sites: a less disturbed site located in the Tsimanampetsotsa National Park and a highly disturbed site partially integrated into a village with associated livestock herding and agriculture.

MATERIALS AND METHODS

Study Sites and Sample Collection

The field study was carried out between 2013 and 2015 in south-western Madagascar at two localities: Andranovao and Miarintsoa (**Figure 1**). Andranovao is at the western foot of the Mahafaly plateau, located in the Tsimanampetsotsa National Park, in south-western Madagascar (24°03′–24°12′S, 43°46′–43°50′E). The vegetation consists of continuous dry spiny forest and spiny thicket (Ratovonamana et al., 2011, 2013). Here, annual rainfall averages 300 mm, with some years being without rain (Ratovonamana et al., 2013). Miarintsoa is located on the limestone plateau about 42 km east of Andranovao at 23°50′ S, / 44°06′ E; 183 m a.s.l. The area around Miarintsoa was largely covered with forest until 1973 but has been heavily degraded to a highly fragmented state since 2010 (Brinkmann et al., 2014); it is now dominated by agricultural fields and pasture with small and scattered forest remnants used intensively for wood collection. The area around Miarintsoa can be described as being land covered with grasses (mainly *Heteropogon contortus*), with woody plant cover of 10%–30% (according to the vegetation

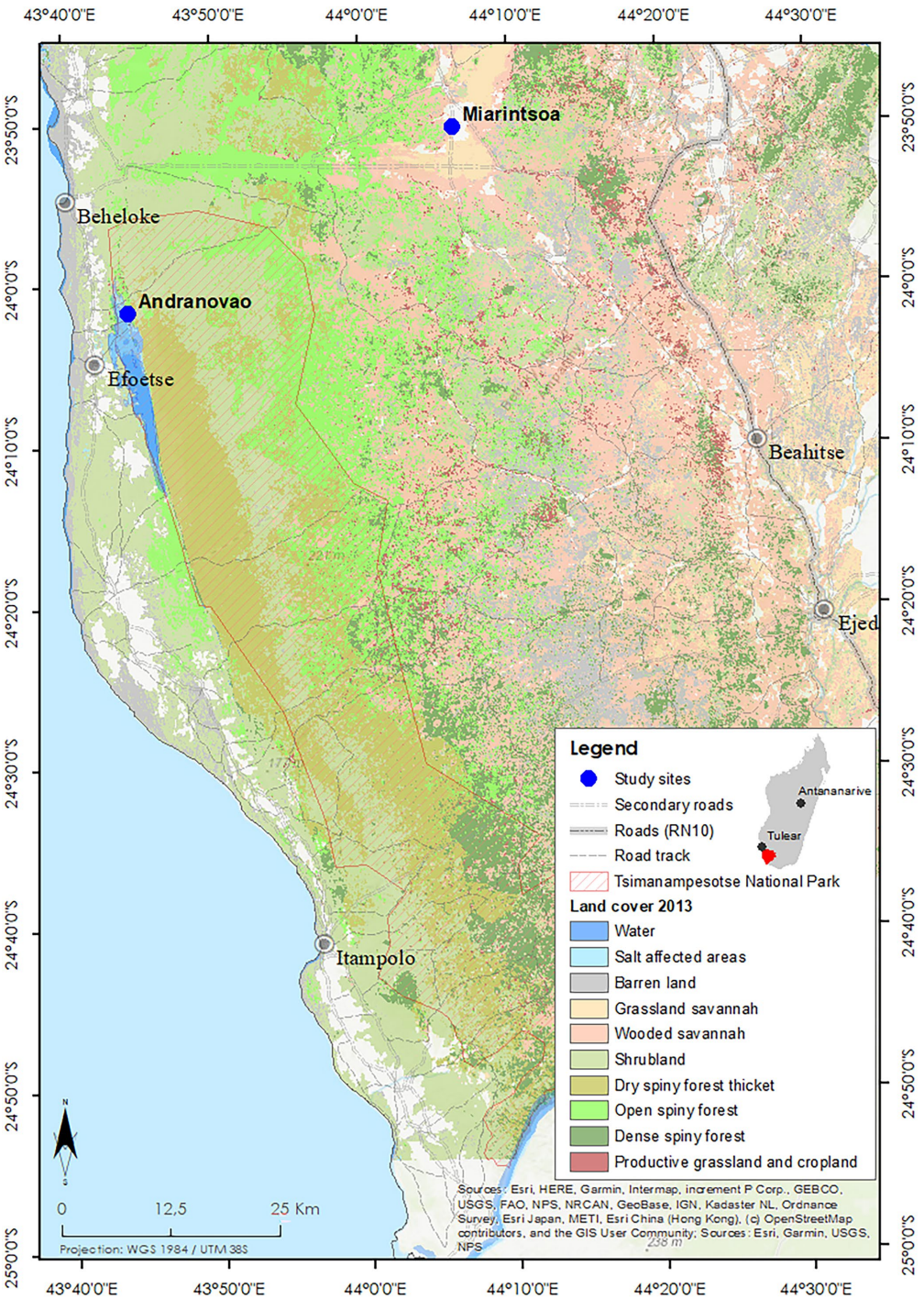

FIGURE 1 | Study area and vegetation cover in south-western Madagascar. The study sites Andranovao and Miarintsoa are marked with blue dots. The Tsimanampetsotsa National Park boundaries and the type of land cover (in 2013) are indicated (adapted from www.sulama.de by Y. R. Ratovonamana).

classification by Brinkmann et al., 2014 and Nopper et al., 2018). On the plateau, annual rainfall ranges between 500 and 800 mm (Ratovonamana et al., 2013).

We used samples from 161 *M. griseorufus* individuals captured with Sherman live-traps (H.B. Sherman Traps, Tallahassee; 7.5 × 7.5 × 30.5 cm) during the same seasons in both study locations following standard protocols (e.g., Bohr et al., 2011). All trapped animals were sexed, measured, weighed, and marked permanently with subdermal transponders. None of the individuals exhibited any signs of illness or disease at the time of capture. Fresh fecal samples (one sample per individual; Andranovao; $n = 113$, Miarintsoa; $n = 47$) were collected either

from the traps or handling bags and preserved in Eppendorf vials filled with 500 µl RNAlater (Life Technologies). After morphological measurement, the animals were released immediately at the site of capture. Upon return to the field station, samples were kept cool (around 20°C) until they could be transported to the nearest city. They were then stored at −20°C before being transported in polystyrene isolation boxes equipped with passive cooling elements to Germany where they were kept at −80°C until DNA extraction.

Bacterial DNA Extraction and 16S rRNA Gene Amplicon Sequencing

Fecal samples were homogenized by using beads in a SpeedMill PLUS Homogenizer (Analytik Jena, Germany; Wasimuddin et al., 2019; Montero et al., 2021). Bacterial genomic DNA was extracted from feces by using NucleoSpin 96 Soil kits (Macherey-Nagel, Germany) according to the manufacturer's instructions. We amplified the hypervariable V4 region of the 16S rRNA gene (291 bp) with the primer pair 515F (5-GTGCCAGCMGCCGCGGTAA-3) and 806R (5-GGAC TACHVGGGTWTCTAAT-3; Kuczynski et al., 2011; Caporaso et al., 2012; Wasimuddin et al., 2020). Following the approach recommended for the Fluidigm System (Access Array™ System for Illumina Sequencing Systems, © Fluidigm Corporation), these primers were tagged with sequences (CS1 forward tag and CS2 reverse tag) complementary to the respective forward or reverse access array barcode primers required for the Illumina platform. The PCR and PCR-product purification were performed as previously reported (Wasimuddin et al., 2019; Montero et al., 2021). After quantification by DropSense (Trinean, US), samples were pooled to give an equal amount of 50 ng DNA and the pool was diluted to 8 pM in hybridization buffer. The final library was paired-end sequenced in a single run on an Illumina® MiSeq platform. Negative controls were included for both the DNA extraction (no sample added) and 16S PCR amplification (with PCR certified water) to test for contamination. No DNA contamination was observed when using DropSense quantification.

Bioinformatics

Demultiplexed reads without barcodes and adapters were received as output from the Illumina sequencing platform. All subsequent analyses were performed within the R environment (R version 3.5.1, R Development Core Team, 2011). For data pre-processing, we followed the *DADA2* pipeline (version 1.10; Callahan et al., 2016). For the dataset, reads were trimmed from both ends based on the quality profile; error rates were obtained from the data by using the parametric error model as implemented in *DADA2*. After the reads had been denoised and merged, chimeric sequences were removed from the dataset by following the "consensus" method. Thus, non-chimeric amplicon sequence variants (ASVs; i.e., sequences differing by as little as one nucleotide) were identified in each sample. The taxonomy of representative ASVs was assigned using the naïve Bayesian classifier method with the SILVA v132 non-redundant (NR) database. Species level was designated by the exact matching (100% identity) of ASVs with database sequences, as previously recommended (Edgar, 2018). The *Phyloseq* (version 1.24.2; McMurdie and Holmes, 2013) package was used for further data processing, with ASVs belonging to chloroplast, mitochondria, archaea, Eukaryota, and unassigned ASVs at the phylum level being removed from the dataset. As *DADA2* might be more sensitive to a low amount of contamination (Caruso et al., 2019), we further removed the ASVs showing less than 10 reads in the overall dataset. We recovered, on average, 52,136 (range 12,081–130,215) high quality reads per sample. In total, 1,503 unique ASVs were detected in the gut microbiomes of gray-brown mouse lemurs ($N = 161$ individuals) after taxonomic assignments. Since we aimed for a minimum depth of 20,000 reads in analyses requiring rarefaction (alpha and beta diversity calculations), we removed the sample with 12,081 reads from these analyses.

Alpha and Beta Diversity Analyses and Identification of Phyla and ASVs Associated With Anthropogenic Disturbance

We investigated the effects of habitat differing in anthropogenic disturbance and of sex on microbial diversity for each sample by using three different alpha diversity indices (number of observed species, Fisher, and Shannon) after rarefying the data to the 23,150 sequences per sample (one sample with 12,081 reads was removed before rarefaction) by using *Phyloseq*. The first alpha diversity index describes the unique taxa count (i.e., ASV and species richness), whereas the Fisher index accounts for the number of species and for abundance and the Shannon index is an abundance-weighted diversity measure. To analyze the association of study site and sex with these alpha diversity matrices, we performed General Linear Modeling (GLM) by using the lme_4 package in R (Bates et al., 2015). We included the study sites (Andranovao; $n = 113$, Miarintsoa; $n = 47$) and sex (male; $n = 69$, female; $n = 89$, not known; $n = 2$) in the model as explanatory variables for each alpha diversity metric table.

Beta diversity analyses were based on calculated Euclidean, Unweighted, and Weighted UniFrac dissimilarity matrices after the data had been rarefied to 23,150 sequences per sample by using *Phyloseq*. The Euclidean distance is commonly used to detect changes in relative composition without using phylogenetic information, whereas Unweighted UniFrac distances account for phylogenetic distances among taxa present and Weighted UniFrac distances additionally include information on taxa abundance. The permutational multivariate analysis of variance (PERMANOVA) was employed as implemented in the *adonis* function of the *vegan* package (version 2.5-2; Oksanen et al., 2018) in order to test the significance of the differences in community composition with 999 permutations. For all beta diversity metrics, we similarly included study site and sex in the models as explanatory variables. To visualize patterns of separation between the different sample categories, principal coordinates analyses (PCoA) were performed based on the Euclidean and Unweighted and Weighted UniFrac dissimilarity matrices. Moreover, we used Mann–Whitney–Wilcoxon tests

to deduce the effect of habitat disturbance on the inter-individual variability of the three beta diversity metrics of lemur individuals.

In order to identify the phyla and ASVs accountable for differences in the microbiome of lemurs inhabiting different habitats, we employed a negative binomial model-based approach available in the *DESeq2* package in R (Love et al., 2014) without rarefaction. Wald tests were performed and only phyla and ASVs remaining significant ($p < 0.01$) after the Benjamini–Hochberg correction were retained. Additionally, the *Random forest* classifier was used to assign the rank of differentially abundant phyla to the microbial community based on the mean decrease in accuracy (Ssekagiri et al., 2017).

Microbial Function Predictions and Identification of Differentially Abundant Functional Pathways

To anticipate functional differences of the gut microbiome of individuals inhabiting the different habitats based on 16S rRNA gene sequencing, we used *PICRUSt2* (Douglas et al., 2020). After normalization for copy number variation as implemented in *PICRUSt2*, the metagenome prediction was carried out by using the KEGG Orthology (KOs) classification. To assess the accuracy of the prediction, weighted Nearest Sequenced Taxon Index (weighted NSTI) scores were calculated. The NSTI score represents the sum of phylogenetic distances of each organism in a sample to its nearest relative in a sequenced bacterial genome. Only sequences with NSTI values of <2, as default, were kept in order to allow better predictions of metagenomes. To investigate the effect of the anthropogenic disturbances on the KEGG composition, we calculated "*gower*" distances after rarefying the data. We applied PERMANOVAs with 999 permutations and included study site and sex of the individual in the model as explanatory variables to explain differences in the *gower* metrics. PCoA plots were used to visualize the pattern of separation of metagenomes based on the *gower* metric between individuals captured in Andranovao or Miarintsoa (not shown). We categorized KOs to major functional pathways by applying the KEGG classification at hierarchy level 3. To identify the pathways that showed differential abundance between Andranovao and Miarintsoa individuals, we used Wald tests implemented in the *DESeq2* package in R (Love et al., 2014) and only pathways that remained significant ($p \leq 0.05$) after the Benjamini–Hochberg correction were reported.

RESULTS

Gut Microbial Diversity and Community Composition of Gray-Brown Mouse Lemurs Differ Between Habitats

The gut bacterial communities of gray-brown mouse lemurs were dominated by the phyla Bacteroidetes (32.3%), Actinobacteria (30.2%), and Firmicutes (25.4%), followed by Proteobacteria (5.9%) and Epsilonbacteraeota (4.3%). We used General Linear Modeling (GLM) to test whether inter-individual differences within three alpha diversity estimates (number of observed species, Fisher, and Shannon) can be explained by host habitat and sex. Habitat had a significant effect on the number of observed species ($p = 0.003$), on Fisher ($p = 0.003$), and on Shannon ($p = 0.016$), but not sex (all $p > 0.05$). All three alpha diversity metrics were lower in individuals from Miarintsoa than Andranovao (**Figure 2**; **Supplementary Table S1**).

To determine whether the host habitat influenced the composition of the gut microbial community, we calculated three beta diversity metrics (Euclidean, unweighted UniFrac, and weighted UniFrac) and included again habitat and sex as explanatory variables in PERMANOVA models. We observed a significant effect of habitat (unweighted UniFrac: $R^2 = 0.046$, $p = 0.001$; weighted UniFrac: $R^2 = 0.033$, $p = 0.001$; Euclidean: $R^2 = 0.035$, $p = 0.001$) but no effect of host sex (unweighted UniFrac: $R^2 = 0.006$, $p = 0.280$; weighted UniFrac: $R^2 = 0.006$, $p = 0.364$; Euclidean: $R^2 = 0.006$, $p = 0.426$) on gut microbial beta diversity estimates (**Figure 3**; **Supplementary Table S2**). Inter-individual beta diversity was significantly increased in lemurs from Miarintsoa compared to those from Andranovao using Euclidean ($p < 0.001$) metric but did not show any significant difference using the two other metrics (unweighted UniFrac; weighted UniFrac; all $p > 0.05$; **Figure 3**).

Relative Abundance of Major Phyla and ASVs Differ Between Habitats

The habitat influenced the relative abundance of taxa at various taxonomic levels. At the higher phylum level, five phyla, namely, Verrucomicrobia, Spirochaetes, Fusobacteria, Lentisphaerae, and Tenericutes, were detected as being differentially abundant ($p < 0.01$) between Andranovao and Miarintsoa individuals. All observed phyla showed a decrease in the relative abundance in Miarintsoa compared with Andranovao (**Figure 4**). Among the detected phyla, Verrucomicrobia was found to be the top ranked phylum according to the Random forest classifier. At the ASV level, we identified 48 ASVs that differed significantly ($p < 0.01$) in abundance in mouse lemurs, with 33 ASVs (68.7%) revealing a lower and 15 ASVs (31.2%) revealing a higher mean abundance in Miarintsoa compared to Andranovao individuals (**Figure 5**; **Supplementary Table S3**).

ASVs that showed a lower abundance in mouse lemurs from Miarintsoa predominantly belonged to the families Prevotellaceae ($n = 9$ ASV), Bacteroidaceae ($n = 3$ ASV), Tannerellaceae ($n = 3$ ASV), Muribaculaceae ($n = 3$ ASV), and genera *Prevotellaceae_NK3B31_group* ($n = 3$ ASV), *Parabacteroides* ($n = 3$ ASV) and *Bacteroides* ($n = 3$ ASV; **Figure 5**; **Supplementary Table S3**). ASVs that showed a higher abundance in Miarintsoa individuals mainly belonged to the families Prevotellaceae ($n = 4$ ASV), Bifidobacteriaceae ($n = 3$ ASV), Veillonellaceae ($n = 3$ ASV), and genera *Prevotellaceae_UCG-001* ($n = 3$ ASV) and *Bifidobacterium* ($n = 3$ ASV; **Figure 5**; **Supplementary Table S3**).

Predicted Metagenomes and Higher Functional Pathways Differ Between Lemurs From Two Sites

We applied a PERMANOVA-based model approach to investigate whether differences in the predicted KEGG orthologs (KOs) occurred in relation to site (habitat disturbance) or sex. In

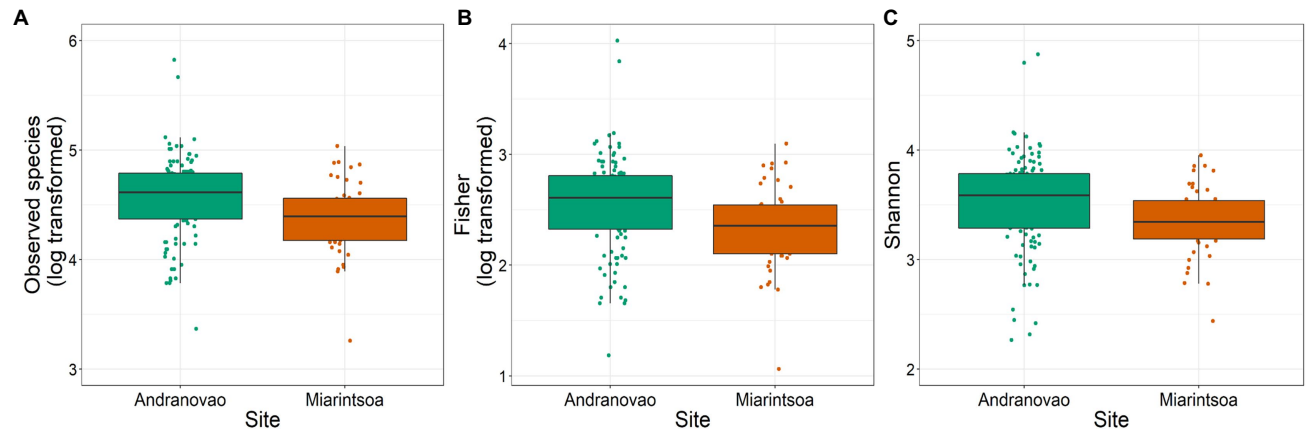

FIGURE 2 | Alpha diversity of gut bacteria in gray-brown mouse lemurs differs between habitats. Effect of study site on **(A)** number of observed species, **(B)** Fisher diversity index, and **(C)** Shannon diversity index of mouse lemur individuals. Individuals from Miarintsoa (red) revealed a lower bacterial diversity than individuals trapped in Andranovao (green; see **Supplementary Table S1** for details).

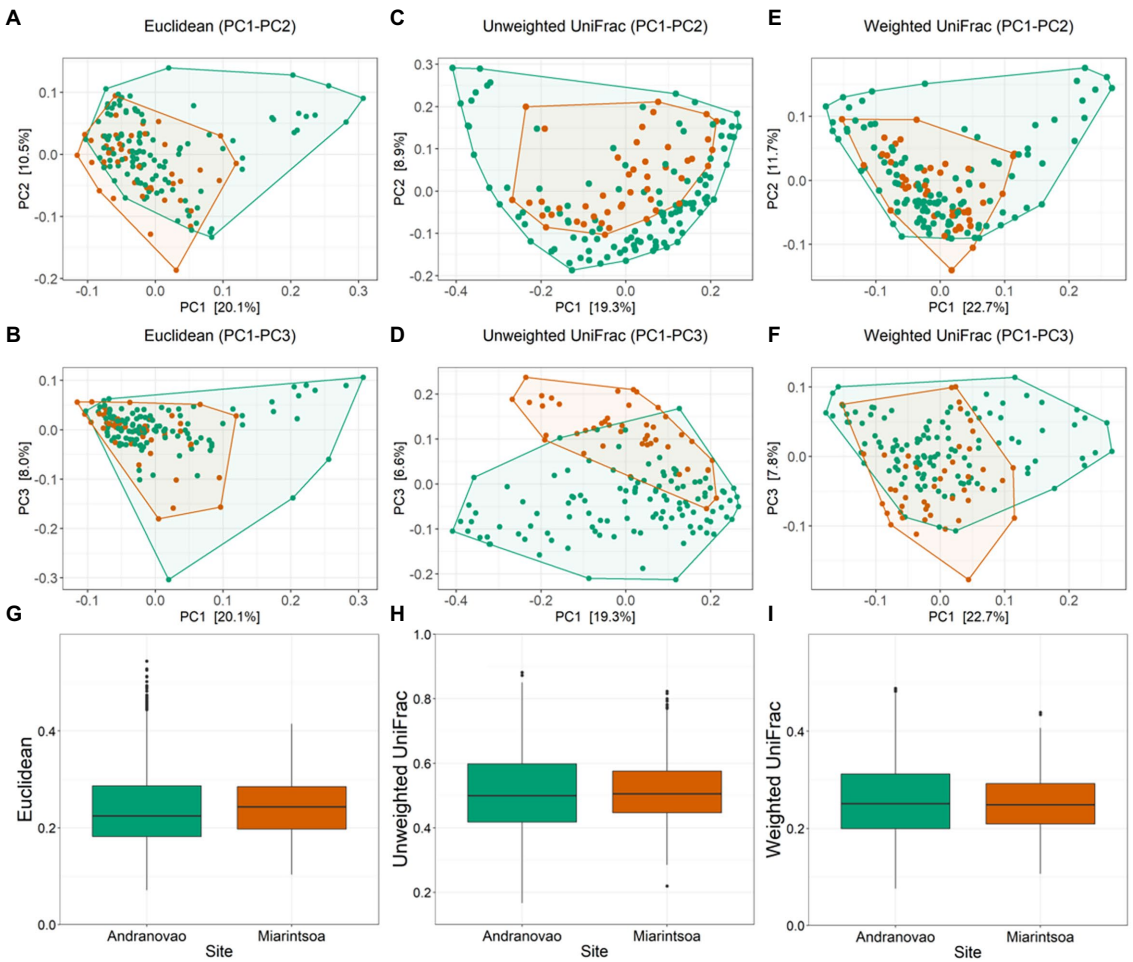

FIGURE 3 | Composition of bacterial community in the gut of mouse lemurs differs between habitats. Principal-coordinate plots of Euclidean [**(A)** PC1–PC2, **(B)** PC1–PC3; Unweighted UniFrac **(C)** PC1–PC2, **(D)** PC1–PC3; and Weighted UniFrac **(E)** PC1–PC2, **(F)** PC1–PC3] metrics in mouse lemurs. Dots and connecting polygons represent bacterial communities in gut of mouse lemurs trapped in Andranovao (green) or Miarintsoa (red). Box plots are showing the inter-individual distances between Andranovao and Miarintsoa individuals using **(G)** Euclidean, **(H)** Unweighted UniFrac, and **(I)** Weighted UniFrac metrics.

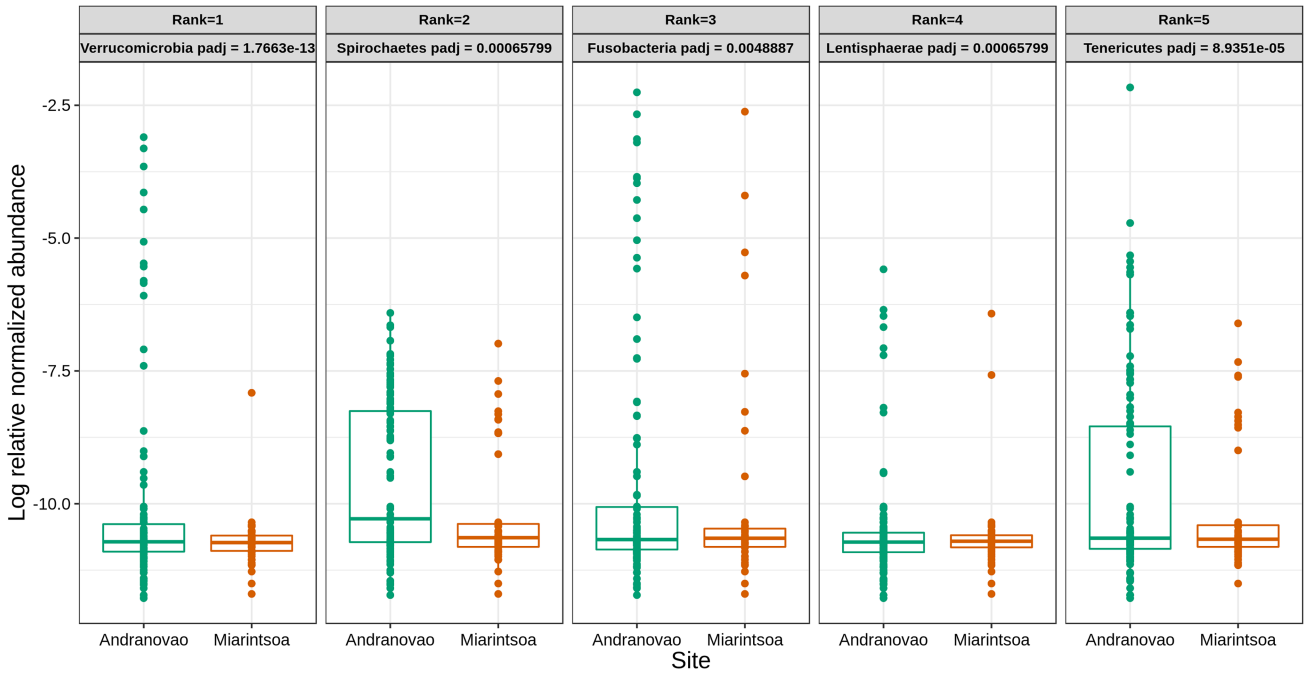

FIGURE 4 | Relative abundance of major bacterial phyla in gut of mouse lemurs differs between habitats. Box plots indicate the effect of study site (Andranovao in green; Miarintsoa in red) on the relative abundance of major phyla in the gut microbiomes of mouse lemurs (all $p < 0.01$). Phyla are arranged according to the assigned rank based on the importance of differentially abundant phyla (see "Materials and Methods" for details).

the model, site ($R^2 = 0.012$, $p = 0.039$) had a significant effect on the *gower* distances, but not sex ($R^2 = 0.003$, $p = 0.941$).

Four predicted higher functional pathways (KEGG) were identified that differed significantly in their abundance between Andranovao and Miarintsoa individuals ($p \leq 0.05$; **Supplementary Figure 1**). At the higher level, all the identified pathways were related to "metabolism." The identified pathway that showed severely lower abundance (25-fold) in Miarintsoa individuals belonged to "Metabolism of terpenoids and polyketides." The metabolism pathway, "Xenobiotics biodegradation and metabolism," also showed a decrease in individuals from Miarintsoa, whereas the abundance of two other metabolic pathways ("Carbohydrate metabolism" and "Metabolism of other amino acids") increased in Miarintsoa compared with Andranovao individuals (**Supplementary Figure 1**).

DISCUSSION

Despite major conservation efforts, Madagascar's unique ecosystems are declining at a vast speed, threatening the home of many endemic lemur species. Whereas most lemur species are highly sensitive to anthropogenic change, others, including our focus species, which is one of the smallest Malagasy primates, namely, the gray-brown mouse lemur (*M. griseorufus*), seem at the first glance to be more resilient, since they can also inhibit human-modified habitats (Hending, 2021). However, whether such resilience also applies to its inner ecosystem, the gut microbiome remains unknown. Nevertheless, increasing numbers of studies emphasize the negative impacts of habitat modification on the homeostasis of the intestinal microbiome, which is key to host health (Amato et al., 2013; Barelli et al., 2015, 2020; Hayakawa et al., 2018; Trosvik et al., 2018). Reduction in overall gut microbial diversity can result in "dysbiosis," i.e., a depletion in bacteria essential for host health and an increase in pathogenic bacteria, both of which can negatively affect the health of gray-brown mouse lemurs (Duvallet et al., 2017).

Our comparison of the gut microbiomes of mouse lemurs inhabiting two different habitats, namely, Andranovao situated in Tsimanampetsotsa National Park and the highly disturbed habitat Miarintsoa surrounded by human settlements, revealed that anthropogenic disturbance was indeed associated with the disruption of the homeostasis of the gut microbiome. This was reflected by a decrease in microbiome alpha and beta diversity and an alteration in microbial community composition in Miarintsoa. Moreover, we observed a decrease in beneficial bacterial taxa and associated shifts in the predicted metabolic functions of the microbiome of the gray-brown mouse lemurs living in the disturbed habitat. Even though Miarintsoa receives more rain per year (~100 mm difference) than Andranovao (about 40 km distance), which might provide a higher abundance and diversity of fruit plants and should give rise to higher gut microbial diversity, we observed a reduction in gut microbial diversity suggesting that the influence of anthropogenic disturbance is greater than that of better food availability attributable to the wetter climate. We did not observe any effect of sex on both alpha and beta gut microbial diversity of mouse lemurs. Defining the role of sex in shaping lemur

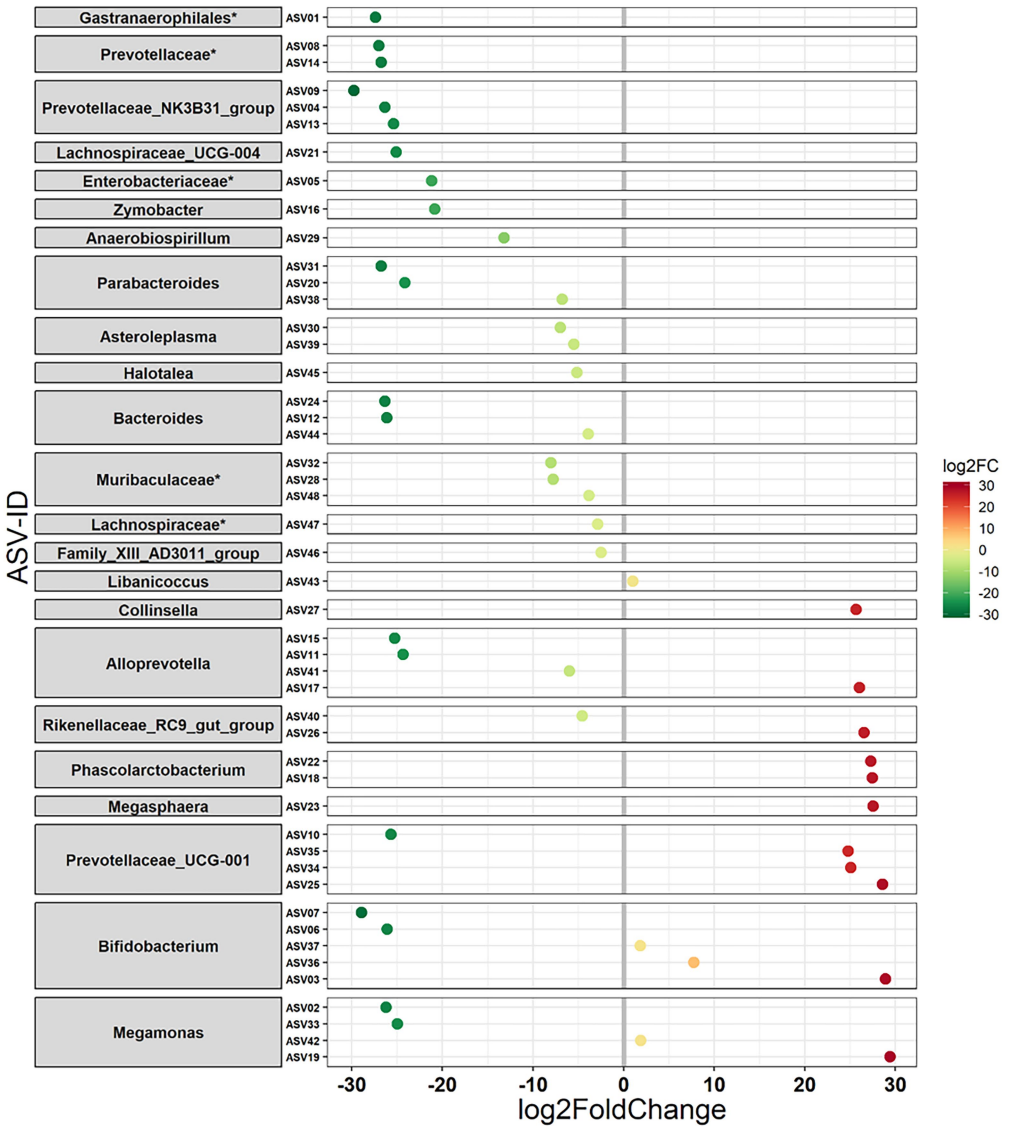

FIGURE 5 | Differential abundance of amplicon sequence variants (ASVs) in mouse lemurs inhabiting two different habitats. ASVs (48 ASVs) that differ in their mean abundance in relation to the habitat of mouse lemurs. Values indicate a log 2-fold (log2FC) decrease (33 ASVs) or increase (15 ASVs) in Miarintsoa individuals. ASVs are arranged according to increasing values of log2FC. The highest possible taxonomic assignment (maximal to the genus level) is shown for each ASV; *includes unclassified ASV/s at genus level.

microbiomes is challenging since earlier studies provided mixed results, reporting either a significant role of sex (Perofsky et al., 2021) or no sex-dependent effects (Bornbusch et al., 2022).

Habitat modifications can stimulate many factors impacting the host microbial community and its functions: (1) diet-associated factors driven by a change in vegetation arising from (micro) climatic, rainfall, or land-use shifts, (2) altered home range sizes caused by habitat disturbance and changes in food items and their distribution, and (3) proximity to humans, livestock and other domestic animals (Wasimuddin et al., 2017; Fackelmann et al., 2021). In Andranovao, the natural vegetation consists of largely intact dry spiny forest and spiny thicket, whereas in Miarintsoa, which usually receives more rainfall, the vegetation is dominated by agricultural fields and pasture with small and scattered forest remnants used intensively for wood collection (Ratovonamana et al., 2011, 2013). Such disturbances in forests over time might constrain the movement of mouse lemurs, restrict its ranging pattern (Bohr et al., 2011; Steffens et al., 2017), and impact its natural diet composition. Reduction and modification in diet diversity have also been suggested to cause the decreases seen in the gut microbial diversity of other non-human primates (Amato et al., 2013; Barelli et al., 2015, 2020; Hayakawa et al., 2018; Trosvik et al., 2018). Similarly, in association with increasing disturbance, an increase in the activity period of mouse lemurs in order to meet their energy demands has been noted in disturbed forests (Fish, 2014). All such

behavioral and physiological changes can disturb the diversity and composition of the gut microbiome of mouse lemurs but cannot be disentangled in this study.

Consistent with the decrease in overall gut microbial diversity in mouse lemurs from Miarintsoa, five phyla (Verrucomicrobia, Spirochaetes, Fusobacteria, Lentisphaerae, and Tenericutes) had a lower relative abundance in the disturbed habitat compared with individuals from the National Park. All of these phyla are common in various non-human primate guts (Clayton et al., 2018). The presence of Verrucomicrobia and Fusobacteria in human guts has proven beneficial for host health (Ghosh and Pramanik, 2021). Interestingly, higher proportions of phyla Verrucomicrobia and Lentisphaerae have been positively correlated with better sleep and high cognitive abilities (Anderson et al., 2017). Thus, the reduced abundance of these two phyla might explain the increased activity in lemurs living in fragmented forests (Fish, 2014). Furthermore, the lower abundance of these phyla makes the host susceptible to other gut disturbances (Kalkeri et al., 2021). Spirochaetes are normally present in the gut microbiomes of humans and other mammals and play an important role in the digestion of plant polysaccharides (Thingholm et al., 2021). Moreover, in baboons, social interactions are crucial for the transmission of gut bacteria, specifically anaerobic and non-spore-forming bacteria such as Spirochaetes, between members of the same social group (Tung et al., 2015). One can speculate that the observed reduced abundances of Spirochaetes in Miarintsoa individuals is attributable to the limited contact that they have with other co-specifics because of habitat disturbance. Little is known so far about the functional role of members of the Tenericutes phylum; however, they are commonly present in the gut of non-human primates, humans, fishes, and insects (Clayton et al., 2018; Wang et al., 2020b). Nevertheless, a deeper understanding of the cause-and-effect relationships for phyla abundance requires further investigations.

Among the ASVs that differed significantly between the Miarintsoa and Andranovao individuals, most ASVs were lower (68%) in mouse lemurs from Miarintsoa. At the family level, these decreasing ASVs predominantly belonged to Prevotellaceae followed by Bacteroidaceae, Tannerellaceae, and Muribaculaceae and, at the genera level, decreasing ASVs were assigned to the *Prevotellaceae_NK3B31_group*, *Parabacteroides,* and *Bacteroides*. The family Prevotellaceae is associated in humans and chimpanzees with their carbohydrate-rich diets; this is also true for gray-brown mouse lemurs whose diet is primarily based on gum and fruits (Moeller et al., 2012). Interestingly, the *Prevotellaceae_NK3B31_group* has been suggested to provide protection against invading pathogens and to reduce diarrhea in piglets (Wang et al., 2020a). Thus, the reduced relative abundance of members of the Prevotellaceae family in mouse lemurs from the disturbed habitat Miarintsoa might be a result of their ingesting a diet less rich in carbohydrates but richer in insects, which ultimately might make mouse lemurs more susceptible to invading pathogens. Bacteroidaceae and its genus *Bacteroides*, and *Parabacteroides* (a genus of Tannerellaceae) are generally considered as beneficial bacteria with numerous benefits to host health (Wexler and Goodman, 2017). They are major producers of many short chain fatty acids in the gut and play an important role in gut homeostasis and immune regulation (Venegas et al., 2019; Wang et al., 2019). A decrease in the abundance of Bacteroidaceae/*Bacteroides* and *Parabacteroides* has been demonstrated in various intestinal disorders (Forbes et al., 2016).

Mouse lemurs represent an important model in aging research because of their high phenotypic plasticity and similarity with human aging (Hozer et al., 2019). Interestingly, members of the Muribaculaceae family are often reported to be associated with aging and longevity (Sibai et al., 2020). Thus, a decrease in the relative abundance of members of the Muribaculaceae in Miarintsoa might be a sign of accelerated aging and/or disturbed biological rhythms in anthropogenically disturbed landscapes. Yet, members of Muribaculaceae are involved in the digestion of complex carbohydrates; thus, the decrease in their abundance in Miarintsoa individuals could be also associated with dietary differences compared to Andranovao individuals. We have also noted an increase of a smaller number of ASVs (31%) mainly belonging to the Prevotellaceae family (genus *Prevotellaceae_UCG-001*), Bifidobacteriaceae family (genus *Bifidobacterium*), and members of the Veillonellaceae family in the gut of mouse lemurs from Miarintsoa. Genera of the Prevotellaceae family are well known for their competitive interactions and thus a decrease in the abundance of some of their members and increase of others is not surprising (Lei et al., 2018). However, the genus *Bifidobacterium* is usually considered as beneficial for host health and therefore, an increase in the abundance of its members in mouse lemurs trapped in a disturbed habitat is surprising (Sun et al., 2020). Members of the Veillonellaceae family are often found in association with gut inflammation (Ling et al., 2016) and hence are more abundant in patients with Inflammatory Bowel Disease, fibrosis, and other disease (Forbes et al., 2016; Lee et al., 2020; Salliss et al., 2021). As with phylum, defining the role of specific ASVs as well as cause-and-effect relationships require carefully planned experimental studies in future. Further, one could argue that the observed changes in microbial taxa could simply mirror the adaptation to newer habitats; however, the decrease in the abundance of beneficial bacterial taxa together with an increase in taxa associated with a diseased state rather implies a loss of homeostasis in the gut microbiomes of mouse lemurs sampled in Miarintsoa; this might adversely affect the health of lemurs and potentially make them more susceptible to diseases.

In accordance with the changes in the microbial community composition, namely, the decrease in bacterial diversity and in the beneficial taxa in mouse lemurs captured in the disturbed habitat, we detected shifts in microbial metabolic functions. All predicted microbial functional pathways differing between lemurs of Miarintsoa and Andranovao sites belonged to metabolism at a higher level. The major pathway that showed severely lower abundance (25-fold) in Miarintsoa individuals was assigned to "Metabolism of terpenoids and polyketides." Terpenoids and polyketides are secondary metabolites from plants and trees and a reduced abundance of functions related to their metabolism might constrain the digestive abilities of the hosts (Grassotti et al., 2021). Similarly, the pathway related to "Xenobiotics biodegradation and metabolism" was also noted to be lower in Miarintsoa individuals. *PICRUSt2* uses a stringent approach and

only taxa with known functions are used for function predictions. This means that the functions associated with new taxa, specifically based on 16S rDNA marker information in the study system could not be predicted. Nevertheless, our analyses provide a broad glimpse of functions of mouse lemur gut microbial community and shifts associated with anthropogenic disturbance.

Because of the crucial services performed by the microbiome with regard to host health, such negative alterations of the gut microbiome of lemurs living in anthropogenically disturbed habitats might impact the health of even a phenotypically resistant species, rendering them susceptible to diseases and ultimately impacting their survival in Madagascar's shrinking biodiversity. Investigation of the gut microbiome represents a non-invasive and simple method for detecting early signs of declining wildlife health and is easy to implement as a diagnostic tool in primate conservation.

DATA AVAILABILITY STATEMENT

The individual gut bacterial 16S rRNA gene sequences are available under NCBI SRA accession ID SRP217185 and can be found online at: https://www.ncbi.nlm.nih.gov/sra/?term=SRP217185.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of the Institute of Zoology of Hamburg University, the University of Antananarivo and Madagascar National Parks.

AUTHOR CONTRIBUTIONS

W, SS, and JG conceived the microbiome study. JG, SR, and YR provided host data. W carried out laboratory experiments, bioinformatics, statistical analyses, and wrote the manuscript. HM contributed to draft scripts. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.911275/full#supplementary-material

Supplementary Figure 1 | Differential abundance of predicted major functional pathways in relation to the habitat of mouse lemurs. Differences in the mean abundance of major functional pathways (by using KEGG classification) between Andranovao and Miarintsoa individuals (Wald tests, $p \leq 0.05$). The values indicate a log 2-fold decrease or increase in Miarintsoa individuals. Functional pathways are arranged according to increasing values of log 2-fold change.

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Multi-Omics Approaches Revealed the Associations of Host Metabolism and Gut Microbiome With Phylogeny and Environmental Adaptation in Mountain Dragons

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The molecular basis enabling the adaptation of animals to spatially heterogeneous environments is a critical clue for understanding the variation, formation, and maintenance of biodiversity in the context of global climate change. Mountain dragons (Agamidae: *Diploderma*) thrive in the Hengduan Mountain Region, a biodiversity hotspot and a typical spatially heterogeneous environment. Here, we compare the liver and muscle metabolome and gut microbiome of 11 geographical populations from three *Diploderma* species (*D. iadinum*, *D. yulongsense*, and *D. vela*) after 7 days acclimation in the same laboratory conditions. Amino acid metabolism, particularly the products of the glutathione cycle, accounted for major interspecies variations, implying its significance in genetic differentiation among mountain dragons. Notably, the cold-dwelling *D. vela* and *D. yulongense* populations tended to have higher glycerophosphate, glycerol-3-phosphocholine, and kinetin levels in their liver, higher carnosine levels in their muscle, and higher Lachnospiraceae levels in their gut. Phylogeny, net primary productivity (NPP), and the temperature had the highest explanation rate to the variations in muscle metabolome, liver metabolome, and gut microbiome, respectively, suggesting heterogeneity of biological systems in response to climatic variations. Therefore, we suggested that the organ heterogeneity in environmental responsiveness might be substantial for mountain dragons to thrive in complicated environments.

Keywords: heterogeneous environment, lizard, local adaptation, multi-omics, organ heterogeneity

INTRODUCTION

Almost all animals live in spatially heterogeneous environments (Keller and Seehausen, 2012). Environmental heterogeneity can drive adaptive divergences between populations at both genetic and physiological levels (Rainey and Travisano, 1998; Valladares et al., 2014). These interpopulation variations play a fundamental role in maintaining a species' genetic or functional diversity and

response to climate change (Charmantier et al., 2008; Zhu et al., 2021c). Moreover, adaptive genetic divergence may result in ecological speciation if it causes some form of reproductive isolation (Schluter, 2000; Rundle and Nosil, 2005). Thus, the physiological strategies and underlying molecular basis of environmental adaptation are critical clues for understanding the formation, maintenance, and variation trends of biodiversity in the context of global climate change.

Reptiles have been suggested to be particularly sensitive to climate change due to their poor dispersal capacity (Urban et al., 2013b). These animals have already experienced extensive declines and extinctions worldwide (Sinervo et al., 2010), and climate change has contributed to these threats (Stuart Simon et al., 2004). Correlative climate envelope models even predict that climate change will cause the extinction of 11–49% of endemic reptiles (Thomas et al., 2004), and 20% of lizard species are expected to extinct by 2080 (Sinervo et al., 2010). However, phenotypic plasticity and genetic adaptation are expected to mitigate some of the negative biotic consequences of climate change (Holt, 1990; Urban et al., 2013a). Thus, good knowledge of an environment's adaptive mechanisms is important for understanding the influences of climate change on these animals and also a precondition for scientific and accurate conservation measures. In this regard, species adapted to typically heterogeneous environments provide a unique opportunity to study how these animals respond to environmental variations. The Hengduan Mountain Region (HMR), located in the southeastern part of the Tibet Plateau, varies spatially in climatic factors (e.g., temperature and precipitation) (Xu et al., 2018), resulting in many different ecosystems (Myers et al., 2000; Lei et al., 2015). It harbors high reptile diversity, including more than ten *Diploderma* (Squamata: Sauria: Agamidae; mountain dragons) species, which are micro-endemic to the dry and hot valleys of HMR (Wang et al., 2020). Currently, the formation of *Diploderma* diversity in HMR has not been studied systematically, and vicariant isolation, ecological divergence, and the low migration capacity of lizards (within 10 km; Southwood and Avens, 2010) are all potential drivers. The distribution areas of the *Diploderma* species are narrow but highly heterogeneous in terms of spatial climatic factors (Zhu et al., 2021c). This makes the *Diploderma* species an ideal model for investigating environmental adaptation at a micro-geographic scale.

Comparative genetics and genomics are major approaches in the study of the mechanisms of environmental adaptation (Savolainen et al., 2013; Kubota et al., 2015). However, the accumulation of stochastic genetic changes in the genome constitutes an obstacle to the screening of environment-related variations, especially for interspecies studies. Additionally, genetic variations may not always explain adaptation processes intuitively due to the limited understanding of their cellular functions (Chang et al., 2020), especially when adaptive traits are determined by multiple genetic loci or mutations are located in non-coding regions. Moreover, not all environmental adaptive traits are caused by changes in DNA sequences; for example, epigenetics, in response to external or environmental factors, can also shape cellular and physiological phenotypic traits by changing cellular gene expression patterns (Bird, 2007; Vogt, 2017). Despite the variability of genetic materials and the complexity of genetic determination, the expression of genetic information in phenotypic traits always relies on the qualitative and quantitative variations in a set of cellular chemical molecules—metabolites, which are the effectors of cellular regulation networks and the molecular basis of phenotypes (Nicholson and Lindon, 2008; Johnson et al., 2016). The whole set of metabolites in a cell, tissue, or organisms is called metabolome, which is at the frontline of the interactions between organisms and the environment (Bundy et al., 2009). Unlike the sequence mutations of genes, the chemical structures of primary metabolites are not easily varied with phylogeny, and species with close phylogenetic relationships likely share the same set of primary metabolites. This facilitates convenient comparisons on environment-related biological constructions and physiological functions between species or populations in the absence of whole-genome data. For example, comparative metabolomics revealed the critical role of metabolic switch in substrates for the thermal adaptation of a Plateau dwelling insect (Zhu et al., 2016; Zhu et al., 2019a). And combined comparative transcriptomic and metabolomic analysis illuminated the involvement of fatty acid metabolism in the highland adaptation across altitudinal songbirds (Xiong et al., 2021). Accordingly, comparative metabolomics provides an alternative approach for studying the adaptation of animals to spatially heterogeneous environments (Shi et al., 2015).

Additionally, the gut microbiome is considered to be the second genome of animals (Zhu et al., 2010). The host genome is highly conserved, and genetic changes within it occur slowly, whereas the gene pool of microbiota is dynamic and can change rapidly in response to the environment by increasing or reducing the abundance of particular microbes, by acquisition of novel microbes, by horizontal gene transfer, and by mutation (Rosenberg and Zilber-Rosenberg, 2018). Increasing numbers of studies have evidenced the contribution of the commensal microbiome to host environmental adaptation (Chevalier et al., 2015; Zhang et al., 2016); a disturbance in the gut microbiome can also lead to animal maladaptation to climate change (Greenspan et al., 2020). For example, gut microbiota promote cold adaptation of Brandt's voles by increasing host thermogenesis through the activation of cAMP–PKA–pCREB signaling (Bo et al., 2019). Seasonal shifts in gut microbiota composition is in favor of utilization of nitrogen and energy in yaks at harsh cold winter, implying essential role of symbiotic microbiota in high altitude adaptation of animals (Guo et al., 2021). Thus, variations in the microbiome are important aspects that should be considered in the environmental adaptation of animals.

In this study, environment-related variations in organ metabolism (i.e., liver and hindlimb muscle) and gut microbial community composition were studied in three *Diploderma* species (*D. iadinum*, *D. yulongense*, and *D. vela*). *D. yulongense* and *D. vela* are phylogenetically closer to each other than to *D. iadinum* (**Figure 1A**; Wang et al., 2020), while *D. iadinum* and *D. vela* are spatially closer to each other (the Lancang River Valley) than to *D. yulongense* (the Jinsha River Valley) (**Figure 1B**). The associations between biological traits and climatic factors (e.g., temperature and precipitation) were

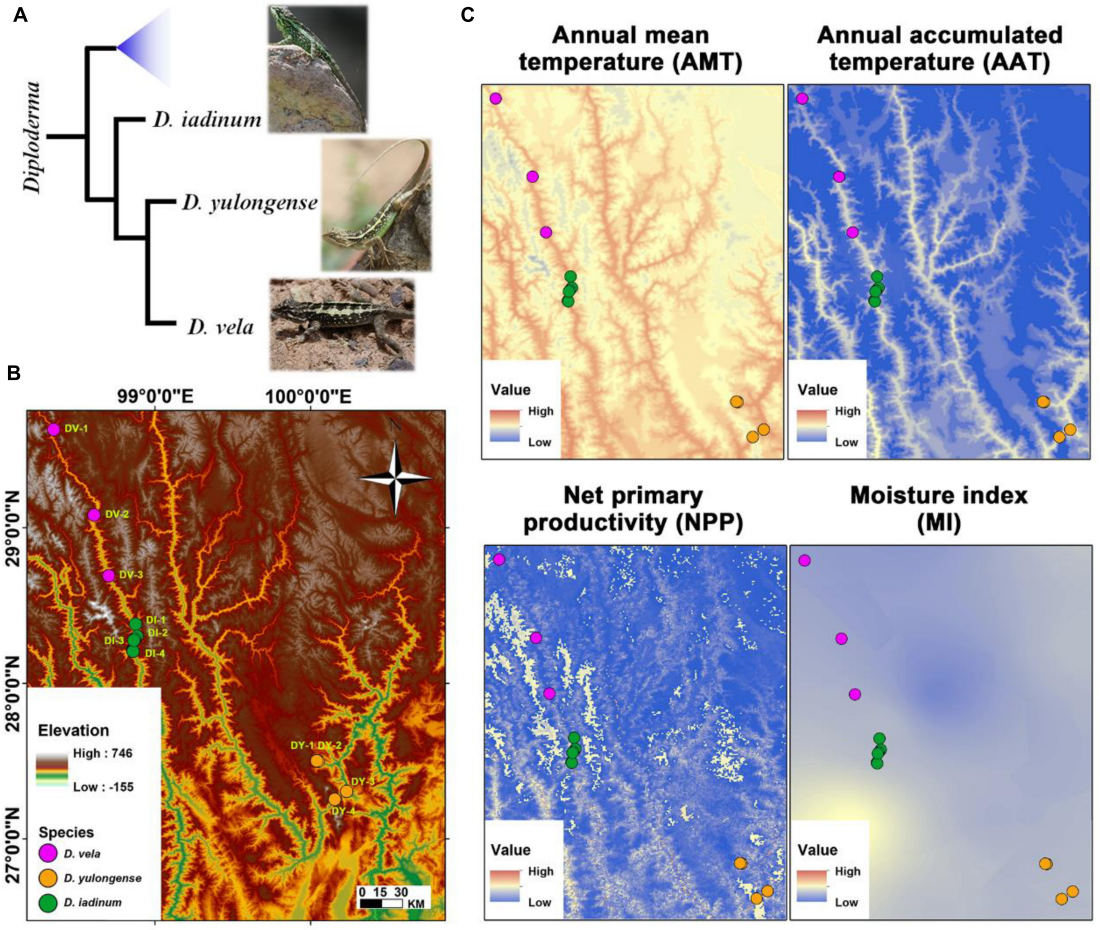

FIGURE 1 | Animal phylogeny, study area, and significant climatic factors. **(A)** The phylogenic relationships of *Diploderma* species (*D. iadinum*, *D. yulongense*, and *D. vela*) (Wang et al., 2020). **(B)** Study area and sample collection sites. **(C)** Maps illustrating the major climatic factors. The white area in the map of net primary productivity (NPP) denotes that data are unavailable.

analyzed at a population level (across species and within species). We raise three scientific questions regarding the contributions of phylogeny and climatic factors to animal metabolic and microbiome variations: (1) What factor is more significant, and whether or not there is any organ heterogeneity? (2) do the metabolome and microbiome vary with climatic factors convergently between species? and (3) which are the biological functions of the environment-related variations and their implications in environmental adaptation?

MATERIALS AND METHODS

Habitats and Animals

The *D. vela* (n = 15) and *D. iadinum* individuals (n = 14) and *D. yulongense* individuals (n = 17) were sampled from the dry and hot valley of the Langcang River and the Jinsha River, respectively, in July 2020 (**Figure 1B** and **Supplementary Table 1**). The sample information is detailed in **Supplementary Table 1**. As the study involved invasive experiments, only males were collected. Considering that the animal metabolome and gut microbiome are likely variable to their instant physiological status (e.g., feeding status) and environmental conditions (e.g., the real-time temperature and moisture at the time of collection), the gut content and tissues were not sampled immediately after collected. Instead, all the individuals were acclimated to the same laboratorial condition (24 ± 1°C, L: D = 12: 12) for 7 days before sample collection to diminish the random variations. Our goal was to reveal the inter-populational divergences in animal metabolome and gut microbiome that were potentially associated with phylogeny and climate. Such an acclimation procedure could ensure the authenticity of the differences between geographical populations, despite some information might be lost during this process. During acclimation, each individual was placed in a 29 × 18 × 10 cm plastic container and fed with mealworm (*Tenebrio molitor* larvae) and tap water daily. The feeding behavior was confirmed by daily observation. Following euthanasia with ether, the liver, hind limb muscle, and gut mucosa (from the intestine to the rectum, pooled) were collected and stored at −80°C. Animal procedures were approved

by the Animal Care and Use Committee of the Chengdu Institute of Biology, Chinese Academy of Sciences (permit number: 2020-AR-JJP-01).

Environmental factors (i.e., annual mean temperature/AMT, annual precipitation/AP, annual accumulated temperature (> 10°C)/AAT, moisture index/MI, and net primary productivity/NPP) were extracted from Resource and Environment Science and Data Center.[1] This information is detailed in **Supplementary Table 1**. The gut microbiome and tissue metabolomics data of *D. vela* have been published previously (Zhu et al., 2021c).

Metabolic Profiling

After grinding in liquid nitrogen, 50 mg tissue powder was transferred into 1.5 ml Eppendorf tubes with 800 μL precooled methanol: acetonitrile = 1:1 (v/v), followed by ultrasonication for 30 min × 2 and incubation at –20°C for 1 h. After centrifugation at 16,000 g for 20 min (4°C), supernates were transferred into new tubes and freeze-dried. Samples were dissolved in 100 μL acetonitrile: water (1: 1, v/v). After centrifugation at 14,000 g for 15 min (4°C), the supernates were ready for analysis. Extracted supernatants were analyzed by LC (1,290 Infinity LC, Agilent) coupled with quadrupole-time-of-flight mass spectrometry (Triple TOF 5,600 +, AB SCIEX). The details in the metabolic profiling followed the methods described by Zhu et al. (2021c). Metabolite data were processed using XCMS software[2] and Microsoft Excel (Microsoft, Redmond, WA, United States). Data of impurity peaks from column bleeds were excluded. Metabolites were identified by a combination of molecular weight comparison (molecular ion peak) and MS/MS spectrum comparison to a standard library. The relative abundances/concentrations of metabolites were presented as the ion intensities of their molecular ion peaks.

16S rRNA Gene-Based (Full Length) Microbiome Analyses

The PowerSoil® DNA Isolation kit (MO BIO Laboratories, Solana Beach, CA, United States) was used to extract DNA from the samples at room temperature according to the manufacturer's protocol. A DNA extraction (blank) control was included during DNA isolation. The integrity of the nucleic acids was determined visually by electrophoresis on a 1.0% agarose gel containing ethidium bromide. The concentration and purity of each DNA extraction was determined using a Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, United States). The whole region of the 16S rRNA gene was amplified with 27F (5 = –AGRGTTTGATYNTGGCTCAG-3 =) and 1492R (5 = –TASGGHTACCTTGTTASGACTT-3 =) primers, following the method described by Zhu et al. (2021c). A DNA extraction (blank) control was also included during PCR reaction. We used the following PCR thermocycling conditions: 95°C for 5 min, 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 60 s, with a final extension step at 72°C for 7 min. The products were purified with MagicPure Size Slection DNA Beads (TransGen Biotech, Beijing, China). High-throughput sequencing was performed using the PacBio platform. Sequencing (including the blank control) was performed by Biomarker Technologies Corporation (Beijing, China). The optimized circular consensus sequences (CCS) were obtained after filtering with the threshold of minPasses ≥ 5, minPredictedAccuracy ≥ 0.9, and length between 1,200 and 1,650 bp (lima v1.7.0 and cutadapt 1.9.1). Amplicon sequence variants (ASVs) were obtained after denoising with dada2 (Callahan et al., 2016). Annotation was conducted by querying against SILVA 132 (Quast et al., 2013), and the taxon summary was shown with QIIME2 2020.6 pipeline (Bolyen et al., 2019). The alpha-diversity (e.g., ACE and Shannon index) was calculated in QIIME 2. The dissimilarity matrices (e.g., unweighted and weighted UniFrac distances) were produced by QIIME2 pipeline.

Statistical Analyses

The influences of phylogeny (presented as genetic distances; Wang et al., 2020) and climatic factors (AMT, ATT, AP, NPP, and MI) on organ metabolome and gut microbiome were analyzed by PERMANOVA (adonis function in the Vegan package) based on R platform (Dixon, 2003). These analyses set metabolites or bacterial taxa abundances as dependent variables, and genetic distances and climatic factors were selected as independent factors. In detail, the beta-diversity of organ metabolomes was presented as Bray-Curtis distance, and the beta-diversity of gut microbiome was presented as Binary Jaccard, Bray-Curtis, Unweighted UniFrac, and Weighted UniFrac distances. The climatic factors and phylogeny (shown as the mean genetic distances to other species) were independent factors. Type I sum of square was used, as our main target was to screen the factors that had the most significant explanation rate to the variations. For each analysis, e.g., influences of phylogeny and climatic factors on beta-diversity of gut microbiota based on Bray-Curtis distances, the model was built by adding independent factors one by one. The sequences of independent factors were determined by ensuring that the factor added at each round could offer the most significant improvement on the total explanation of the total variations of dependent factors. The significance of each independent factors to the model was checked at threshold of $p < 0.05$. This method could avoid the interference of variable autocorrelation on models and reflect the importance order of factors.

Metabolites and bacterial taxa varied with phylogeny or climatic factors were screened. The metabolites or bacteria associated with phylogeny met the threshold of significant difference between any two species (at $p < 0.05$, Mann–Whitney *U*-test). Pearson correlations and Spearman correlations was used simultaneously to screen the metabolites and gut bacteria associated with climatic factors. Valid pairwise correlations met the threshold of $q < 0.05$ or $q < 0.01$ (Pearson correlation and BH correction) and $p < 0.05$ (Spearman correlation). The potential bacterial functions and phenotypes were predicted with PICRUSt2 (Douglas et al., 2020) and BugBase (Ward et al., 2017). However, PiCRUST was developed for human microbiome function and should certainly be used with caution for host groups outside the Mammalia. ANCOVA was

[1] http://www.resdc.cn/Default.Aspx

[2] https://xcmsonline.scripps.edu

TABLE 1 | The permutation ANOVAs on liver and muscle metabolomes (Bray-Curtis distance, permutations = 9,999) for the best models.

Tissue	Factors	F model	R^2	*p*
Liver	NPP	6.0752	0.1263	0.0001
	Phylogeny	3.4673	0.0721	0.0025
	AAT	1.9947	0.0415	0.0492
	AP	2.1221	0.0441	0.0371
Muscle	Phylogeny	8.8817	0.1748	0.0001
	AAT	2.6577	0.0523	0.0060
	AP	3.1607	0.0622	0.0027
	AMT	1.8952	0.0373	0.0438

conducted to analyze the variations in gut bacterial alpha-diversity and bacterial functions. The intraspecies differences in organ metabolome and gut microbiome between populations were analyzed by PERMANOVA (adonis function in the Vegan package). Principal coordinate analyses (PCoA, based on dissimilarity matrices) were used to visualize the dissimilarity of beta-diversity. Metabolite enrichment analyses were conducted using MetaboAnalyst 5.0.[3] The Spearman correlation coefficients were calculated for each metabolite–bacteria pair. To ensure the reliability of the results, the coefficients were calculated across all the samples from different species, as well as limited for the samples from the same species. Valid metabolite–bacteria correlations should meet $p < 0.001$ across samples and $p < 0.01$ at least in two species. Correlation networks were constructed on Cytoscape 3.5.0. Other graphs were drawn using Graphpad prism 5 and ggplot2, an R package (Wickham, 2009).

RESULTS

Environmental Heterogeneity of the Diploderma Distribution Range

The climatic factors (i.e., AMT, AAT, NPP, AP, and MI) vary spatially across the distribution range of *Diploderma* species (**Figure 1C** and **Supplementary Table 1**). The AMT and AAT share similar variation pattern, which is different from that of NPP and AP (**Supplementary Figure 1**). The collection sites for *D. vela* tended to have lower temperature, MI, and NPP than that of the other two species, and the intraspecies variations in temperature is prominent for *D. vela* and *D. yulongense*.

Variations in Organ Metabolome Across Species

D. iadinum, *D. yulongense*, and *D. vela* differed significantly in their liver and hindlimb metabolomes (**Supplementary Table 2**). However, for the liver metabolome, *D. yulongense* was more similar to *D. iadinum* than to *D. vela* (**Supplementary Figure 2**), despite *D. yulongense* and *D. vela* being more adjacent in phylogeny. In fact, while phylogeny contributed significantly to variations in liver metabolome ($R^2 = 0.072$, $p = 0.0025$), NPP explained the variations better than phylogeny ($R^2 = 0.126$, $p = 0.0001$) (PERMANOVA; **Table 1** and **Figure 2A**). The similarity between species was consistent with their genetic distances (**Supplementary Figure 2**). Additionally, phylogeny had the highest explanation rate ($R^2 = 0.175$, $p = 0.0001$) regarding the variations in muscle metabolome (PERMANOVA, **Table 1** and **Figure 2A**). The other significant contributors included AAT and AP.

Most phylogeny-associated metabolites did not overlap with those associated with climatic factors (**Supplementary Figure 3A**). In both liver and muscle, phylogeny-associated metabolites were primarily amino acids and their derivates, most of which shared similar variation patterns across species (metabolite abundance: *D. iadinum* > *D. yulongense* > *D. vela*) (**Figure 2B**). Functional enrichment analyses against the KEGG database highlighted the potential involvement of hepatic purine metabolism, glycine and serine metabolism, ammonia recycling, and methionine metabolism in genetic differentiation of *Diploderma* species (**Supplementary Figure 3B**). Notably, three gamma-glutamyl dipeptides (i.e., gamma-glutamylglutamine, gamma-glutamylmethionine, and gamma-glutamyltyrosine) were shared by the liver and muscle.

NPP and AAT were the primary climatic factors associated with the variation in the liver metabolome. Hepatic metabolites showing strong correlations ($q < 0.01$, Pearson correlation; $p < 0.05$, Spearman correlation) with AAT included glycerol-3-phosphocholine, glycerophosphate, kinetin, and fructose (**Figure 3A**). Their levels were higher in individuals that inhabited cooler environments (**Figure 3B**). Compared to other climatic factors, there were more metabolites associated with NPP in the liver (**Supplementary Figure 3A**); this finding is consistent with the high explanation rate of NPP for liver metabolome. These metabolites highlighted metabolism, nicotinamide metabolism, ammonia recycling, urea cycle, and numerous amino acid metabolisms (**Supplementary Figure 3B**). The abundance of nicotinamide, NAD, and AMP, which participated in the same metabolic reaction (interconversion between nicotinamide and NAD), showed correlations with NPP level (**Figure 3A**). Lower NPP was associated with higher NAD and lower nicotinamide in the liver and vice versa (**Figure 3C**).

AAT and AP were the major climatic factors associated with variations in muscle metabolome. Enrichment analysis highlighted the potential association between muscle histamine metabolism oxidation of branched-chain fatty acids and environmental AAT (**Supplementary Figure 3B**). Notably, muscle carnosine and perseitol showed a strong negative correlation with environmental AAT, while taurine and kinetin riboside positively correlated with AAT (**Figure 3D**). Muscle metabolites associating with AP included persecutor, O-phosphothreonine, and histamine (**Figure 3A**). Only a few metabolites correlated with environmental AAT and NPP simultaneously (**Supplementary Figure 4**).

Variations in Gut Microbiome Across Species

Proteobacteria, Firmicutes, and Bacteroidetes dominated the gut microbiome of *Diploderma* species (**Figure 4A**), and

[3]https://www.metaboanalyst.ca/faces/ModuleView.xhtml

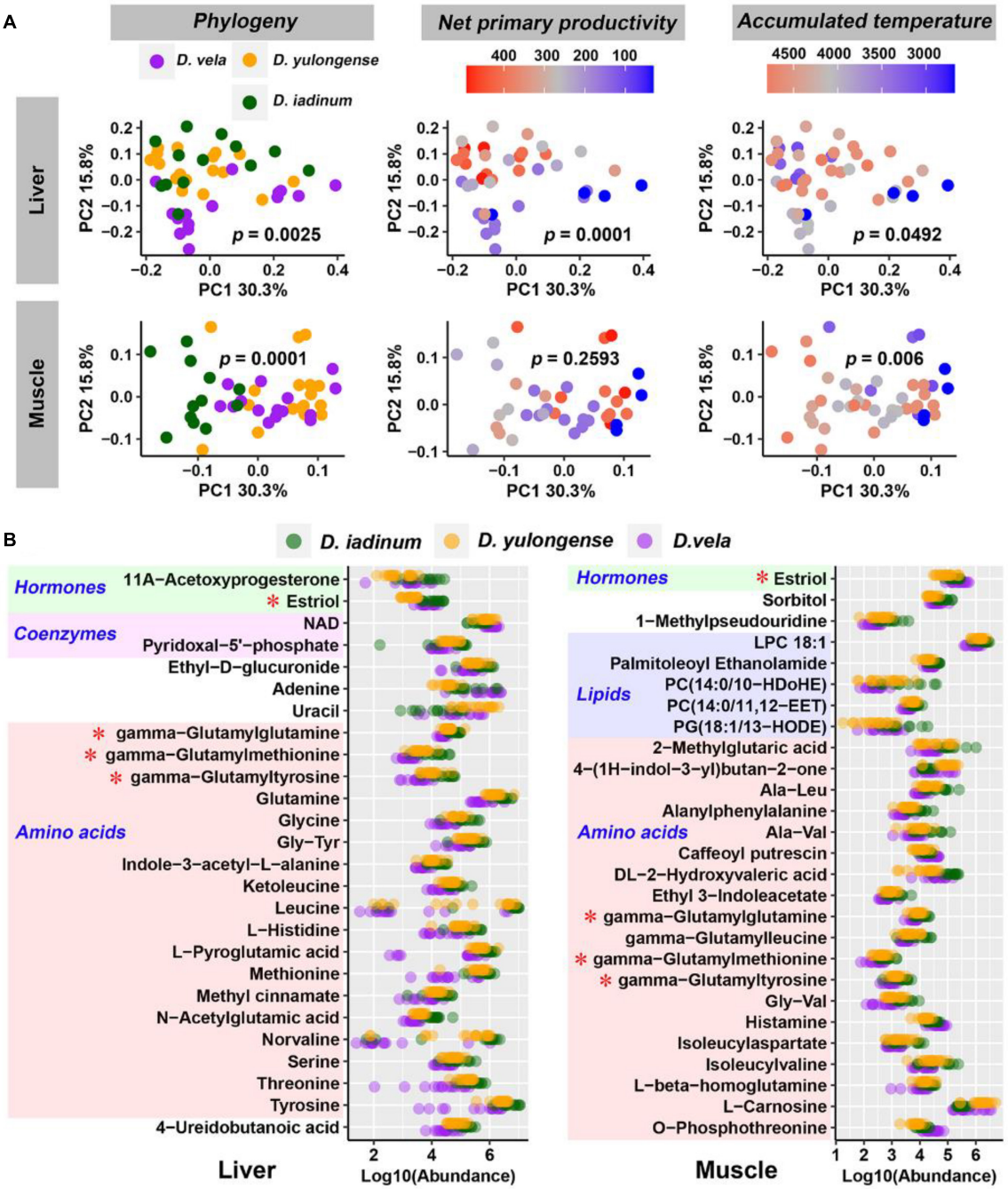

FIGURE 2 | Influences of host phylogeny and climatic factors on the liver and muscle metabolic profiles. **(A)** PCoA scatter plots presenting the significance of phylogeny and climatic factors on variations in organ metabolome. The *p*-values of PERMANOVA were labeled. **(B)** Metabolites varied in abundance between any two *Diploderma* species ($p < 0.05$, Mann–Whitney *U*-test). Red asterisks denote the metabolites shared by the liver and muscle.

Enterobacteriaceae, Bacteroidaceae, and Lachnospiraceae were their most abundant bacterial families (**Figure 4B**). Neither species nor climatic factors had significant influences on the alpha-diversity of the gut microbiota (ANCOVA, **Supplementary Table 3**). The interspecies similarity of bacterial community structure was not consistent with the genetic distances (unweighted UniFrac distance and weighted UniFrac distance, **Figure 4C**). When bacterial phylogenetic relations were not considered (Binary Jaccard and Bray-Curtis distances), phylogeny was the primary contributor to the variation in gut microbiota and environmental AMT, NPP, and MI significant contributors (PERMANOVA, **Table 2** and **Figure 4D**). When

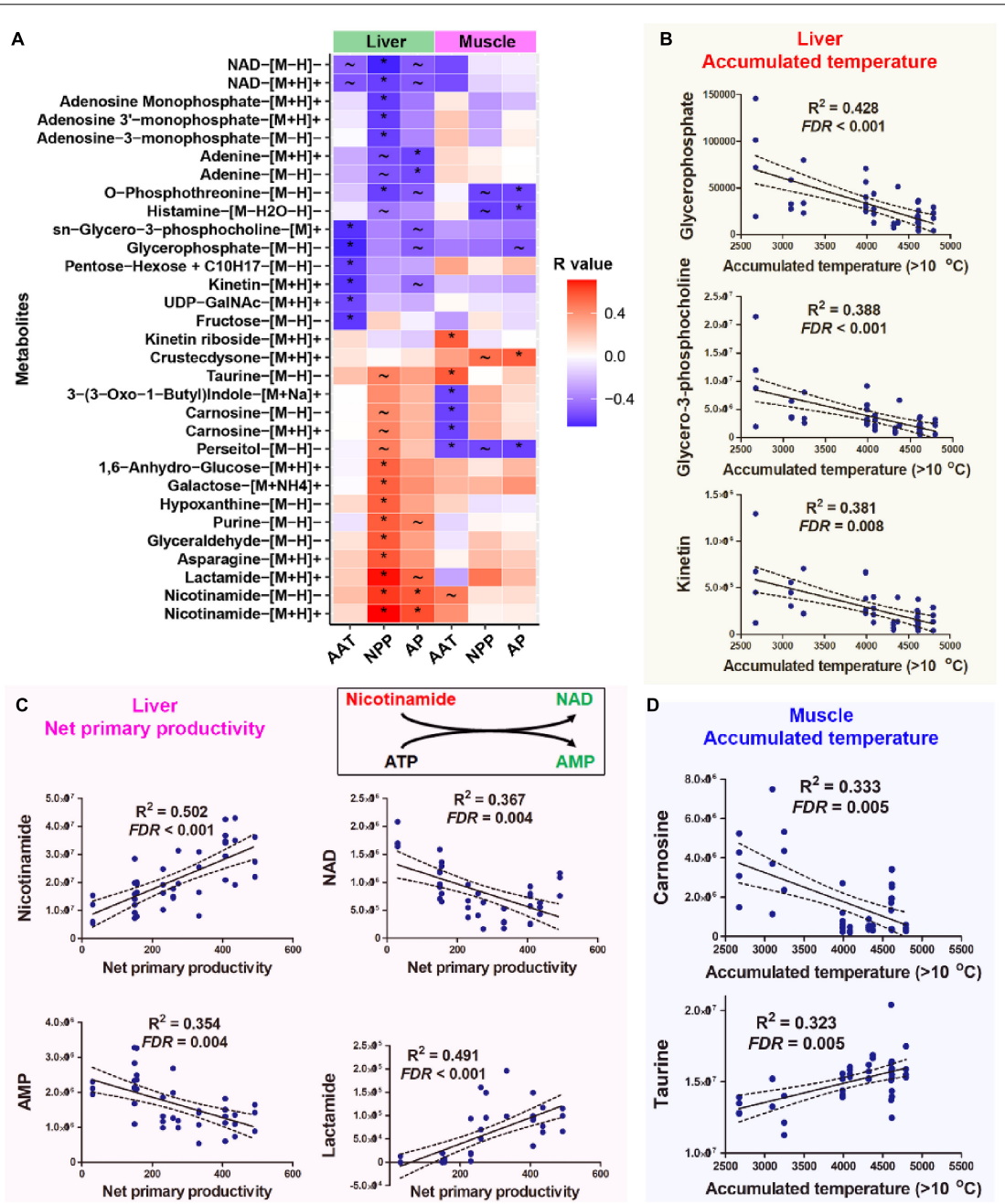

FIGURE 3 | Host metabolites associated with climatic factors. **(A)** Heatmap presenting the correlations (*R*-value) between host metabolites and climatic factors. Climatic factors were limited to those contributing significantly to the variations in organ metabolome ($p < 0.05$, PERMANOVA). The metabolites presented were significantly correlated with ($q < 0.01$, Pearson correlation; $p < 0.05$, Spearman correlation) at least one climatic factor. *$q < 0.01$ for Pearson correlation and $p < 0.05$ for Spearman correlation; ~$q < 0.05$ for Pearson correlation and $p < 0.05$ for Spearman correlation. **(B)** Correlations between AAT and liver metabolites. **(C)** Correlations between NPP and liver metabolites. **(D)** Correlations between AAT and muscle metabolites. The abundances of metabolites were presented as peak area, which had no unit.

bacterial phylogenetic relations were considered, phylogeny still had the highest explanation rate (R^2 = 0.122) to the gut microbiota community if ASV abundance was ignored (unweighted UniFrac distance). However, when ASV abundance was taken into consideration (weighted UniFrac distance), AMT was the only significant contributor (R^2 = 0.125) to the variation in the gut microbiota community (**Table 2** and **Figure 4D**). Additionally, interspecies differences no longer existed (**Supplementary Table 2**).No bacterial groups or ASVs showed significant variations between species (Kruskal–Wallis test at the

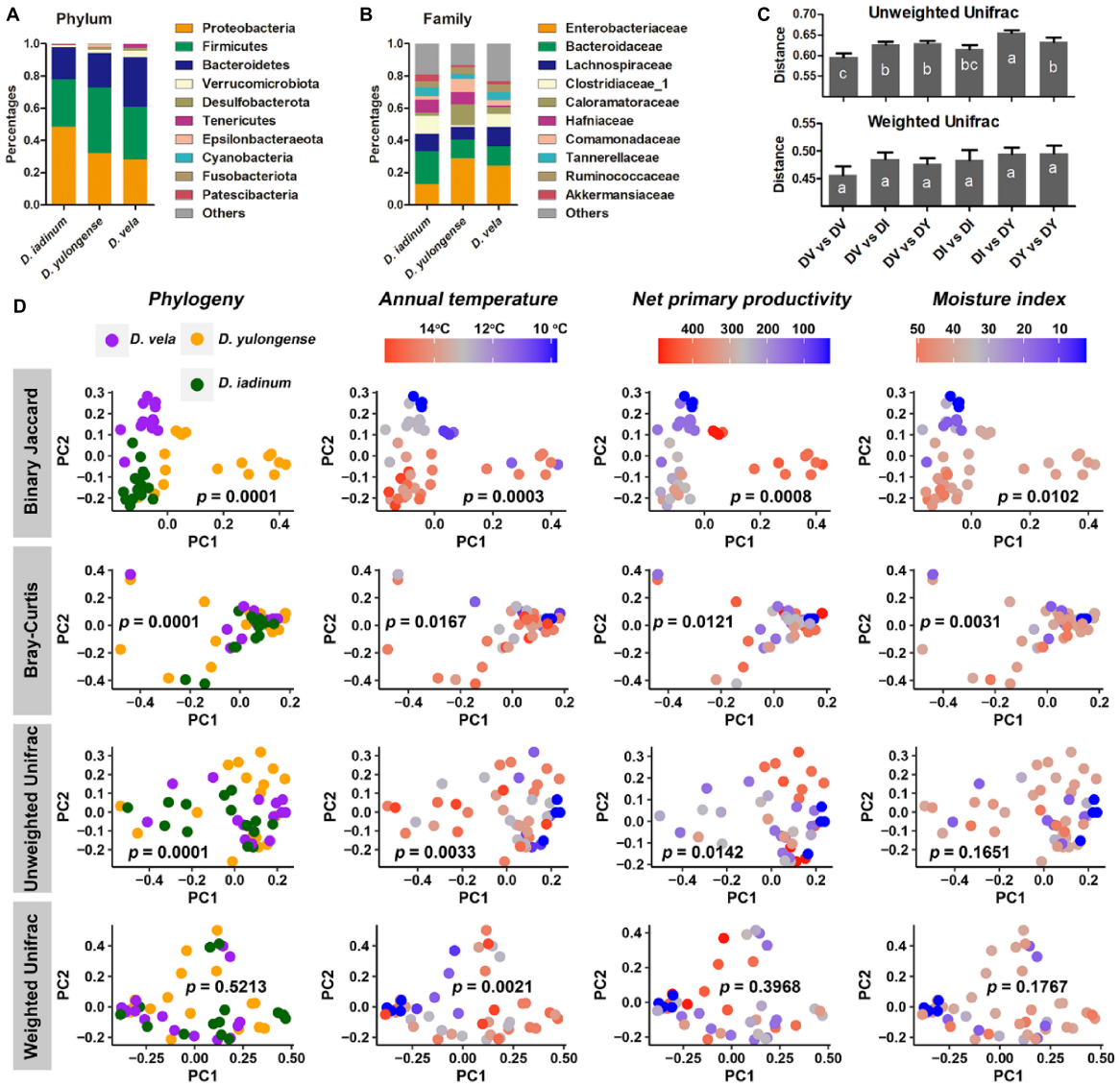

FIGURE 4 | Influences of host phylogeny and climatic factors on the gut bacterial community structure of mountain dragons. **(A,B)** Composition of the microbiome at phylum **(A)** and family **(B)** levels. **(C)** Interspecies distances of the gut microbiome. **(D)** PCoA scatter plots presenting the significance of phylogeny and climatic factors on variations in the gut microbiome (interpopulation analyses across species). The *p*-values of PERMANOVA were labeled.

threshold of $p < 0.05$). Instead, many bacterial groups, including Lachnospiraceae, Desulfovibrionaceae, and Veillonellaceae families, negatively correlated with environmental AMT, while Proteobacteria and Gammaproteobacteria positively correlated with it (**Figures 5A,B**). Notably, *Intestinimonas butyriciproducens* negatively correlated with environmental AMT, NPP, and MI ($q < 0.05$ in Pearson correlation and $p < 0.05$ in Spearman correlation, **Figure 5C**). There were no significant associations between any environmental factors and bacterial functions predicted by COG[4] and KEGG databases.[5] However, environmental MI was positively correlated with some bacterial phenotypes (i.e., potentially pathogenic, contains mobile elements, and facultative anaerobic) predicted by BugBase (**Supplementary Figure 5**).

Intraspecies Variations Associated With Thermal Adaptation

For both *D. vela and D. yulongense*, there are populations (DV1, DY1, and DY2) inhabiting environments with more than 2°C lower in AMT than other populations from the same species (**Figure 6A** and **Supplementary Figure 6**). These populations were classified to be cold-dwelling populations (AMT < 11°C), while the other populations of these two species were classified as warm-dwelling populations (AMT > 12°C).

[4]http://www.ncbi.nlm.nih.gov/COG

[5]https://www.kegg.jp/kegg/

TABLE 2 | The permutation ANOVAs on symbiotic microbiota (permutations = 9,999) for the best models.

Distance type	Factors	F model	R^2	*p*
Binary Jaccard	Phylogeny	2.3528	0.10652	0.0001
	AMT	1.5025	0.03401	0.0003
	NPP	1.4711	0.03330	0.0008
	MI	1.2865	0.02912	0.0102
Bray-Curtis	Phylogeny	1.8353	0.08564	0.0001
	MI	1.4406	0.03361	0.0031
	NPP	1.3532	0.03157	0.0121
	AMT	1.3262	0.03094	0.0167
Unweighted UniFrac	Phylogeny	2.8262	0.12229	0.0001
	AMT	2.2056	0.04772	0.0033
	NPP	1.8763	0.04059	0.0142
Weighted UniFrac	AMT	5.5076	0.12475	0.0021

Pairwise distances were calculated between cold-dwelling and warm-dwelling populations of these two species (detailed in **Figures 6B,C**). For liver metabolome, the distance between two cold-dwelling populations was longer than that between the two warm-dwelling populations (**Figure 6B**). A topological network suggested that the orientations from warm- to cold-dwelling populations were contradictory between these two species (**Figure 6C**). The distance between the two cold-dwelling populations was maintained for muscle metabolome compared to that between warm-dwelling populations (**Figure 6B**). The topological network suggested a paralleled variation orientation of cold adaptation between these two species. The weighted UniFrac distance between two cold-dwelling populations was the shortest (**Figure 6B**). Its topological network indicated a convergence of gut microbiota in cold-dwelling populations (**Figure 6C**).

The organ metabolites or gut bacteria that differed between cold- and warm-dwelling populations in both species were screened (**Figure 6D**). For the liver metabolome, most of these metabolites exhibited divergent variation trends between *D. vela* and *D. yulongense* (**Figure 6D**), except for glycerophosphate, glycerol-3-phosphocholine, and fructose (**Figure 6E**). For the muscle metabolome, however, more metabolites exhibited consensus variation trends between the two species (**Figure 6D**), including carnosine and 2′-deoxycytidine (**Figure 6E**). For gut microbiomes, all screened taxa presented consensus variation trends between *D. vela* and *D. yulongense*. This included the Lachnospiraceae and Veillonellaceae (**Figures 6D,E**).

Correlation Networks Analysis

The correlations ($q < 0.05$ for Pearson correlation and $p < 0.05$ for Spearman correlation) between organ metabolites, gut bacterial taxa, and environmental factors were analyzed, and ten sub-networks were constructed (**Supplementary Figure 7**). Liver core metabolites (correlated with more than four bacterial taxa) included glycerophosphate, propionyl carnitine, kinetin, carnitine, creatinine, glutaric acid, 3-phenyllactic acid, and 4-guanidinobutyric acid. Muscle core metabolites included taurine, cholest-4,6-dien-3-one, kinetin-riboside, and beta-homothreonine. Core bacterial taxa were p.Proteobacteria, c.Gammaproteobacteria, o.Enterobacterales, f.Comamonadaceae, g.Comamonas, and o.Burkholderiales. Notably, metabolites associated with environmental AAT and bacteria associated with AMT were involved in the same correlation network.

DISCUSSION

The Primary Factors Shaping the Organ Metabolome and Gut Microbiome

The most significant determinators on muscle, liver, and gut microbiome of *Diploderma* were phylogeny, NPP, and temperature, respectively. The influence of phylogeny on metabolism is predictable, as species with distant phylogenic relationships accumulate more genetic divergences and thus a more differentiated metabolism (Ma and Zeng, 2004). The metabolic pattern in muscle is associated with locomotive endurance, explosive force, and the locomotor mode of animals and these physiological functions play critical roles in speciation (Hedrick et al., 2020). An interesting finding was that the impact of phylogeny on metabolome exhibited organ heterogeneity. This might be explained by their different sets of organ-specific enzymes (e.g., isoenzymes), which might exhibit different evolutionary rates or varied expression plasticity in response to different environments (Pazzagli et al., 1998; Kryuchkova-Mostacci and Robinson-Rechavi, 2015). Many studies highlight that host phylogeny and diet are the two main factors influencing animal gut microbiota (Ley et al., 2008a,b). Consistently, our study also suggested that the gut bacterial taxa differed between *Diploderma* species (unweighted UniFrac distance). However, when taxon abundance was considered, host phylogeny was no longer a determinator. These results suggested that host phylogeny and environmental variations were mainly responsible for the gut microbiome's taxonomic and abundance variations.

NPP was a significant determinator for the liver and muscle metabolomes and gut microbiome of mountain dragons. The relevance between primary productivity and the overall metabolic rate has been well documented in vertebrates (Lovegrove, 2000; Shi et al., 2015), and evidence from field studies suggests that a large fraction of observed variations in animal metabolism is attributable to variations in primary productivity rather than direct temperature (Tieleman et al., 2003). This is consistent with our observations in *Diploderma*, whose organ metabolome varies with environmental NPP significantly. As all the individuals in our study had been provided with enough food for 7 days, this association was unlikely due to their different nutrition statuses. Alternatively, it implied potential evolutionary adaptation or adaptive plastic response. In the *Diploderma* lizards, the associations between NPP and metabolome were more significant in the liver than in the muscle. This is reasonable, as the liver plays a central role in metabolic regulation to meet the energy requirements of different organs (Han et al., 2016; Zhu et al., 2021b), and acts a major storage space for resources in fish, amphibians, and reptiles (Derickson, 1976;

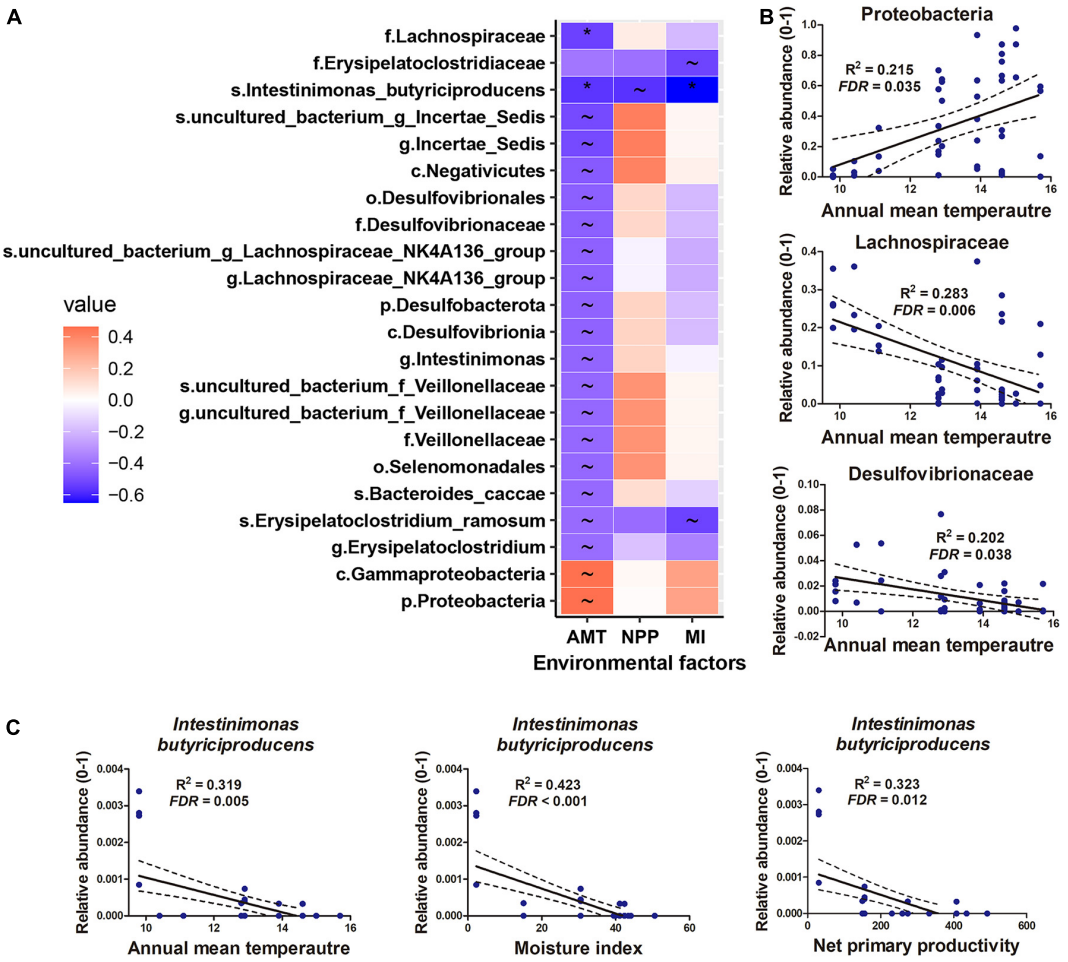

FIGURE 5 | Gut microbiomes associated with climatic factors. **(A)** Heatmap presenting the correlations (*R*-value) between gut microbes and climatic factors. Climatic factors were limited to those contributing significantly to the variations in the gut microbiome ($p < 0.05$, PERMANOVA). *$q < 0.01$ for Pearson correlation and $p < 0.05$ for Spearman correlation; ~$q < 0.05$ for Pearson correlation and $p < 0.05$ for Spearman correlation. **(B,C)** Correlations between microbes and environmental factors.

Zhu et al., 2019b). The associations between environmental NPP and the gut microbiome of mountain dragons might be mediated by food availability and diet compositions, both of which could shape the host's gut microbiome (Ley et al., 2008a,b).

Like NPP, temperature could also influence the liver and muscle metabolome and gut microbiome of mountain dragons. Environmental temperature imposes selective solid pressure on animals. The metabolome is at the forefront of life in coping with thermal stress. For example, the accumulation of cryoprotectants (e.g., glycerol and proline) and antioxidant metabolites (e.g., glutathione) reinforces the tolerance of thermal-acclimated animals to freezing (Koštál et al., 2012). In *Diploderma* lizards, the muscle was more responsive to variations in environmental temperatures than the liver. Cold-dwelling *D. vela* and *D. yulongense* individuals shared more metabolic variations in the muscle than the liver when compared to their respective warm-dwelling counterparts. The gut bacterial community varied with environmental temperature most significantly in mountain dragons. The influence of temperature on the animal commensal microbiome has been supported by many studies (Fan et al., 2013; Zhu et al., 2021a), and fluctuations in symbiotic microbes have been suggested to play a role in host thermal adaptation (Bo et al., 2019; Guo et al., 2021). Interestingly, we observed convergence in the gut microbiome of cold-dwelling populations from two *Diploderma* species. Diet-driven convergence of the gut microbiome is common to animals (Muegge et al., 2011), while evidence for environment-related convergences of the commensal microbiome is relatively scarce (Zhang et al., 2016). Whether temperature influenced the gut microbiome of *Diploderma* directly required further investigations. For example, it is possible that microorganisms adapted to cold conditions are less diverse than those in warm places or because the diet in cold areas is less varied, which determines a more homogeneous microbiota in these conditions.

Collectively, our results suggested that organ systems might differ in their variability, whether genetic or plastic, to

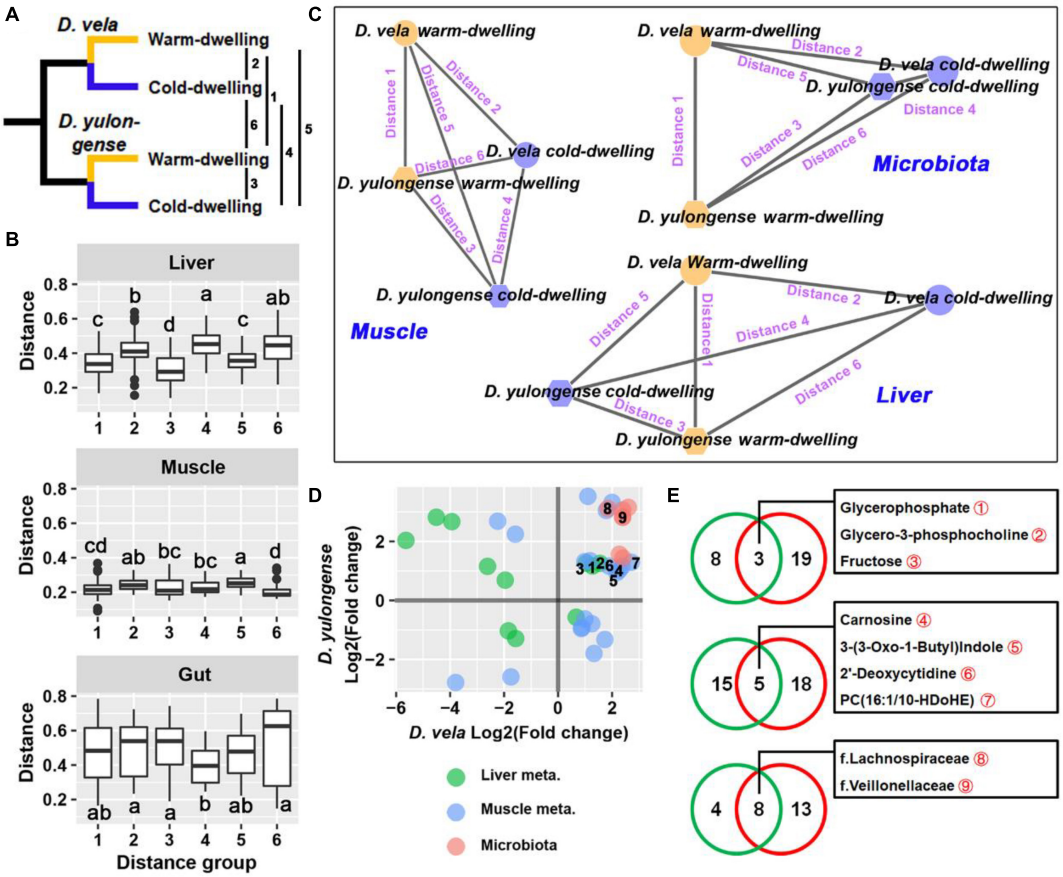

FIGURE 6 | Intraspecies comparisons between cold- and warm-dwelling populations. **(A)** *D. vela* and *D. yulongense* individuals could be divided into cold- (DV-1, DY-1, and DY-2) and warm-dwelling populations (DV-2, DV-3, DY-3, and DY-4) according to the annual temperature. The distances between populations ranged from 1 to 6. **(B)** Distances between cold- and warm-dwelling species. Metabolome: Bray-Curtis distance; microbiome: weighted UniFrac distance. **(C)** Topological networks present the relative distances between groups. Note that a cold environment drives the opposite, parallel, and concurrent variations in the liver, muscle, and gut microbiome, respectively, between *D. vela* and *D. yulongense*. **(D)** Point plot presenting the variation patterns of metabolites and microbes, which differed significantly between cold- and warm-dwelling populations in both species. The horizontal and vertical axes denote the fold changes of cold- to warm-dwelling populations in *D. vela* and *D. yulongense*, respectively. Points with numeric numbers are annotated in panel **(E)**. **(E)** Venn plots presenting the number of metabolites associated with the environmental temperature. Green circle, metabolites or microbes screened by correlation analyses across species ($q < 0.05$ for Pearson correlation and $p < 0.05$ for Spearman correlation); red circle, metabolites or microbes filtered by intraspecies comparisons between cold- and warm-dwelling populations ($p < 0.05$ for Mann–Whitney *U*-test in both species).

climatic factors. Muscle metabolism predominantly reflects the phylogenetic relationships between species and has the lowest environmental variations. By contrast, liver metabolism exhibits a higher correspondence to environmental factors, especially NPP, but phylogeny is still a significant determinator in the liver metabolome. For the gut microbiome, however, the quantitative traits of the microbiome were only associated with environmental temperature (see a graphic summary in **Figure 7**).

Potential Metabolic Adaptation to a Spatially Heterogeneous Environment

Amino acids and dipeptides were highly variable between species, implying that amino acid metabolism underwent remarkable genetic differentiation during the speciation. Notably, three gamma-glutamyl dipeptides (i.e., gamma-glutamylglutamine, gamma-glutamylmethionine, and gamma-glutamyltyrosine) differed between species in both liver and muscle. These metabolites are products of the glutathione cycle, and their levels indicate the oxidative status in tissue (Zierer et al., 2016). Additionally, gamma-glutamylglutamine plays a role in regulating neurobehavioral, e.g., fearfulness (Puurunen et al., 2018). Their variations implied physiological and behavioral divergences during the speciation of mountain dragons. Although their variation patterns between populations were not correlated with current climatic factors, they might be associated with ecological speciation from the perspective of historical climates or presumably involved in topographical adaptations.

The inverse variations in nicotinamide and NAD in the liver were the most significant metabolic variations associated with NPP. Populations with low environmental NPP had higher NAD and lower nicotinamide levels. Nicotinamide is

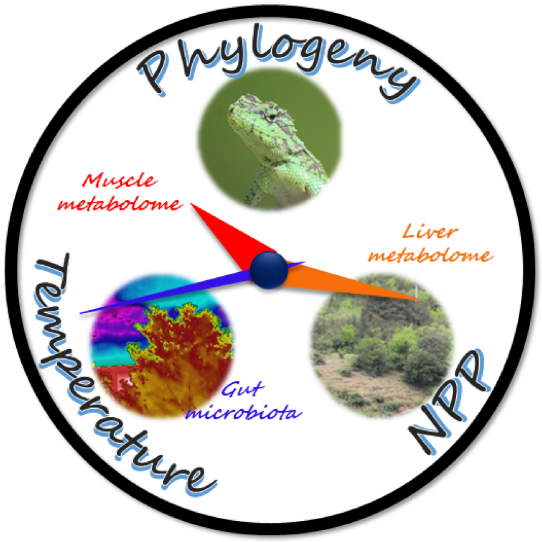

FIGURE 7 | Schematic diagram of the results. The hour, minute, and second hands of the clock symbolize the muscle, liver, and gut microbiomes of the mountain dragons, respectively, and they are pointing to their respective major determinators. The movement speed of the clock hands symbolizes the variability, whether genetic or plastic, of organ metabolome and gut microbiome to the climatic factors. The faster they move, the more variable they are.

the precursor for NAD biosynthesis (Magni et al., 2004). The cellular NAD level is a critical target for regulating energy expenditure (Cantó et al., 2009). And higher NAD levels are indicative of a metabolic shift from energy storage/anabolism to energy mobilization/catabolism, and vice versa (Revollo et al., 2007). Additionally, NAD is required for the catalytic activity of sirtuins (Imai and Armstrong, 2000; Houtkooper et al., 2010), the deacetylases that promote glucose and lipid metabolism and mediate metabolic transcriptional adaptations linked to nutrition scarcity (Imai and Armstrong, 2000; Canto et al., 2012). Accordingly, the negative correlation between NAD level NPP might be a compensation strategy for the spatial variations in NPP.

The reorganization of phospholipid composition is a common strategy for thermal adaptation in animals (Reynolds et al., 2014). The levels of hepatic glycerol-3-phosphocholine and glycerophosphate, two intermediates in phospholipid metabolism, were negatively correlated with environmental AAT in mountain dragons. These two metabolites were again highlighted in intraspecies comparisons, showing a higher abundance in the cold-dwelling populations. It suggests that phospholipid metabolism might be involved in the adaptation of mountain dragons to spatial variations in temperatures. The hepatic kinetin level was also negatively correlated with environmental AAT. This metabolite was reported to be a cryoprotectant for animal cells with antioxidation function (Zadeh Hashem and Eslami, 2018). Cold-dwelling populations tended to have higher muscle carnosine levels at both interspecies and intraspecies levels. Carnosine is highly concentrated in muscle with biological activities including antioxidation and preventing the formation of advanced glycation end-products (Boldyrev et al., 2013). Notably, carnosine can promote the heat denaturation of glycated protein (Yeargans and Seidler, 2003); thus, we presumed that a higher carnosine level could compensate for the reduced capacity in clearing glycated protein at cold conditions.

Association of Gut Microbes With Spatially Heterogeneous Environments

Gut Proteobacteria and Gammaproteobacteria abundance was positively correlated with environmental AMT. The variation trends of Proteobacteria vary between animal taxa. In insects, temperature increases have been associated with increased relative abundances of Proteobacteria (Moghadam et al., 2018). In the gut microbiome of the Chinese giant salamander, however, the abundance of Proteobacteria decreased with a rise in temperature (Zhu et al., 2021a). These variations' biological significance or outcomes to the host have not been illuminated. In contrast to Proteobacteria, the families Lachnospiraceae, Desulfovibrionaceae, and Veillonellaceae tended to be more abundant in cold-dwelling populations of mountain dragons. Members of the Lachnospiraceae family are suggested to be beneficial to the host (Meehan and Beiko, 2014) by promoting short-chain fatty acids, converting primary bile acids to secondary ones, and facilitating colonization resistance against intestinal pathogens (Sorbara et al., 2020). Therefore, its enrichment in the gut of cold-dwelling *Diploderma* populations could be beneficial to the host in adapting to a challenging environment. Our results also suggested that increasing environmental moisture was accompanied by an increase in potential pathogenic and facultative anaerobic bacteria in the gut of mountain dragons (**Supplementary Figure 5**). Warming and high humidity favor the spread of pathogens in this environment (Bosch et al., 2007). Further studies are required to confirm the association between gut pathogen abundance and environmental moisture in mountain dragons and clarify how this association may influence the existence and distribution of mountain dragons in the HMR. Another noticeable bacterium in mountain dragons was *Intestinimonas butyriciproducens* due to its properties of butyrate production and host metabolic regulation (Kang, 2018). Its abundance negatively correlates with environmental temperature, moisture, and primary productivity. Further functional studies are required to give a mechanistic insight into the role of symbiotic microbiota in host adaptation to environmental variations.

Our results highlighted robust quantitative correlations between host metabolite levels and gut microbe abundances in *Diploderma* lizards, suggesting intimate interactions between host metabolism and gut microbes. Many of these metabolites and microbes were also associated with climatic factors, particularly temperature. It implied coordinated variations in host metabolism and gut microbiota with climatic factors. Thus, our results indicated the significance of the concept of holobiont in investigating the influence of climate on biodiversity.

CONCLUSION

Here, we investigated the relationship between variations in organ metabolism and gut microbiota and climatic factors in mountain dragons. The host metabolomes and gut microbiome displayed distinct variability with environmental variations, and their variations were associated with different climatic characteristics. This organ heterogeneity might be important for mountain dragons to thrive in complicated environments. We also observed convergence in the gut microbiome of cold-dwelling populations between species. Our results using the multi-omics approach provided some details regarding the interaction between holobiont and the environment, which might shed some light on the mechanisms underlying evolutionary adaptation in animals.

DATA AVAILABILITY STATEMENT

The raw data of 16S rRNA gene sequences have been submitted to Genome Sequence Archive (CRA005166) at https://ngdc.cncb.ac.cn/gsub/.

AUTHOR CONTRIBUTIONS

WZ, JJ, and LZ conceived the project. YQ and XS collected the samples. WZ, YQ, and XS performed the experiments. WZ, XW, LC, and JL analyzed the data. WZ and LZ wrote the manuscript. All authors approved the final version of the manuscript.

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Seasonal and Soil Microbiota Effects on the Adaptive Strategies of Wild Goitered Gazelles Based on the Gut Microbiota

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Seasonal variation in extreme environments is a threat to endangered species. The gut microbiota is important in the adaptive strategies of wild herbivores, and herbivores will contact the soil microbiota when they are feeding. However, there are no studies about the effects of soil microbiota on the gut microbiota of wild herbivores. Understanding the seasonal adaptive strategies of wild herbivores based on their gut microbiota and the effects of soil microbiota on the herbivorous gut microbiota is indispensable for making optimal conservation recommendations. To address those issues, we compared the diversity and functions of gut microbiota in goitered gazelles between winter and summer with a non-invasive fecal sampling method from the Qaidam Basin based on 16S rRNA V3–V4 regions. The data showed that seasonal variations caused the significant changes in gut microbiota at α-and β-diversity levels. The main gut microbial function was "Metabolism." It showed significant seasonal changes. The goitered gazelles adapted to the seasonal changes by increasing the relative abundance of *Firmicutes*, *Christensenellaceae*, *Bacteroides* and the function about "Metabolism" in the winter to improve the adaptability. We also compared the effects of soil microbiota on the gut microbiota between winter and summer, covering source tracking analysis and the seasonal differences in ecological assembly processes. The contribution of soil microbiota on the gut microbiota of goitered gazelles was 5.3095% and 15.6347% in winter and summer, respectively, which was greater than on species of animals living underground. Seasonal variation also influenced the ecological processes of microbiota both in the gut and soil. Due to the differences in environments, the ecological processes between fecal microbiota and soil microbiota showed significant differences, and they were dominated by stochastic processes and deterministic processes, respectively. The soil microbiota has contributed to the gut microbiota, but not a decisive factor. Our research laid the foundation on the seasonal and soil microbiota effects on the adaptive strategies of goitered gazelles, and is the first study to explain the soil microbiota influence on the gut microbiota of wild herbivores.

Keywords: *Gazella subgutturosa*, gut microbiota, soil microbiota, ecological process, adaptation

INTRODUCTION

Gut microbiota plays an essential role in the health and adaptation of the herbivores (Ding et al., 2018; Wu et al., 2019; Cui et al., 2020). Seasonal variation is the main factor causing changes in gut microbiota. Herbivores in extreme environments have evolved seasonal adaptive strategies based on their gut microbiota (Guo et al., 2021). Understanding the relationship between host adaptability and gut microbial seasonal variation is important for managing the conservation of wild herbivores in extreme environments. The significant seasonal changes in the external environment can change the gut microbial diversity (Ren et al., 2017; Smits et al., 2017). For example, diet is a major factor that affects seasonal variations in the gut. Seasonal dietary changes may be related to the different functions of gut microbiota between the wet and dry seasons in the Hadza (Smits et al., 2017). Under the same feeding conditions and environment, Sprague–Dawley male rats showed seasonal gut microbial variations (Liu et al., 2018). Gut microbiota seasonal changes can help the host adapt to environmental changes. Seasonal variation in gut microbiota may help the wild Tibetan macaque adapt to diet changes and provide sufficient energy for them in extreme climates. In winter, the increase in the relative abundance of *Proteobacteria* and *Succinivibrio* of the wild Tibetan macaque is associated with energy accumulation and utilization (Sun et al., 2016). *Treponema*, whose relative abundance is higher in winter, can help the yak degrade plant polysaccharides from hay or from a concentrated diet. The relative abundance of *Butyrivibrio_2* is higher in summer, which is of benefit for the yak to degrade complex carbohydrates and protein (Ma et al., 2019). Seasonal changes are closely related to changes in gut microbial diversity, and as a result, changes in gut microbiota can be used as indicators to reflect the adaptability of the host (Bergmann et al., 2015; Xu et al., 2015; Xue et al., 2015; Chen et al., 2017; Hicks et al., 2018).

The goitered gazelle, *G. subgutturosa* (Güldenstaedt, 1780), is mainly distributed in arid and subarid areas, including shrubland, grassland and desert. There are fewer than 49,000 individuals of goitered gazelle, and it is listed as a vulnerable species based on IUCN red list. Climate change and temperature extremes are among its main threats (IUCN SSC Antelope Specialist Group, 2017). In the Qinghai-Tibet plateau, goitered gazelles are mainly found in the Qaidam Basin. The goitered gazelle is the representative herbivore of the Qaidam Basin and is the foundation for the ecological balance of this area. The Qaidam Basin is located in the eastern portion of the Qinghai-Tibet plateau. The climate is characterized by evaporation greater than rainfall, cold and long winters and hot and dry summers, the altitude ranges from 2,600 to 5,500 m (Shen, 1998; Shi et al., 2005; Wei et al., 2014; Li et al., 2017a). In this location, goitered gazelles must adapt to large seasonal temperature differences and drought. Goitered gazelles can adapt to the environment seasonal variations by adjusting water evaporation and the size of organs, including the heart, liver and brain (Ostrowski et al., 2006). Goitered gazelle is a representative ungulate species that can be used to study adaptations to arid environment. However, prior studies on the adaptability of goitered gazelles have mainly focused on ecological adaptation and behavioral characteristics (Blank et al., 2012, 2015; Zhang et al., 2021). The main research sites have been in Middle Asia and the Xinjiang Uygur Autonomous Regions. There have been no systematic studies on the adaptation of goitered gazelle to the seasonal changes based on gut microbiota in the extreme environment of the Qaidam Basin. Therefore, understanding those issues is the basis for the conservation of goitered gazelles in the Qaidam Basin and also an urgent problem to be solved now.

The environment of Keke Town in Qinghai Province has the universal characters of the Qaidam Basin environment, and it is a representative site of the Qaidam Basin. The goitered gazelle population in Keke Town is large, and it is a major population within the Qaidam Basin. The gazelles in this population do not migrate and are present in both summer and winter. Some goitered gazelle populations in Xinjiang embark on long-distance migration to cope with seasonal environmental variation (Sun et al., 2002), but we have not observed the population in Keke Town migrating over long distances. The goitered gazelle population in Keke town may have a different strategy to adapt to seasonal variation. It is unknown how the Keke population has adapted to the seasonal variations in its environment. We believe that a study on the seasonal variations in gut microbiota of the goitered gazelle population in Keke Town is an representativeness for studying the seasonal adaptation strategies of wild herbivores in extreme environments. This research expands knowledge of both this population and the entire species.

The goitered gazelle mainly feeds on *Chenopodiaceae* and *Gramineae* (Xu et al., 2008), so goitered gazelles are likely in contact with the surface of the soil when they are feeding. During feeding, the gazelle may ingest microorganisms from the soil. More attention is given to the direct impact of climate change on wildlife, but herbivores frequently contact the soil when eating, and soil is also a factor that can influence changes in herbivorous gut microbiota. There is no known analysis on the source tracking between the gut microbiota of herbivores and soil microbiota. Microbial diversity in soil shows different characters and seasonal changes, and seasonal changes in soil microbiota may benefit the survival and adaptation of associated animals (Garcia-Alvarez and Ibañez, 1994; Guimaraes et al., 2020; Ishaq et al., 2020). For example, *Gemmatimonadetes* in soil has seasonal variations and uses these to adapt to changes in the soil environment (DeBruyn et al., 2011). Soil may also explain the gut microbiological properties of the host (Grieneisen et al., 2019; Hannula et al., 2019). However, most studies on seasonal changes of soil microbiota are related to crops and climate changes, and few focus on animal conservation.

The ecological aspects of communities are important in revealing the process of community formation, including the microbial community of soil and the animal gut. Deterministic and stochastic processes are the dominant components of community formation. Deterministic processes

involve biotic and abiotic factors, and stochastic processes involve factors under which all species are ecologically equivalent (Mo et al., 2021). The dominant causes (deterministic or stochastic processes) in the assembly process of gut microbiota from different wild animals are changed in different environments (Li et al., 2019b, 2022). However, there are few studies about the ecological assembly process of gut microbiota, not to mention their effect on the host of gut microbiota. This gap needs to be filled. We speculated that the gut microbial formation process of goitered gazelles in Keke Town may show seasonal variations to adapt to the seasonal changes in the Qaidam Basin.

In this study, we set out to solve two scientific problems. The first one is what are the seasonal changes of gut microbiota in goitered gazelles, and how do goitered gazelles adapt to environmental seasonal changes based on gut microbiota? The second one is the effects of soil microbiota on seasonal adaptation of gut microbiota in goitered gazelles. Here, we collected 78 fecal samples from goitered gazelles from Qaidam Basin, using a non-invasive method. We used 16S rRNA V3-V4 regions to compare the differences of microbiota in feces and soils between winter and summer and describe the seasonal variations and the effects of soil microbiota on fecal microbiota of goitered gazelle. This study describes the seasonal adaptability of goitered gazelle populations in Qaidam Basin, and the data are beneficial to the conservation and management of this species.

MATERIALS AND METHODS

Ethics Statement

All experiments, including the sample collection methods, followed the principles of the Ethical Committee for Experimental Animal Welfare of the Northwest Institute of Plateau Biology.

Sample Collection

All the samples were collected in Keke Town of Wulan County, Qinghai Province, China (36.97°N 98.04°E). In the winter, a total of 47 fresh fecal pellets of goitered gazelles (one per individual) and 8 soil samples were collected on 3–4 December 2020. In the summer, a total of 31 fresh fecal pellets from goitered gazelles (one per individual) and 6 soil samples were collected on 12 August 2021.

According to our investigation, the goitered gazelles in Keke Town usually go to the lake to drink water after sunrise and mostly defecate near the lake. So our sampling time was after sunrise and before noon. The goitered gazelles' fecal pellets were concentrated rather than scattered on the ground, allowing them to be identified as individuals rather than groups. In the winter, fresh fecal pellets were either moist and warm, or they had a frosty surface but were moist on the inside. In the summer, fresh fecal pellets were either moist and warm, or slightly dry on the surface but moist on the inside (**Figure 1**). We tried to select inside fecal pellets that were moist and not stuck to the soil, and we collected at least three fecal pellets (more than 0.5 g) from each individual. Each individual fresh fecal sample was collected with disposable polyethylene (PE) gloves to avoid contamination. Individual fecal samples were placed in ziplock bags (one per individual), labeled with water-proof and alcohol-proof label pen, and then stored in liquid nitrogen for less than 14 days.

During soil sampling, we first collected topsoil from three different sites; the distance between them was about 100 m. Then we mixed the three topsoils and passed them through a 100 mesh sieve to remove rocks and dead plant material. Finally, what we had was 1 soil sample. We repeated the above steps for each soil sample. To make the soil samples representative, the distance between each soil sample we obtained was more than 500 m. All the soil samples were labeled, stored in cryopreservation tubes and stored in liquid nitrogen for fewer than 14 days. Prolonged storage of fecal samples and soil samples was at −80°C.

DNA Extraction, Amplification, and Sequencing

Library preparation and sequencing were performed at the Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Total DNA was extracted with the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, United States) using manufacturer instructions. All of the DNA samples were qualified and determined with a NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, DE, United States). The 16S rRNA V3-V4 regions were amplified with primers 338F(5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R(5′-GGACTACHVGGGTWTCTAAT-3′). PCR reactions were performed in 20 μl and included 4 μl of 5× TransStart FastPfu buffer (Thermo Fisher Scientific, Wilmington, DE, United States), 0.4 μl of TransStart FastPfu DNA Polymerase (Thermo Fisher Scientific, Wilmington, DE, United States), 10 ng of extracted DNA as template, 0.8 μl of 5 μM each primer, 2 μl of 2.5 mM deoxynucleoside triphosphates (dNTPs) and additional ddH_2O up to 20 μl. The PCR protocol was: 30 s at 95°C, 30 s at 55°C, and 45 s at 72°C for 27 cycles. All PCR products were mixed and assessed by 2% agarose gel electrophoresis, purified with the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) based on manufacturer instructions. The purified amplicons were sequenced on the Illumina MiSeq sequencing platform (Illumina, San Diego, United States). A total of 92 samples underwent this analysis. The raw data are available from the Sequence Read Archive (SRA) under the accession number: PRJNA 825477.

The Bioinformatics Pipeline

The bioinformatics pipeline was mainly conducted in QIIME2 (Bolyen et al., 2019). In brief, after demultiplexed according to the specific barcode sequences of each sample, the resulting sequences were merged using FLASH (v1.2.11) software (Magoč and Salzberg, 2011) and quality filtered with fastp (0.19.6; Chen et al., 2018). The DADA2 (*via* q2-dada2 plugin) was used to denoise the sequences with recommended parameters (Callahan et al., 2016) to obtain raw amplicon sequence variant (ASV) table and raw ASV representative sequences.

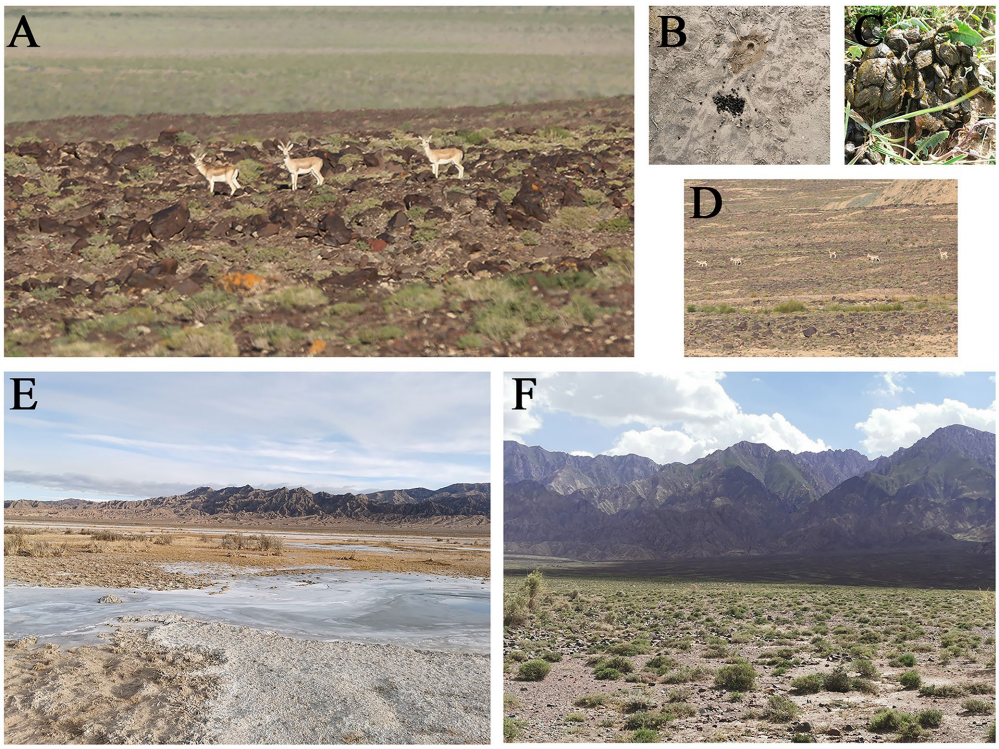

FIGURE 1 | (A) The picture of goitered gazelles in Keke Town; **(B)** The fresh feces of one individual of goitered gazelle in the winter; **(C)** The fresh feces of one individual of goitered gazelle in the summer; **(D)** The picture of a group of goitered gazelles in Keke Town; **(E)** The sampling area of Keke Town in the winter; **(F)** The sampling area of Keke Town in the summer.

Based on the curated SILVA SSU NR99 (version 138) database,[1] the reference sequence annotation and curation pipeline (RESCRIPt) were used to prepare a QIIME2 compatible amplicon-specific naïve Bayes classifier to improve the quality of classification (Robeson et al., 2021), following the protocol suggested by the author.[2] Taxonomic classification was performed with Q2-feature-classifier plugin (0.8 confidence). The taxonomy-based filtering was used to remove the ASVs that belong to mitochondria, chloroplast, or archaea. ASVs with relative abundance lower than 0.01% as well as present in fewer than five samples were also excluded. The ASV table was than rarefied to the minimum sequencing depth of all samples and used for further analyses except FEAST analysis, which required a raw ASV table.

The Venn diagram was analyzed using package "stats" (R Core Team, 2019). The pairwise comparisons of microbiota between gut and soil at the phylum level, family level, and genus level were all calculated based on the Wilcoxon rank-sum test. Alpha diversity indexes (Shannon and Simpson) were calculated using the package "vegan" (Oksanen et al., 2019), and compared by Wilcoxon rank-sum test between any two groups using package "stats" (R Core Team, 2019). All distance-based analyses were performed based on the respective Bray-Curtis distances, which was calculated by package "vegan" (Oksanen et al., 2019). The non-metric multidimensional scaling (NMDS), analysis of similarities (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) were performed using the package "vegan" with 999 permutations (Oksanen et al., 2019), and visualized with the package "ggplot2" (Wickham, 2016). LefSe (linear discriminant analysis effect size) analysis was performed using LefSe software (LDA score = 4; Segata et al., 2011). All the relevant bioinformatic analyses were performed on the free online platform of Majorbio Cloud Platform (Shanghai Majorbio Bio-pharm Technology Co., Ltd., Shanghai, China).

Source Tracking Analysis

We used the fast expectation–maximization microbial source tracking (FEAST) to reveal the origins of the fecal microbiota of goitered gazelles (Shenhav et al., 2019). We considered the fecal microbiota of every sample as a sink, and the soil microbiota of each corresponding sampling site were considered a source. Fecal microbiota in each season were source tracked to soil microbiota in the corresponding season. Taxa that could not be classified to the input sources were categorized as unknowns. The parameters were COVERAGE = 12,234, EM_iterations = 10,000,000 in both two FEAST analysis.

[1]https://www.arb-silva.de/fileadmin/silva_databases/release_138/Exports/SILVA_138_SSURef_NR99_tax_silva.fasta.gz

[2]https://forum.qiime2.org/t/processing-filtering-and-evaluating-the-silva-database-and-other-reference-sequence-data-with-rescript/15494

The Ecological Assembly Process of Microbiota in Gut and Soil

We estimated contributions of the stochastic and deterministic assembly processes in the microbiota of gut and soil with the modified stochasticity ratio (MST). MST values > 0.5 and < 0.5 indicated that the dominant assembly processes were deterministic process and stochastic process, respectively (Ning et al., 2019). The MST were calculated using "NST" (normalized stochasticity ratio) package with 30,000 simulations in R and Rstudio (Zhou et al., 2014; Ning et al., 2019) followed the protocol that author suggested.

With the neutral community model, we assessed the effects of stochastic processes on the microbiota of gut and soil and evaluated the goodness of fit to the model by non-linear least-squares (Sloan et al., 2006; Östman et al., 2010). This model was performed with the package "Minpack.lm" in R and Rstudio.[3] The R^2 values were <1. When the R^2 values increased, the predominance of the stochastic process was also greatly increased.

To test the clustering or overdispersion of microbiota in gut and soil, we examined the deviation of each observed metric from the average of the null model [checkerboard score (C-score); Stone and Roberts, 1990]. The standardized effect size (SES) included standardized values to comparisons among assemblages and was calculated under the null model. The C-score was assessed based on 30,000 simulations by the sequential swap randomization algorithm with the "EcoSimR" package in R and Rstudio[4] (Stone and Roberts, 1990; Mo et al., 2021). The values of SES indicate the strength of the effect of deterministic processes on the assemblage (Li et al., 2017b; Mo et al., 2021).

RESULTS

Comparison of Gut Microbial Diversity in Goitered Gazelles Between Winter and Summer

There were 3,558 ASVs shared between winter and summer, and there were more unique ASVs in the winter (603 ASVs). In the summer, there were 323 unique ASVs (**Figure 2A**).

In the winter and summer, the top 5 phyla in relative abundance were *Firmicutes* (78.95% ± 4.66%, 75.15% ± 5.51%), *Bacteroidota* (17.02% ± 4.60%, 18.60% ± 6.49%), *Actinobacteriota* (0.83% ± 0.79%, 4.03% ± 3.93%), *Patescibacteria* (0.63% ± 0.44%, 1.13% ± 1.0%), *Verrucomicrobiota* (0.96% ± 1.10%, 0.66% ± 0.71%). Only the relative abundance of *Bacteroidota* and *Verrucomicrobiota* showed no significant seasonal differences ($p > 0.05$; **Figure 2B**).

At family level, the top 5 families in relative abundance were *Oscillospiraceae* (17.34% ± 2.30%, 16.67% ± 3.54%), *Lachnospiraceae* (17.70% ± 2.68%, 16.42% ± 3.61%), *Eubacterium_coprostanoligenes_group* (6.74% ± 1.43%, 9.90% ± 4.74%), *Rikenellaceae* (6.32% ± 1.57%, 8.18% ± 3.23%), *UCG-010* (7.77% ± 3.93%, 4.74% ± 1.80%). Only the relative abundance of *Lachnospiraceae* and *Oscillospiraceae* showed no significant differences between winter and summer ($p > 0.05$; **Figure 2C**).

At genus level, the top 5 bacteria in relative abundance were *UCG-005* (12.56% ± 2.38%, 12.91% ± 3.41%), *unclassified_f__Lachnospiraceae* (13.47% ± 2.16%, 11.10% ± 2.80%), *norank_f__Eubacterium_coprostanoligenes_group* (6.74 %± 1.43%, 9.90% ± 4.75%), *Christensenellaceae_R-7_group* (6.99% ± 1.31%, 4.83 %± 1.58%) and *norank_f__UCG-010* (7.77% ± 3.93%, 4.74% ± 1.80%). Only the relative abundance of *UCG-005* showed no significant differences between winter and summer ($p > 0.05$; **Figure 2D**).

The relative abundance of *Firmicutes* was significantly higher in the winter ($p < 0.05$), and the *Bacteroidota* was higher in the summer, showed no significant differences between winter and summer ($p > 0.05$). The ratio of *Firmicutes*/*Bacteroidota* was higher in the summer but there was no significant differences between summer and winter ($p > 0.05$).

At α-diversity level, based on Shannon index (Winter = 6.1904 ± 0.1859; Summer = 5.8871 ± 0.2335) and Simpson index (Winter = 0.0052 ± 0.0080; Summer = 0.0084 ± 0.0035), the α-diversity of gut microbiota in the winter was significantly higher ($p < 0.05$; **Figures 3A,B**).

At β-diversity, the gut microbial diversities between winter and summer showed significant differences based on PERMANOVA (Permutational MANOVA) analysis ($R^2 = 0.1261$; $p < 0.05$; **Appendix 1**). The Anosim analysis at the ASV level ($R = 0.9087$, $p = 0.001$) supports this result. The NMDS analysis also showed that there was obvious separation in gut microbiota between winter and summer (**Figure 3C**). The LEFSe (Linear discriminant analysis Effect Size) analysis indicates that a total of 15 bacteria are biomarkers, including *Firmicutes*, *Actinobacteriota*, *Eubacterium_coprostanoligenes_group*, *Christensenellaceae* and *Christensenellaceae_R-7_group* (**Figure 3D**).

Differences of Gut Microbial Functions in Goitered Gazelles Between Winter and Summer

The main function is "Metabolism" both in the winter and summer and the relative abundance are all above 58%. The following are functions of "Environmental Information Processing" and "Genetic Information Processing" based on KEGG database with Tax4Fun. The relative abundance of functions about "Metabolism" and "Cellular Processes" is significantly higher in the winter, and that of "Genetic Information Processing" is significantly lower in the winter ($p < 0.05$). There were no significant differences in functions about "Environmental Information Processing" between winter and summer ($p > 0.05$; **Table 1**).

At pathway level 3, the main function was "ABC transporters," whose relative abundance was all above 10% in the two seasons. The following are "Two-component system" (ko02020), "Aminoacyl-tRNA biosynthesis" (ko00970), and "Purine metabolism" (ko00230), whose relative abundance were greater than 3% in both seasons. All the top 10 functions showed significant differences between winter and summer. Only the relative abundance of "Two-component system" was significantly higher in the winter ($p < 0.05$; **Table 2**).

[3]https://cran.r-project.org/web/packages/minpack.lm/index.html
[4]https://github.com/GotelliLab/EcoSimR/

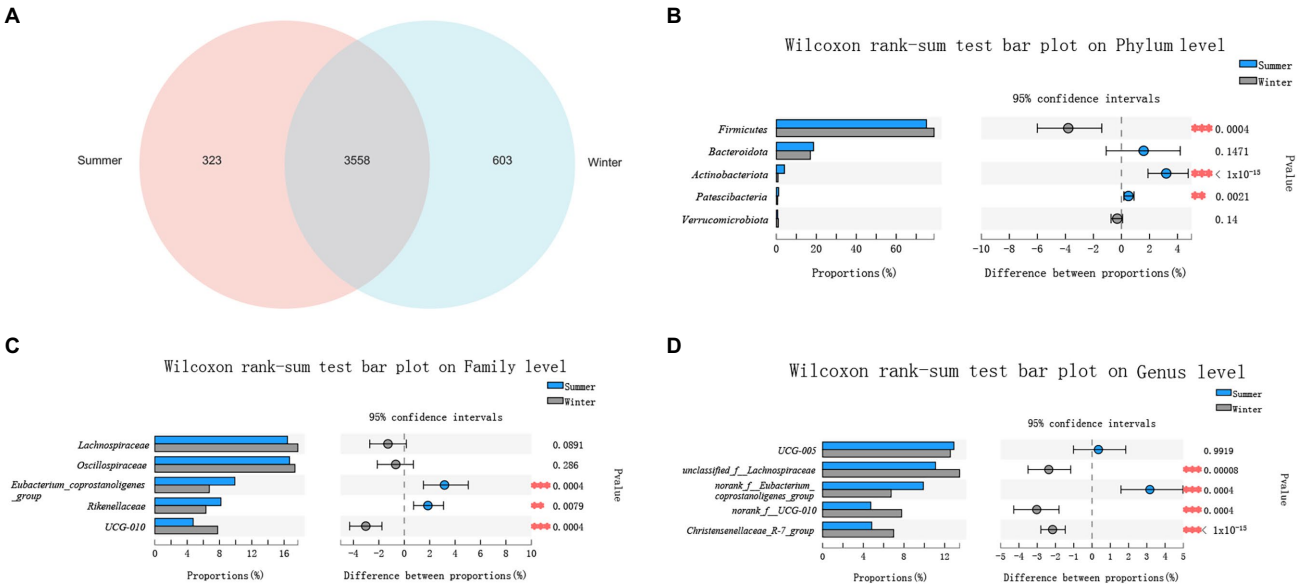

FIGURE 2 | Comparison of gut microbial diversity between winter and summer. **(A)** A Venn diagram at amplicon sequence variant (ASV) level; **(B)** Top 5 phyla; **(C)** Top 5 families; **(D)** Top 5 genera. Significant differences in gut microbiota between winter and summer are indicated with value of *p* and asterisks (** if $0.001 < p < 0.01$ and *** if $p < 0.001$).

Taxonomic Differences of Soil Microbial Diversity Between Winter and Summer

Between winter and summer, a total of 337 ASVs were shared in soil microbiota. There were more unique ASVs (464) in summer, and only 317 unique ASVs present in the winter. This is the opposite of the results seen in the fecal microbiota (**Figure 4A**).

The top 5 bacteria were *Actinobacteriota* (33.68% ± 16.79%, 5.87% ± 2.41%), *Deinococcota* (5.99% ± 12.57%, 8.25% ± 3.10%), *Proteobacteria* (39.97% ± 17.16%, 25.60% ± 12.06%), *Bacteroidota* (5.06% ± 8.67%, 33.80% ± 20.26%), *Cyanobacteria* (6.50% ± 9.77%%, 9.97 ± 21.28%) at phylum level between winter and summer. Only the relative abundance of *Proteobacteria* and *Cyanobacteria* showed no significant differences between winter and summer ($p > 0.05$; **Figure 4B**).

At family level, the top 4 families were *Balneolaceae* (2.98% ± 8.22%， 25.21% ± 18.01%), *Burkholderiaceae* (15.45% ± 27.68%, 0.02% ± 0.05%), *Rhodobacteraceae* (7.39% ± 5.12%, 7.39% ± 4.33%), *Trueperaceae* (5.99% ± 12.57%， 8.25% ± 3.10%), *Phormidiaceae* (5.97% ± 9.29%， 9.01% ± 21.29%) between winter and summer. Only the relative abundance of *Phormidiaceae* and *Rhodobacteraceae* showed no seasonal significant differences ($p > 0.05$; **Figure 4C**).

At genus level, the top 5 genera were *norank_f__Balneolaceae* (2.88% ± 8.03%, 18.18% ± 13.97%), *Burkholderia-Caballeronia-Paraburkholderia* (15.12% ± 27.10%, 0), *Truepera* (5.99% ± 12.57%, 8.25% ± 3.10%), *Tychonema_CCAP_1459-11B* (5.97% ± 9.29%, 9.01% ± 21.29%), *Halanaerobium* (0.01% ± 0.02%, 7.78% ± 15.51%) between winter and summer. Only the relative abundance of *Tychonema_CCAP_1459-11B* showed no significant seasonal differences ($p > 0.05$; **Figure 4D**).

Among these bacteria from phylum to genus, only the relative abundance of *Actinobacteriota*, *Burkholderiaceae* and *Burkholderia-Caballeronia-Paraburkholderia* were significantly higher in the winter ($p < 0.05$; **Figures 4B–D**).

The α-diversity of soil microbiota was higher in the summer based on Shannon index (Winter = 3.5721 ± 0.9662; Summer = 3.6492 ± 0.7680), and Simpson index (Winter = 0.1137 ± 0.1515; Summer = 0.1072 ± 0.1010), there were no significant differences present ($p > 0.05$; **Figures 5A,B**).

At β-diversity, based on PERMANOVA analysis, the diversity of soil microbiota showed significant differences between winter and summer at ASV level ($R^2 = 0.3059$; $p < 0.05$; **Appendix 2**). The Anosim analysis also indicated that inter-groups differences were greater than intra-groups differences ($R = 0.6085$; $p = 0.0040$) at the ASV level.

Source Tracking Analysis of Fecal Microbiota From Soil Microbiota

The total contribution ratio of soil microbiota to fecal microbiota in the winter ranged from 4.12% to 6.84%, and the average contribution was ~5.31%. In the summer, the soil microbiota contributed more to the gut microbiota of goitered gazelle. The contribution rate ranged from 10.35% to 25.56% and the average value was 15.63%. Based on a *t*-test, there was a significant difference in soil microbiota contribution between winter and summer ($p < 0.05$).

Deterministic and Stochastic Processes in the Gut and Soil

The MST distribution in fecal microbiota from the winter and summer both exceeded the threshold value (0.5). This suggests that the dominant ecological process in fecal microbiota is a stochastic process. The MST values of soil microbiota from

the winter and summer were both less than 0.5, indicating that the dominant ecological process is deterministic (**Figure 5**). The C-score results also revealed that soil microbiota in the winter showed the highest standardized effect size (SES), followed by soil microbiota in the summer, fecal microbiota in the summer and fecal microbiota in the winter (**Figure 6**).

A higher SES value indicates a stronger deterministic process, and this result means the strongest deterministic process occurs in the winter soil microbiota. The neutral community model (NCM) showed that the microbiota in the gut and soil are weakly influenced by stochastic processes. The values of R^2 decreased from winter to summer and the values of Nm

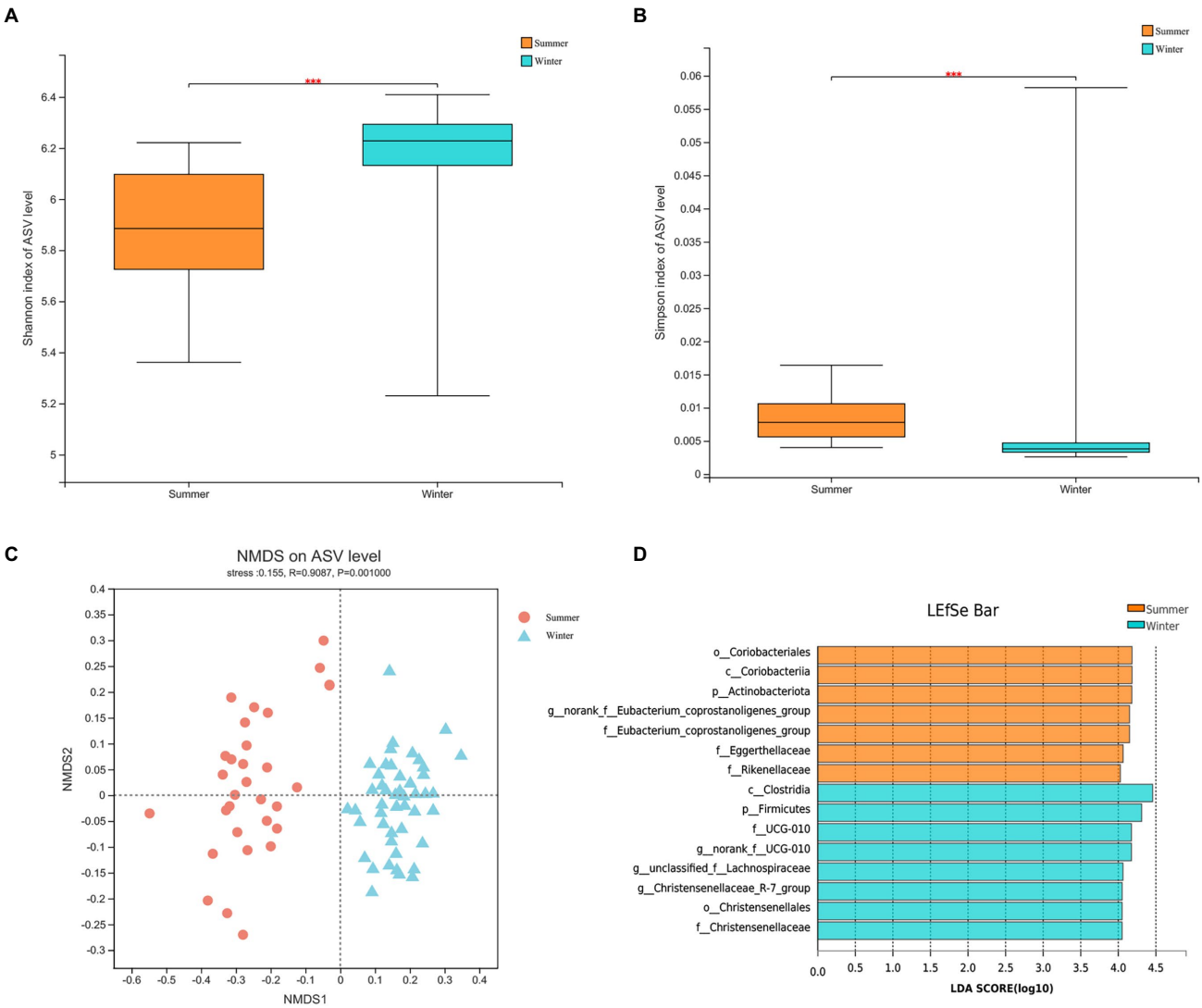

FIGURE 3 | The α-diversity between winter and summer in gut microbiota: **(A)** Shannon index at amplicon sequence variant (ASV) level; **(B)** Simpson index at ASV level; **(C)** The non-metric multidimensional scaling (NMDS) analysis of gut microbiota between winter and summer; **(D)** The biomarkers of gut microbiota based on linear discriminant analysis effect size (LEFSe) analysis between winter and summer from phylum to genus.

TABLE 1 | The comparison of relative abundance of functions in gut microbiota between winter and summer at level 1 based on KEGG database.

Pathway level 1	Cellular processes	Environmental information processing	Genetic information processing	Human diseases	Metabolism	Organismal systems
Winter	3.17%	19.35%	13.97%	2.41%	60.05%	0.91%
Summer	1.88%	19.47%	17.01%	2.38%	58.02%	1.14%
Value of *p*	2.03×10^{-11}	0.357431	7.85×10^{-11}	0.5239	6.24×10^{-12}	7.06×10^{-12}

TABLE 2 | The comparison of relative abundance of functions in gut microbiota between winter and summer at level 3 based on KEGG database.

Pathway level 3	Description	Winter	Summer	Value of *p*
ko02010	ABC transporters	10.84%	11.94%	1.08×10^{-7}
ko00970	Aminoacyl-tRNA biosynthesis	3.40%	4.46%	1.63×10^{-11}
ko00230	Purine metabolism	3.74%	4.43%	$1.29 \times 10–^{11}$
ko02020	Two-component system	5.51%	4.23%	1.71×10^{-10}
ko00240	Pyrimidine metabolism	2.76%	3.44%	6.52×10^{-11}
ko03010	Ribosome	2.11%	2.71%	4.15×10^{-11}
ko00550	Peptidoglycan biosynthesis	2.04%	2.61%	9.34×10^{-11}
ko00500	Starch and sucrose metabolism	2.02%	2.58%	3.88×10^{-10}
ko00520	Amino sugar and nucleotide sugar metabolism	2.20%	2.48%	8.84×10^{-7}
ko02060	Phosphotransferase system (PTS)	2.03%	2.34%	0.0007

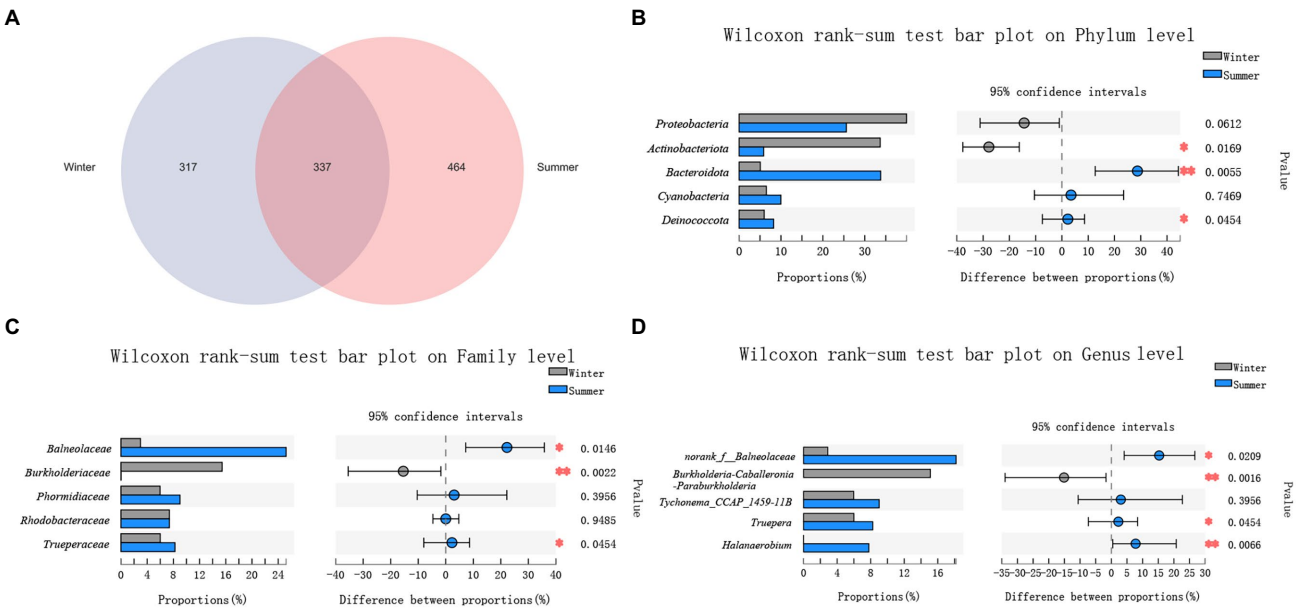

FIGURE 4 | Comparison of soil microbial diversity between winter and summer. **(A)** A Venn diagram at amplicon sequence variant (ASV) level; **(B)** Top 5 phyla; **(C)** Top 5 families; **(D)** Top 5 genera. Significant differences in soil microbiota between winter and summer are indicated with value of *p* and asterisks (* if $0.01 < p < 0.05$ and ** if $0.001 < p < 0.01$).

increased from winter to summer, which is associated with diffusivity (**Figures 7A–D**).

DISCUSSION

Seasonal Adaptation Strategies of Goitered Gazelles Based on Gut Microbiota

Between winter and summer, the gut microbial diversity showed significant differences. This result was consistent with other wildlife studies including musk deer and white-lipped deer on the Qinghai-Tibetan plateau (Jiang et al., 2021; Li et al., 2022). For herbivores in extreme environments, seasonal variation in gut microbiota is an essential adaptive strategy. It allows for the efficient digestion of food and sufficient energy while maintaining a constant body temperature, which is essential for winter survival.

Firmicutes and *Bacteroidota* are the dominant bacteria in gut microbiota of goitered gazelles, and this is common in other herbivores (Ilina et al., 2021; Liu et al., 2021b). *Firmicutes* are involved in the digestion of cellulose and energy metabolism and are indispensable to herbivores (Chen et al., 2017; Zhao et al., 2018). In this study, the relative abundance of *Firmicutes* was significantly higher in winter, indicating that *Firmicutes* may be important in the winter adaptation of goitered gazelles. *Christensenellaceae* bacteria are associated with the body mass index (BMI) of the host and negatively correlated with visceral fat mass and trunk fat (Waters and Ley, 2019). The relative abundance of *Christensenellaceae* was significantly higher in the winter, and this suggested that the visceral fat mass and trunk fat are lower in the winter than in the summer. Because

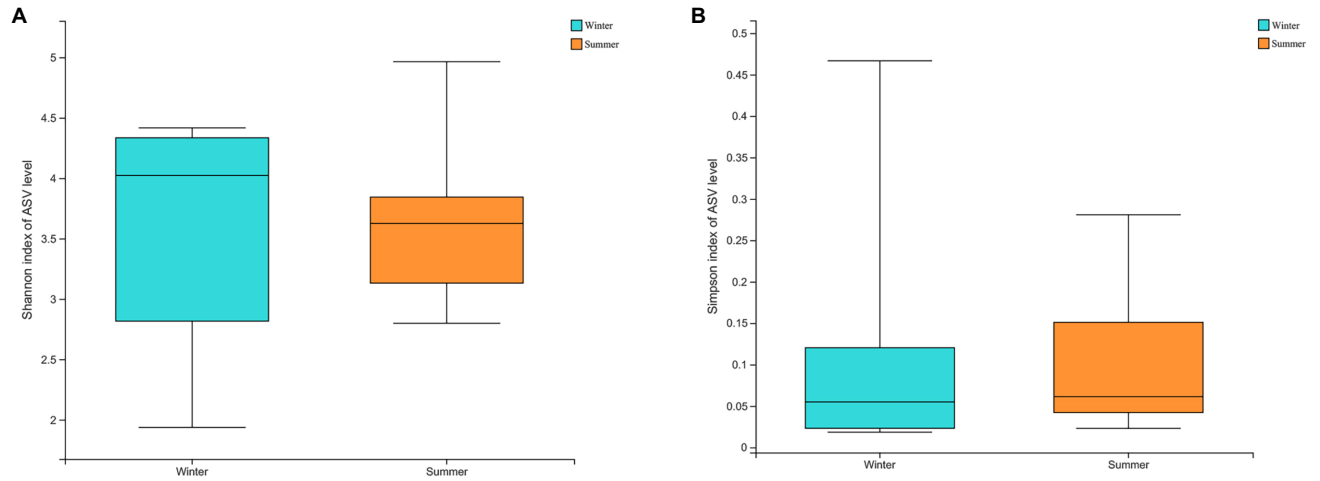

FIGURE 5 | The α-diversity between winter and summer in soil microbiota: **(A)** Shannon index at amplicon sequence variant (ASV) level; **(B)** Simpson index at ASV level.

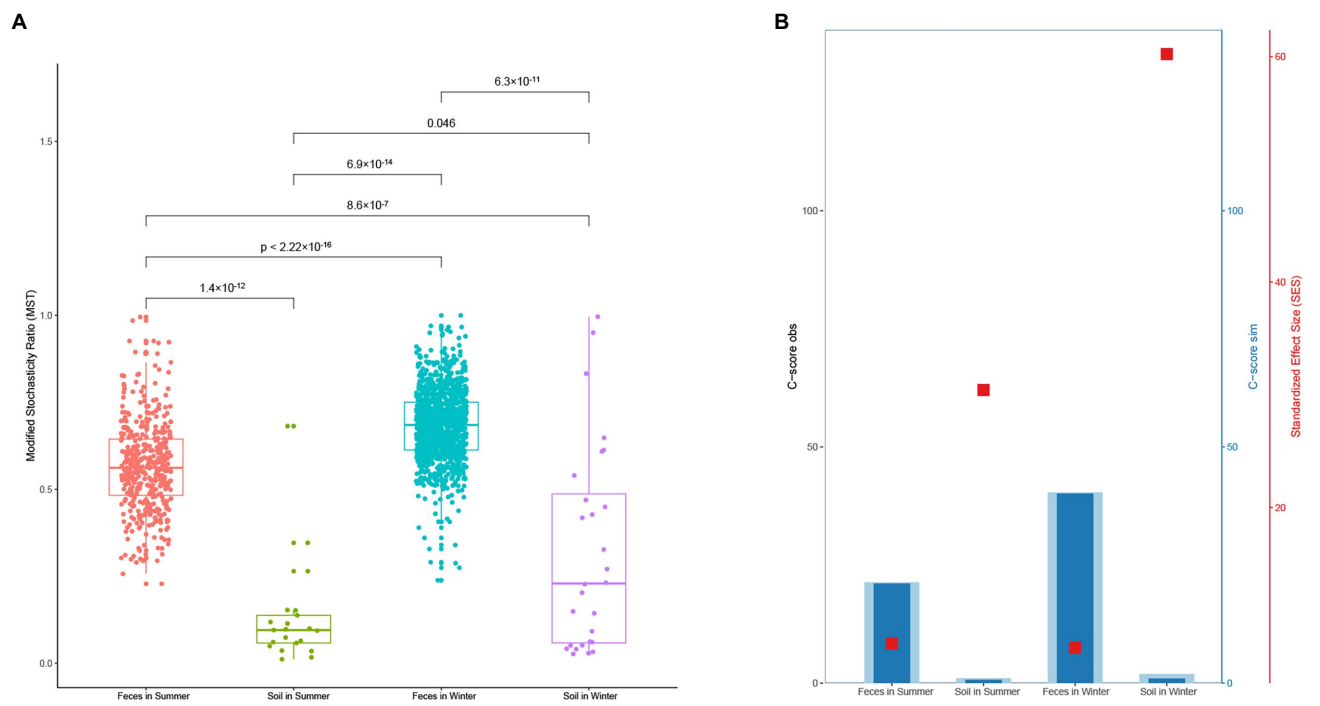

FIGURE 6 | (A) Modified stochasticity ratio (MST) analysis of microbiota in gut and soil between winter and summer. **(B)** Checkerboard score (C-score) of microbiota in gut and soil between winter and summer based on the null model.

Christensenellaceae are associated with healthy glucose metabolism (Waters and Ley, 2019), they could promote the glucose conversion into energy to aid winter survival of goitered gazelles. The relative abundance of *Bacteroides* is also significantly higher in the winter, and this may be related to carbohydrate metabolism to enhance the nutrient utilization of the goitered gazelles (Li et al., 2017b). The relative abundance of "Metabolism" functions is significantly higher in the winter to maintain the basal metabolism of the goitered gazelles. To metabolize high-fat food in the summer, the relative abundance of *Rikenellaceae* was significantly higher in the summer (Daniel et al., 2014). Goitered gazelles in Keke Town can therefore use gut microbiota to enhance their seasonal adaptations, especially in the winter.

The ratio of *Firmicutes*/*Bacteroidota* can reflect the nutrition status of the host. When food has a low carbohydrate

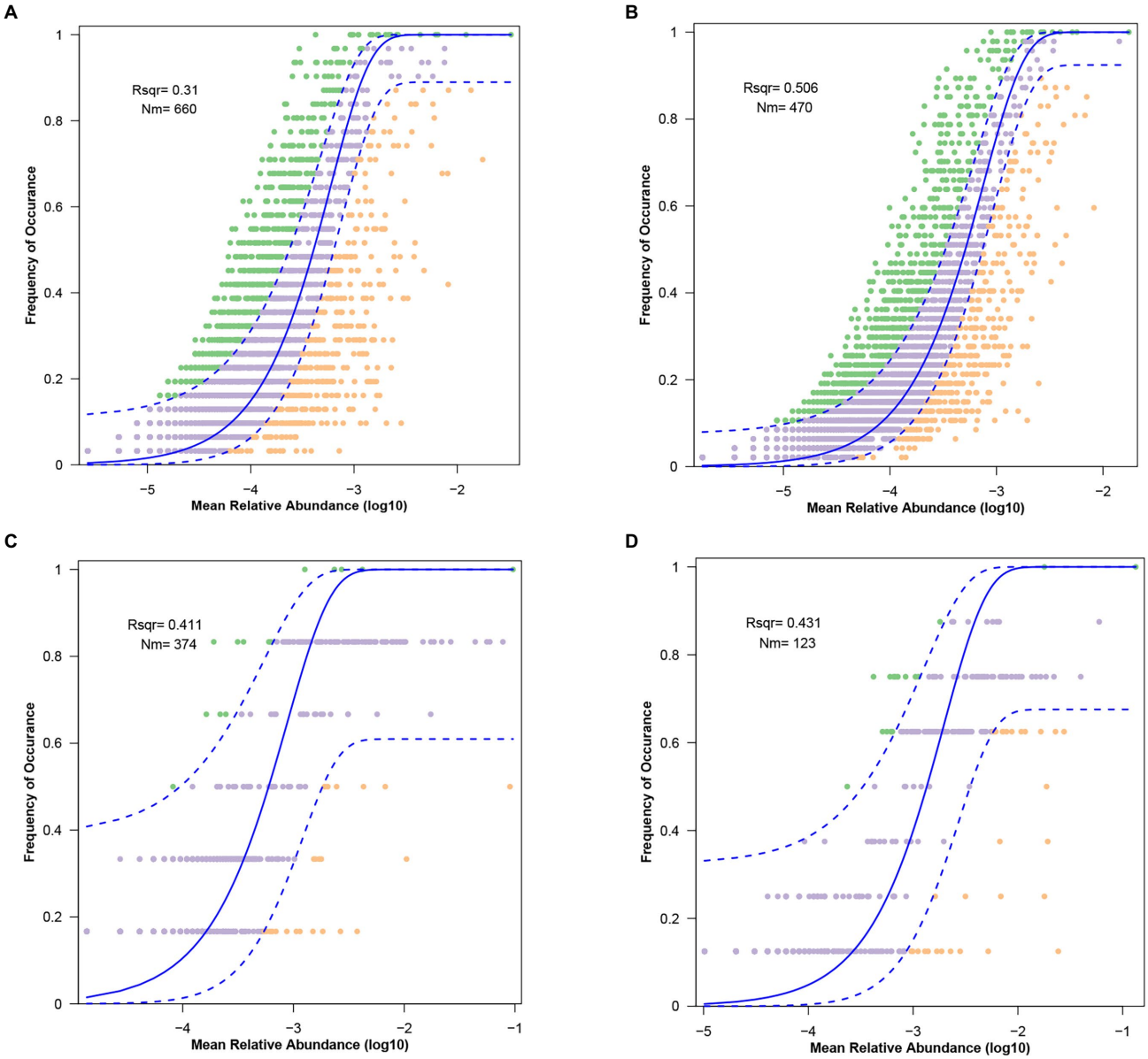

FIGURE 7 | The predicted occurrence frequencies for **(A)** gut microbiota from summer, **(B)** gut microbiota from winter, **(C)** soil microbiota from summer, and **(B)** soil microbiota from winter. The solid blue line indicates the best fit to the neutral community model (NCM), the dashed blue line means 95% confidence intervals around the NCM prediction.

content, the ratio decreases (De Filippo et al., 2010). However, in goitered gazelles, the gut microbiota ratios showed no significant differences between winter and summer. This means there is no significant seasonal variation in the nutritional status of goitered gazelles at Keke Town, and there is no significant decline in their nutritional status during the winter. We speculated that the goitered gazelle maintains the seasonal balance of nutrition by regulating the relative abundance of different bacteria in the gut.

Effects of Seasonal Dietary Changes on the Gut Microbiota of Goitered Gazelles

Diet is the main factor that affects gut microbial diversity, and seasonal dietary changes contribute to the seasonal changes of gut microbiota (Zhang et al., 2014; Li et al., 2019a; Guo et al., 2021; Huang and Liao, 2021). In the winter, there are 17 plant species consumed by goitered gazelles while 30 plant species are consumed in the summer (Xu et al., 2008). The α-diversity of gut microbiota in the winter was higher than in the summer. A healthy, diverse

diet promotes diverse gut microbiota (Zmora et al., 2018). However, our result was not consistent with this general conclusion. There are two explanations for this phenomenon. The first one is that the seasonal dietary changes of goitered gazelles in Keke Town are different from those of Xinjiang. Maybe even the opposite. The second one is that it is a compensatory measure. More bacteria with more functional genes that can improve nutrition status with a large number of microorganisms in the winter and maintain the nutritional balance between winter and summer. For example, the relative abundance of "Metabolism" significant higher in the winter, it may exist to maintain stable metabolic levels. However, the seasonal dietary changes and the specific compensation mechanism all need further study.

Effects of Soil Microbiota on the Gut Microbiota of Goitered Gazelles

No previous studies have evaluated the contribution of soil microbiota to herbivores in the Qinghai-Tibetan plateau. We found that the gut microbiota of goitered gazelles had more microbiota from soil than zokors that live underground (Liu et al., 2021a). This suggests that goitered gazelles contact the soil when feeding and obtain microorganisms from the soil. The gut microbiota of goitered gazelle is clearly affected by soil microbiota. There were significantly more bacteria in the gut that originated from soil microbiota in the summer than in the winter. This indicated that goitered gazelles have more contact with soil or lick more soil in the summer, but this is not consistent with the results of higher diversity of gut microbiota in the winter. We believe that the intake mass of soil microorganisms is the result of voluntary selection by goitered gazelles. *Firmicutes* in the soil showed no significant differences between winter and summer, which is different from what happens in the gut. However, the relative abundance of *Bacteroidota* showed a significant increase in soil in the summer, but no significant differences were seen in the gut microbiota of goitered gazelles between winter and summer. It is possible that *Firmicutes* and *Bacteroidota* in soil have no seasonal effect on the gut microbiota of goitered gazelles, and these two dominant bacteria are affected by other factors. However, soil microbiota effects on goitered gazelles at Keke Town need further study.

Seasonal Variations of Ecological Assembly Processes of Microbiota Communities in the Gut and Soil

The seasonal variations of ecological assembly processes of microbiota communities in the gut and soil are the parts of the contributions that elucidate the seasonal changes of microbiota in the gut and soil. We found that seasonal variations have an important influence on the assembly of microbiota in the gut of goitered gazelles and the soil by affecting the balance between deterministic and stochastic processes. The ecological processes between fecal microbiota and soil microbiota showed significant differences, and they were dominated by stochastic processes and deterministic processes, respectively.

Gut microbiota of goitered gazelles changes rapidly to maintain homeostasis within the intestinal environment. These changes include reproduction, death and exchanges of a large number of microorganisms, resulting in the dominance of the stochastic process. The soil environments on the Qaidam Basin are relatively stable, and the changes in ground temperature and precipitation are relatively slow (Shen, 1998; Zeng et al., 2020). Thus, the soil microbiota remains in a relatively dynamic balance. However, the soil microbiota may be exposed to relatively high physiological stress, including high altitude, cold and drought (Shen, 1998). Therefore, they are more competitive, resulting in the dominance of deterministic processes.

The seasonal variation of fecal microbiota explained by stochastic processes decreased from 50.6% in the winter to 31% in the summer. The same phenomena also appeared in the seasonal changes of soil microbiota, which decreased from 43.1% in the winter to 41.1% in the summer. One possible explanation is related to the seasonal variations of environments. In the summer, goitered gazelles consume more plant species, and they can obtain better nutrition in the summer than in the winter (Xu et al., 2008). This situation is beneficial for the growth and reproduction of gut microbiota, which would increase the physiological stress of gut microbiota of goitered gazelles. The average temperature, precipitation and sunshine hours all increase in the summer (Xu et al., 2020), which is beneficial to the growth and reproduction of soil microbiota. These conditions would also increase the physiological stress of soil microbiota. Lower physiological stress may let microbiota both in gut and soil grow and reproduce freely (Mo et al., 2021); therefore, the influences of stochastic processes are relatively decreased in the summer.

Due to different environmental factors, the dominant ecological processes and seasonal variation of microbiota in the gut and soil were different. The microbial seasonal changes of ecological assembly processes in gut and soil were the result of adaptation to the seasonal changes of the external environment. Although soil microbiota contributed a relatively large proportion to the gut microbial composition of the goitered gazelle, the ecological assembly processes of the microbiota between gut and soil changed obviously due to the huge difference in environments. The host can filter and select certain microbes from the external environment (Kohl, 2020). Therefore, in this study, the soil microbiota may not have a decisive effect on the seasonal variation of gut microbial diversity in goitered gazelles. Filtering and the gut environment of goitered gazelles may be the prior factors for the seasonal variation in the gut microbiota to the soil microbiota.

CONCLUSION

The gut microbial diversity and functions showed significant seasonal differences in goitered gazelles. Goitered gazelle adapted to the winter environment by increasing the relative abundance of *Firmicutes*, *Christensenellaceae*, and *Bacteroides* to improve

energy utilization. The ratio of *Firmicutes*/*Bacteroidota* showed no significant nutritional differences in goitered gazelles between winter and summer. These are the adaptive strategies that gut microbiota helps the host maintain a nutritional balance between winter and summer. Soil microbiota contribute to the gut microbiota of goitered gazelles relatively more based on source tracking analysis, and the seasonal changes in soil microbiota can have an influence on the diversity of gut microbiota. It is possible that goitered gazelles lick the soil to obtain microorganisms to maintain homeostasis. Due to the difference in assembly processes and physiology stresses, the dominant ecological processes in the gut are stochastic, and in soil, they are deterministic. Soil microbiota does not have a decisive effect on the gut microbiota of goitered gazelles. Goitered gazelles on the Qinghai-Tibetan plateau have co-evolved with their gut microbiota to survive. Perhaps filtering and the gut environment of goitered gazelles are the prior factors for the seasonal variation in the gut microbiota to the soil microbiota.

These results provide information useful for the conservation of goitered gazelles and also contribute to the analysis of environmental effects on gut microbiota. Understanding the seasonal adaptive strategies of goitered gazelles on the Qaidam Basin is beneficial to making specific conservation measures, and the goitered gazelle is a good example for studying the adaptations of animals in extreme environments.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material.**

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethical Committee for Experimental Animals' Welfare of Northwest Institute of Plateau Biology.

AUTHOR CONTRIBUTIONS

WQ and PS wrote the manuscript and analyzed the data. WQ contributed to sampling and laboratory experiments. SZ designed the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.918090/full#supplementary-material

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High-Altitude Drives the Convergent Evolution of Alpha Diversity and Indicator Microbiota in the Gut Microbiomes of Ungulates

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Convergent evolution is an important sector of evolutionary biology. High-altitude environments are one of the extreme environments for animals, especially in the Qinghai Tibet Plateau, driving the inquiry of whether, under broader phylogeny, high-altitude factors drive the convergent evolution of Artiodactyla and Perissodactyla gut microbiomes. Therefore, we profiled the gut microbiome of Artiodactyla and Perissodactyla at high and low altitudes using 16S rRNA gene sequencing. According to cluster analyses, the gut microbiome compositions of high-altitude Artiodactyla and Perissodactyla were not grouped together and were far from those of low-altitude Artiodactyla and Perissodactyla. The Wilcoxon's test in high-altitude ungulates showed significantly higher Sobs and Shannon indices than in low-altitude ungulates. At the phylum level, Firmicutes and Patescibacteria were significantly enriched in the gut microbiomes of high-altitude ungulates, which also displayed a higher Firmicutes/Bacteroidetes value than low-altitude ungulates. At the family level, Ruminococcaceae, Christensenellaceae, and Saccharimonadaceae were significantly enriched in the gut microbiomes of high-altitude ungulates. Our results also indicated that the OH and FH groups shared two significantly enriched genera, *Christensenellaceae_R_7_group* and *Candidatus_Saccharimonas*. These findings indicated that a high altitude cannot surpass the order level to drive the convergent evolution of ungulate gut microbiome composition but can drive the convergent evolution of alpha diversity and indicator microbiota in the gut microbiome of ungulates. Overall, this study provides a novel perspective for understanding the adaptation of ungulates to high-altitude environments.

Keywords: gut microbiome, ungulates, phylogeny, high-altitude, convergent evolution

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INTRODUCTION

The gut microbiome can provide energy, in the form of short-chain fatty acids, to its host and maintain the gut homeostatic balance (Gonçalves et al., 2018; Markowiak-Kopeć and Śliżewska, 2020). Many factors affect gut microbiome composition and function, including diet (Huang et al., 2022), phylogeny (Wang et al., 2019; Gregor et al., 2022), and environment

(Gacesa et al., 2022; Wang et al., 2022). When a factor surpasses the other influencing factors, the gut microbiome converges to adapt toward this dominant factor. Giant panda (*Ailuropoda melanoleuca*) and red panda (*Ailurus fulgens*) are typical models for convergent evolution studies. Huang et al. (2021) found that their bamboo diet, rather than host phylogeny, is the dominant driver of gut microbiome convergence within these species. Surprisingly, giant pandas and bamboo-eating insects share strikingly similar gut microbiota (Yao et al., 2021). Owing to their diets, whales have similar gut microbiome compositions to that of carnivores (Wu et al., 2022). Similarly, the gut microbiomes of many myrmecophagous species, such as anteaters, aardvarks, and aardwolves, are clustered in the same group, although they belong to different orders (Delsuc et al., 2014).

In addition to animals with specialized diets, species with closer phylogenetic relationships or similar behavior have similar gut microbiome compositions (Gregor et al., 2022). Amato et al. (2019) found that host phylogeny outweighs the dietary niche in structuring primate gut microbiomes. The phylogeny of water bird species and their gut microbiome hierarchical tree evinced phylosymbiosis (Laviad-Shitrit et al., 2019). Flight behavior drives the convergent evolution of the gut microbiome in bats and birds, where Proteobacteria are the dominant bacterial species (Song et al., 2020).

Many scholars have focused on the environmental impacts to the gastrointestinal microbiome of different species, especially in high-altitude environments. For example, based on a principal component analysis plot of high-altitude rumen, such as yak and Tibetan sheep, microbiome compositions are clustered, with significantly enriched VFA-yielding pathways (Zhang et al., 2016). The Firmicutes/Bacteroidetes ratio in high-altitude blue sheep and European mouflon is higher than in low-altitude blue sheep and European mouflon, and principal component analysis and non-metric multidimensional scaling (NMDS) plots have shown that the gut microbiomes of blue sheep and European mouflon at high-altitude are more similar than those of the same species at low altitude (Sun et al., 2019). Ma et al. (2019) found that Tibetan antelopes (*Pantholops hodgsonii*), Tibetan sheep (*Ovis aries*), and Tibetan wild ass (*Equus kiang*) had similar gut microbiome compositions, and Tibetan antelopes and sheep were annotated to carbohydrate and energy metabolism. In these studies, significantly enriched microbiota and metabolic pathways were related to animal energy acquisition. High altitudes involve extreme environmental conditions, such as low oxygen (Wang et al., 2021), thus, driving the convergent evolution of gastrointestinal microbiome compositions and functions, which can produce more energy to maintain life activities in harsh plateau environments.

Ungulates are widely distributed, easy to domesticate and breed, and can adapt to a variety of environments (Mas-Coma et al., 2009). Some studies have shown that the feeding niche overlap of high-altitude (Qinghai-Tibet Plateau) ungulates is low in such high-altitude areas (Harris and Miller, 1995; Shrestha et al., 2005; Cao et al., 2009; Shi et al., 2016). There are abundant wild ungulates on the Tibetan Plateau, including Artiodactyla, such as yak (*Bos grunniens*), Tibetan antelopes (*P. hodgsonii*), and blue sheep (*Pseudois nayaur*), and Perissodactyla, such as Tibetan wild ass (*E. kiang*). Ungulates represent an excellent model for studying the convergent evolution of high-altitude gut microbiomes. Many scholars have conducted in-depth research on the convergent evolution of ungulates in different altitude environments based on microbiomics; however, in these studies, the species phylogeny is closely related at the family or genus level. Further, several of these studies have significant limitations; for example, Ma et al. (2019) did not compare the gut microbiome of *E. kiang* at different altitudes and performed cluster analyses between groups. Under broader phylogeny, high altitudes drive the convergent evolution of ungulate gut microbiomes; however, this issue remains unclear. Based on these previous studies, we hypothesized that high altitudes drive the convergent evolution of ungulate gut microbiome diversity patterns, core microbiota, composition, and indicator species at each classification level. Therefore, we used the 16S rRNA gene to compare the gut microbiomes of high- and low-altitude ungulates. We aim to reveal the how influential altitude and phylogeny are to ungulate microbiome compositions at different altitudes.

MATERIALS AND METHODS

Sample Collection

Samples of wild and captive ungulates were collected from Golmud (Qinghai Province, China) and Jinan (Shandong Province, China; Jinan wildlife and Jinan zoos), respectively. We collected samples from different wild ungulates and observed distinct individuals, immediately collecting excrement-core samples. Three observation points (point 1, *B. grunniens* and *E. kiang*; point 2, *P. nayaur*; and point 3, *P. hodgsonii*) along a straight-line distance of more than 100 km were considered. *B. grunniens* mainly feed on sedges in summer and graminoids in winter; *E. kiang* primarily consumes forbs in summer and browse in winter, while *P. hodgsonii* and *P. nayaur* primarily feed on graminoids and sedges in summer, respectively (Harris and Miller, 1995; Shi et al., 2016). Shi et al. (2016) observed that dietary overlaps were generally low among *P. hodgsonii*, *B. grunniens*, and *E. kiang* based on their Pianka's indices. Captive ungulates ranged from 3 to 8 years old, were healthy, and had not received any medications within 3 months of sampling. The *B. grunniens*, *P. nayaur*, and *E. kiang* from the two zoos were fed approximately the same diets, consisting of cotton grass, alfalfa, carrots, and pellet feed. Information of all samples is detailed in **Supplementary Tables S1**, **S2**. All wild and captive samples were placed in sterile tubes and stored at −20°C during transit to Qufu Normal University, China, where all samples were frozen at −80°C until sequencing. This methodology followed the ethical standards of the Qufu Normal University Animal Care and Use Committee (Permit Number: 2022-020).

DNA Extraction and High-Throughput Sequencing

Bacterial DNA was extracted from the samples with HiPure Stool DNA Mini Kits (Magen, Guangzhou, China) following

the manufacturer's protocols. We used a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, United States of America) to measure the DNA to ensure concentrations were higher than 20 ng/μl. The V3–V4 hypervariable regions of bacterial 16S rRNA genes were amplified using PCR primers (forward primer: CTACGGGNGGCWGCAG; reverse primer: GACTACHVGGGTATCTAATCC; Wu et al., 2016). We performed PCR amplification in a 50 μl reaction mixture containing 1.5 μl of 2.5 mM dNTPs, 10 μl of 5 × Q5 Reaction Buffer, 10 μl of 5 × Q5 High GC Enhancer, 0.2 μl of Q5 High-Fidelity DNA Polymerase, 1.5 μl of the forward and reverse primers, and 25.3 μl of DNA. The PCR conditions were as follows: pre-denaturation at 95°C for 5 min, with 30 cycles of 1 min at 95°C for denaturation, 30 cycles of 1 min at 60°C for annealing, 30 cycles of 1 min at 72°C for elongation, and a final elongation at 72°C for 5 min. A TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, United States of America) was used to generate the DNA libraries, and the DNA library quality was quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, United States of America) and FEMTO Pulse system (Agilent Technologies, Santa Clara, CA, United States of America). The DNA library was sequenced on an Illumina HiSeq platform (PE 250, United States of America) with a 250 bp.

Sequence Processing and Statistical Analyses

The pair-ended reads were combined with raw tags using FLASH (v. 1.2.7; Magoč and Salzberg, 2011), and chimeric sequences were eliminated from them using the UCHIME algorithm (Edgar et al., 2011) to obtain effective tags. Based on a 16S rRNA sequence similarity at or greater than 97%, we performed UPARSE (v. 9.2.64; Edgar, 2013) to cluster the effective tags into operational taxonomic units (OTUs), and used the Mothur (Schloss et al., 2009) and SILVA databases (v 138.1; Quast et al., 2012) to annotate species with a threshold of 0.8–1. Alpha diversity [that is, the observed number of OTUs (Sobs), Chao1, Simpson, Shannon, and Good's coverages] and beta diversity (that is, the Bray-Curtis distance) were calculated using the QIIME package (v. 1.9.1; Caporaso et al., 2010). Rarefaction curve and species accumulation boxplot analyses were performed with the Tutools platform (http://www.cloudtutu.com), a free online data analysis website. Principal coordinate analysis (PCoA), non-metric multidimensional scaling (NMDS), and the unweighted pair group method with arithmetic mean (UPGMA) were plotted using the ggplot2 package in R software (v 2.15.3). We used the Vega package in R software (v. 2.15.3) to perform Wilcoxon's test ($p < 0.05$) and analysis of similarities, and used LDA Effect Size software (LDA score > 4, $p < 0.05$; White et al., 2009) to detect bacteria with significant differences between the groups.

RESULTS

Overview of the Sequencing Data

We obtained 4,374,777 effective tags from 41 samples with an average of 106,702 effective tags per sample. With a 97% similarity criterion threshold, sample HA-9 showed the highest number of OTUs among all samples (2,560 OTUs), whereas sample HT-1 showed the lowest number of OTUs (1,161 OTUs; **Supplementary Table S3**, Sobs). Other alpha diversity indices are listed in **Supplementary Table S3** (including Chao1, Simpson, Shannon, and Good's coverages). The species accumulation boxplot and 41 rarefaction curves approached a plateau, suggesting that the number of samples and sequencing depth were sufficient for experimental analyses (**Figure 1**). Furthermore, the Good's coverage index of each sample was higher than 95%, which also showed that the bacterial communities in the samples effectively represented those in the ungulate gut.

Cluster Analyses

Since food composition differed between wild and captive ungulates, we added the published data of blue sheep (Group B) and European Mouflon (Group M); (samples were collected from the Xining wildlife zoo) into the cluster analyses to eliminate dietary effects. The ungulates of the Xining wildlife zoo were fed approximately the same diets as those of the Jinan wild zoo and Jinan zoo [Sun et al., 2019; **Supplementary Table S4**, with previously published sample information (Sun et al., 2019)]. Based on the relative abundance at the phylum level and the Bray–Curtis distance, we selected the top 10 phyla to generate a UPGMA tree (**Figure 2**). Artiodactyla species (groups HT, HB, HY, M, B, LB, and LY) were grouped together, and those from Perissodactyla (groups HA and LA) were clustered into another clade. The Artiodactyla and Perissodactyla clades diverged at high and low altitudes. PCoA (**Figure 3**) and NMDS analysis (stress = 0.051, **Figure 4**) also showed that the gut microbiome compositions of Artiodactyla differed from those of Perissodactyla, and the gut microbiome compositions of high-altitude Artiodactyla (groups HT, HB, HY, M, and B) and Perissodactyla (group HA) differed greatly from those of low-altitude Artiodactyla (groups LB and LY) and Perissodactyla (group LA), respectively. These results showed that high altitudes could not surpass the order level to drive the convergent evolution of ungulate gut microbiome composition. Next, we eliminated dietary composition effects between wild and captive ungulates. Thus, we observed that the similarity of the gut microbiome composition corresponding to the HT, HB, HY, M, and B groups was higher than that corresponding to the LB, LY, and LA groups. This implied that the main influencing factors were altitude and phylogeny, and not diet. Therefore, based on these two influencing factors, we classified high-altitude Artiodactyla (groups HT, HB, and HY), high-altitude Perissodactyla (group HA), low-altitude Artiodactyla (groups LB and LY), and low-altitude Perissodactyla (group LA) as groups the FH, OH, FL, and OL groups, respectively.

The analysis of similarities (ANOSIM) demonstrated that the gut microbiome compositions of the OH and OL groups ($R = 0.9695$, $p = 0.001$) and the FH and FL groups ($R = 0.7028$, $p = 0.001$) showed significantly different unweighted uniFrac distances. This observation indicated that the division of these groups was reasonable.

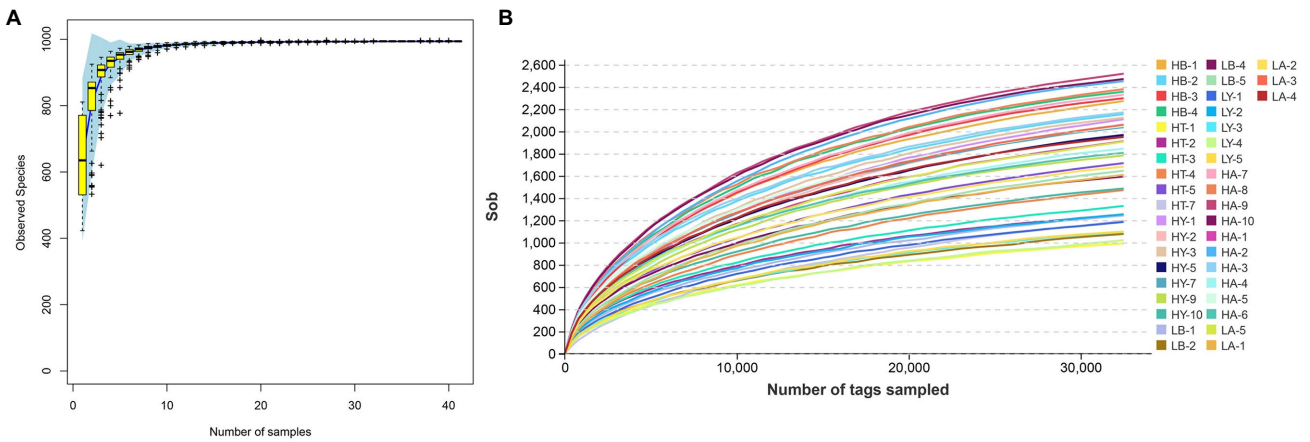

FIGURE 1 | Species accumulation boxplot **(A)** and rarefaction curves **(B)**. In the species accumulation boxplot, the abscissa is the sample size, and the ordinate is the observed species. In the rarefaction curves, the abscissa is the number of sequencing samples randomly chosen from the sample, and the ordinate is the number of OTUs (Sobs).

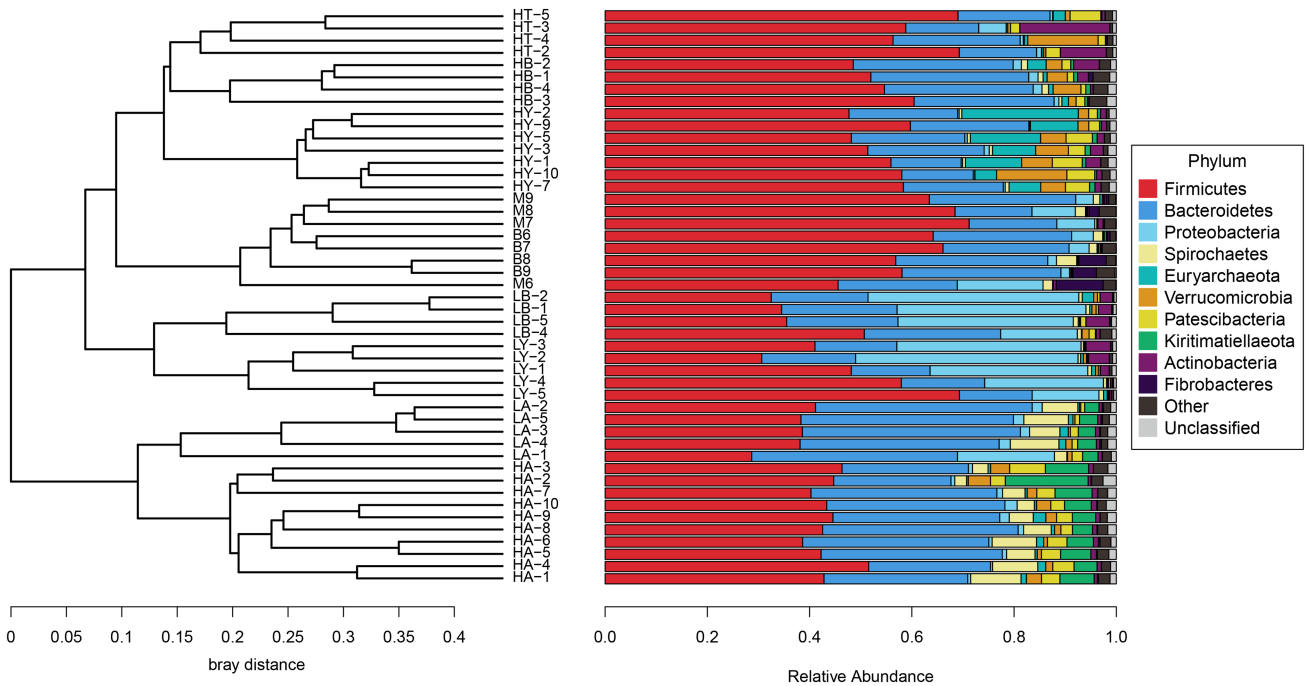

FIGURE 2 | Cluster analysis with the Bray–Curtis distance. The unweighted pair group method with arithmetic mean (UPGMA) tree is generate based on the top 10 phyla.

Gut Microbiome Composition

Overall, 25 phyla, 49 classes, 99 orders, 170 families, 366 genera, and 136 species were detected in the bacterial microbiome communities of the Perissodactyla and Artiodactyla samples.

At the phylum level (top five abundance phyla), Firmicutes (F) and Bacteroidetes (B) dominated in groups such as FH (F, 53.77%; B, 20.57%), FL (F, 45.48%; B, 18.92%), OH (F, 43.71%; B, 31.35%), and OL (F, 38.98%; B, 41.5%). Other major phyla were Actinobacteria (7.99%), Euryarchaeota (5.27%), and Verrucomicrobia (4.13%) in the FH group; Proteobacteria (30.46%), Actinobacteria (2.41%), and Spirochaetes (0.72%) in the FL group; Kiritimatiellaeota (6.79%), Spirochaetes (5.62%), and Patescibacteria (3.68%) in the OH group; and Spirochaetes (6.71%), Proteobacteria (5.39%), and Kiritimatiellaeota (3.28%) in the OL group (**Figure 5A**).

At the family level, the top 10 families accounted for more than 59.26% of the four groups (FH: 69.19%; FL: 82.74%; OH: 59.26%; and OL: 66.72%). Among these 10 families, Ruminococcaceae was the most abundant in groups FH (29.98%) and OH (19.38%).

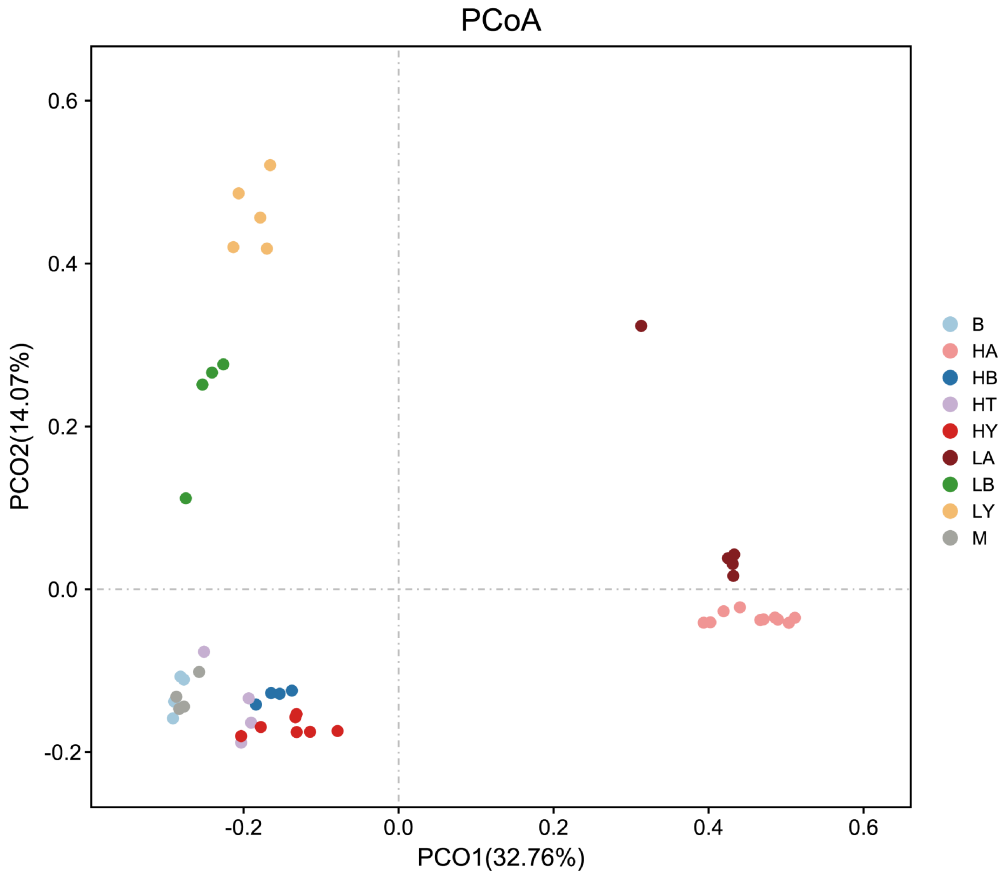

FIGURE 3 | Principal coordinates analysis (PCoA) of gut microbiome composition. Each symbol represents the gut microbiome of a group. HA, high-altitude Tibetan wild ass; HB, high-altitude blue sheep; HT, high-altitude Tibetan antelope; HY, high-altitude yak; LA, low-altitude Tibetan wild ass; LB, low-altitude blue sheep; LY, low-altitude yak. B, blue sheep from Xining wildlife zoo, M, European Mouflon from Xining wildlife zoo.

Moraxellaceae (27.49%) was the most abundant in the FL group, and F082 (16.81%) was the most abundant in the OL group (**Figure 5B**). Additionally, at the genus level, *Ruminococcaceae_UCG-005* (9.44%) was the most abundant genus in the FH group, *Acinetobacter* (27.39%) showed predominance in the FL group, while *Rikenellaceae_RC9_gut_group* showed predominance in the OH (7.63%) and OL (7.70%) groups (**Figure 5C**).

Analysis of Discrepancies for Between Groups

Wilcoxon's test of Sobs (FH group vs. FL group, $p = 0.003$; OH group vs. OL group, $p = 0.03$) and Shannon (FH group vs. FL group, $p = 0.001$; OH group vs. OL group, $p = 0.008$) indices are shown in the boxplot (**Figure 6**). The gut microbiome diversity and richness of groups FH and OH were significantly higher than those of the low-altitude groups (FL and OL).

We used LDA Effect Size analysis (LDA score > 4, $p < 0.05$) to identify the indicator microbiota (biomarker) between groups (groups FH and FL and groups OH and OL). At the phylum level, Firmicutes and Patescibacteria were significantly enriched in the FH and FL groups. Verrucomicrobia and Euryarchaeota were significantly enriched in the FH group. Meanwhile, Kiritimatiellaeota were more abundant in the OH group than in the OL group, Proteobacteria were significantly enriched in groups FL and OL, and Bacteroidetes were more abundant in the OL group than in the OH group (**Figure 7**).

At the family level, Ruminococcaceae, Christensenellaceae, and Saccharimonadaceae were significantly enriched in groups FH and OH; Akkermansiaceae, Lachnospiraceae, FamilyXIII, and Methanobacteriaceae were significantly enriched in the FH group. Moraxellaceae was significantly enriched in the FL and OL groups; Planococcaceae and Enterobacteriaceae were more abundant in the FL group than in the FH group; and F082 was more abundant in the OL group than in the OH group (**Figure 7**). At the genus level, *Christensenellaceae_R_7_group* and *Candidatus_Saccharimonas* were significantly enriched in the FH and OH groups. However, *Ruminococcaceae_UCG-013*, *Ruminococcaceae_UCG-005*, *Methanobrevibacter*, and *Akkermansia* were more abundant in the FH group than in the FL group. Our results also indicated that *Escherichia_Shiella*, *Acinetobacter*, *Solibacillus*, and *Planococcus* were more abundant in the FL group than in the FH group, and *Acinetobacter* was more abundant in the OL group than in the OH group (**Figure 7**).

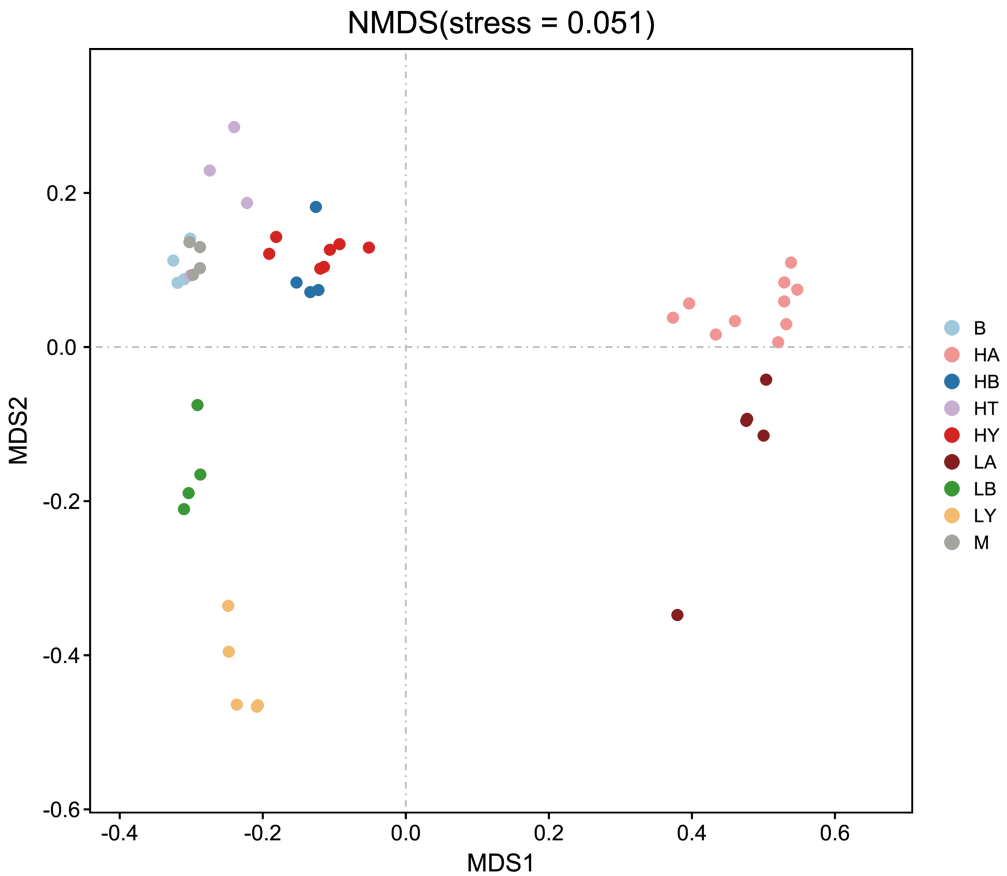

FIGURE 4 | Non-metric multidimensional scaling (NMDS) of gut microbiome composition. Each symbol represents the gut microbiome of a group. HA, high-altitude Tibetan wild ass; HB, high-altitude blue sheep; HT, high-altitude Tibetan antelope; HY, high-altitude yak; LA, low-altitude Tibetan wild ass; LB, low-altitude blue sheep; LY low-altitude yak. B, blue sheep from Xining wildlife zoo, M, European Mouflon from Xining wildlife zoo.

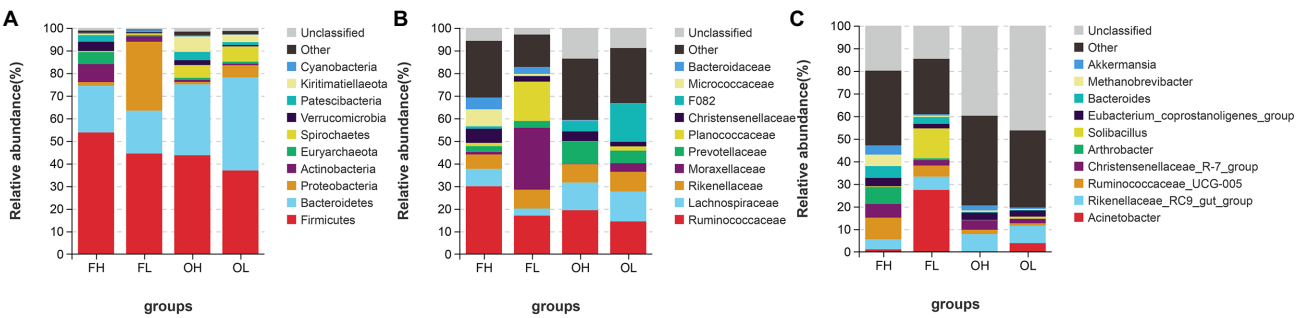

FIGURE 5 | Gut microbiome composition of ungulates at the phylum **(A)**, family **(B)**, and genus **(C)** levels. FH, high-altitude Artiodactyla (including groups HB, HT, and HY); FL, low-altitude Artiodactyla (including groups LB and LY); OH, high-altitude Perissodactyla (group HA); OL, low-altitude Perissodactyla (group LA).

Wilcoxon's test was used to analyze the Firmicutes/Bacteroidetes (F/B) values between the groups. **Supplementary Figure S1** shows that the F/B values of the high-altitude groups (FH and OH) were higher than those of the low-altitude groups (FL and OL; group FH vs. group FL, $p > 0.05$) and that of the OH group was significantly higher than that of the OL group ($p < 0.01$).

DISCUSSION

The gut microbiome is important for the ability of the host to adapt to various factors, including temperature, niche, and diet (Bo et al., 2019; Greene et al., 2020), which can provide energy and nutrients and maintain gut homeostasis in the host (Cani and Delzenne, 2007; Chung and Kasper, 2010;

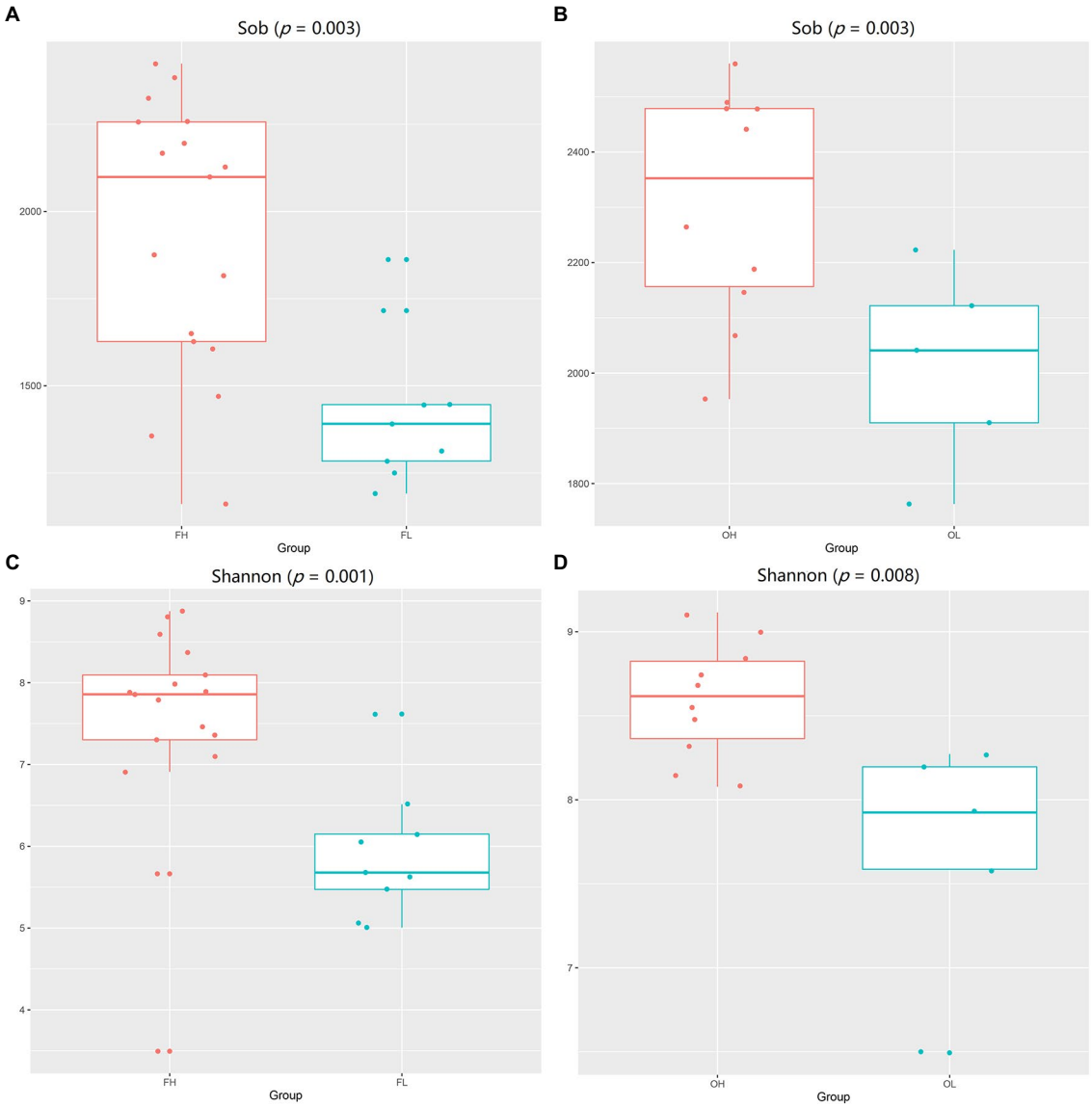

FIGURE 6 | Wilcoxon's test of alpha-diversity (Sobs and Shannon index) of gut microbiome between groups. **(A)** Sob index of group FH vs. group FL; **(B)** Sob index of group OH vs. group OL; **(C)** Shannon index of group FH vs. group FL; **(D)** Shannon index of group OH vs. group OL. FH, high-altitude Artiodactyla (including groups HB, HT, and HY); FL, low-altitude Artiodactyla (including groups LB and LY); OH, high-altitude Perissodactyla (group HA); OL, low-altitude Perissodactyla (group LA).

Wu et al., 2017). Many scholars have studied the adaptation mechanism of animals to high altitudes through the gut microbiome (Mazel, 2019; Xu et al., 2020), especially ungulates (Zhang et al., 2016, 2020; Sun et al., 2019). However, phylogeny has had little effect on their gut microbiome compositions in previous studies, because the phylogeny of ungulates was similar at the family, subfamily, and genus levels. Therefore, under broader phylogeny, this research explored the factors driving the convergent evolution of the gut microbiome of ungulates at different altitudes.

Using 16S rRNA high-throughput sequencing, we described the gut microbiome compositions of ungulates at different altitudes. At the phylum level, Firmicutes and Bacteroidetes were the dominant phyla in all samples (**Figure 5**). These results were consistent with other gut microbiomes of ungulates, such as the *E. kiang* (Gao et al., 2020), *B. grunniens* (Guo et al., 2021), *P. hodgsonii* (Bai et al., 2018), and *P. nayaur* (Sun et al., 2019).

According to the UPGMA tree, we found that groups HA, HB, HT, and HY were not clustered in the clade, while Artiodactyla (groups HB, HT, HY, LY, M, B, and LB) were clustered together. Ungulates with close phylogenetic relationships had similar gut microbiome compositions. These results revealed that high altitudes could not surpass the order level to drive the convergent evolution of the relative abundance composition of the ungulate.

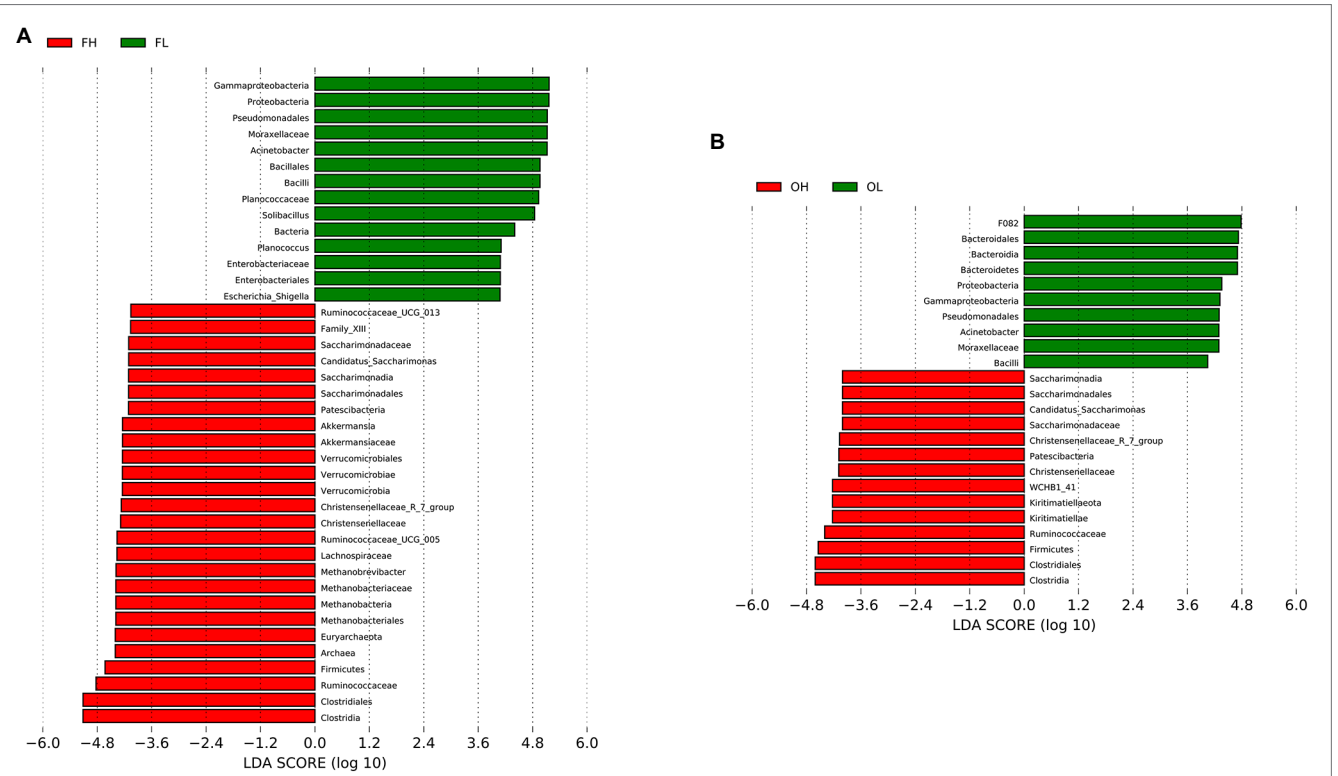

FIGURE 7 | LEfSe (LDA Effect Size) analysis in ungulates between different altitudes. **(A)** Group FH (high-altitude Artiodactyla, including groups HB, HT, and HY) vs. group FL (low-altitude Artiodactyla, including groups LB and LY); **(B)** Group OH (high-altitude Perissodactyla, group HA) vs. group OL (low-altitude Perissodactyla, group LA).

Phylogeny is one of the main factors affecting the gut microbiome composition of species (Benson et al., 2010; Zhu et al., 2021); for example, a study of 59 neotropical bird species found that the phylogeny factor was most frequently significant and explained most of the variation (Hird et al., 2015). In different niches, non-primates with close phylogenetic relationships possess similar gut microbiome compositions (Amato et al., 2019). Furthermore, the gut microbiome composition of Artiodactyla and Perissodactyla differed at high or low altitudes, and groups HB, HT, HY, M, and B clustered together through PCoA and NMDS plots. This showed that high altitudes drove the convergent evolution of ungulate gut microbiome composition at the order level. Notwithstanding, altitude is the main factor that affects the relative microbiome composition of ungulates. This is because, even though the diets of corresponding to the Xining wildlife zoo groups (the M and B groups) and the FL group were similar in composition, they did not group together.

High-altitude groups (OH and FH) were significantly higher than the low-altitude groups (FL and OL) in the Wilcoxon's test of Sobs and Shannon. Higher gut bacterial diversity and richness generally correlate with a healthy and stable host gut microbiome (Petersen and Round, 2014; Waite and Taylor, 2014). High altitudes drove the gut microbiome of Artiodactyla and Perissodactyla to produce the same pattern of alpha diversity.

In this study, the OH and FH groups harbored large proportions of Firmicutes, which can decompose fibers and cellulose to provide volatile fatty acids to the host (Allison et al., 1962; Dai et al., 2015). Additionally, the F/B values of the high-altitude groups showed an increasing trend compared with that of the low-altitude groups. These high F/B values indicate that the host has a large body size and fat (Magne et al., 2020; Wolf et al., 2021). Many researchers have found that the F/B value of high-altitude ungulate gastrointestinal tract microbiomes was significantly higher than that of low-altitude ungulates (Ma et al., 2019; Sun et al., 2019). A high F/B ratio is beneficial for high-altitude ungulate energy harvesting (Zhang et al., 2016; Lan et al., 2017). The relative abundances of Patescibacteria in the OH and FH groups were higher than those in the OL and FL groups. Nitrate reductase (EC 1.7.99.4) positively predicts nitrite reductase (NO-forming, EC 1.7.2.1) and nitric-oxide reductase (cytochrome c, EC 1.7.2.5) in Patescibacteria, which has a synergistic effect on denitrification (Yang et al., 2022). Denitrification bacteria can reduce methane gas production in cow rumen liquids (Megga et al., 2017), as methane is a rumen fermentation by-product from methanogenic archaea, which leads to energy loss (Morgavi et al., 2010). Patescibacteria might reduce the energy loss of ungulates in high-altitude environments.

The relative abundance of Verrucomicrobia and Euryarchaeota was significantly higher in the FH group than in the OH group. Verrucomicrobia exists in the gut mucosa of healthy individuals and has anti-inflammatory effects (Fujio-Vejar et al., 2017) while maintaining glucose homeostasis in the host

microbiome (Lindenberg et al., 2019). Euryarchaeota can promote carbohydrate fermentation to produce short-chain fatty acids, which play an important role in the gut ecosystem (Chaudhary et al., 2018). Kiritimatiellaeota is the major microbiota in horse (Edwards et al., 2020) and wild gelada (Baniel et al., 2021) hindguts and exists in the rumen of sheep (Wang et al., 2017) and cattle (Ribeiro et al., 2017). We speculate that Kiritimatiellaeota plays an important role in the digestion of herbivorous species, which requires further functional verification.

Ruminococcaceae, Christensenellaceae, and Saccharimonadaceae were characterized by a significant increase in the OH and FH groups. Ruminococcaceae and Christensenellaceae were the top 10 families in groups OH and FH. Ruminococcaceae is related to the decomposition of cellulose and starch (Andersen-Ranberg et al., 2018) and can produce acetic and formic acids (Ze et al., 2012). Christensenellaceae is an efficient sugar-fermenting family that can decompose glucose into acetic and butyric acids and can decompose cellulose (Morotomi et al., 2012). Meanwhile, Saccharimonadaceae is associated with immune response (Zhao et al., 2020). FamilyXIII is associated with the maintenance of gut health (Zhao et al., 2020), and Lachnospiraceae contained several cellulolytic/fibrolytic genera, and its abundance increased in the FH group, which is conducive to obtaining energy and maintaining homeostasis for the host in extreme environments (Biddle et al., 2013).

The OH and FH groups shared two significantly enriched genera, *Christensenellaceae_R_7_group* and *Candidatus_Saccharimonas*. The function of *Christensenellaceae_R_7_group* is the same as that of Christensenellaceae (Morotomi et al., 2012). Additionally, *Candidatus_Saccharimonas* species are positively correlated with propionic acid and butyric acid levels (Xia et al., 2022) and play an important role in maintaining gut functions (Xie et al., 2021). The relative abundance of the genus *Akkermansia* was greater in the FH group than in the other groups. Previous studies have shown that *Akkermansia* can consolidate the intestinal barrier and regulate immune functions (Zhao et al., 2022). Thus, the significantly enriched genera can help the high-altitude ungulates cope in extreme environments.

Based on the above results, the significantly enriched microbiota in high-altitude ungulates play an important role in disintegrating dietary fiber and cellulose, gaining energy, and maintaining gut homeostasis. High-altitude factors cannot surpass phylogeny to drive the convergent evolution of gut microbiome composition in ungulates, and high-altitude factors drive convergent evolution of alpha diversity and indicator microbiota in the gut microbiome of Artiodactyla and Perissodactyla at high altitudes. However, our understanding of the enriched microbiota and species level is still limited considering the limitations of 16S rRNA sequencing technology. Thus, in future studies, it would be necessary to employ metagenomics and metabolomics comprehensively to clarify the composition and function of enriched microbiota, especially at the species level, in ungulates at different altitudes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA837737.

ETHICS STATEMENT

The animal study was reviewed and approved by Qufu Normal University Animal Care and Use Committee (Permit Number: 2022-020).

AUTHOR CONTRIBUTIONS

XWa: conceptualization, methodology, data curation, software, and writing-original draft preparation. XWu: methodology and writing—review and editing. YS: writing—review and editing. YG and YL: sample provision. QW: conceptualization and resources. XM: visualization. YD, SZ, and ZZ: conceptualization. GS: data curation. LL and BL: investigation. HZ: conceptualization and writing—review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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An amateur gut microbial configuration formed in giant panda for striving to digest cellulose in bamboo: Systematic evidence from intestinal digestive enzymes, functional genes and microbial structures

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The giant panda has been considered to maximize nutritional intake including protein and soluble carbohydrates in bamboo, but it has spent almost entire life with the high-cellulose diet. Whether giant panda is still helpless about digesting bamboo cellulose or not is always contentious among many researchers around the world. The work has systematically clarified this issue from the perspectives of digestive enzymes, functional genes, and microbial structures in giant panda gut. The intestinal cellulase activities of panda increase with bamboo consumption, performing that the endoglucanase activity of adults reaches 10-fold that of pandas first consuming bamboo. More abundance and types of microbial endoglucanase genes occur in bamboo-diet giant panda gut, and the corresponding GH5 gene cluster is still efficiently transcribed. Gut microbes possessing cellulose-degrading genes, belong to the phylum Firmicutes and some Bacteroidetes, but their structural and functional configurations are insufficient to completely degrade cellulose. Therefore, giant panda is striving to digest cellulose in bamboo, but this adaptation is incomplete. This is probably related to the short straight carnivore-like gut structure of the giant panda, preventing the colonization of some efficient functional but anaerobic-preferred flora.

KEYWORDS

giant panda, gut microbiome, cellulose digestion, cellulases activity, dietary adaptation

Introduction

Since the 1960s, protection of the giant panda has been undertaken and gradually expanded in China, especially after the construction of the Giant Panda National Park in 2017. There has been marked improvement in the breeding capacity, recovery of severely fragmented habitats, and a reduction in the high inbreeding risk of the population (Kang and Li, 2019; Song et al., 2021). However, the vulnerability of the giant panda remains a global concern, because they have an exclusively bamboo diet despite a carnivore-like intestine and a very poor ability to digest bamboo.

Unlike herbivorous mammals, which have rumens to ferment cellulose, the giant panda relies solely on the microbial flora in its hindgut (Li et al., 2010; Wei et al., 2019). When researchers have studied the gut microbiome of the giant panda after dietary shift, they have detected no colonization by large cellulose-degrading microbial populations in this specialized species (Zhan et al., 2020). However, in a study that analyzed the fecal microbial flora of bamboo-consuming animals, including the adult giant panda and two other species, McKenney et al. (2018) have shown that all three species shared some specialized flora during their long-term adaptation. An association analysis has identified a group of bacteria in the giant panda related to the genus *Clostridium* as candidates for the degradation of cellulose (Zhu et al., 2018; Guo et al., 2020), but their production of the appropriate digestive enzymes has not yet been demonstrated. The data are still insufficient to explain that this microbial flora assists in the giant panda's digestion of cellulose, but reflect that the gut flora may possess an adaptive process to the bamboo diet of giant panda.

Zhan et al. (2020) have investigated the ability of the gut microbial flora to digest cellulose by analyzing the microbial genome of the giant panda during its dietary shift during weaning, and found a succession of glycoside hydrolase genes appropriate for cellulose degradation. However, Zhang et al. (2018) have conducted a similar investigation according to different sequencing and analysis methods, but proposed that the giant panda might live on the hemicellulose in bamboo, rather than on the cellulose. Metagenomic analyses of the adaptive changes that allow giant panda to digest bamboo have produced different results in various studies. To clarify the actual ability of the gut flora of the panda to digest cellulose, there is still a need for efficient sequencing technologies with larger data volume and higher sequence quality, combined with relevant research into the transcription of microbial cellulose-degrading genes in the giant panda.

Some studies have also reported the apparent digestibility of cellulose in the adult giant panda (Wang et al., 2006; Lin et al., 2020) or compared the activities of cellulases secreted by the gut flora with those of other herbivores (Guo et al., 2018). However, to fully understand whether the adaptation to a high-cellulose diet occurs in the giant panda, how the panda's ability to digest cellulose changes during its growth, and what determines its ability (or incomplete ability) to digest bamboo, a systematic study that combines analyses of enzymes, gut microbes, and the abundances and expression of functional genes in its different developmental stages is necessary.

The dietary shift is a notable stage in the giant panda's development, occurring between 8 months and 2.5 years of age, and the death rate of the giant panda is very high in this period, mainly with symptoms of gastrointestinal diseases (Zhou et al., 2021). Several studies have identified some defects including reduced microbial diversity and the lack of a dominant cellulose-degrading flora or genes encoding cellulases, in the giant panda during the dietary shift compared with those in the rumen of cattle (Suen et al., 2010; Guo et al., 2020; Jin et al., 2020; Zhan et al., 2020). The systematic analysis from the enzymes, gut microbes and functional genes can comprehensively determine whether it is the most vulnerable stage, during the pandas' developmental process due to their inability to digest cellulose.

In this study, several pandas at different stages of their development (including cubs and during the dietary shift process, sub-adult and adult, respectively) have been selected for sample collection to: (1) analyze the changes in cellulase activities during the dietary shift and growth periods, reflecting adaption to a high-cellulose diet; (2) profile microbial cellulose-degrading genes by advanced multi-omics technologies, exploring the molecular adaptive response of cellulose digestion; and (3) identify the cellulose-degrading contributors, paralleling the panda's adaptive change to digesting cellulose. The results may systematically and hierarchically explain the current adaptive development of giant panda for the digestion of bamboo (also including wild giant panda, because they also experience dietary transition from milk to bamboo).

Materials and methods

Sample collection

Fecal samples were collected from individual captive giant pandas at different developmental stages (n = 22) at the China Conservation and Research Center (Dujiangyan, China), Chongqing Zoo (Chongqin, China), and Shanghai Zoo (Shanghai, China). Detailed information on these individuals is given in **Supplementary Table 1**. All samples were immediately stored at 0–4°C before enzyme assays and at <−20°C before DNA extraction, and were frozen in liquid nitrogen before the extraction of total RNA. The age and dietary composition of the giant pandas at different developmental stages are

TABLE 1 Feeding and dietary information of giant panda at different developmental stages.

Developmental stage	Age	Dietary composition
Cub	**0∼1 year**	**Milk-fed** (mainly ate milk, licking a little coarse pastry and bamboo shoots)
The dietary shift stage	**1∼2 years**	**Dietary shift** (milk consumption decreased and bamboo consumption increased, with bamboo gradually excreted in the feces)
Sub-adult	**>2∼6 years**	**Bamboo-fed** (mainly ate bamboo, supplemented with little bamboo shoots, coarse pastry, apple and carrot)
Adult	**>6 years**	

shown in **Table 1**. The health status of the pandas was monitored during the sampling period and no abnormalities were detected.

Enzyme activity assays

A 5 g sample of giant panda stool was stored at 4°C. It was then placed in 50 mL of citrate buffer solution (pH 4.8), mixed thoroughly, and centrifuged at 4000 rpm (10 min) to obtain the crude cellulase solution. Carboxymethyl cellulose solution (1%, 10 mL) was added to 5 mL of enzyme solution. The mixture was stored at 40°C for 30 min before the endoglucanase activity was measured at 540 nm with the 3,5-dinitrosalicylic acid (DNS) colorimetry method (Ghose, 1987). Microcrystalline cellulose (100 mg) was mixed with 10 mL of satellite salt buffer solution (pH 4.8), and then reacted with 5 mL of the above enzyme solution for 1 day at 40°C. The exoglucanase activity was measured at 540 nm with the DNS method (Han and Chen, 2010; Alawlaqi and Alharbi, 2020). The activity of crude protease was measured with the Folin method at pH 7.5 (SB/T 10317-1999) (SB/T 10317-1999, 1999), and the amylase activity was analyzed by measuring the production of reducing sugar from soluble starch with DNS (Stroparo et al., 2012). We set up parallel experiments (n = 3), using a no-substrate control (in which distilled water replaced the substrate solution).

DNA extraction and sequencing

The frozen stool samples from the giant pandas were thawed on ice packs. A contamination-free core part of each sample (10 g/day) was taken from five stool samples collected on five consecutive days with a clean, sterile pharmacy spoon. The samples from each individual were mixed for later analysis. The DNA was extracted from the fecal samples with the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, United States). The extracted DNA was stored at <−20°C before analysis. The details of the DNA extraction, and 16S rRNA and internal transcribed spacer 1 (ITS-1) sequence PCR amplification are available in the **Supplementary Material**. The raw reads were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database.

16S rRNA, internal transcribed spacer 1, and metagenomic sequence analyses

A pyrosequencing analysis was performed on the Illumina MiSeq PE300 platform by Shanghai Majorbio Technology Co., Ltd. (Shanghai, China). The raw fastq files for the 16S rRNA and ITS rRNA were demultiplexed and quality-filtered with fastp version 0.19.6 (Chen et al., 2018) and merged with FLASH (version 1.2.11) (Magoč and Salzberg, 2011). The operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE (version 7.1) (Edgar et al., 2011). Chimeric sequences were identified and removed with UCHIME. The taxonomy of representative sequences of each OTU was analyzed with RDP Classifier version 2.11 (Wang et al., 2007) against the 16S rRNA (Silva version 132) and ITS (UNITE version 8.0) databases with a confidence threshold of 0.7.

The concentration and purity of the extracted fecal DNA (samples from before, during, and after the dietary shift; n = 6 total) were determined with a TBS-380 Mini-Fluorometer (Turner BioSystems, San Francisco, CA, United States) and a NanoDrop 2000 spectrophotometer (NanoDrop, Wilmington, DE, United States), respectively. A paired-end library was constructed with the NEXTFLEX^{TM} Rapid DNA-Seq Kit (Bioo Scientific, Austin, TX, United States). Paired-end sequencing was performed on the Illumina NovaSeq platform (Illumina Inc., San Diego, CA, United States) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) using the NovaSeq Reagent Kit. The paired-end Illumina reads were qualified with Transcriptomic Sequence Analysis Fastp (version 0.20.0) (Chen et al., 2018). The metagenomic data were assembled with MEGAHIT (version 1.1.2) (Li et al., 2015). The open reading frames in each assembled contig were predicted with MetaGene (Noguchi et al., 2006). The predicted ORFs (≥100 bp) were retrieved and translated (by NCBI). A non-redundant gene catalog was constructed with CD-HIT (version 4.6.1) with 90% sequence identity and 90% coverage (Fu et al., 2012). After quality control, the remaining reads were mapped to the gene sets with SOAPaligner (version 2.21) with 95% identity (Li et al., 2008), and the gene abundance in each sample was evaluated. Representative sequences from the NR gene

catalog were aligned to those in different functional databases for taxonomic annotation (following best-hit approach) and KEGG annotation by DIAMOND (version 0.8.35) at an optimized e-value cutoff of 1e–5, and carbohydrate-active enzyme annotation *via* HMMER (version 3.1. b2) with an e-value cutoff of 1e–5 (Buchfink et al., 2015). The criteria for DNA quality and 16S, ITS-1, and metagenomic sequencing are available in the **Supplementary Material**, together with details of the construction of the paired-end library and the annotation of sequences.

Transcriptome sequencing analysis

The total RNA was extracted from the fresh giant panda fecal samples with the RNA PowerSoil® Total RNA Isolation Kit (Mo Bio Laboratories Inc.) and immediately stored in liquid nitrogen. The rRNA was removed with the magnetic bead method to isolate the mRNA. A cDNA library was constructed with the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina Inc.). The reads were qualified on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Beijing, China) with the Agilent High Sensitivity DNA Kit, and quantified on the Promega QuantiFluormeter (Promega Corporation, Beijing, China) with the Quant iT^{TM} PicoGreen^{TM} dsDNA Assay Kit. Paired-end sequencing was performed on the Illumina NovaSeq platform (Illumina Inc.) at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China) using the NovaSeq 6000 S4 Reagent Kit (2 bp × 150 bp). The raw reads were deposited in the NCBI Sequence Read Archive (SRA) database. The details of the criteria for RNA quality and the construction of the paired-end library and annotation of sequences are available in the **Supplementary Material**.

Statistical analysis

All data on digestive enzyme activities, the abundances of genes and transcripts, and microbial α-diversity in the panda feces were calculated with OriginPro 8 SR0 (version 8.0724). Significant differences in α-diversity between groups were detected with Welch's *t*-test. The R language vegan package was used to draw the OTU community bar charts and heatmaps. The Bray–Curtis method was used to calculate the distance between two samples and to quantify the differences in the species abundance distributions among samples. The OTU abundance table was standardized with Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt 2), a software package for the functional prediction of amplified 16S rRNA sequences, which removes the effect of the copy number of the 16S rRNA gene in the species genome. Each OTU corresponding to a phylogenetic lineage in this software was then used to annotate the Clusters of Orthologous Genes and KEGG functions of the OTUs to produce the OTU annotation information for each functional level and the abundance information for each function. PICRUSt 2 was also used to analyze the fungal data.

Results

Enzyme activities and properties related to bamboo digestion in giant panda at different developmental stages

In considering the adaptation of the giant panda to a diet of bamboo (with a predominant cellulose content) during its development, the activities of endoglucanase and exoglucanase were examined in individual pandas at different developmental stages, using carboxymethyl cellulose and microcrystalline cellulose as substrates, respectively. The enzyme activities were measured as the production of reducing sugar (**Figures 1A,B**). These enzymes were two key cellulases, hydrolyzing the non-crystalline and crystalline regions of the cellulose molecule, respectively (Attigani et al., 2016). Surprisingly, during the development of the giant panda, both the endoglucanase and exoglucanase activities were initially high in cubs, but decreased during the dietary shift process. They slowly recovered, and thus presented *U*-shaped activity profiles, as did the other digestive enzymes in the feces, e.g., amylase and crude proteases. However, the activities of both cellulases did not recover as well as those of the other enzymes (**Figures 1C,D**), and the exoglucanase activity was always 1–2 two orders of magnitude lower than the endoglucanase activity (**Figure 1**).

Changes in all the microbial enzyme activities indicate that the gut flora formed in childhood are under great pressure to digest the high-cellulose diet when the panda first begins to consume bamboo, reflecting the difficulty faced by the giant panda in transition to a bamboo diet. However, giant panda gradually partially digests bamboo cellulose with the assistance of its microbiome.

Cellulase activity was further monitored at different pH and temperature in the giant panda at different developmental stages to explore the changes in the properties and types of microbial cellulases in giant pandas during the adaptation to eating bamboo. The relationship between endoglucanase activity and pH was clearer in the adults on a bamboo diet. Endoglucanase of adults showed the highest activity at pH 5, which was similar to the fecal pH (**Figure 2**). However, the endoglucanases in the milk-fed cubs did not respond in any way to pH (**Figure 2A**). This means that the properties and types of endoglucanase in adults might differ from those in cubs, and

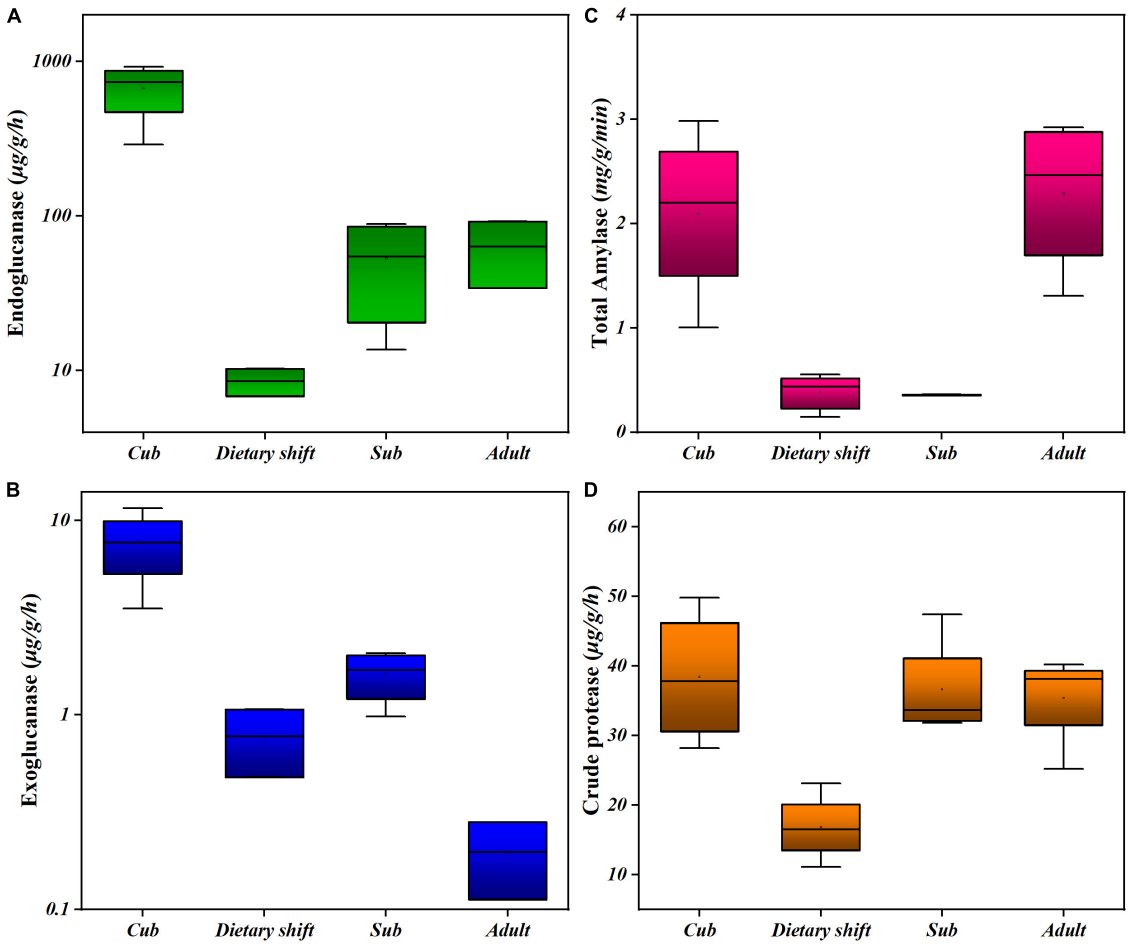

FIGURE 1
The activities of different digestive enzymes in giant panda during its development. **(A)** Endoglucanse activity; **(B)** exoglucanase activity; **(C)** total amylase activity; **(D)** crude protease activity. Each box has reflected the range of digestive activities of individual pandas ($n > 3$) at the specific developmental stage.

the endoglucanase in adults may occur to digest cellulose in bamboo.

Genes encoding lignocellulose-decomposing enzymes in gut microbial genome and transcriptome of giant panda

To explain why the ability of the giant panda to digest bamboo cellulose is still low and why it cannot completely adapt to a bamboo diet, we analyzed the abundances of cellulolytic genes and their transcription in the giant panda at different developmental stages.

The data set of non-redundant genes (total value = 724 019) collected from all metagenomic samples was annotated with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The metabolic level 2 categories showed that genes related to carbohydrate metabolism (total value = 31 130) were most abundant in the gut microbiome of giant panda, while polysaccharide metabolism accounted for only 21% and even less was related to the degradation of cellulose and hemicellulose. Cellulose and hemicellulose are both more readily degraded than lignin in the lignocellulose complex (Wilhelm et al., 2019; Zhang et al., 2019). Amino acid metabolism (total value = 21 368) and nucleotide metabolism (total value = 12 767) followed. Therefore, we focused on the key enzyme genes involved in the hydrolysis of cellulose and hemicellulose, including endoglucanase (EC 3.2.1.4), endoxylanase (EC 3.2.1.8), and β-D-xylosidase (EC 3.2.1.37) the frequency and relative abundance of which were indeed much less than α-amylase (EC 3.2.1.1) (**Figures 3A–D**).

Consistent with the results of the enzyme activity analysis, no genes encoding exoglucanase (EC 3.2.1.91) were predicted and had disappeared from the microbial genome. The abundances of key hemicellulose-degrading genes were always

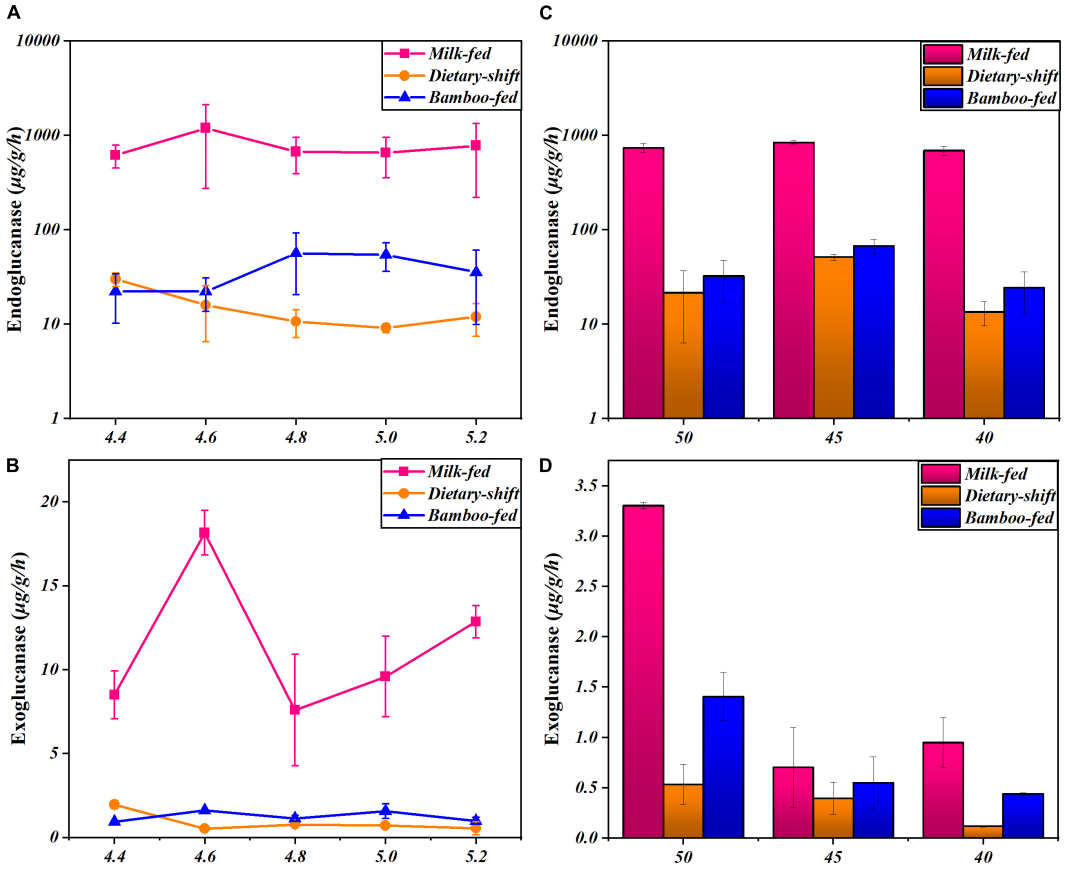

FIGURE 2
The activities of endoglucanase and exoglucanase in giant panda at different developmental stages under different pH and temperature conditions. **(A,B)** Represented respectively the activities of endoglucanase and exoglucanase of the giant pandas on milk-fed diet, dietary-shift process and bamboo-fed diet, under different pH at 4.4∼5.2 (T = 40°C); **(C,D)** represented respectively the activities of endoglucanase and exoglucanase of giant pandas at different dietary stages under different temperature at 40∼50°C (pH 4.8). Each point or column has reflected the range of digestive activities of individuals (n > 3) at the specific developmental stage and culture condition.

1–2 orders of magnitude lower than those of the cellulose-degrading genes during the development of the giant panda. As shown in **Figures 3B,E**, the frequencies and relative abundances of endoglucanase (EC 3.2.1.4)-encoding genes increased during the panda's development and with bamboo consumption (reaching to nearly 0.01%, gene count/total gene count), although the relative abundance of endoglucanase in the adult giant panda was still only one-half that in herbivores (Guo et al., 2018).

Alignment of the above-mentioned NR genes to CAZy database identified 377 different CAZy families and subtypes, including 7134 genes encoding 184 glycoside hydrolases (GHs) and subtypes. Surprisingly, the numbers and types of GHs corresponding to endoglucanase (EC 3.2.1.4) also increased in the giant panda after it adopted a bamboo diet, including the two subtypes of GH5 (GH5_2 and GH5_4) and GH9 (**Figure 3F**). GH5 is always more abundant during the development of the giant panda (Zhan et al., 2020), with a relative abundance of 0.0056 among the GHs (gene count/total gene count of GHs) (**Supplementary Table 2**). The genes encoding GH9 were only found after consumption of a bamboo diet, but were not abundant, with a relative abundance of 0.0019 among the GHs (**Supplementary Table 2**). GH9 has been reported to degrade complex celluloses (Vita et al., 2019).

In an analysis of the transcriptome, GH5, GH13, GH16, and GH43 were the only GHs transcribed in the gut flora of the bamboo-fed giant pandas (**Figure 3G**), and among these, only GH5 is reported to degrade cellulose, with a relative abundance of 0.0094 among the GH transcripts. No GH9 transcripts were detected. However, the transcripts of GH13 and GH43, which encode proteins responsible for oligosaccharide degradation, were more abundant (Dai et al., 2014).

These results suggested that the relative abundance and type of genes encoding endoglucanase (EC 3.2.1.4) were much greater in the bamboo-fed giant panda, which could endow the giant panda with the ability to digest bamboo cellulose.

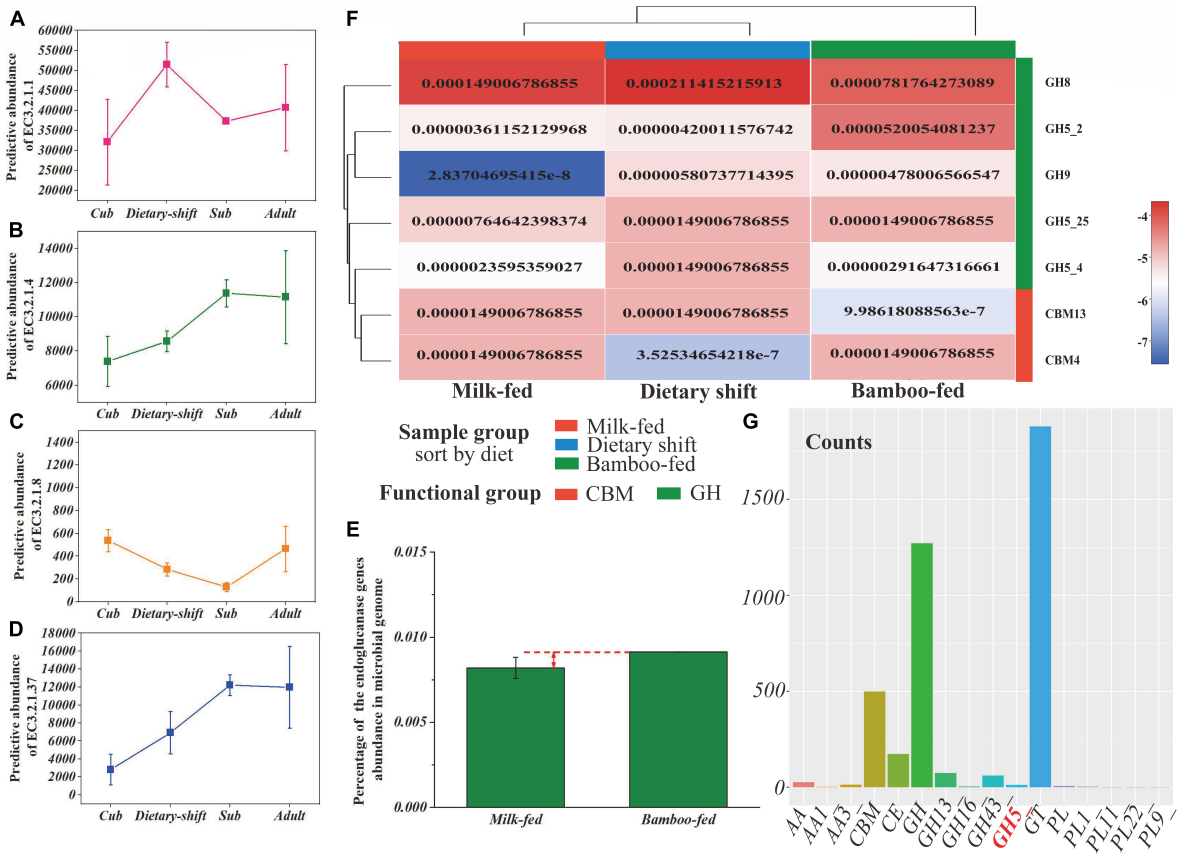

FIGURE 3
The gene frequency and abundance of cellulase for giant panda during the dietary adaptation process and the transcription efficiency of GHs in gut flora. **(A–D)** Represented the gene frequency of α-amylase (EC 3.2.1.1), endoglucanase (EC 3.2.1.4), endoxylanase (EC 3.2.1.8), and β-D-xylosidase (EC 3.2.1.37), respectively, of the giant panda at cub, dietary-shift, sub-adult and adult stages; **(E)** showed the heat map of GHs genes corresponding to cellulose degradation in the giant panda with different dietary compositions, calculated with the relative abundance in genome; **(F)** represented the percentage of endoglucanase (EC 3.2.1.4) gene abundance in the giant panda on milk-fed diet and bamboo-fed diet, and **(G)** represented the counts of gene transcripts of all classes of carbohydrate-active enzymes and their main families of gut microbiome in the bamboo-fed giant panda.

However, the cellulase activity in the giant panda might still be restricted by the lack of available genes for the hydrolysis of the complex celluloses in bamboo.

Gut microbial diversity and composition of giant panda at different developmental stage and the microbial contributors for digesting bamboo

The occurrence and accumulation of cellulase genes in the gut microbial genome of the giant panda, together with GH5 transcripts in the gut microbial transcriptome, indicate that the gut flora of the giant panda contributes to its adaptation to a bamboo diet. The changes in the structure and composition of the gut flora were further studied, especially the cellulolytic contributors to the panda's digestion of cellulose as the cub matures into an adult.

In an α-diversity analysis, both Shannon's and Simpson's index varied significantly ($P < 0.05$) between the cub and the sub-adult, indicating that the microbial diversity became more complex during the dietary shift process. The Shannon's index of the gut microbiome then decreased significantly ($P < 0.05$, Figures 4A,B). A β-diversity analysis indicated that the differences in the gut microbial structure among individuals increased after the dietary shift (Figure 4C). It seems that the gut microbes of the giant panda form a single-diversity and sensitive community under the influence of the individual environment, when the giant panda first mainly consumes bamboo.

An investigation of the gut microbial composition showed that the phyla Firmicutes and Proteobacteria were always predominant, with the relative abundance of >90%. Among them, five genera (*Streptococcus*, *Clostridium*, *Megasphaera*, *Blautia*, and *Weissella*) of Firmicutes and three genera (*Escherichia-Shigella*, *Acinetobacter*, and *Klebsiella*) of

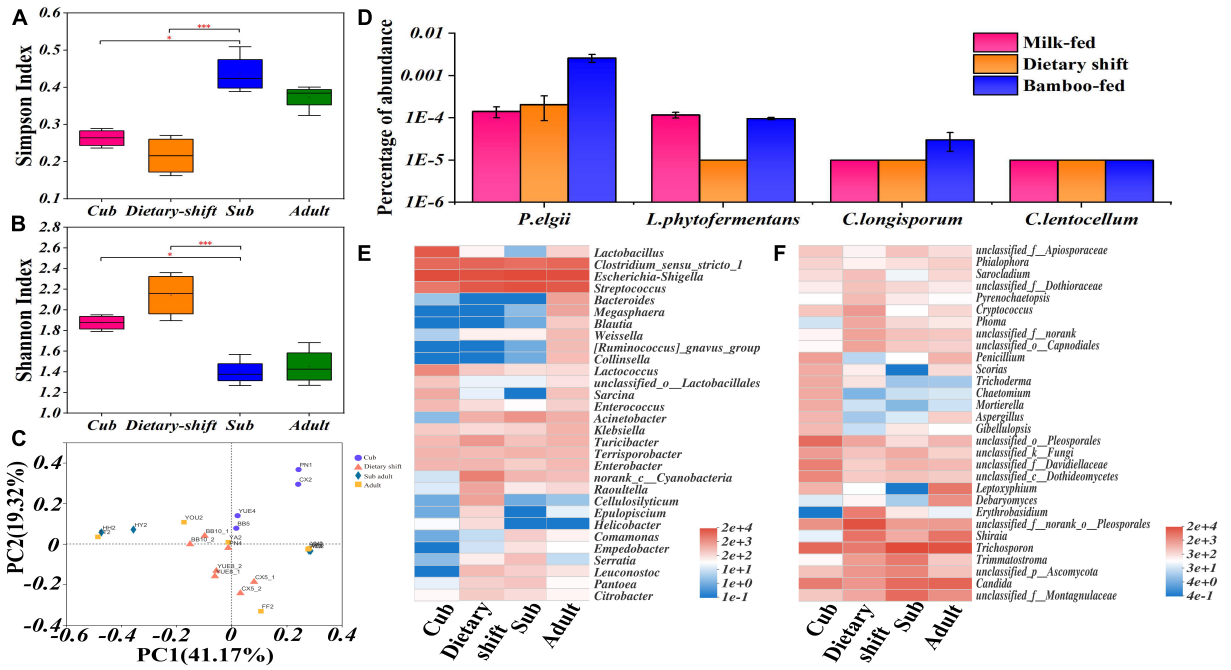

FIGURE 4
The diversity and structure of gut flora and the relative abundance of microbial populations possessing endoglucanase genes in giant panda at different developmental stages along with the adaptation to bamboo diet. **(A,B)** Represented the α analysis of Simpson's and Shannon's Index in growth of the giant panda, respectively, and also have shown the significant difference ($0.01 < P \leq 0.05$, marked with *; $0.001 < P \leq 0.01$, marked with **; $P \leq 0.001$, marked with ***) of these indexes among different stages; **(C)** β diversity analysis based on Bray-Curtis method has quantified the differences in the species abundance distributions among the samples at different developmental stages; **(D)** has shown the relative abundance of microbial populations possessing endoglucanase (EC 3.2.1.4) genes in the giant panda at different dietary stages; **(E,F)** were heat maps that have shown the succession of bacterial and fungal composition respectively in the giant panda.

Proteobacteria had increased relative abundance in the giant panda with bamboo consumption. The Bacteroidetes and Actinobacteria increased in adult giant panda with the relative abundance of 4%. The phyla Basidiomycota and Ascomycota were the dominant fungi in giant panda and increased abundance to 98% in adults. The genus *Trichosporon* of Basidiomycota and *Candida*, *Trimmatostroma*, Pleosporales and Montagnulaceae of Ascomycota were more common. However, the more abundant *Trichosporon* and *Candida* have been reported as pathogens in mammals, thus a hostile relationship might exist between fungi and the giant panda. There was no obvious dominant resident in fungal community and no positive correlation with the development of giant panda (**Figure 4** and **Supplementary Figures 1**, **2**).

According to the metagenomic analysis, a specific set of genes encoding endoglucanase (total value = 96) was collected from the above total non-redundant gene catalog and aligned to NR database for taxonomic annotation (**Supplementary Table 3**). The gut bacterial species possessing endoglucanase genes mainly included *Paenibacillus elgii*, *Lachnoclostridium phytofermentans*, *Clostridium longisporum*, and *Cellulosilyticum lentocellum* (**Figure 4D**). Among these species, *P. elgii*, *L. phytofermentans*, and *C. longisporum*, belonging to Firmicutes, were enriched in the giant panda on a bamboo diet, but all had a relative abundance <0.01% (**Figure 4D**). *Cellulosilyticum*, in the phylum Bacteroidetes, occurred momentarily in the dietary-shift period (**Figures 4D,E**), but was not detected in the sub-adult or adult giant panda; therefore, this genus was likely not able to colonize in a bamboo-fed giant panda. Members of the phylum Bacteroidetes are reported to degrade lignocellulose in herbivores at the genus level (Zhu et al., 2011; Yang et al., 2018; Guder and Krishna, 2019), such as the genus *Bacteroides* which has increased in the adult giant panda with a relative abundance of 2.25%. Low levels of cellulose-degrading fungi, such as *Aspergillus* (0.73%), *Penicillium* (1.67%), and *Trichoderma* (0.02%) were also detected (Carvalho et al., 2013; Gaurav et al., 2017; **Figure 4F**); however, these species were more abundant in cubs and even higher than in adults. In contrast, bacteria might be more important for the adaptation to bamboo diet of giant panda.

This study further found that the predominant species in Firmicutes and Proteobacteria, such as *Escherichia coli*, *Clostridium dakarense* and *Clostridium botulinum*, *Turicibacter sanguinis*, and *Terrisporobacter glycolicus* actively participated in soluble carbohydrate and amino

acid metabolism; consistent with the analysis of digestive enzyme activity. In particular, according to the taxonomic annotation of genes involved in the amino acid metabolism, the species *E. coli*, *C. dakarense*, *C. botulinum, and T. sanguinis* contributed to the metabolism of 13 amino acid, such as alanine, aspartate, glutamate, arginine, proline, phenylalanine, and methionine, which were also the main composition of amino acid in bamboo.

In general, gut bacteria have been involved in assisting giant panda to adapt to eating bamboo by gradually enriching the cellulolytic community to digest bamboo cellulose. In addition, some dominant bacteria might cooperate with cellulolytic flora to mainly metabolize proteins and soluble carbohydrate in bamboo for giant panda.

Discussion

Delayed but increased expression of cellulose-degrading enzymes in the giant panda during adaptation to cellulose

The analysis showed that the changes in activities of endoglucanase and exoglucanase presented *U*-shaped profiles. Both cellulases increased in bamboo-fed giant pandas after dietary shift. However, compared with protease and amylase that returned abilities to previous levels in sub-adults or adults, cellulases activities in adults were still an order of magnitude worse than previously, showing delayed expression in giant panda with bamboo consumption.

When adapting to a new fibrous diet (e.g., bamboo, forage, or gluten), the activities of fiber-digesting enzymes in the guts of the giant panda, herbivores, and omnivores all show *U*-shaped enzyme activity profiles. Therefore, some adaptive process occurs in every mammal. The endoglucanase activity of the giant panda increased 10-fold in adults compared with when they first consumed bamboo, and that of herbivores increased 25-fold in adults (Hao et al., 2021). In addition, the gliadinase activity of omnivores increased two-fold in adults (Fernandez-Perez et al., 2020). The giant panda showed a more positively adaptive change to digesting bamboo cellulose than omnivores showed, but its ability was still far behind that of herbivores, and the activities of both endoglucanase and exoglucanase were always lower during the growth of the giant panda than in herbivores (Guo et al., 2018; Hao et al., 2021). There is no doubt that the lack of exoglucanase, which directly breaks down the tough structure of bamboo cellulose, may be one factor limiting the complete degradation of cellulose by giant panda.

With regard to cellulases activities being maintained at high levels in milk-fed cubs in this study, a similar phenomenon has also been detected in humans and many other mammals before weaning (e.g., gliadinase in humans and cellulases in calves and pandas). This is speculated to be related to early development and derived from maternal inheritance or early diet (Fernandez-Perez et al., 2020; Hao et al., 2021). The later cellulase properties, expression profiles and the structure of the gut flora may not be consistent with those in the milk-fed cubs in terms of the degradation of cellulose.

The properties (optimal pH) of endoglucanase in adults differ from those in cubs, which means they are different types of endoglucanase. Hao et al. (2021) have also shown that in calves, the nature of their cellulases might vary with weaning and the consumption of forage, and that the ruminal pH also changed with this dietary change. The optimal pH of endoglucanase tends to be weakly acidic in both herbivores and bamboo-fed giant pandas, close to the ruminal pH (Hao et al., 2021). Therefore, it was speculated that after the pandas began to consume bamboo, they tried to produce new cellulases that differed from those expressed in milk-fed cubs, in an attempt to adapt the intestinal environment to the degradation of new types of cellulose. This phenomenon is worthy of further study.

Neither the abundance nor transcription of lignocellulose-decomposing genes in the gut microbial genome are sufficient for the giant panda to fully degrade bamboo cellulose

Gut microbial metagenomic analysis showed that the abundance of genes encoding endoglucanase (EC 3.2.1.4) was higher than for other lignocellulose-decomposing enzymes in the giant panda and increased with bamboo consumption. The two subtypes of GH5 (GH5_2 and GH5_4) and GH9 corresponding to endoglucanase (EC 3.2.1.4) have been detected in the microbial genome of bamboo-fed giant pandas, and GH9 is more helpful for hydrolyzing complex cellulose (Vita et al., 2019).

However, genes encoding GH9 (with a relative abundance of >0.01 among the GHs) were more abundant in herbivores, both with (e.g., cow) and without (e.g., elephant) rumens, than in the giant panda, and that more diverse suites of genes encoding GHs and carbohydrate-binding modules (CBMs) for lignocellulose degradation (such as GH44, GH45, GH48, GH74, CBM4, CBM6, CBM37, and CBM44 for cellulose degradation and GH10, GH26, GH28, GH53, CBM8, and CBM46 for hemicellulose degradation) were enriched and expressed during the growth and transition of herbivores to a plant-based diet (**Supplementary Table 2**; Shinkai et al., 2016; Ilmberger et al., 2017; Bohra et al., 2019).

Analysis of the transcriptome showed that only GH5 gene cluster maintained efficient transcription in bamboo-fed giant pandas for hydrolyzing cellulose. Compared with transcriptional silencing of GH9 in the giant panda, the transcriptional efficiency of GH9 was higher in herbivores than in the giant panda, accounting for 0.023–0.043 of GH transcripts (**Supplementary Table 2**; Dai et al., 2014). The transcriptional efficiency of GH5, encoding the most abundant cellulase GH, does not differ markedly between the giant panda and herbivores after they begin to consume plants (Shinkai et al., 2016). Moreover, the subtypes of GH5 found in the giant panda could potentially degrade cellulose and hemicellulose simultaneously (Glasgow et al., 2020; Pauchet et al., 2020).

However, compared with other herbivores, the giant panda lacks genes encoding more types of lignocellulose-decomposing enzymes for the hydrolysis of the complex celluloses in bamboo, in terms of their abundance and transcriptional efficiency. This largely explained the giant panda's poor ability to adapt to the dietary shift and to fully degrade bamboo cellulose.

A primary and amateur cellulose-degrading microbial community is formed to adaptively digest cellulose in the short carnivore-like gut of the giant panda

Consistent with the findings of Guo et al. (2020), the diversity of the gut flora decreased significantly in the giant panda after the dietary shift to that of sub-adults. Reduced diversity in the gut microbial population and gradually weakened complex interactions in the microbial community indicate adverse effects on the intestinal health of many animals (Tulstrup et al., 2018; Fassarella et al., 2021). They might also hinder the giant panda's adaptation to high-cellulose diet, inducing the crisis observed in the giant panda. In addition, combined with the results of β-diversity analysis, the structure of the gut flora was immature and fragile at that time.

The dominant bacterial genera in adult pandas were still *Streptococcus*, *Escherichia–Shigella*, and *Clostridium*, which have long histories of survival in the human gut and those of other omnivores. These taxa have adequate strategies for obtaining energy (Berlemont and Martiny, 2013); probably including in high-cellulose niches (Williams et al., 2013; Song et al., 2017). Therefore, it was considered that the gut microorganisms of the giant panda are still predominantly in a struggle for survival, and have not yet become a specialized bamboo-degrading community. In this study, the predominant species preferred to metabolize the small amounts of protein and soluble carbohydrate in bamboo.

Some cellulose-degrading microbial populations were gradually enriched in the adult giant panda, but their abundance and activities were insufficient to support a high-cellulose bamboo diet. In herbivores, the ratio of Firmicutes to Bacteroidetes is an important index of the ability to digest plant material, and is on average 1:1 in different herbivorous animals. However, the proportion of Bacteroidetes in the giant panda is smaller (Güllert et al., 2016), which might explain the lack of a stable, strictly anaerobic gut environment, because its short intestine limits the length of stay for food (Megahed et al., 2019). In response to plant polysaccharides, the GHs in herbivores are expressed by a complex network of Bacteroidetes, Fibrobacteres, and Firmicutes (Jiang et al., 2011; Hao et al., 2021), whereas the cellulase GHs in the giant panda were mainly expressed by Firmicutes, and the community of cellulolytic microbes was still elementary and simple.

According to the systematic analysis and discussion of digestive enzymes, functional genes and microbial community, both the gut microbial structure and function have been developed to digest bamboo cellulose in the giant panda. However, their configurations were not able to fully degrade cellulose. Therefore, the giant panda cannot completely adapt to bamboo diet by only relying on cellulose-degrading flora.

Potential strategies of the giant panda to maintain its normal growth and development without completely degrading bamboo cellulose

The studies reported above have indicated that the adaptive succession of the gut flora in the intestinal environment of the giant panda is still immature and amateur, and cannot fully convert cellulose into small-molecule nutrients like those in herbivores. It was anticipated that the microbial community had potential to produce new types of cellulases and continuously enhance its ability to digest bamboo. At present, the increased expression of endoglucanase might damage the bamboo cell walls and help release the intracellular degradable nutrients to the giant panda, which is likely to be one of the most important efforts by the giant panda to cope with bamboo diet. Some researchers have reported that the giant panda preferentially eats bamboo leaves where the main component is cellulose and there is accumulated crude protein, crude fat, sugar, and minerals (He, 2010; Wu et al., 2017).

To acquire even a small amount of energy and nutrient in bamboo, the giant panda must eat a large quantity (up to tens of kilograms per day). Because mucus is discharged by the giant panda after it foregoes a bamboo diet for a while, we inferred that a large amount of mucus generated in the intestine might protect the panda's gastrointestinal tract, preventing damage by tough bamboo fiber. Whether the secretory mucus was actually an important strategy allowing its consumption of a lot of bamboo and/or has other functions, such as promoting the absorption of bamboo nutrients, required further research.

Conclusion

From our results and the other research discussed above, several conclusions can be drawn:

1. During its dietary shift process, the giant panda gradually adapts to a bamboo diet, especially enhancing its ability to digest bamboo cellulose, but the activities of cellulases derived from gut microbes are still insufficient to convert cellulose to available nutrients.
2. A primary and amateur cellulolytic microbiota has been formed in giant panda, while the colonization of some efficient but anaerobic-preferred flora is limited by the short carnivore-like gut, resulting in the low abundance and transcription of cellulase genes and currently incomplete digestion of bamboo cellulose.
3. The giant panda appears most vulnerable during its dietary shift, especially when it first predominantly eats bamboo, because the intestinal flora structure is immature, so that the activities of digestive enzymes and the transcription of functional genes are lowest in this period.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA808648.

Author contributions

MZ, AW, YZ, and SZ performed the material preparation. MZ, AW, XF, and JZ planned the experiments. MZ and XF performed the data collection and analysis. MZ wrote the first draft of the manuscript. LW revised the manuscript critically. YY, EP, and LW participated in the planning and coordination of the study. All authors commented on previous versions of the manuscript and read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Gut microbiomes of cyprinid fish exhibit host-species symbiosis along gut trait and diet

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Teleost omnivorous fish that coexist partially sharing resources are likely to modify their gut traits and microbiome as a feedback mechanism between ecological processes and evolution. However, we do not understand how the core gut microbiome supports the metabolic capacity of the host and regulates digestive functions in specialized omnivorous fish gut traits. Therefore, we evaluated the gut microbiome of eight omnivorous fish from a single family (i.e., Cyprinidae) in the current study. We examined the correlation between host phylogeny, diet composition, and intestinal morphological traits related to the intestinal microbiome. The results indicated that cyprinid fish with similar relative gut lengths had considerable gut microbiome similarity. Notably, the SL (short relative gut length) group, as zoobenthos and zooplankton specialists, was abundant in Proteobacteria and was less abundant in Firmicutes than in the ML (medium relative gut length) and LL (long relative gut length) groups. These fish could extract nutrients from aquatic plants and algae. Additionally, we found the relative abundance of *Clostridium* and *Romboutsia* to be positively correlated with host relative gut length but negatively correlated with the relative abundance of *Cetobacterium*, *Plesiomonas*, *Bacteroides*, and *Lactobacillus*, and host-relative gut length. We also show a positive linear relationship between host gut microbiome carbohydrate metabolism and relative gut length, while the amino acid and lipid metabolism of the gut microbiome was negatively correlated with host-relative gut length. In addition, omnivorous species competing for resources improve their ecological adaptability through the specialization of gut length, which is closely related to variation in the synergy of the gut microbiome. Above all, specialized gut microbiota and associated gut morphologies enable fish to variably tolerate resource fluctuation and improve the utilization efficiency of nutrient extraction from challenging food resources.

KEYWORDS

gut microbiome, Cyprinidae, coexistence, gut length, metabolism

Introduction

The co-evolution of animals and microbes within their guts facilitates their radiation into a wide variety of habitats (Muegge et al., 2011). In the long-term evolutionary process, hosts and microbes cooperate and interact, eventually forming an intimate symbiotic relationship (Suzuki, 2017). Over the past decade, our understanding of the diversity and functions of host-associated microbial communities has dramatically expanded (Ley et al., 2008; McFall-Ngai et al., 2013; Ye et al., 2014; Rennison et al., 2019). Notably, the adaptive capacity of an animal species is not determined solely by the host genome but must also include the vast genetic repertoire of the microbiome (Rosenberg and Zilber-Rosenberg, 2018). To gain a better understanding of the role of the gut microbiome in animal fitness, microbiome research is now considered a major area of research in ecology and evolution (Levin et al., 2021). Vertebrates consume a large array of food items, and their guts and microbiome reflect a complexity influenced by diet and genetics (Karasov and Douglas, 2013; Youngblut et al., 2019). Moreover, fish comprise the largest vertebrate group, with a wide spectrum of host habitats, physiology, and ecological strategies (Parris et al., 2016). Specifically, teleost fish are an invaluable repertoire of host species suitable for the study of factors shaping animal-associated microbiomes (Sylvain et al., 2020). Givens et al. (2015) found that some core shares OTUs in the guts of fish species. These may be important contributors to fish gut functions, such as digestion, nutrient absorption, and immune response. Several studies have shown that fish microbial effects can not only support the important role of microbes in promoting or enhancing fish adaptation but also potentially facilitate diversification (Huang et al., 2020; Liu et al., 2022). Moreover, numerous published studies have indicated that fish gut microbiota can be affected by factors, such as host diet, habitat, and genotypes (Li et al., 2020; Kang et al., 2022). However, these factors are often interrelated, and their effects on the fish gut microbiome tend to be complex and highly confounded.

Fish have specific gut traits that depend on their diet (Egerton et al., 2018). Nevertheless, it is not clear what gut trait specialization means for the gut microbiomes of fish. Fish gut length is one of the most important specialized gut traits in the aquatic ecosystem and can result in changes to the ecological interactions between species and their "fitness" in the community (Post and Palkovacs, 2009; Vasseur et al., 2011; Barabás and D'Andrea, 2016; Pastore et al., 2021). It is widely recognized that fish gut length can be affected by evolved differences in the diet as well as phylogenetic history (German and Horn, 2006; Zandonà et al., 2015). Longer guts are often observed in herbivorous fish, while shorter guts are often found in carnivorous fish (German and Horn, 2006). Nevertheless, omnivorous fish have a more diverse diet than herbivorous and carnivorous fish and the trophic niches overlapped. For instance, grass carp is considered an herbivorous fish. However, relevant findings show that the grass carp feeds primarily on Streptophyta and facultatively on Arthropoda, Rotifera, and Ascomycota, indicating that the grass carp is an omnivorous and only partially herbivorous fish (Zhang et al., 2021). Furthermore, the length of the gut determines the duration of food retention, whereas building and maintaining a long gut have high evolutionary and physiological costs. There is a trade-off between the benefits of nutrient acquisition and the costs of maintaining a long gut in a host (Ghilardi et al., 2021). A relevant study demonstrated that both evolutionary history and plasticity in diet quality can drive variation in fish gut length and the fish gut microbiome (Escalas et al., 2021). In recent years, an intensive study of wild fish gut microbes has mainly focused on the diversities and complexities of gut microbiota communities in wild fish species with different genotype and trophic levels (Liu et al., 2016; Escalas et al., 2021; Ofek et al., 2021). However, there is still limited information on relationship between the microbiomes of omnivorous fish hosts gut microbiome and their gut length.

Cyprinidae has more than 2,000 freshwater fish species, is widely distributed, and is one of the most diverse taxonomic groups in Cypriniformes. Cyprinid fish are composed of an abundance of omnivorous fish species that show incredible diversity, leading to resource specialization in various species, yet the gut length of these same species shows marked generality and plasticity, maintaining the ability to process a diversified diet. Thus, Cyprinidae is considered a good taxanomic group for exploring the gut microbiome of omnivorous fish in response to gut length specialization. However, the overall role of diet and anatomy in shaping the gut microbiome remains to be unfolded, especially within families characterized by ecological diversification. Given the general close relationship between the composition of the gut microbiome and digestion strategy, we hypothesized that there could be a possible link between gut length and gut microbiome in omnivorous fish, which have a diversified diet and a wide trophic niche. To assess our hypotheses, we utilized a high-throughput 16S ribosomal RNA (rRNA) sequencing to assess the gut microbiome of eight Cyprinidae species, namely, Grass carp (*Ctenopharyngodon idellus*, GC); Black Amur bream (*Megalobrama terminalis*, BA); Silver carp (*Hypophthalmichthys molitrix*, SC); Barbel chub (*Squaliobarbus curriculus*, BC); Bleeker's yellow tail (*Xenocypris davidi*, XD); Topmouth culter (*Culter alburnus*, TC); Mud carp (*Cirrhinus molitorell*a, MC), and Common carp (*Cyprinus carpio*, CA) from a single location with the same cohabitated environment to gain insights into how host evolutionary history, diet, and gut anatomy are related to the gut microbiome. Additionally, studying the gut microbiomes of eight Cyprinidae species is greatly important for further understanding of symbiotic interactions between fish hosts and their microbes.

Materials and methods

Fish sample collection

In this study, eight wild Cyprinidae fish species were collected from the Xiniu reservoir (one of the cascades in the Lianjiang River, owning 12 cascades. Fragmented fluvial habitats compress fish's living space and lead to a relatively independent environment in different cascades, which is supposed to be an ideal minor natural ecosystem) in Lianjiang River, Guangdong Province, China in July 2021 (**Figure 1**). The Lianjiang River is the largest tributary of the Beijiang River, and has 12 cascades. We captured 80 fish using gillnets (10 specimens for each species). Basic environmental information of sample sites is provided in **Supplementary Table 1**. Body length (BL, to the nearest 1 mm) was measured from the length from the tip of the mandible to the base of the caudal fin; Body weight (BW was measured to the nearest 1 g), Gut morphology was characterized using gut length (mm), and relative gut length (gut length/body length) was measured. Relative gut length was used to reduce the effect of individual differences on gut length. Based on the discrepancy in relative gut length, eight cyprinid fish were divided into three groups, the SL (short: relative gut length < 2); ML (medium: $2 \leq$ relative gut length < 4); and LL (long: relative gut length ≥ 4) groups. In order to investigate the fish gut microbiota, three or four fish were randomly selected for sequencing from eight species samples. Prior to dissection, fish were euthanized with an overdose of MS 222 (3-aminobenzoic acid ethyl ester methanesulfonate, Sigma, Germany). All procedures for the handling of wild freshwater fish species were approved by the institutional animal care. To prevent contamination from the skin surface, and eliminate transient bacteria, the whole intestinal tract of an individual fish sample was dissected with sterile instruments and quickly washed in 75% ethanol and sterile water. The obtained gut contents that were utilized for deoxyribonucleic acid (DNA) extraction were immediately put into liquid nitrogen and then transferred to an ultra-low temperature freezer and stored at −80°C until use.

Deoxyribonucleic acid extraction and amplification

Approximately, 0.2 g of each sample was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Valencia, United States). All DNA extracts were stored at −80°C until use. The V3-V4 hypervariable region of the bacterial 16S rRNA genes was amplified using the specific primer pairs 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, United States). Total DNA from the gut of different fish samples was sent to Novogene Bioinformatics Technology, Co., Ltd., Beijing, China for further sequencing analysis.

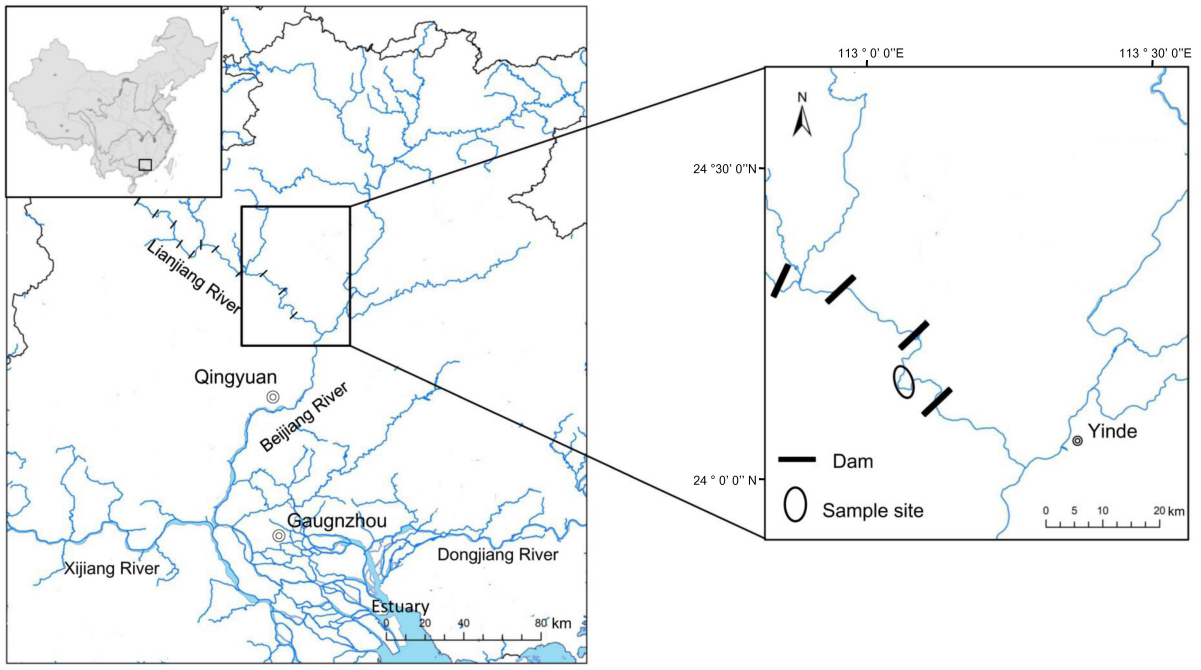

FIGURE 1
Location of the sample sites.

High-throughput sequencing analysis

Sequencing libraries were created using the Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific, United States) according to the manufacturer's instructions. The library quality was assessed on a Qubit@ 2.0 Fluorometer (Thermo Scientific, United States). After that, the library was sequenced, and single-end reads were generated. Quality filtering of the raw reads was performed under specific filtering conditions to obtain high-quality clean reads according to the cut-adapt quality-controlled process (Martin, 2011). The tags were compared with the reference database using the UCHIME algorithm to detect chimera sequences (Edgar et al., 2011; Haas et al., 2011). Sequence analysis was performed using UPARSE 7.1 software (Edgar, 2013). Sequences with equal or greater than 97% similarity were assigned to the same operational taxonomic unit (OTU). The most abundant sequence for each OTU was selected as a representative sequence. To minimize the effects of sequencing depth on alpha and beta diversity measure, the number of 16S rRNA gene sequences from each sample was rarefied to 20,000, which still yielded an average Good's coverage of 99.09%. To predict the microbial function of the bacterial communities on the gut contents of different samples, PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was utilized to analyze the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways at Levels 2 and 3 (Douglas et al., 2020).

Phylogenetic analysis of fish

Phylogenetic analysis of eight Cyprinid species (grass carp, black Amur bream, silver carp, barbel chub, bleeker's yellowtail, topmouth culter, mud carp, and common carp) based on cytochrome c subunit I (CO1) gene was done using the MEGA program (version 7.0) (Kumar et al., 2016).

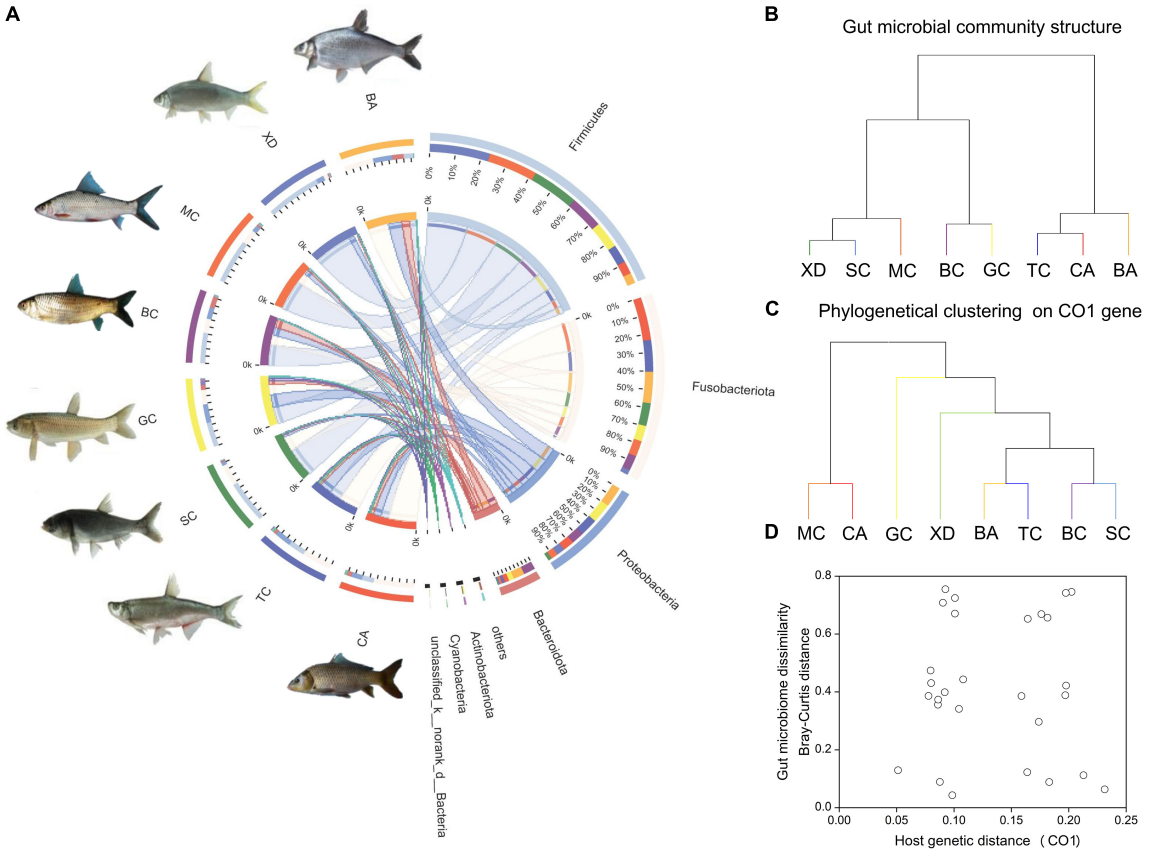

FIGURE 2
An overview of the data. **(A)** A circos chart indicating microbial communities in fish gut samples at the phylum level. The inner circular diagram shows the relative abundance of different phyla in different cyprinid fish gut samples. Only those with mean relative abundance more than 1% for phylum are shown. Sequences that could not be assigned at the phylum level were marked as "Unclassified"; **(B)** hierarchical clustering of gut microbiome of the different cyprinid fish based on Bray-Curtis distance; **(C)** phylogenetical clustering based on host genetic distance (the CO1 gene); **(D)** relationship between fish gut microbiome dissimilarity based on Bray-Curtis distance and host genetic distance (the CO1 gene). BA, Black Amur bream; BC, Barbel chub; CA, Common carp; GC, Grass carp; MC, Mud carp; SC, Silver carp; TC, Topmouth culter; XD, Bleeker's yellow tail.

TABLE 1 An overview of fish samples, including biological information and dietary composition.

Host	*n*	Body weight	Body length	Gut length	Gut length/body length ratio	Relative gut length level	Dietary composition		
							Dominant food item	Facultative food item	References
TC	10	330 ± 10.54	249 ± 15.41	214 ± 22.98	0.86	SL	Zooplankton (crustaceans); zoobenthos (insects); small fish	Other aquatic plant (vascular plant).	Zheng, 1989; Zhou and Zhang, 2005; Froese and Pauly, 2022
CA	10	473 ± 21.65	248 ± 16.98	345 ± 31.25	1.39	SL	Zoobenthos (aquatic insects; mollusks); Zooplankton (cladocerans; crustaceans)	Phytoplankton (algae); other aquatic plant (terrestrial plants).	Zheng, 1989; Lu, 1990; Froese and Pauly, 2022
BC	10	323 ± 23.61	269 ± 20.41	557 ± 48.97	2.08	ML	Zooplankton (fish eggs/larvae; planktonic crustaceans); Phytoplankton (algae)	Zoobenthos (aquatic insects).	Zheng, 1989; Lu, 1990
GC	10	319 ± 20.48	253 ± 16.47	552 ± 38.95	2.18	ML	Other aquatic plant (terrestrial plants); phytoplankton (algae)	Zooplankton (invertebrates); zoobenthos (aquatic insects).	Lu, 1990; Zhang et al., 2021; Froese and Pauly, 2022
BA	10	316 ± 18.97	229 ± 15.87	657 ± 50.14	2.87	ML	Zooplankton (crustaceans); zoobenthos (mollusk)	other aquatic plant (terrestrial plants); Phytoplankton (algae).	Lu, 1990; Xia et al., 2017
XD	10	271 ± 23.41	199 ± 20.14	1147 ± 106.72	5.77	LL	Phytoplankton (algae); Other aquatic plant (terrestrial plants)	Zooplankton (invertebrates); zoobenthos (aquatic insects).	Zheng, 1989; Lu, 1990; Froese and Pauly, 2022
SC	10	408 ± 28.64	276 ± 22.98	1779 ± 123.12	6.44	LL	Phytoplankton (algae); zooplankton (invertebrates)	Zoobenthos (aquatic insects).	Zheng, 1989; Lu, 1990; Froese and Pauly, 2022
MC	10	339 ± 19.52	244 ± 17.54	2432 ± 198.64	9.98	LL	Phytoplankton (algae); zooplankton (invertebrates)	Zoobenthos (invertebrates); other aquatic plant (terrestrial plants).	Zheng, 1989; Lu, 1990; Froese and Pauly, 2022

Relative gut length groups (SL: gut length/body length < 2; ML: $2 \leq$ gut length/body length < 4; LL: gut length/body length ≥ 4). BA, Black Amur bream; BC, Barbel chub; CA, Common carp; GC, Grass carp; MC, Mud carp; SC, Silver carp; TC, Topmouth culter; XD, Bleeker's yellow tail.

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The corresponding gene sequences were downloaded from GenBank (accession numbers in **Supplementary Table 2**). The concatenated sequences of the mitochondrial genes were aligned using ClustalW in MEGA (pairwise and multiple alignment parameters). The phylogenetic tree was constructed based on the aligned DNA sequences by the neighbor-joining method using the Kimura 2-parameter model in MEGA, and then pairwise genetic distance among eight cyprinid species was calculated using MEGA (Kumar et al., 2016).

Statistical analysis

The unweighted pair-group method with arithmetic means (UPGMA) clustering was performed to interpret the Bray-Curtis distance matrix using average linkage, which is a hierarchical clustering method. It was performed using QIIME software (Version 1.9.1). Then, we used Mantel tests implemented in the *R* software package to assess the relationships between host phylogenetic and microbiome dissimilarity matrixes (Goslee and Urban, 2007). Based on the OTU information, alpha-diversity indices, including the Shannon index and ACE richness index, were calculated using QIIME (version 1.9.1). The shared and unique OTUs of different groups were also represented by a Scale-Venn diagram. A one-way ANOVA test was used in SPSS Statistics 28.0 to evaluate if the differences in alpha diversity between groups were significant. A *p*-value below0.05 was used to determine statistical significance. Significance tests of the bacterial community composition with analysis of similarities (ANOSIM) were computed with 999 permutations using the VEGAN package and carried out on the significance of defined categories based on Bray-Curtis distances using the OTU table (Clarke, 1993). Power analysis for the ANOVA-based and permutation-based ANOSIM test was used to estimate sample size (Cohen, 1977; Wang and Zhang, 2021; **Supplementary Figures 1, 2**). Adonis analysis (on parametric MANOVA) based on Bray-Curtis distances was used to explain the different grouping factors for differences of samples, and the statistical significance of division was analyzed for significance using a permutation test (QIIME version 1.9.1). Non-metric multidimensional scaling (NMDS) was used to analyze the differences in the bacterial community composition of different groups across orders and phyla based on the Bray-Curtis distance matrix using the

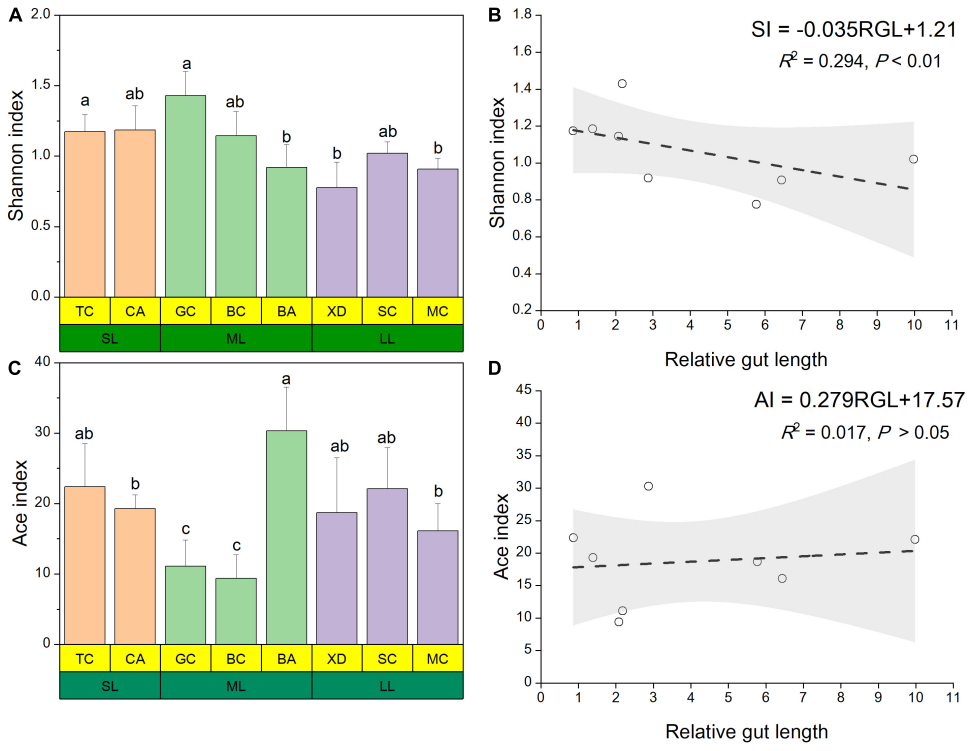

FIGURE 3
Alpha diversity results of the gut microbial community pertaining for the eight different fish species at three relative gut length levels. The Shannon **(A)** and Ace **(C)** index of gut microbiota composition from the eight different fish species at three relative gut length levels. Samples marked different lowercase letters indicate significant differences (a > b > c; $p < 0.05$) among different fish species. Linear regression of the Shannon **(B)** and Ace **(D)** index among eight cyprinid fish species vs. mean of their relative gut length with 95% confidence interval. BA, Black Amur bream; BC, Barbel chub; CA, Common carp; GC, Grass carp; MC, Mud carp; SC, Silver carp; TC, Topmouth culter; XD, Bleeker's yellow tail; RGL, Relative gut length; SI, Shannon index; AI, Ace index.

VEGAN package (Lozupone and Knight, 2005). A principal coordinate analysis (PCoA) was conducted to explain the difference between the KOs of different groups of relative gut length by using the WGCNA package, stats, and ggplot2. Here, we used the *R* implementation of the procedure (version 3.1.14). For correlation analysis, the relationships between the relative gut length and alpha diversity or the relative abundance of core genera relative abundance and digestion-related bacterial gene functions were investigated separately, and the *p*-values under0.05 were considered indicative of significant pairwise relationships.

Results

Relationships between host and core microbiome dissimilarity

The gut bacteria of the eight different cyprinid fish species showed remarkable differences. At the phylum level, the gut bacteria of TC, CA, and BA were dominated by Fusobacteriota (41.7–57.3%); those of BC and GC were characterized by Firmicutes (35.3–43.9%) and Proteobacteria (19.–26.8%), and those of XD, SC, and MC were dominated by Firmicutes (63.8–73.6%) (**Figure 2A**). It is evident from the results that, overall, with the same diet type and gut trait, the community composition was similar (**Table 1** and **Figure 2B**). We generated an UPGMA tree based on 16S rDNA gene sequences from the gut microbial communities (**Figure 2B**). Phylogenetic analysis of eight Cyprinid species based on the cytochrome c subunit I (CO1) gene was indicated in **Figure 2C**. As shown in **Figure 2D**, the CO1 genetic distance between fish species was not correlated with their microbiome dissimilarity ($p > 0.05$).

Association between cyprinid gut microbiome and specialized gut traits

The microbial complexity in eight fish species was estimated on the basis of alpha-diversity (Shannon and Ace indices), and it showed distinct differences (**Figures 3A,C**). The Shannon index of the SL (short-relative gut length) and ML (medium-relative gut length) groups was larger than that of the LL (long-relative gut length) group, whereas there were no significant differences among the three groups. It should be noted that alpha-diversity indices exhibit obvious differences even in the similar relative gut length level. We observed that there was a linear relationship between Shannon index and relative gut length, while no significant correlation was found between Ace index and relative gut length (**Figures 3B,D**). Furthermore, similarities of the microbial community composition between fish samples were compared by NMDS based on Bray-Curtis distance (**Figure 4A**). The XD, SC, and MC samples formed a cluster and distinctly separated from the cluster of CA and TC samples, while others were located in the middle of them. In addition, we established significant differences of gut microbiome among different fish species (ANOSIM analysis: $R = 0.244$, $p = 0.015$; Adonis analysis: $R^2 = 0.495$, $p = 0.006$). Pearson correlation analysis between gut trait and dominant phylum in cyprinid fish species showed that the gut length and relative gut length were significantly correlated with the relative abundance

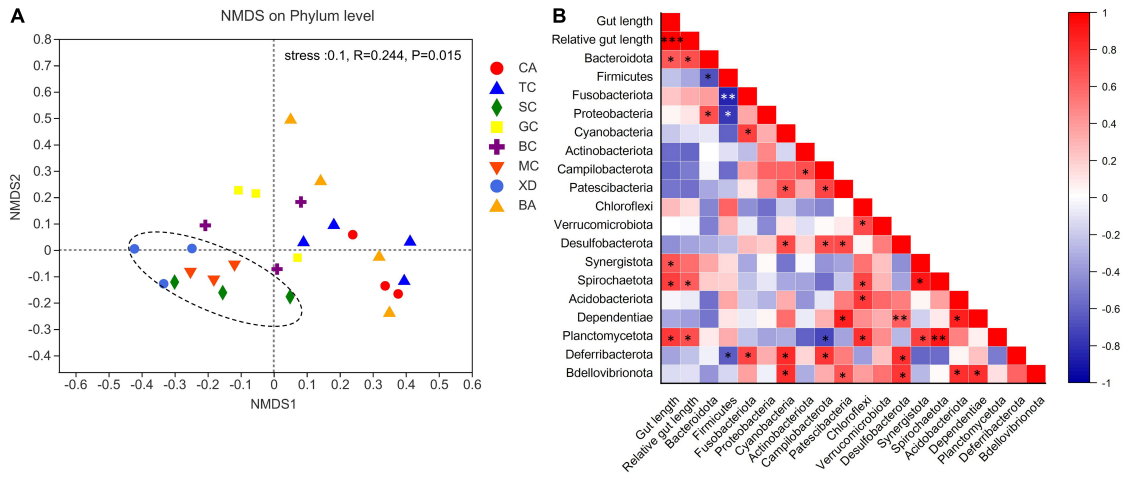

FIGURE 4
An overview of the data. **(A)** NMDS (non-metric multidimensional scaling) based on bray curtis distance matrix, demonstrating different fish samples. Significance tests of the bacterial community composition with analysis of similarities (ANOSIMm), indicating the significance of defined species based on Bray-Curtis distance. The individual samples are color-coordinated according to the different species. **(B)** Pearson correlation analysis between relative gut length and dominant phylum in the eight fish species *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. BA, Black Amur bream; BC, Barbel chub; CA, Common carp; GC, Grass carp; MC, Mud carp; SC, Silver carp; TC, Topmouth culter; XD, Bleeker's yellow tail.

of Bacteroidota and Spirochaetota (Figure 4B). Additionally, there was a negative relationship between the Firmicutes and Proteobacteria and Fusobacteriota, while Proteobacteria showed a positive relationship with Bacteroidota in all the fish samples.

Gut microbiome differentiation along the relative gut length

The most abundant taxa of bacteria in each relative gut length group were observed at the phylum and order levels (Figures 5A,B). Firmicutes were the most abundant in the XD, MC, and SC groups, whereas the most abundant phylum of the CA and TC groups was the Fusobacteriota (Figure 5A). According to Figure 5B, Fusobacteriota was observed as the dominant order of the CA and TC groups, while the dominant orders of the SC and XD groups were Lachnospirales. Moreover, we compared similarities of the microbial community composition among the three groups by NMDS based on Bray-Curtis distance at the phylum and order levels (Figure 6A). At the phylum level, SL samples formed a cluster and distinctly separated from the cluster of LL samples, while ML samples were located in the middle of them (ANOSIM analysis: $R = 0.331$, $p = 0.001$; Adonis analysis: $R^2 = 0.332$, $p = 0.001$). Nevertheless, SL samples separated from the cluster of the LL and ML samples, which were close (Anosim analysis: $R = 0.219$, $p = 0.002$; Adnois analysis: $R^2 = 0.251$, $p = 0.001$). Furthermore, the Venn diagram revealed that the three groups shared 17 phyla, whereas the ML group shared the fewest unique phyla. The number of common orders presented in all groups was 71, and unique orders for each group varied from 15 to 25 (Figure 6B). The ML group shared more orders with the LL group than with the SL group. A one-way ANOVA analysis of the dominant microbiome among the three groups showed significant differences in Firmicutes, Proteobacteria, Planctomycetota, and Dependentiae ($p < 0.05$) (Figure 6C and Table 2). At the order level, the relative abundance of Chitinophagales in the SL group was the highest, whereas the relative abundance of Erysipelotrichales, Bacteroidales, and Osillospirales was the lowest among the three groups ($p < 0.05$). The relative abundance of Babeliales in

FIGURE 5
Comparison of the bacterial community in the different relative gut length groups. Dominant gut microbiota composition in the different groups at the phylum **(A)** and order **(B)** level; each bar represents average relative abundance of each bacterial taxon within a group at the phylum and order level. BA, Black Amur bream; BC, Barbel chub; CA, Common carp; GC, Grass carp; MC, Mud carp; SC, Silver carp; TC, Topmouth culter; XD, Bleeker's yellow tail.

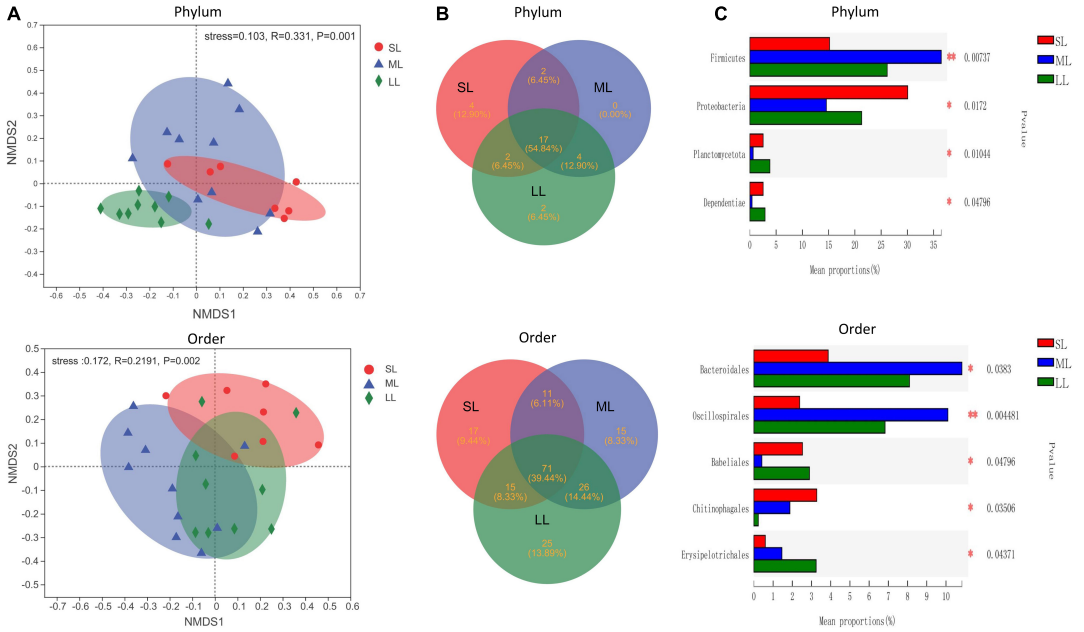

FIGURE 6
An overview of the data. **(A)** NMDS based on the bray curtis distance matrix demonstrating different fish samples at the phylum and order level. The individual sample is color-coordinated according to the different gut length groups; significance tests of the bacterial community composition with analysis of similarities (ANOSIM), indicating the significance of groups based on Bray-Curtis distances. **(B)** The Venn diagram illustrating the shared and unique phylum and orders in the different gut length groups. **(C)** One-way ANOVA analysis demonstrating significant differences of gut bacterial phyla and order in the different gut length groups. Samples marked by an asterisk indicate significant differences (*$p < 0.05$; **$p < 0.01$) among relative gut length groups.

the ML group was much lower than that of the LL groups ($p < 0.05$) (Figure 6C and Table 2). In addition, it is evident from the result that there was a positive linear relationship between the relative abundance of *Clostridium* and *Romboutsia* and host relative gut length, whereas a negative correlation between the relative abundance of *Cetobacterium*, *Plesiomonas*, *Bacteroides*, and *Lactobacillus* and the host relative gut length was observed (Figure 7).

TABLE 2 One-way ANOVA analysis of differences of gut bacterial phyla and order in the different relative gut length groups.

	SL vs. ML *P*-value	ML vs. LL *P*-value	SL vs. LL *P*-value
Phylum			
p__Firmicutes	0.157	0.001**	0.001**
p__Proteobacteria	0.807	0.094	0.004**
p__Planctomycetota	0.107	0.013*	0.085
p__Dependentiae	0.107	0.010*	0.459
Order			
o__Bacteroidales	0.048*	0.130	0.042*
o__Oscillospirales	0.039*	0.137	0.037*
o__Babeliales	0.107	0.010*	0.244
o__Chitinophagales	0.046*	0.870	0.044*
o__Erysipelotrichales	0.047*	0.025*	0.008**

*Means significant difference between two populations ($p < 0.05$). **Means very significant difference between two populations ($p < 0.01$).

Predicted gut microbiome digestion-related function using phylogenetic investigation of communities by reconstruction of unobserved states

Here, we aimed to investigate a possible link between gut traits and gut microbiome digestion-related functional profiles by utilizing the PICRUSt pipeline. KEGG ortholog groups (KOs) were predicted by PICRUSt. PCoA based on KOs (Level 3) revealed that there was an obvious distinct separation of functional gene distribution between the SL and LL groups (Figure 8A). We identified 23 pathways related to digestion, including carbohydrate, amino acid, and lipid metabolism, of which 19 pathways showed significant differences in relative abundance among the three groups (Figure 8B). Some of the amino acid metabolism pathways (i.e., alanine, aspartate, glutamate, valine, leucine, and proline pathways) were highly abundant in the SL group. For some pathways related to carbohydrate metabolism, starch and sucrose metabolism was more enriched in the LL group than in the SL group, while fructose, mannose, and pentose metabolism was more enriched in the ML group. Some pathways related to lipid metabolism (i.e., fatty acid degradation and glycerolipid and ether fatty acid metabolism)

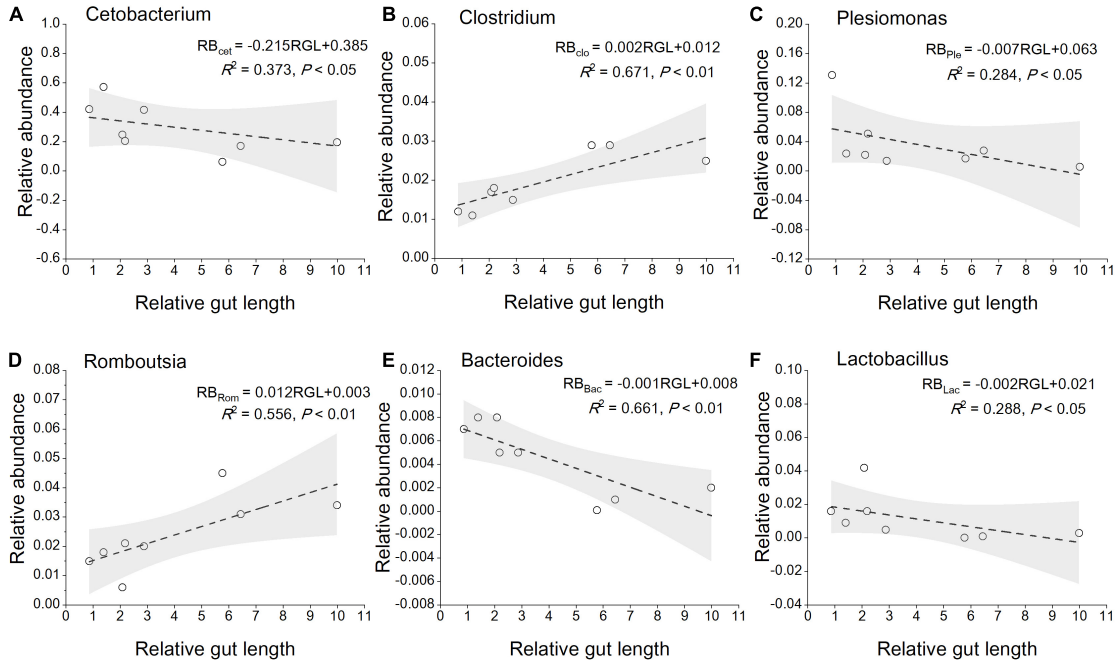

FIGURE 7
Linear regression of relative abundance of gut core microbiome (the genera level) among eight cyprinid fish species vs. mean of their relative gut length, with 95% confidence interval. **(A)** Cetobacterium; **(B)** clostridium; **(C)** plesiomonas; **(D)** romboutsia; **(E)** bacteroides; **(F)** lactobacillus. RGL, Relative gut length; RB, Relative abundance; Cet, Cetobacterium; Clo, Clostridium; Ple, Plesiomonas; Rom, Romboutsia; Bac, Bacteroides; Lac, Lactobacillus.

were more enriched in the SL group than in the ML and LL groups. There was a positive linear relationship between gut microbiome carbohydrate metabolism and relative gut length, whereas a negative correlation between gut microbiome amino acid, lipid metabolism, and relative gut length was observed (**Figure 8C**).

Discussion

In this study, the dominant microbiome groups in eight cyprinid species were Firmicutes, Fusobacteriota, Proteobacteria, and Bacteroidetes. These four phyla represented more than 80% of the sequences, which is consistent with a previous research, indicating the commonality of many fish gut communities (Ghanbari et al., 2015). Moreover, the relative abundance of the same phylum in the different species showed an obvious divergence (**Figure 2A**). Several studies have established that fish gut microbial community compositions are distinct among different species, even among those co-habiting in the same environment (Eichmiller et al., 2016; Baldo et al., 2017). Differentiation of the fish gut microbiome can frequently be predicted by host variable exogenous diet, environmental conditions, and phylogeny (Fishelson et al., 1985; Su et al., 2020; Liu et al., 2021). In this study, we preliminarily investigated the relationship between cyprinid fish hosts CO1 genetic distance and gut microbiome dissimilarity. No obvious correlation signal was observed, which is consistent with the study of Escalas et al. (2021). Relevant research has shown that host genetic relatedness based on variation in the CO1 gene showed no significant association between similarity in the composition of the gut microbial community and host phylogenetic distance by investigating gut microbiota of 85 different fish species (Kim et al., 2021). The fish gut microbiome is affected by multiple complex factors, especially in the natural aquatic ecosystem. Levin et al. (2021) also found that the composition, diversity, and function of gut microbes in wild animals are closely related to host species, habitat, dietary habits, circadian rhythms, and social structure by analyzing the gut microbiota of over 180 species in the wild. Specifically, the host dietary strategy was also considered as one of the most important factors to influence the gut microbiome (Ley et al., 2008; Youngblut et al., 2019). Regardless of the phylogenetic distance among hosts, the dissimilarities of gut microbial communities between fish taxa were randomly distributed (Kim et al., 2021). Therefore, we speculated that it is hard to discern whether host genetic factors have a direct effect on the physiological control of the gut microbiome.

For alpha-diversity analysis, we found that the Shannon index of fish samples was negatively associated with their relative gut length (**Figure 3B**). These correlations were relatively low but significant. Relevant research demonstrated that gut

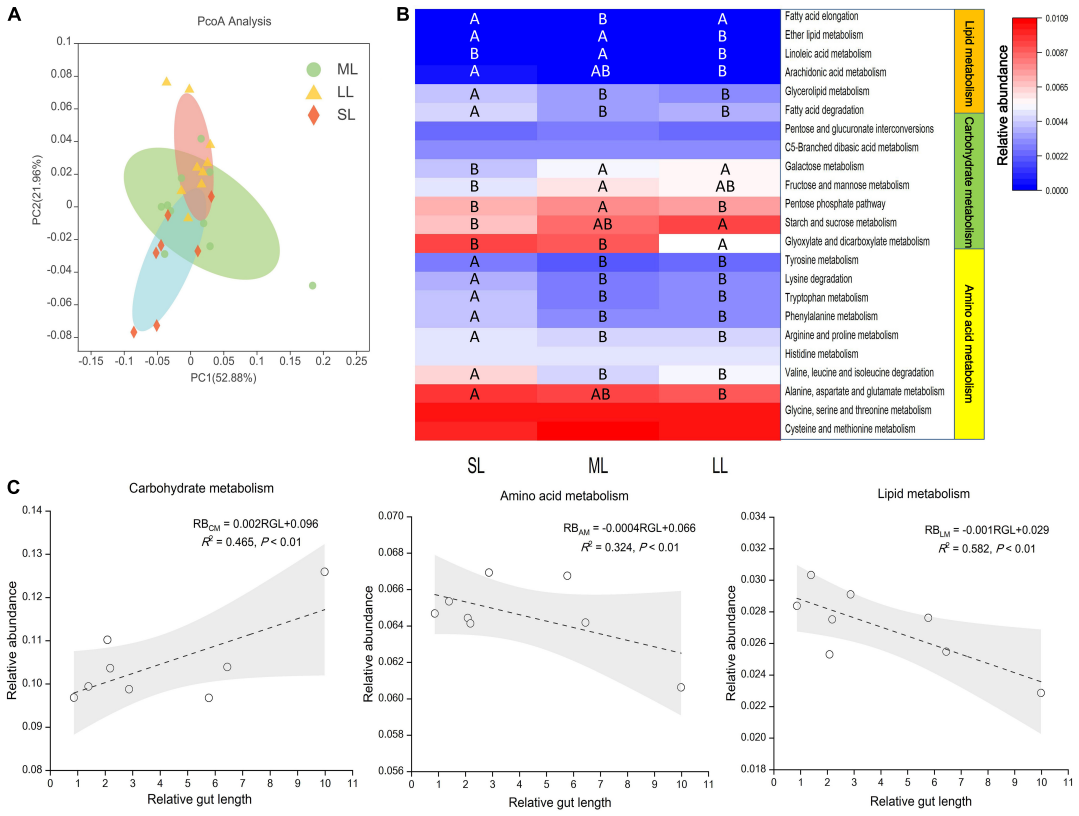

FIGURE 8
KEGG categories derived from the 16S rRNA sequences of the fish gut microbiomes by PICRUSt. **(A)** PCoA of the binary Jaccard dissimilarity of the functional profiles (Level 3). **(B)** The Heatmap presenting the relative abundance of digestion-related bacterial gene functions among the three gut length groups. Samples marked different capital letters indicate significant differences (A > B > C; $p < 0.05$) among relative gut length groups. **(C)** Linear regression of relative abundance of digestion-related bacterial gene functions among eight Cyprinidae fish species vs. mean of their relative gut length, with 95% confidence interval.

morphology and diet were the strongest determinants of the gut microbiome in Sparidae (Escalas et al., 2021). Fish gut length and relative gut length were found to be conserved in host phylogeny and were regarded as an important specialized trait for exogenous resource utilization (Greene et al., 2020). Here, we found a clear separation between XD, SC, and MC samples and CA and TC samples (**Figure 4A**), indicating that omnivorous fish gut microbiome had a close relationship with their relative gut length. Gut length is central to one of the most important organismal processes, namely, the digestion of prey sourced from the environment, and, as such, it is likely to close links to functional roles (Ghilardi et al., 2021). Furthermore, gut length has already been proved to be negatively correlated with the trophic level in fish (Elliott and Bellwood, 2003). In the current study, we found fish gut length and relative gut length to be significantly correlated with the relative abundance of Bacteroidota. In general, consumers with longer guts can acquire more energy and nutrients from low nutritional food (i.e., aquatic plants and algae). Some previous studies have shown that Bacteroidota can assist the host by utilizing exogenous nutrition by the efficient degradation of polysaccharides (i.e., cellulose), which the host alone would not be able to degrade efficiently (Glenwright et al., 2017; Greene et al., 2020).

At the beta-diversity level, the gut microbiome of the SL and LL groups had an obvious differentiation, potentially indicating differences in the competition and utilization of exogenous nutrition (Roggenbuck et al., 2014; Greene et al., 2020). The SL group includes zoobenthos and zooplankton specialists, and their gut microbiota comprised more abundant Proteobacteria and less-abundant Firmicutes than the ML and LL groups (**Figure 6C**). It has been found that the function of these Proteobacteria is typically associated with shorter gastrointestinal systems, and only rarely with plant fiber (Dill-McFarland et al., 2016; Greene et al., 2019). Additionally, related research indicates that Proteobacteria are dominant in carnivorous fish (Kim et al., 2007; Merrifield et al., 2009; Liu et al., 2016). Firmicutes were more abundant than Proteobacteria in hosts with herbivorous diet (Gharechahi et al., 2021). In this study, we identified a dramatically different relative abundance of Oscillospirales and Chitinophagales among the three groups (**Figure 6C**). Further research has

proved that Oscillospirales can help the host to ferment complex plant carbohydrates (Konikoff and Gophna, 2016; Gharechahi et al., 2021). As shown by previous research, some species in the order Chitinophagales can degrade chitin and organic matter (Hou et al., 2021). We found a negative correlation between gut core microbiome (*Cetobacterium*, *Bacteroides*, *Lactobacillus*) and relative gut length. According to previous research, *Cetobacterium* in the gut of fish host is closely linked to host protein digestion (Larsen et al., 2014; Liu et al., 2016). One vital gut microflora, *Lactobacillus*, can effectively help some animal hosts degrade polyunsaturated fatty acids (Falcinelli et al., 2015). Moreover, *Bacteroides* plays an important role in fish host glucose and lipid metabolism (Bjursell et al., 2011). The relative abundance of *Clostridium* increased with relative gut length, indicating potential enhancement of fish host carbohydrate metabolism, such as in the degradation of plant polysaccharides (Larsen et al., 2014).

Further prediction analysis of microbial metabolic function indicated that the LL group had a weaker amino acid and lipid metabolism, and stronger carbohydrate metabolism than the SL group. Our result also provides evidence of a positive linear relationship between gut microbiome carbohydrate metabolism and relative gut length, and a negative correlation between gut microbiome amino acid lipid metabolism, and relative gut length (**Figure 8C**). The result is consistent with the idea that longer gut length is associated with lower nutrient levels (Olsson et al., 2007; Zandonà et al., 2015; Ghilardi et al., 2021). Related research reveals that intestinal microbes play an important role in host nutrient metabolism, reflecting the host's utilization of an exogenous diet (Huang et al., 2020; Zhu et al., 2021). On the whole, we found discrepancy in the gut microbiota of multiple trophic niches overlapping species, which could potentially reflect exogenous resources utilized efficiency of these species in similar habitat and environments (De León et al., 2014).

Cyprinid fish species may coexist because they have differentiated resource use and thus do not significantly interact, or they can coexist with partially overlapping resources use if they are nearly equivalent in their average competitive abilities (Yang and Hui, 2020). Dietary partitioning is one axis by which sympatric species avoid competition, and is typically documented by cataloging diets to show a minimal overlap (German and Horn, 2006; Ghilardi et al., 2021; Herrera et al., 2022). Omnivorous species that compete with each other for resources improve their ecological adaptability through gut trait regulation. A relevant researcher has suggested that morphological variation can promote bat dietary niche evolution (Chang et al., 2019). Gut length and associated gut microbiota specialization are closely related to changes in host dietary niche differentiation as compensation for species coexistence (Saavedra et al., 2017; Yang and Hui, 2020). In addition, the gut microbiome that inhabits fish guts is metabolically versatile, and, *via* its role in digesting substrates otherwise unavailable to hosts, they can help the host to expand their dietary options (Greene et al., 2020). Specifically, specialized gut microbiota and associated gut morphology enable fish in the LL group to variably tolerate resource fluctuation and support nutrient extraction from challenging resources (e.g., metabolizing plant secondary compounds or recalcitrant fibers), perhaps ultimately facilitating host species diversity and specialized feeding ecologies. Additionally, intestinal microbes in different fish species showed significant different utilization efficiency for exogenous food resources, and this may be one of the potential factors in improving host ecological adaptability in aquatic ecosystems. Nevertheless, how the underlying effects of the gut microbiome affect multi-fish species coexistence and trophic differentiation is still unclear. Therefore, the functional role of the colonization of symbiosis microbiome in fish host ecological adaptability needs further evaluation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary material**.

Ethics statement

The animal study was reviewed and approved by the methods involving animals in this study were conducted in accordance with the Laboratory Animal Management Principles of China. All experimental protocols were approved by the Ethics Committee of the Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences.

Author contributions

YQL: data collection, conceptualization, data curation and analysis, and writing—original draft. XL: writing—review and editing. JL: funding acquisition and supervision. YFL: formal analysis. SZ: data collection. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Diet drives the gut microbiome composition and assembly processes in winter migratory birds in the Poyang Lake wetland, China

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The complex gut bacterial communities may facilitate the function, distribution, and diversity of birds. For migratory birds, long-distance traveling poses selection pressures on their gut microbiota, ultimately affecting the birds' health, fitness, ecology, and evolution. However, our understanding of mechanisms that underlie the assembly of the gut microbiome of migratory birds is limited. In this study, the gut microbiota of winter migratory birds in the Poyang Lake wetland was characterized using MiSeq sequencing of 16S rRNA genes. The sampled bird included herbivorous, carnivorous, and omnivorous birds from a total of 17 species of 8 families. Our results showed that the gut microbiota of migratory birds was dominated by four major bacterial phyla: Firmicutes (47.8%), Proteobacteria (18.2%), Fusobacteria (12.6%), and Bacteroidetes (9.1%). Dietary specialization outweighed the phylogeny of birds as an important factor governing the gut microbiome, mainly through regulating the deterministic processes of homogeneous selection and stochastic processes of homogeneous dispersal balance. Moreover, the omnivorous had more bacterial diversity than the herbivorous and carnivorous. Microbial networks for the gut microbiome of the herbivorous and carnivorous were less integrated, i.e., had lower average node degree and greater decreased network stability upon node attack removal than those of the omnivorous birds. Our findings advance the understanding of host-microbiota interactions and the evolution of migratory bird dietary flexibility and diversification.

KEYWORDS

microbiome, host phylogeny, ecological assembly, network robustness, migratory birds

Introduction

Migratory birds travel a long distance annually between the breeding grounds, resting areas, and wintering grounds (Kreisinger et al., 2017). This unique behavior exposes migratory birds to complex diets and varying living environments, which may affect the host–microbe interactions and lead to changes in the gut microbial community

(McCormick et al., 2013; Grond et al., 2018; Bodawatta et al., 2021). The avian gut microbiome is essential in improving host bird fitness, adaptability, and evolution (Grond et al., 2018). Therefore, understanding factors that impact the assembly of the gut microbiome can help to establish a compressive view of the interactions between migratory birds and their environment. This knowledge will improve our understanding of the health and fitness of migratory birds, particularly in the context of enhanced environmental disturbances and global change.

Both host phylogeny and diet are major regulators of gut microbiota (Hird et al., 2015; Lewis et al., 2016; Zhang et al., 2021). Hosts can serve as a "habitat filter" allowing preferentially colonizing specific microbes in the gut that can provide mutual benefits to the host (John et al., 2010; Stegen et al., 2013; Sieber et al., 2019). Host phylogeny of birds has been found to explain most of the variation in gut microbial composition, followed closely by ecological variables, such as local habitat and diet (Hird et al., 2014; Waite and Taylor, 2014). Several scientists have attempted to link gut microbial community composition to avian phylogenetic identity but yielded no congruent conclusions (Capunitan et al., 2020; Trevellineet et al., 2020). Moreover, a recent study found weak signatures of phylosymbiosis among 15 species of cranes (family Gruidae) housed in the same captive environment and maintained on identical diets (Trevellineet et al., 2020).

Diets are considered one of the main sources of microbial colonizers for guts, as they provide nutrients to both hosts and gut microbes while requiring different digestive requirements assisted by specialized microbial communities (Laparra and Sanz, 2010; Grond et al., 2018). In general, migratory birds are exposed to a wider range of diets (food-associated microorganisms) than nonmigratory birds. Hence, the change in diet due to geographical variation may affect the gut microbiome of migratory birds greater than nonmigratory birds (Grond et al., 2018). We hypothesized that diets play more significant roles than host phylogeny in the gut microbial community of migratory birds. However, which processes drive the assembly and shift of migratory bird gut microbiome remains unknown.

Differential selection of microbes by the host is assumed to drive variation in microbial communities (Mazel et al., 2018). Host hosts may be exposed to the same pool of potential microbial colonizers but then filter and select certain microbes to persist as their symbionts. However, some theoretical studies have suggested that for certain host-associated habitats, such as the vertebrate gut, host selection may not fully explain the observed patterns (Mazel et al., 2018); other ecological and evolutionary processes may also be contributing factors (Kohl, 2020). Stegen et al. (2013) proposed five main mechanisms of microbial community assembly, including variable selection, homogeneous selection, homogeneous dispersal, dispersal limitation, and undominated processes (Stegen et al., 2013). Selection may cause communities to converge if they undergo similar environmental conditions (homogeneous selection) or diverge if they undergo distinct environmental conditions (variable selection; Chase and Myers, 2011; Ge et al., 2021). Depending on the magnitude of dispersal, it can also converge or diverge communities. Ecological drift results in stochastic population fluctuation of component species in communities by chance birth and death events and, thus, generally disperses communities (Ge et al., 2021). Dietary specialization can impact the assembly of the gut microbial community in migratory birds by enhancing the variable selection contribution of specific processes (Kohl, 2020).

We tested the hypothesis using samples from Poyang Lake (PYL; 28°22′-29°45′N, 115°47′-116°45′E) wetland. PYL wetland is one of the world's six major wetland systems and is located in the lower and middle reaches of the Yangtze River, China (Mei et al., 2016). PYL serves as a critical wintering habitat for migratory birds along the East Asian-Australasian Flyway, which supports about 76 species and 500, 000 individual migratory birds during the wintering period each year (Wang et al., 2016; Yang et al., 2020).

Materials and methods

Sample collection

This work was performed at the Nanjishan Wetland Nature Reserve (NJNR) and Duchang Migratory bird Nature Reserve (DCNR), which are located in the southwestern and northeastern parts of PYL, respectively (Figure 1). Most of the migratory birds [e.g., Siberian cranes (*Grus leucogetanus*), oriental storks (*Ciconia boyciana*), and swan geese (*Anser cygnoides*)] were distributed in the NJNR and DCNR (Barter et al., 2005; Aharon-Rotman et al., 2017), without significant site differences. These migratory birds typically arrive at PYL starting in October and do not depart until the following March (Xia et al., 2016). There is no geographical isolation between the two sites, and migratory birds can fly freely in between.

Fresh feces of migratory birds were collected from January 2019 to February 2020; this sampling time overlapped with the ongoing NJNR and DCNR monitoring projects. During sampling, locations with large flocks of single bird populations (i.e., > 200 individuals) were chosen for the bird feces collection. All fresh feces were collected and transferred promptly into sterile tubes after the birds had flown away. Specifically, solid feces (excreted by herbivorous and omnivorous birds) were directly picked up from the ground by hands with sterile gloves, while liquid feces (excreted by carnivorous birds) were collected by gently scraping (avoiding obvious soil particles) the soil surface using a sterilized spoon. The freshness of fecal samples was confirmed by observation of instant defecation and the presence of a high moisture sheen (Cox et al., 2005). To ensure samples from multiple individuals, we collected feces at least 2 m away from each other and changed disposable gloves for each sample (Cao et al., 2020). One hundred and eighty samples from 17 species were frozen on dry ice immediately and then stored at −80°C until DNA extraction. Migratory bird species associated with feces samples

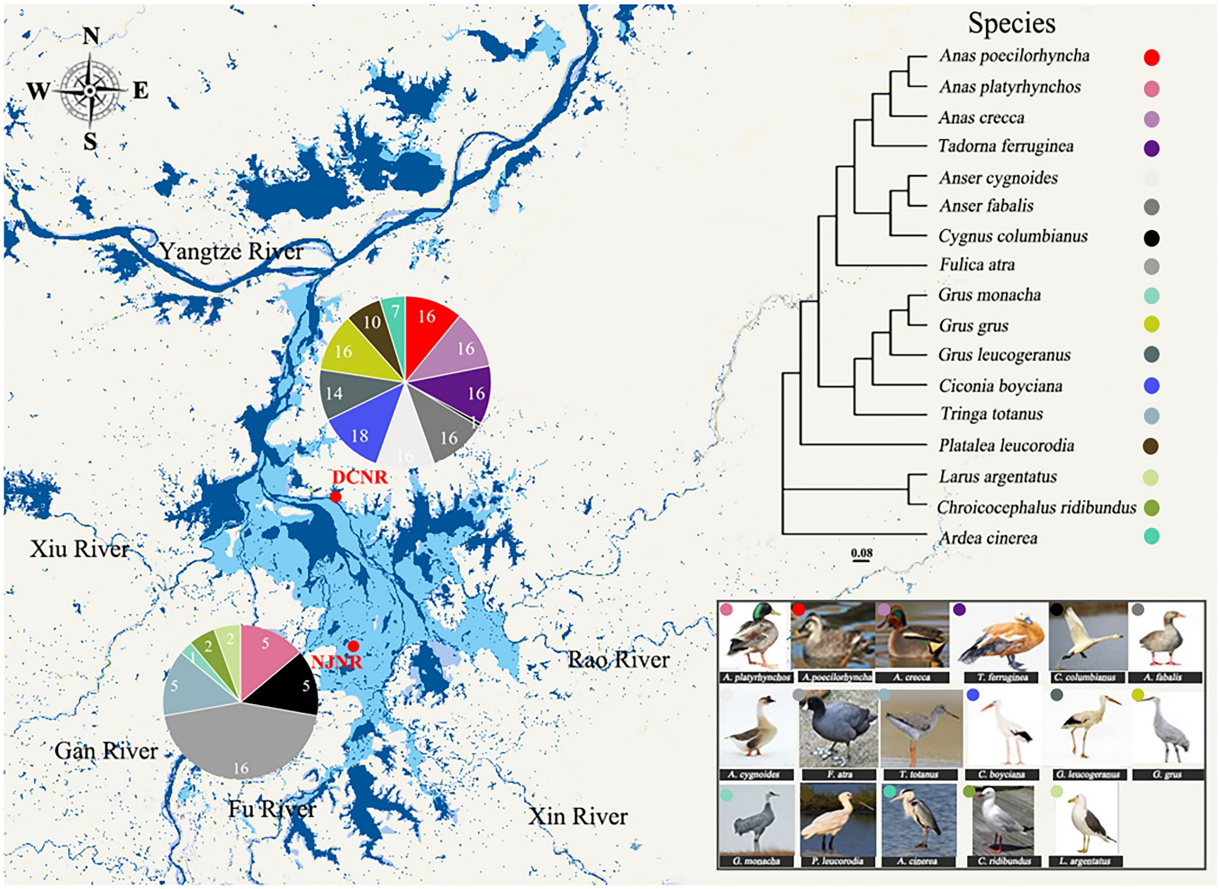

FIGURE 1
Overview of migratory bird feces samples collected from the Poyang Lake (PYL). Pie charts represent the number of fecal samples from each species at a different site. A phylogenetic tree based on partial mitochondrial gene sequences shows the lineage of the 17 bird species.

were identified with the assistance of NJNR and DCNR protection station personnel based on methods described by Barter et al. (2005) and a waterbird survey manual (Bai et al., 2021). Since the samples were collected by picking up the feces of migratory birds, the effects of age and gender on gut microbes were not considered in this study.

Host phylogeny and classification by diets

The complete mitochondrial 16S rRNA and cytochrome b (Cyt b) gene homologous sequences of migratory bird species were obtained using the National Center for Biotechnology Information (NCBI) blast server.[1] A phylogenetic tree was created using the Neighbor-Joining algorithm using MEGA X (Kumar et al., 2018).[2] Sequences of the 16S rRNA region were aligned using MAFFT (Katoh et al., 2002), and the host phylogeny of birds was constructed using Fast Tree (Price et al., 2010). According to their main foraging areas and diets in PYL, the migratory birds were further classified into three guilds: herbivorous, omnivorous, and carnivorous birds (Barzen et al., 2009; Wang et al., 2013; Xia et al., 2016).

DNA extractions and high-throughput sequencing of fecal microorganisms

DNA was extracted from fresh feces (0.2 g) using the Power Fecal DNA Isolation Kit (MOBIO, United States), following the manufacturer's instructions (Vo and Jedlicka, 2014). Extracted DNA was quantified using a NanoDrop ND-2000 spectrophotometer (Thermo-Fisher Scientific, California, USA) and then sent to Majorbio Co. Ltd. (Shanghai, China) for 16S rRNA gene (V3-V4 hypervariable region: 338F 5′ACTCCTACGGGAGGCAGCA3′ and 806R 5′ GGACTACHVGGGTWTCTAAT 3′) paired-end sequencing (2 × 300 bp reads) using the Illumina MiSeq platform (Mori et al.,

1 http://blast.ncbi.nlm.nih.gov/Blast.cgi
2 https://www.megasoftware.net

2014). Raw sequences of partial 16S rRNA genes were deposited in the NCBI Sequence Read Archive (SRA) under accession no. PRJNA736842.

Sequence analysis

The raw data of Illumina sequences were demultiplexed, quality-filtered, and merged using QIIME2 (v2020.02; Bolyen et al., 2019).[3] The processed sequences were used to reconstruct amplicon sequence variants (ASVs) by the Divisive Amplicon Denoising Algorithm (DADA 2; Callahan et al., 2016). Obtained ASVs were taxonomically annotated by the pre-trained Naive Bayes classifier (Bokulich et al., 2018) based on the database SILVA 138 at a confidence level of 70%. Afterward, non-bacterial sequences such as mitochondrial and chloroplasts were removed according to the annotation results (Glöckner, 2019). The PICRUSt2 (V2.4.1; Douglas et al., 2020) was used to predict the functional potentials of bacterial communities based on 16S rRNA gene sequences, abundance, and the Integrated Microbial Genomes (IMG).[4]

Statistical analysis

Potential differences in microbial communities among samples were analyzed using nonmetric multidimensional scaling (NMDS) based Bray-Curtis and weighted UniFrac distances using the vegan package in R software (R Core Team, 2016). The alpha-diversity indices, including Shannon, Chao1, Simpson, and Faith's phylogenetic diversity (Faith's PD), were calculated based on the ASV level by "adiv," an R package to analyze biodiversity in ecology (Pavoine, 2021). Furthermore, the phylogenetic generalized linear mixed models were used to test the effect of diet and sampling time on the alpha diversity of the gut microbiome using the R package "lme4" (Douglas et al., 2015). The phylosymbiosis testing was used to examine the potential role of host phylogeny in microbiome structures (Trevellineet et al., 2020; Bodawatta et al., 2021). The values range from-1 (perfect clustering of dissimilar samples) to 1 (perfect clustering of similar samples), with 0 indicating a perfect random association between microbial communities and host phylogeny. The patterns of phylosymbiosis were used to compare the gut microbiota dendrograms with the bird phylogeny *via* the Robinson–Foulds and matching cluster metrics with 100,000 random trees using the Python script (Brooks et al., 2016). To compare the alpha diversity of the multiple diet groups, we did Kruskal–Wallis rank-sum test followed by a pairwise Wilcoxon rank-sum test with the adjustment of p values by Benjamini–Hochberg FDR correction at 0.05. A heatmap was generated using R at the phylum level.

The microbial diversity analyses at the ASVs level were performed with sample size and coverage-based integrations of interpolation (rarefaction) and extrapolation (prediction) of the Hill numbers (Hill, 1973; Alberdi and Gilbert, 2019; Roswell et al., 2021) using the R packages "iNEXT" and "iNextPD" (Chao et al., 2014, 2015; Hsieh et al., 2016). Hill numbers were computed using the scaling parameter q (Jost, 2007), corresponding to increasing weight on the species abundance and phylogenetic diversity indices. The larger the q value, the greater the importance of ASVs attributed to the abundance of ASVs. When $q = 0$, the rare species (those with low abundance) were counted; when $q = 1$, the common species (those with medium-high abundances) were measured; when $q = 2$, the dominant species (those with very high abundance) were counted.

To predict the potential interactions among individual ASVs, the phylogenetic molecular ecology networks (pMENs) were constructed at the ASV level using random matrix theory (RMT) models after Pearson correlation estimation (Zhou et al., 2011). All molecular ecology networks (MENs) were constructed based on Pearson correlations of the log-transformed ASV abundance, followed by an RMT-based approach that automatically determines the correlation cut-off threshold (Luo et al., 2007; Zhou et al., 2013). To reduce the complexity of the datasets, the ASVs that presented in no more than five samples were removed before the network constructions (Waite et al., 2018). A set of topological features, including average clustering coefficient, average path length, network diameter, graph density, average degree, and modularity, was calculated using "igraph" in the R package (Csardi and Nepusz, 2006). The network plot was visualized with Gephi 0.9.2 software (Bastian et al., 2009). Each node represented one ASV, and each edge represented a strong and significant correlation between two ASVs. Network stability was evaluated by removing nodes in the static network to estimate how quickly robustness degraded, and network robustness was assessed by the nodes' degree distribution and natural connectivity. The node removal was followed by the random repetitive principle (Peng and Wu, 2016).

Quantification of ecological processes of microbial communities

Mean-nearest-taxon-distance (MNTD) and the nearest-taxon-index (NTI) were used to characterize the phylogenetic community composition of each sample using "mntd" and "ses. Mntd" in package "Picante" by R (Webb et al., 2002). Nearest-taxon-index quantified the number of standard deviations that the observed MNTD from the mean of the null distribution (999 randomizations). The abundance-weighted mean was then taken across these phylogenetic distances. Null model distribution of βMNTD (Pawlowsky-Glahn and Buccianti, 2011) was built by shuffling bacterial taxa among the tips of the phylogenetic tree or between different communities using randomization and permutation analyses. The β-nearest taxon index(βNTI), a

3 http://www.qiime2.org

4 https://img.jgi.doe.gov/cgi-bin/m/main.cgi/m/main.cgi

standardized measure of the βMNTD, was then generated by comparing the observed and the null distribution of βMNTD, using the following formula: βNTI = (βMNTDobs-βMNTDnull)/sd ($βMNTD_{null}$).

To examine the potential effects of stochastic and deterministic processes in the assembly of gut microbial communities, we calculated the Levins' niche breadth (B) index using the "niche width" function in the R package "spaa" (Zhang, 2016). A given ASV with a high B value represents a wide habitat niche breadth. The community-level B value was calculated as the average of B values from all taxa in one given community (Jiao et al., 2019; Mo et al., 2021).

Results

Classification of migratory birds

A total of 180 fecal samples were collected. They were from a total of 17 unique bird species of 8 families, including Ardeidae, Anatidae, Ciconiidae, Gruidae, Laridae, Rallidae, Scolopacidae, and Threskiornithidae (Table 1). The branching pattern of the host phylogeny tree illustrated the evolutionary relationships of these species, with the *Anas poecilorhyncha* (APo) and *Anas platyrhynchos* (APl) clustered together as the most closely related but distant from other species (Figure 1). According to the main foraging areas and diets, the populations were divided into three dietary groups: herbivorous (6 species, 69 individuals), omnivorous (5 species, 69 individuals), and carnivorous (6 species, 42 individuals). The herbivorous exploit the stems and leaves of *Carex*, the tubers of *Vallisneria* spp., and *Polygonum criopolitanum*, which are abundant in the PYL wetland. In contrast, carnivores use water invertebrates and fish as their main food. The omnivorous feed on food which the herbivorous and carnivorous also forage.

Bird gut microbiome diversity

A total of 5,460,000 high-quality 16S rRNA gene sequences were recovered, and these were assigned to 6,129 unique amplicon sequence variants (ASVs) after rarefaction. The dilution curve showed an adequate sequencing depth covering most of the diversity of the fecal bacteria community (Supplementary Figure S1). Based on these ASVs, the alpha-diversity indices of the gut microbiome were calculated (Figure 2A, Kruskal–Waillis test, FDR-adjusted $p = 0.001$). Results of phylogenetic generalized linear mixed models revealed that diet had a significant effect on the α-diversity of the gut microbiome ($p = 0.009$). Furthermore, omnivorous species had higher gut microbiome diversity than herbivorous and carnivorous species based on Shannon, Chao1, and Faith's PD indices. Nonetheless, the α-diversity of gut microbes was significantly different among diet groups, which was mainly caused by rare taxa (Figure 2B, Kruskal–Waillis test, FDR-adjusted $p = 0.001$) as revealed by the value of the order parameter (q). Although there was considerable variation among individuals of the same bird population, the species-based difference in α-diversity of gut microbes was still significant. *Larus ridibundus* (LR) had the lowest, but *Anas crecca* (ACr) had the highest gut microbial diversity based on both Shannon and Chao1 indices (Supplementary Table S1; Supplementary Figure S2).

The gut microbial ASVs of the herbivorous were different from those of the carnivorous based on the results of ANOSIM and ADONIS analyses (Supplementary Table S2). The gut microbial community has the greatest difference between herbivorous and carnivorous. Permutational multivariate analysis of variance (PERMANOVA) was performed to determine the relative importance of host phylogeny, diet, sampling time, and their interaction in explaining microbial composition. The results revealed that diet was a major predictor for the gut microbial community of migratory birds ($r^2 = 0.201$, $p = 0.001$), whereas host phylogeny was a weaker predictor ($r^2 = 0.141$, $p = 0.001$). The effect of sampling time is small ($r^2 = 0.014$, $p = 0.001$), which was ignored in the subsequent analysis. The interaction of host phylogeny and diet had a stronger relationship with gut microbial composition than either host species or habitat alone ($r^2 = 0.300$, $p = 0.001$). In NMDS analysis, the gut microbial assemblage was clustered by diet groups (Figure 2C). Based on the result of topological congruence, the bird phylogeny was not associated with the microbial dendrograms (nRF = 1.0, $p = 1.0$; Figure 2D).

Bird gut microbial taxonomic composition

All ASVs of the gut microbiome were restricted within nine bacterial phyla, and four were the most dominant (> 5% of average sequences), including Firmicutes (47.8% of sequences on average), Proteobacteria (18.2%), Fusobacteria (12.6%), and Bacteroidetes (9.1%), accounted for 87.7% of the total sequences collectively (Figure 3A; Supplementary Figure S3). Firmicutes was the most abundant phylum in the gut microbiome in all three diet groups and accounted for 58.0% in the herbivorous, 36.0% in the omnivorous, and 50.0% in the carnivorous. The relative abundance of Fusobacteria in omnivorous (16.5%) and carnivorous (19.8%) birds' gut microbiome was higher than in the herbivorous (4.4%; Supplementary Figure S2; Krauskal–Waillis test, $p = 0.001$). Furthermore, the results of the heatmap based on Bray–Curtis distance (Figure 3B) revealed the dissimilarity of gut microbial communities among bird species. Firmicutes was the most abundant phylum in the gut microbial communities of all bird species. Deferribacterota, Dadabacteria and Sumerlaeota were the most abundant in ACi. Samples from the same diet groups (i.e., CB and LA) tended to have similar gut microbial structures unrelated to the host phylogeny (Figure 3B).

A total of 1,005 genera were identified in the gut microbiome. The most dominant genera were *Clostridium* (Firmicutes phylum; 9.6% of sequences on average), *Catellicoccus* (Firmicutes; 5.7%),

TABLE 1 Migratory bird species associated information in this study, including general diet, taxonomy, abbreviations, common names, and the number of samples; localities related to samples were mapped in Figure 1.

Diet	Family	Species	Species abbreviations	Common name	Sample size
Herbivorous	*Anatidae*	*Anser fabalis*	AF	Bean goose	16
	Anatidae	*Anser cygnoides*	ACy	Swan goose	16
	Anatidae	*Cygnus columbianus*	CC	Tundra swan	6
	Gruidea	*Grus leucogeranus*	GL	Siberian crane	14
	Gruidea	*Grus grus*	GG	Common crane	16
	Gruidea	*Grus monacha*	GM	Hooded crane	1
Omnivorous	*Anatidae*	*Anas crecca*	ACr	Common teal	16
	Anatidae	*Anas platyrhynchos*	APl	Mallard	5
	Anatidae	*Anas poecilorhyncha*	APo	Spot-billed duck	16
	Anatidae	*Tadorna ferruginea*	TF	Ruddy shelduck	16
	Rallidae	*Fulica atra*	FA	Common coot	16
Carnivorous	*Ardeidae*	*Ardea cinerea*	ACi	Grey heron	7
	Ciconiidae	*Ciconia boyciana*	CB	Oriental stork	16
	Laridae	*Larus argentatus*	LA	Herring gull	2
	Laridae	*Larus ridibundus*	LR	Black-headed gull	2
	Scolopacidae	*Tringa totanus*	TT	Common redshank	5
	Threskiornithidae	*Platalea leucorodia*	PL	Eurasian spoonbill	10
Total 3	8	17	17	–	180

Campylobacter (Proteobacteria; 5.4%), *Escherichia* (Proteobacteria; 2.5%), and *Helicobacter* (Epsilonbacteraeota; 2.7%). These genera were found in all samples. Among diet groups, *Catellicoccus* was the most dominant genus identified in the omnivorous (11.9%) and carnivorous (10.9%), and their abundance in the herbivorous was low (<0.1%). *Clostridium* was the most dominant genus identified in the herbivorous (17.5%), their abundance in the gut of omnivorous (5.9%) and carnivorous birds (4.9%) were significantly lower (Supplementary Figure S3). Additionally, *Streptococcus* and *Campylobacter*, as frequently found species, were identified in avian guts.

A total of 3,871 ASVs for the herbivorous, 4,925 ASVs for the omnivorous, and 3,778 ASVs for the carnivorous were recovered (Figure 3C). Among these, 830 gut ASVs (13.5%, a total of 6,129 ASVs) shared among the three diet groups (Figure 3C). These shared ASVs mainly belonged to Proteobacteria (28.3%), Firmicutes (23.8%), and Bacteroidota (12.7%). The core ASVs were defined as those which occurred in over 50% of migratory birds in each diet group. The core ASVs were identified in the herbivorous (29 ASVs), omnivorous (34 ASVs), and carnivorous (8 ASVs), which differed among different diet groups (Supplementary Table S3). Only two core ASVs belonging to *Rhodococcus-erythropolis* (Actinobacteria) and *Ralstonia* (Proteobacteria) were shared by all diet groups (Figure 3D).

Complexity and stability of gut microbial communities

The pMENs of the gut microbiome at the ASV level among the different bird species were evaluated based on Pearson's correlation coefficient (Figure 4A; Table 2). The overall topology indices showed that the distribution of all network connectivity fitted well with the power-law model (the range of R^2 value was from 0.812 to 0.914). The omnivorous group had more edges than the carnivorous group in the networks. The average path length ranged from 4.629 to 9.392, demonstrating that the average network distance was variable among diet groups. The gut microbial community of the omnivores had the highest node connectivity, with an average degree of 4.169. Meanwhile, the node connectivity of the gut microbial community was the lowest in the omnivorous, with an average degree of 3.055. The value of the modularity index of the three groups was higher than 0.7, suggesting that each network had a modular structure. In pMENs, a higher proportion of negative associations were observed in the omnivorous than in the dietary specialization birds.

Microbial networks for the herbivorous and carnivorous were less integrated than the omnivorous, with a lower average node degree (Figure 4B; Table 2) and greater decreased network stability upon node attack removal (Figure 4C; Table 2). The result showed that the gut microbiome of the omnivorous had the most complex associations, followed by the herbivorous and carnivorous.

Relative importance of deterministic and stochastic processes

Based on the metric of the weighted bacterial community assembly (βNTI) analysis, stochastic processes (58.9%) contributed more to the community composition of migratory birds than the deterministic process (41.1%, Figure 5A; Supplementary Figure S4A). For diet groups, the relative

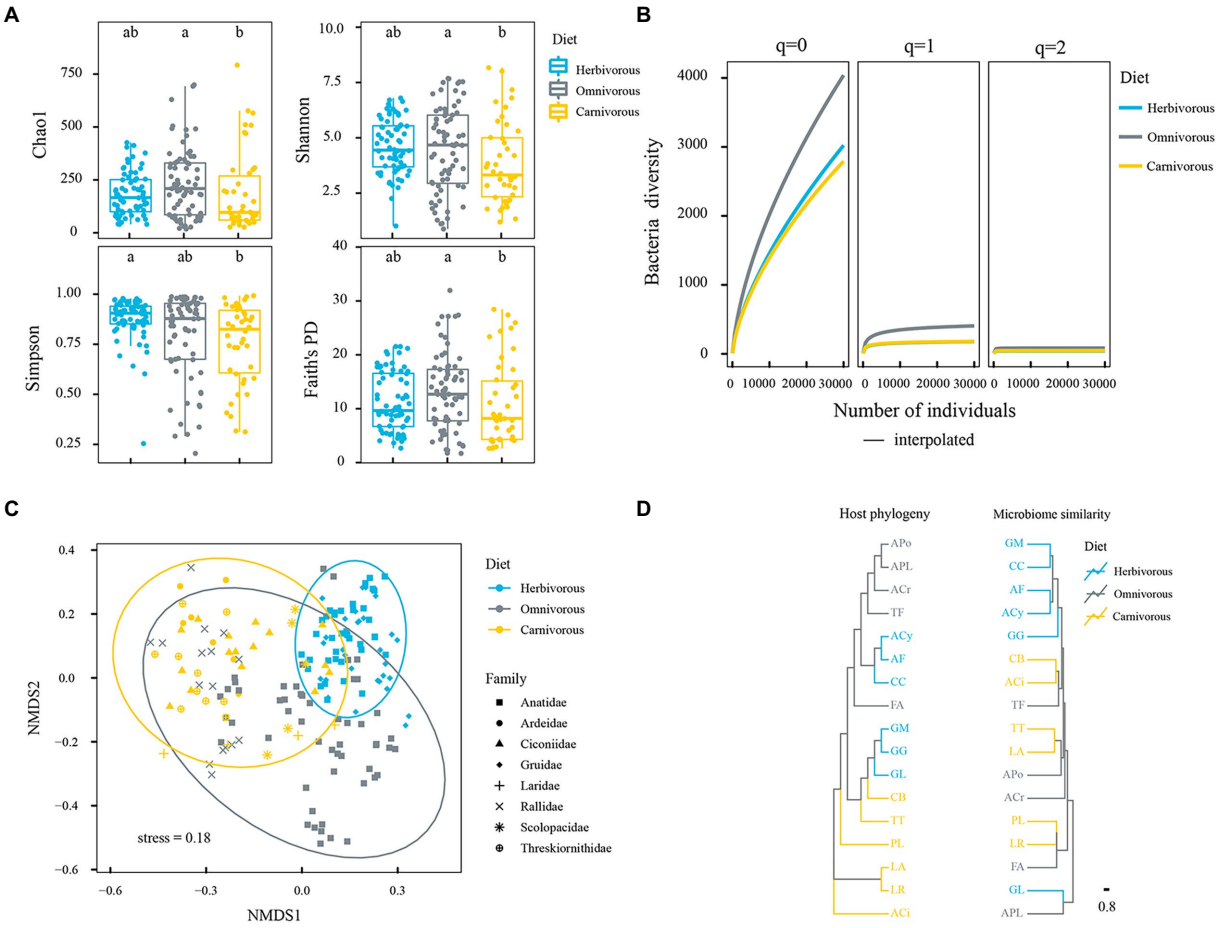

FIGURE 2
Gut microbial diversity among three diet groups. **(A)** The index of Shannon, Simpson, Chao 1, and Faith's phylogenetic diversity for each diet group. Results are shown as box and whiskers, the different letters above columns represent the significant difference ($p<0.05$) by Mann–Whitney *U*-tests adjusted for multiple comparisons; **(B)** coverage based on rarefaction/extrapolation curves of the Hill numbers estimated for three values of the order parameter ($q=0$, $q=1$, and $q=2$). The *x*-axis represents the coverage (that estimates the completeness of the sampling), and the *y*-axis represents the Hill number estimates. 95% confidence interval is also reported; **(C)**: NMDS patterns among diet groups. Ellipses denote a 95% confidence level; **(D)** host phylogeny dendrogram (left panel) obtained using the migratory birds' partial mitochondrial DNA gene sequences compared to dendrogram (right panel) of weighted UniFrac of gut microbiome at ASVs level. The colours correspond to the trophic categories. Blue represents herbivorous, gray represents omnivorous and yellow represents carnivorous.

contribution of stochastic processes explained 63.1% of the gut microbiome assembly in the omnivorous, 57.1% of the gut microbiome assembly in the carnivorous, and 40.5% of the gut microbiome assembly in the herbivorous (Figure 5A; Supplementary Figure S4A). Homogeneous selection (relative contribution, 47.8% in herbivorous birds; 35.8% in carnivorous birds) was the major process driving the gut microbiome to convergence, while homogeneous dispersal (30.6%) was the major process driving omnivorous birds' gut microbiome to convergence (Figure 5A; Supplementary Figure S4A). The drift contribution was much higher for the carnivorous (28.6%) than the herbivorous (5.2%, Figure 5A). The deterministic processes became more critical with increasing dietary specialization. Furthermore, gut microbiome exhibited significantly wider niche breadths in the herbivorous than in the carnivorous/ omnivorous ($p<0.05$; Figure 5B; Supplementary Figure S4B).

Predictions of the function of the gut microbiome

Potential functions of all ASVs were annotated using the PICRUSt2. The majority of functions were clustered into metabolism (11 pathways), cellular processes (4 pathways), genetic information processing (4 pathways), environmental information processing (2 pathways), and organismal systems (1 pathway; Figure 6A). Among them, carbohydrate metabolism and amino acid metabolism were the two main metabolic pathways, and their relative abundance accounted for more than 5% of the whole process (Figure 6A). Most metabolic pathways were similar across the three diet groups ($p>0.05$; Figure 6A).

The relationships between the β-nearest taxon index (βNTI) and functional Bray–Curtis dissimilarity were used to infer the impact of deterministic/stochastic assembly processes on the metabolic

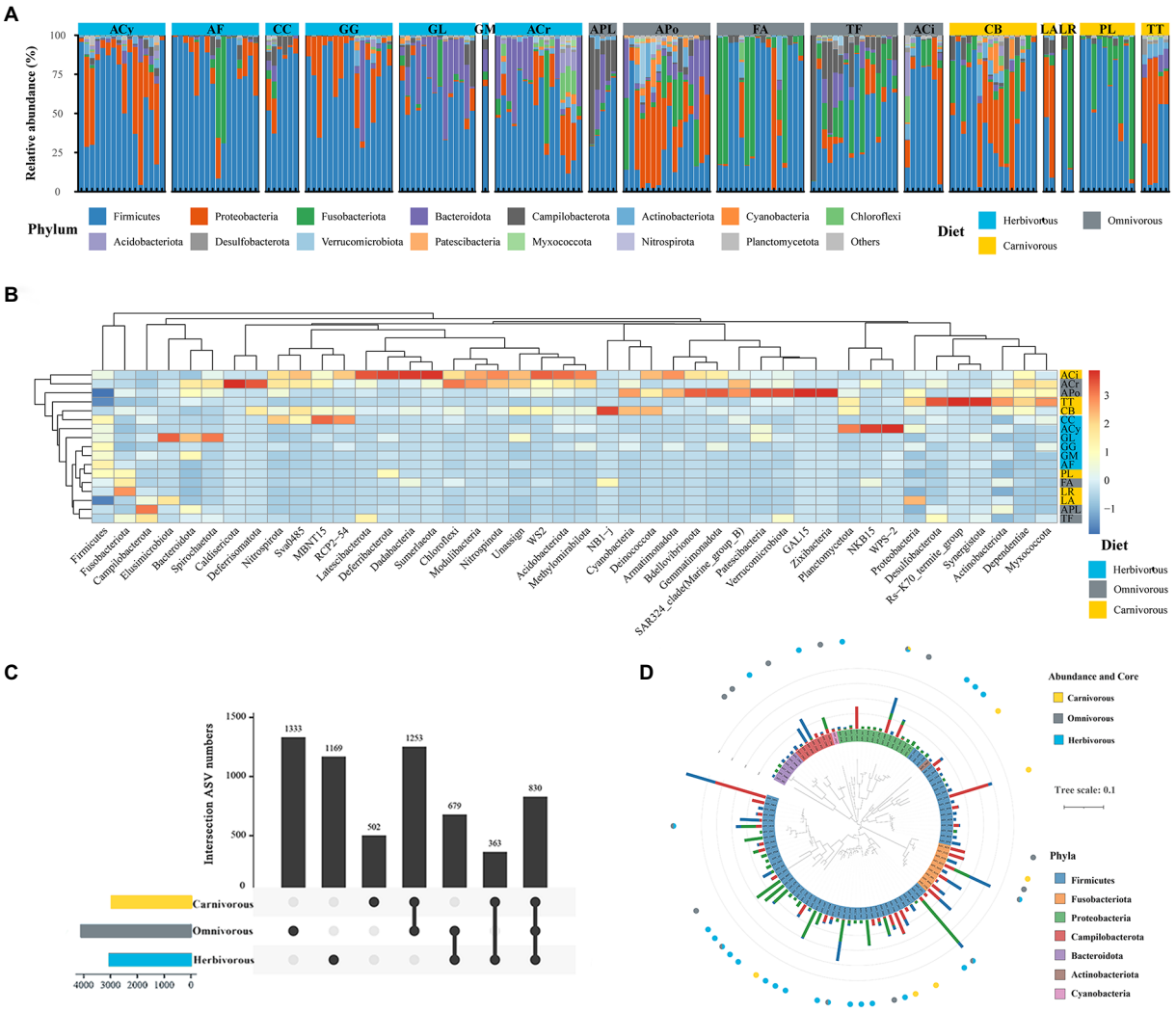

FIGURE 3
Comparison of gut microbiome among different species. **(A)** Abundance; **(B)** heatmap analysis based on Bray–Curtis distance; **(C)** the number of ASVs that are unique and shared among gut microbiome of three groups; **(D)** Phylogenetic tree of the 100 ASVs with a relative abundance of 0.1% in at least one sample. Bars showed the mean relative abundance of the ASVs. The different diets were labelled with blue, gray and yellow, respectively.

functions of the ASVs. The metabolic functions of the ASVs were significantly positively correlated with βNTI (Figure 6B).

Discussion

Diversification and function of gut microbiome among birds

Birds show a remarkable evolutionary diversification of strategies from dietary complex omnivorous mixtures as the ancestral trait to dietary specificity including herbivores and carnivores (Langen and Owens, 2002). In line with prior surveys of mammalian host species (Ley et al., 2008a,b; Perofsky et al., 2019), the omnivorous in this study exhibited the greatest gut microbial diversity, followed by the herbivorous and carnivorous (Figure 2). The diversity of the gut microbiota of migratory birds was correlated with a range of variations in food selection and diet could alter the gut microbial communities (Wu et al., 2011, 2018a,b). The microbial diversity and its versatility may allow the halobiont to function more optimally and adapt rapidly to changing conditions (Yachi and Loreau, 1999).

The predicted function of the gut microbiome reflected differences in carbohydrate and amino acid utilization associated with diet groups of migratory birds (Figure 6A). As found for the herbivorous, the gut microbiome was enriched for folivorous pathways associated with increased plant fiber degradation, such as metabolism of cofactors and vitamins, and lipid metabolism (Moschen et al., 2012; Yang et al., 2016). Along with the carnivorous (Perofsky et al., 2019), the omnivorous microbiome was also

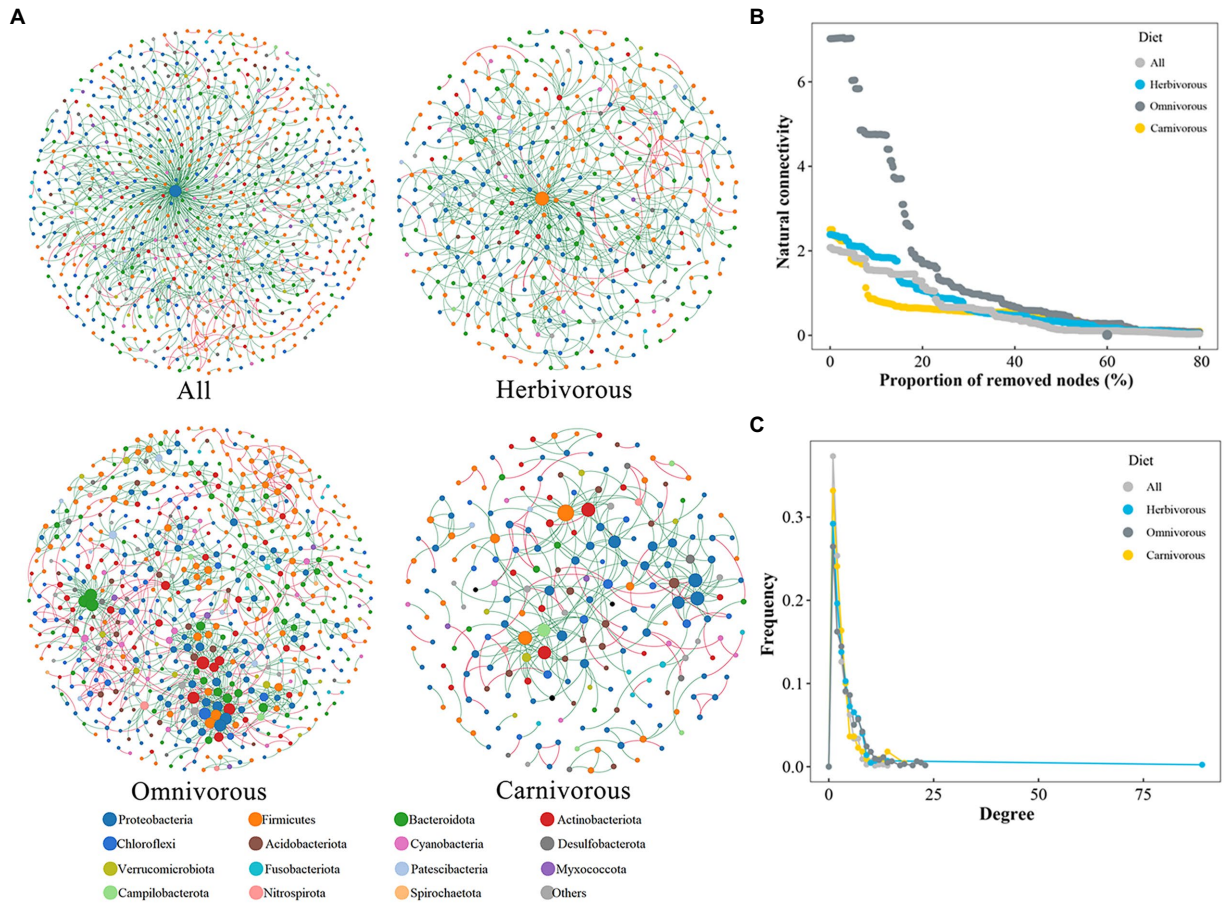

FIGURE 4
The phylogenetic molecular ecology networks and stability (pMENs) based on correlation analysis. **(A)** Networks among gut microbial ASVs. The network nodes are colored according to the taxonomy at the phylum level. A connection indicates a statistically significant (FDR-corrected *p*-value <0.01) positive correlation (red) or a negative correlation (green). The size of each node is proportional to the relative abundance of the ASVs; the thickness of a connection between two nodes (i.e., an edge) is proportional to the value of Spearman's correlation coefficient; **(B)** node degree distribution of the networks. **(C)** The robustness of gut microbiome network.

TABLE 2 The topological features of the gut microbial networks from the herbivorous, omnivorous and carnivorous.

Treatments	Average degree	Diameter	Modularity	Clustering coefficient	Average path length	Node	Edge	*R* square of power-law	Positive/ negative
Herbivorous	3.547	4.07	0.764	0.177	4.791	428	759	0.812	51/708
Omnivorous	4.169	6.712	0.828	0.221	9.392	606	1,284	0.863	313/971
Carnivorous	3.055	3.739	0.761	0.176	4.529	220	336	0.914	76/260

enriched in pathways related to energy metabolism, translation, and glycan biosynthesis and metabolism. However, there have been insufficient dietary studies of the omnivorous to compare the differences in plant and animal consumption. In the omnivorous, most of the identified core ASVs, i.e., *Lactobacillus, Bacteroides plebeius,* and *Hydrogenophaga,* in our study were reported to perform essential functions in nutrition uptake, health promotion, and pathogen defense for their hosts (Thomas et al., 2011; Grond et al., 2019). In all diet groups, the shared core ASV, i.e., *Rhodococcus-erythropolis*, can produce enzymes that allow them to carry out an enormous number of bioconversions and degradations (Carvalho and Fonseca, 2005). Despite highly divergent gut microbiota compositions among dietary groups (Figure 2A), predicted community functional traits were quite similar among the different dietary groups (Figure 6A), indicating a high degree of functional redundancy among migratory birds' gut microbiome (Lozupone et al., 2012). It is noted that our conclusions concerning diet-associated signals in gut metagenomes of migratory birds are predictive, future research on the function of the gut microbiome is required to obtain a clear picture.

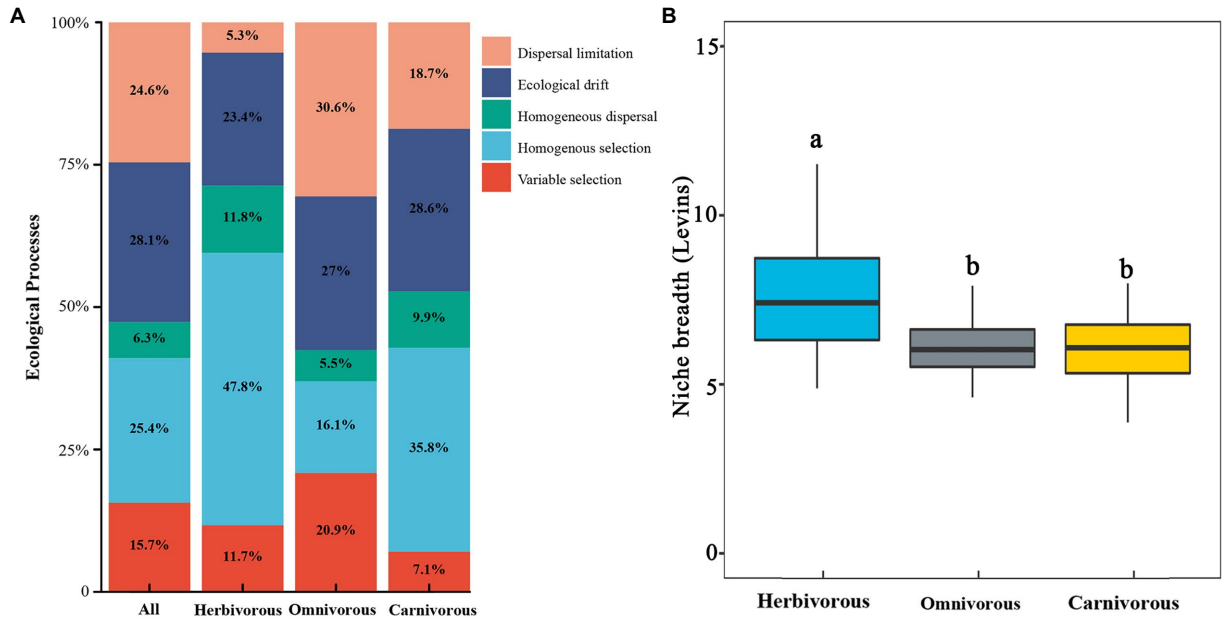

FIGURE 5
Ecological processes about the gut microbial community assembly. **(A)** Quantification of ecological processes governing the microbial community assembly and turnover for diets groups. The percentages are relative contributions of each process; **(B)** comparison of mean habitat niche breadth among herbivorous, carnivorous, and omnivorous birds. The different letters indicate a significant difference (p <0.05 level using Tukey's test).

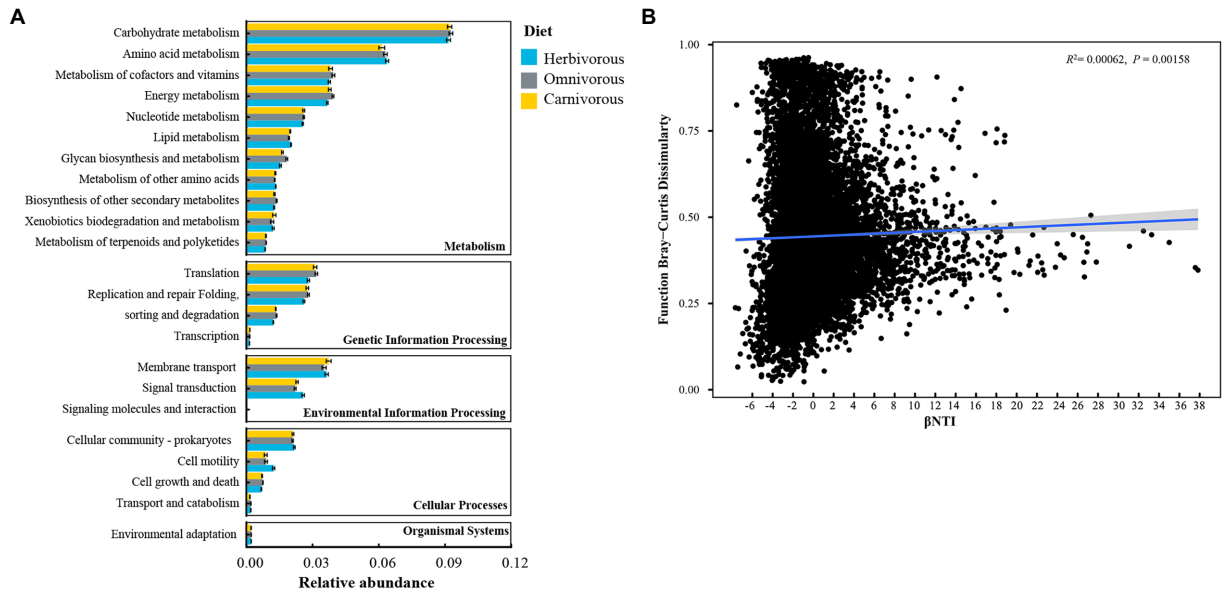

FIGURE 6
Predictions about the function during the microbial community assembly based on amplicon sequence variants (ASVs). **(A)** Level 2 KEGG orthologue functional predictions annotated by PICRUSt2; **(B)** the correction between βNTI and the function. Linear regression models (shown as blue lines) and associated correlation coefficients are provided on each panel.

Diet as the dominant factor contributing to variation in the gut microbiome

This study is an initial effort to understand the gut microbial community of migratory birds in the PYL wetland. Our results revealed that the gut microbiome was similar among the same diet groups (Table 2; Figure 2), and both diet and host phylogeny play important roles in the gut microbial community assembly of migratory birds. The effect value of diet combined with host phylogeny on the difference in gut microbiome was 30.0%, and diet

(20.1%) was identified as the dominant factor. Diets for migratory birds are a compound factor. Migratory birds change diets during migration, which makes them subjected to different local microorganisms associated with food sources. Local diet as a potential driver of gut microbiome assembly has been shown in a study of the gut microbiota of songbirds, which identified similar gut microbial communities within and among species during the stopovers of the songbirds along the migratory route (Lewis et al., 2017). Different diets also require distinct digestive mechanisms and may need specialized microbial communities in the gut to assist in effective digestion (Grond et al., 2018). Microbes originating from diets are the major sources of microbial colonizers for gut microbes. Selective filtering at the colonization stage may be controlled by the host or microbe characteristics and stochastic processes (Bisson et al., 2009; Sieber et al., 2019).

Effects of dietary specialization on the gut microbial communities

Network analysis provided another view of microbial interactions and ecological rules for community assembly (Grond et al., 2019; Song et al., 2020). Although higher in similarity threshold, the networks of the gut microbiome in dietary specialized (herbivorous and carnivorous) birds showed lower numbers of nodes, edges, and lower average connectivity ($p < 0.05$) than those of the omnivorous (Table 2). This indicates fewer interactions in dietary specialized birds than those in the omnivorous, which implies that biodiversity plays a role in the ecosystem stability in microbial communities (Feng et al., 2017). These results implicate that dietary specialization drove gut microbial communities toward a single direction, resulting in relatively less diverse gut microbiota (Ge et al., 2021). Besides, the gut microbiome of the herbivorous occupied a wide variety of niches (i.e., utilized an array of resources; Figure 4B), which was expected to be metabolically more flexible at the community level than one with a narrow niche breadth (Wu et al., 2018a,b; Jiao et al., 2020). Microbiomes with very different taxonomic compositions could share a substantial functional similarity, which is generally believed to play a role in the stability of microbial functions during disturbances. The functional redundancy in the microbial community may provide an environmental health advantage (Le Chatelier et al., 2013; Blakeley-Ruiz et al., 2019). Therefore, the herbivorous can adapt to changes in food sources.

Dietary specialization significantly altered the assembly processes that shape the gut microbiome of migratory birds. For example, the dispersal limitation decreased, and homogeneous selection increased in the dietary specialization birds (Figure 4A). This result suggests that the dispersal limitation leads to a relatively decentralized accumulation of gut microbial diversity in migratory birds with different diets. Specialized diets narrowed the range of food and foraging environments (Wiens, 2011; Shafquat et al., 2014; Burns et al., 2015), which limits the dispersal of microorganisms (Ley et al., 2008a,b; Muegge et al., 2011). Furthermore, migratory birds formed different ecological niches, which reduced the contact among birds (Ge et al., 2021). Additionally, the homogeneous selection may reflect the choice of the gut microbiome in the same inner intestinal tract environment, which maintained the similarity in different species of the same diet (Philipp and Moran, 2013). Dietary specialization had a larger βNTI value than the omnivorous (Figure 5A; Supplementary Figure S4A; Figure 6B). We speculate that after a long period, when the cumulative historical stochasticity causes a substantial change of host genetic identity, the host genetic isolation occurs and results in different host-bacterium specificity (Ge et al., 2021). The specialized function of gut microbes could lead to less available nutrients and more competition for microorganisms. But in birds, each organism specializes in different diets, and there would be less competition.

Conclusion

This study evaluated the contributions of diet and host phylogeny on the assembly of the gut microbiome of migratory birds. Diet was identified as the dominant factor contributing to observed variations of gut microbial structures among migratory birds. Omnivorous species had significantly higher gut microbiome diversity than herbivorous and carnivorous species. Dietary specialization also affected the balance between the deterministic and stochastic assembly of the gut microbiome. Stochastic processes were decreased as dietary specialization increased, indicating an increasing influence of selective processes. These insights could advance our understanding of host-microbiota interactions and the evolution of migratory birds' dietary flexibility and diversification.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA736842.

Author contributions

BW: conceptualization, methodology, data curation, formal analysis, visualization, and writing—original draft. HZ: data curation, formal analysis, and visualization. YL: investigation and formal analysis. LR: resources and revision. ZK: investigation, revision, and formal analysis. XM: revision and supervision. LW: writing—review and editing, funding acquisition, and supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.973469/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Rarefaction curves of the gut microbiome.

SUPPLEMENTARY FIGURE S2
Comparison of gut microbial diversity of 17 species of birds. The index of Shannon, Simpson, Chao 1, and Faith's phylogenetic diversity for each species. Differences in diversity were evaluated using the Kruskal test adjusted for multiple comparisons.

SUPPLEMENTARY FIGURE S3
Phylum: Pie charts of microbial composition for the abundant phyla (>1%). Genus: Pie charts of microbial composition for the abundant genera (>1%).

SUPPLEMENTARY FIGURE S4
(A) Quantification of ecological processes governing the microbial community assembly and turnover in the gut microbial communities. **(B)** Comparison of average habitat niche breadth of gut microbial species.

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Giant pandas' staple food bamboo phyllosphere fungal community and its influencing factors

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Giant pandas have developed a series of foraging strategies to adapt to their special bamboo diets. Although bamboo is an important food resource for giant pandas in Liziping National Nature Reserve (Liziping NR), China, there are relatively few studies on their phyllosphere fungal community and its influencing factors. Herein, we used ITS1 amplification and metagenomic sequencing to analyze the phyllosphere fungi diversity and functions (KEGG, CAZyme, and antibiotic resistance gene) and explore the influencing factors for the three giant pandas foraging bamboo species (*Arundinaria spanostachya*, AS; *Yushania lineolate*, YL; and *Fargesia ferax*, FF) over different seasons (spring vs. autumn) in Liziping NR, China. We found that Ascomycota and Basidiomycota were the most dominant phyla in the bamboo phyllosphere. The alpha diversity (e.g., the Sobs index and Shannon index) was relatively higher in autumn samples than in spring samples, and the community structure differed significantly between the three bamboo species in spring and autumn. Some biotic and abiotic variables (e.g., the elevation and mean base diameter of bamboo) significantly influenced the abundance, diversity, and community structure of the bamboo phyllosphere fungal community. Moreover, the functional analysis showed the differences in the glycoside hydrolase community and antibiotic resistance gene (ARG) profile between spring and autumn samples. Co–occurrence network modeling suggested that AS phyllosphere fungal communities in autumn employed a much more complex network than that in spring, and the abundance of multidrug, tetracycline, and glycopeptide resistance genes was high and closely correlated with other ARGs. These results indicate that fungal community's abundance, diversity, and community structure are mainly affected by the season, host species, and elevation. The season and host species are major factors affecting the biological functions (KEGG and CAZyme), ARGs, and interactions between sympatric bacterial and fungal communities in bamboo phyllosphere. This integrated study can provide a reference basis for the seasonal management of bamboo resources foraged by wild giant pandas, and predict the risk of antibiotic resistance in

bamboo phyllosphere fungal flora in Liziping NR (Xiaoxiangling mountains), China.

KEYWORDS

giant panda, staple food bamboo, phyllosphere fungi, metagenome, ARGs, Liziping NR

Introduction

The giant panda (*Ailuropoda melanoleuca*) belongs to the order carnivore and has a typical carnivore gastrointestinal tract. Fossil evidence has shown that the giant panda at least 7 Myr ago started to forage bamboo (Jin et al., 2007; Wang X. M. et al., 2022). As the umami taste receptor gene *Tas1r1* had lost its function and other reasons by about 4.2 Myr ago (Zhao et al., 2010), it is clear that giant pandas had already completed their dietary switch by about 2.0–2.4 Myr ago (Jin et al., 2007).

To adapt to the special bamboo diet, giant pandas have developed a series of foraging strategies, including the evolution of pseudo-thumbs, seasonal foraging, feeding point selection, habitat selection, etc. (Endo et al., 1999; Wei et al., 1999; Hong et al., 2015, 2016; Hu et al., 2017). However, in different seasons and mountain regions, giant pandas feed on different parts of bamboo and different kinds of bamboo; however, bamboo leaves are still their predominant food throughout the year (Wei et al., 2015). For example, giant pandas forage bamboo leaves most of the year except May–July in the Qinling Mountains. (Wu et al., 2017). Despite the fact that giant pandas only feed on bamboo, they have not evolved any genome-encoding enzymes specific for cellulose digestion (Hu et al., 2017). Thus, bamboo leaves are an important reason driving the composition and changes of intestinal flora of giant pandas; imbalances in this intestinal flora can lead to gastrointestinal disease, which is the most common cause of death in captive and wild giant pandas (Tun et al., 2014).

As an important food source for giant pandas, bamboo leaves have different nutrient and microbial compositions as compared with bamboo shoots, bamboo stems, and branches (Wei et al., 2015; Wu et al., 2017; Long et al., 2021). The health status of bamboo leaves, including the differences in seasonal nutrition and microorganisms in bamboo phyllosphere, could be the reason for the variations in giant pandas' seasonal activities and physiology. Using the traditional pathology method, Xu et al. (2014) found that the microbial community composition of phyllosphere differed among different bamboo species, and microbial groups showed seasonal differences. There were also differences in species composition and frequency of endophytic bacteria and fungi in bamboo leaves (Helander et al., 2013), while endophytic fungi in *Phyllostachys heteroclada* differ in the leaf and branch (Zhou et al., 2017). Using high-throughput amplicon sequencing, Jin et al. (2020) found that the richness and diversity of bacteria and fungi differed significantly between bamboo species.

The phyllosphere is one of the most diverse ecosystems on earth (Lindow and Leveau, 2002; Corinne et al., 2016), inhabited by bacteria, archaea, fungi, and protists (Arnold et al., 2003; Lindow and Brandl, 2003). Bacteria are the most abundant colonizers in leaves. However, phyllosphere fungal colonizers are incredibly diverse (Lindow and Brandl, 2003; Finkel et al., 2011; U'Ren et al., 2012). During the growing season, bacteria dominate, yeasts followed, and filamentous fungi accounted for the least (John and Robin, 2000; Redford and Fierer, 2009). The phyllosphere community composition is significantly impacted by biological and abiotic factors, such as the host species, season, temperature, humidity, host attributes, etc. (John and Robin, 2000; Lindow and Brandl, 2003; Paulino-Lima et al., 2013; Kembel et al., 2014; Copeland et al., 2015).

As an important part of the ecosystem, phyllosphere microorganisms affect plant surface properties (Lindow and Brandl, 2003), which have a key role in the degradation of organic matter, nutrient conversion, and energy flow (Rastogi et al., 2013; Zhao et al., 2013; Horton et al., 2014). Phyllosphere fungi, which can prevent herbivores from eating plants, protect plants from all kinds of pathogens and also make the plant tolerant against abiotic stress (Rehman et al., 2021). The ability to degrade cellulose is widespread in fungi, and is particularly well represented in Ascomycota and Basidiomycota (Boer et al., 2005). In a paddy environment, Fan et al. (2019) found an indigenous fungus, *Aspergillus cvjetkovicii*, which exerted an effect of strong antagonism on *Magnaporthe oryzae* and a promoting effect on rice. Zahn and Amend (2019) found that phyllosphere fungi alter the timing and allocation of reproduction in *Arabidopsis*, resulting in later flowering and higher seed quality. However, with the abuse of antibiotics and the rapid spread and development of antibiotic resistance, antibiotic resistance has become one of the world's greatest public health dangers (Chen et al., 2019; Pei et al., 2019). Unfortunately, phyllosphere is increasingly known as reservoirs of antibiotic resistance genes (Sun et al., 2021).

Most microorganisms do not exist in isolation, but live in the same space and share common substrates; these common substrates lead to either synergistic or antagonistic interactions, which directly or indirectly affect the growth of host plants and the absorption and utilization of nutrients (Mille-Lindblom et al., 2006; Schmidt et al., 2015; Ma et al., 2020). The interaction of bacteria and fungi can change soil structure and fertility, improve plant adaptability, and enhance the absorption and utilization of plants for nutrients and water (Porras-Soriano et al., 2009;

Tang et al., 2020). Bacteria and fungi also work together to promote the rumen digestion of grass (Lee et al., 2000).

As mentioned above, only three studies have been conducted on the phyllosphere microflora of giant pandas' staple food, bamboo (Helander et al., 2013; Jin et al., 2020; Long et al., 2021). Even fewer have been conducted on the phyllosphere microorganisms of bamboo leaves. Our previous research has focused on environmental factors influencing phyllosphere bacterial communities in bamboo in Liziping National Nature Reserve (Liziping NR), Sichuan, China (Long et al., 2021). According to the results of the Fourth National Giant Panda Survey, there are 22 wild giant pandas in the reserve (Sichuan Forestry Bureau, 2015). As of 2019, 1 rescued giant panda and 9 captive pandas were released into the reserve, alongside 8 surviving giant pandas (Hong et al., 2019). In this study, therefore, we focus on the phyllosphere fungal communities in giant pandas' staple food bamboo and their influencing factors using next-generation sequencing (NGS) technology (ITS1 region amplicon sequencing and whole-genome shotgun sequencing). This study aimed to (i) compare interspecies differences and seasonal variations in the composition, diversity, and community structure of phyllosphere fungal communities in three bamboo species (*Arundinaria spanostachya*, AS; *Yushania lineolate*, YL; and *Fargesia ferax*, FF); (ii) explore the ecological factors that influence the phyllosphere fungal community changes; (iii) investigate the interactions between fungi and bacteria in bamboo phyllospheres using co-occurrence network analysis; (iv) reveal the differences in biological functions (KEGG and carbohydrate-active enzyme functions, CAZyme) of AS phyllosphere fungi between spring and autumn; and (v) obtain antibiotic resistance gene (ARG) abundances of the AS phyllosphere fungal community and their differences between spring and autumn, determining their interactive relationship. Our research results could provide a reference basis for the restoration, protection, and management of bamboo resources in wild giant panda habitats.

Materials and methods

Study area

Liziping NR (28°51′02″–29°08′42″N, 102°10′33″–102°29′07″E) is situated in Shimian County, Sichuan Province, China (Figure 1), and lies in the southwest region of the Sichuan Basin; the largest tributary of the Minjiang River; and the southeast region of the Gongga Mountains. The reserve covers an area of about 47,885 hm^2; the elevation of the reserve is from 1,330 m to 4,551 m. The annual mean temperature and annual mean precipitation are 11.7°C–14.4°C and 800–1,250 mm, respectively. As the altitude increases, the vegetation types of the reserve transition from evergreen broadleaved forest and evergreen broadleaved mixed forest to coniferous and deciduous broadleaved mixed forest and evergreen coniferous forest, and subsequently, to alpine shrub and alpine meadow (Hong et al., 2015). AS, YL, and FF are the three main species foraged by giant pandas in the reserve. The most widely distributed species is AS, accounting for 38.08% of the total area of bamboo for giant pandas in this mountainous region (Sichuan Forestry Bureau, 2015). YL and FF account for 28.02% and 12.48% of giant pandas' diets, respectively (Sichuan Forestry Bureau, 2015). AS is mainly distributed in regions higher than 2,500 m above sea level, and YL and FF are mainly distributed below 2,800 m above sea level. AS was the main food year-round, YL was partial food for winter, and FF was occasional food (Xie and Hong, 2020; Long et al., 2021).

Experimental design

Field surveys and sampling were conducted in May (spring) and October (autumn) in 2020 (Figure 1). First of all, 4 transect lines were set up in AS, YL, and FF forests, respectively. The transect lines were set along the elevation gradient, and each transect line was separated by more than 200 m. Second, 3–5 survey plots (20 m × 20 m) were set on each transect line, and adjacent survey plots with an altitude distance of more than 50 m were arranged on the same transect. The bamboo species, latitude and longitude, altitude, tree layer, shrub layer, and other related variables were recorded and measured in each survey plot. Then, 1 bamboo plot (1 m × 1 m) was set at the central point of each survey plot and 5 m east and south of the central point, and the related variable data of bamboo layers were measured and recorded (Supplementary Table S1).

Sample collection and DNA extraction

For each survey plot, no <200 g of mixed bamboo leaves was collected using sterile gloves. Samples were immediately transported to the laboratory within 2 h after collection and stored in a −20°C freezer. Every 200 g sample was sterile transferred to a plastic bag (24 cm × 35 cm) containing 200 ml of sterile precooled TE-buffer (10 mM Tris, 1 mM EDTA, and pH 7.5); subsequently, 0.05% Tween-80 was added (Hong et al., 2017). The samples were cleaned to collect microbiota; each sample was shaken, eddy current, and ultrasonically stirred in a TE-buffer for 5 min, and the plastic bags were then stored in ice water (at ~4°C) for each treatment step. An ultrasonic scrubber with a frequency of 40 kHz (Shanghai Kudos Instrument Co., Shanghai, China) was used to remove microorganisms from bamboo leaves. The leaves were filtered through three layers of sterile nylon mesh for cell suspension. After filtration, the cell suspension was placed in four 50 ml tubes and centrifuged at 4°C for 15 min with the rate of 2,200 × g to remove the supernatant and obtain the fungus slime. Multi-tube mycelium was collected in a 2.0 ml reaction tube and washed twice with TE-buffer. The mud was immediately frozen at −80°C until DNA was extracted.

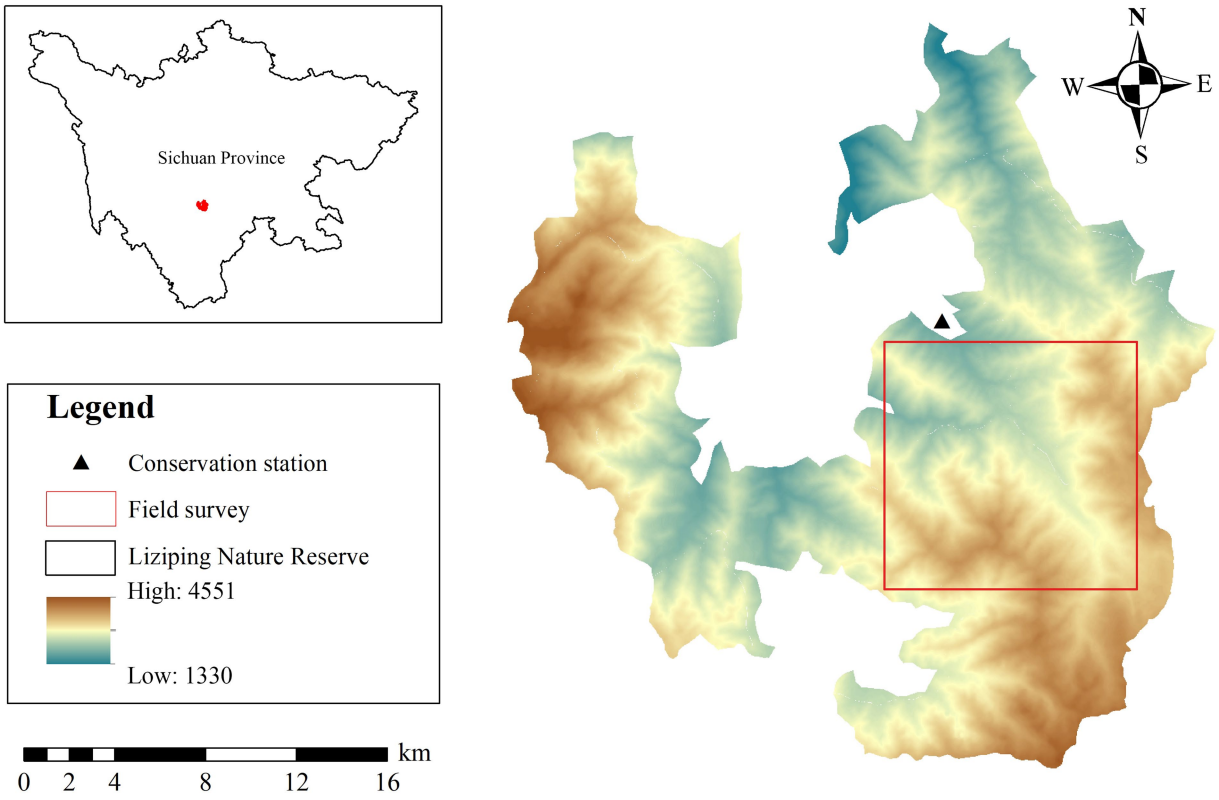

FIGURE 1
Study area in Liziping National Nature Reserve, China.

The DNA was extracted using the E.Z.N.A.TM Soil DNA Kit (Omega, Norcross, GA, United States) using the manufacturer's specifications; however, the following modifications were made: the frozen cell pellets were suspended in a 1 ml Kit-supplied SLX Mlus buffer containing 500 mg glass beads and ground at 65 Hz for 90 s on Tissue Lyser-24 (Jingxin, China). Cell fragment suspensions were immediately disposed of in accordance with instructions in the kit manual. Finally, the total DNA was obtained using an elution buffer of 50 µl.

ITS1 amplification, quantification, and sequencing

The first internal transcribed spacer (ITS1) region of the fungus was amplified. Polymerase chain reaction (PCR) amplified 35 cycles of the target marker gene. Error-corrected 12 bp barcode primers were used for each sample to allow sample multiplexing. PCR products from all samples were quantified using PicoGreen dsDNA analysis and were aggregated at equimolar concentrations. Each library was submitted to Mega Biotech on the Illumina MiSeq PE300 platform. The raw reads have been deposited in the NCBI Sequence Read Archive (SRA) database, with accession number PRJNA718425.

Sequence data analysis

FASTP (V0.20.0) was used to perform multiplex isolation and quality screening of the original ITS1 region sequencing reads (Chen et al., 2018) and using FLASH (V1.2.7) for merging. UPARSE (V7.1) was used to cluster the operational taxonomic units (OTU) of the sequences based on 97% similarity, and single sequences and chimera were removed. Each OTU representative sequence was classified and analyzed using the RDP Classifier (V2.2) and ITS database (e.g., Unite V8.0), with a confidence threshold of 0.7. To better convey the biological information of these samples, bar charts were used to visualize the average relative abundance of fungal communities at the phylum and genus levels.

Statistical analysis

First, the Kruskal–Wallis H test and Wilcoxon rank-sum test were used to detect differences in the abundance of dominant fungi at the phylum and genus levels, respectively. This was conducted among different bamboo species in the same season and the same bamboo species in different seasons. We used the linear discriminant analysis (LDA) effect size (LEfSe;

Segata et al., 2011) to identify the taxa that characterized the differences among three bamboo species (LDA score ≥ 3.5). The Sobs index and Shannon index were calculated for each sample using Motherur (V1.30.1) to estimate the species abundance and diversity of different bamboo species in spring and autumn, and the Wilcoxon rank-sum test was used to determine the significant level. Then, a Principlal Coordinate Analysis (PCoA) distance matrix based on the (un)weighted UniFrac method was used to evaluate the fungal community structure. Permutational MANOVA (PERMANOVA) was used to analyze the effects of different bamboo species and seasons on the fungal community structure based on the Bray–Curtis distance matrix.

Secondly, using the Spearman correlation analysis method, analysis of the relative abundance of fungal community and environmental factors (Supplementary Table S1). To investigate the spatial correlation between environmental factors and fungal communities, the Mantel test (based on the OUT level) was used to analyze the spatial correlation between the distance matrix of environmental factors and the UniFrac distance matrix of fungi. The canonical correspondence analysis (CCA) was used to determine samples, environmental factors, and the relationship between the fungi flora. The relationship between Alpha (the Sobs index and Shannon index) and Beta diversity (Bray-Curtis distance) and environmental factors was analyzed by the linear regression method.

Finally, the FUNGuild (Fungi Functional Guild) prediction method was used to predict the functional composition of fungi (Nguyen et al., 2016).

Metagenome sequencing and analysis

Whole-genome shotgun sequencing using the Illumina sequencing HiSeq platform. The raw sequencing data were filtered for low-quality and host genomic bases using MetaGene (Sun et al., 2018). Through the CD-HIT (V4.6.1) for the sample prediction of gene sequence clustering (parameters: 90% identity and 90% coverage), non-redundant gene sets were constructed. SOAPaligner (V2.21) was used (Li et al., 2008; Fu et al., 2012), mapped the quality read data of each sample to the non-redundant gene set (parameter: 95% identity), and counted the abundance of genes in the sample. Non-redundant genes sets were compared with the NCBI NR database using DIAMOND (V0.8.35; Buchfink et al., 2015; parameter: Blastp. *E*-value ≤ 1E-5) to obtain the taxonomic annotation from the NR library. DIAMOND (V0.8.35) was used to map the non-redundant gene sets to Kyoto Encyclopedia of Genes and Genomes database to annotate the KEGG function. Non-redundant gene sets were compared with the CAZy database using HMMER (V3.1b2; parameter: Hmmscan. *E*-value ≤ 1E-5) to annotate carbohydrate-active enzyme. DIAMOND (V0.8.35) was used to map the non-redundant gene sets into CARD to obtain the annotation results for fungal antibiotic resistance genes (ARGs) in AS phyllospheres.

LEfSe was used to determine the relative abundance characteristics of the KEGG Level 2 (LDA score ≥ 2), CAZyme (LDA score ≥ 3), and CARD databases (LDA score ≥ 3.5) of the AS phyllosphere fungal community between spring and autumn (Segata et al., 2011).

Co-occurrence network analysis

Using the OTU data of bacteria and fungi obtained by sequencing, OTUs with relative abundances >0.1% were selected. Network analyses were performed in R. Gephi (Version 0.9.2) used to construct microbial co-occurrence networks among AS, YL, and FF in spring and autumn. Antibiotic resistance genes with relative abundances >0.1% were selected, and network analyses were also performed in R. Gephi (Version 0.9.2) used to construct microbial co-occurrence network in AS. If the Spearman's correlation coefficient $|\rho|$ is not <0.8, and p values are no more than 0.01, suggests that a correlation between two items was considered statistically robust (Quintela-Baluja et al., 2019).

Results

Phyllosphere fungal alpha and beta diversity

A maximum of 19 samples were collected from each bamboo species in each study season (Supplementary Table S2). A total of 101 bamboo samples and 6,749,062 reads were obtained. The average target ITS1 reads of each sample were 66,822 ± 6,323. A total of 8,769 OTUs were obtained by clustering 97% similarity, among which 3,278 OTUs were shared among the three bamboo species (Supplementary Figure S1A). The fungal OTUs in the three bamboo species in autumn were significantly higher than those in spring (Supplementary Figures S1B, S2). Among the three bamboo species, FF had the highest number of OTUs in spring and autumn (Supplementary Figure S1A).

Taxonomic analysis classified fungal OTUs into 9 phyla and 1,053 genera. Most of the phyllosphere fungal community belonged to the phyla Ascomycota in all samples (Figure 2A). The rest comprised Basidiomycota and other phyla. The relative abundance of the phyla Ascomycota and Basidiomycota were not significantly different among the three bamboo species (Figure 2A, Supplementary Figures S4A,B; Supplementary Table S3). The relative abundance of the phylum Basidiomycota in YL phyllosphere was higher in spring than in autumn, and the relative abundance of the phylum Ascomycota in YL phyllosphere was lower in spring than in autumn (Figure 2A; Supplementary Table S3).

At the genus level, unclassified_p__Ascomycota, *Trichomerium*, unclassified_c__Sordariomycetes, unclassified_o__Helotiales, unclassified_o__Pleosporales, and *Lapidomyces* were the dominant phyllosphere fungi (Supplementary Figure S3). In

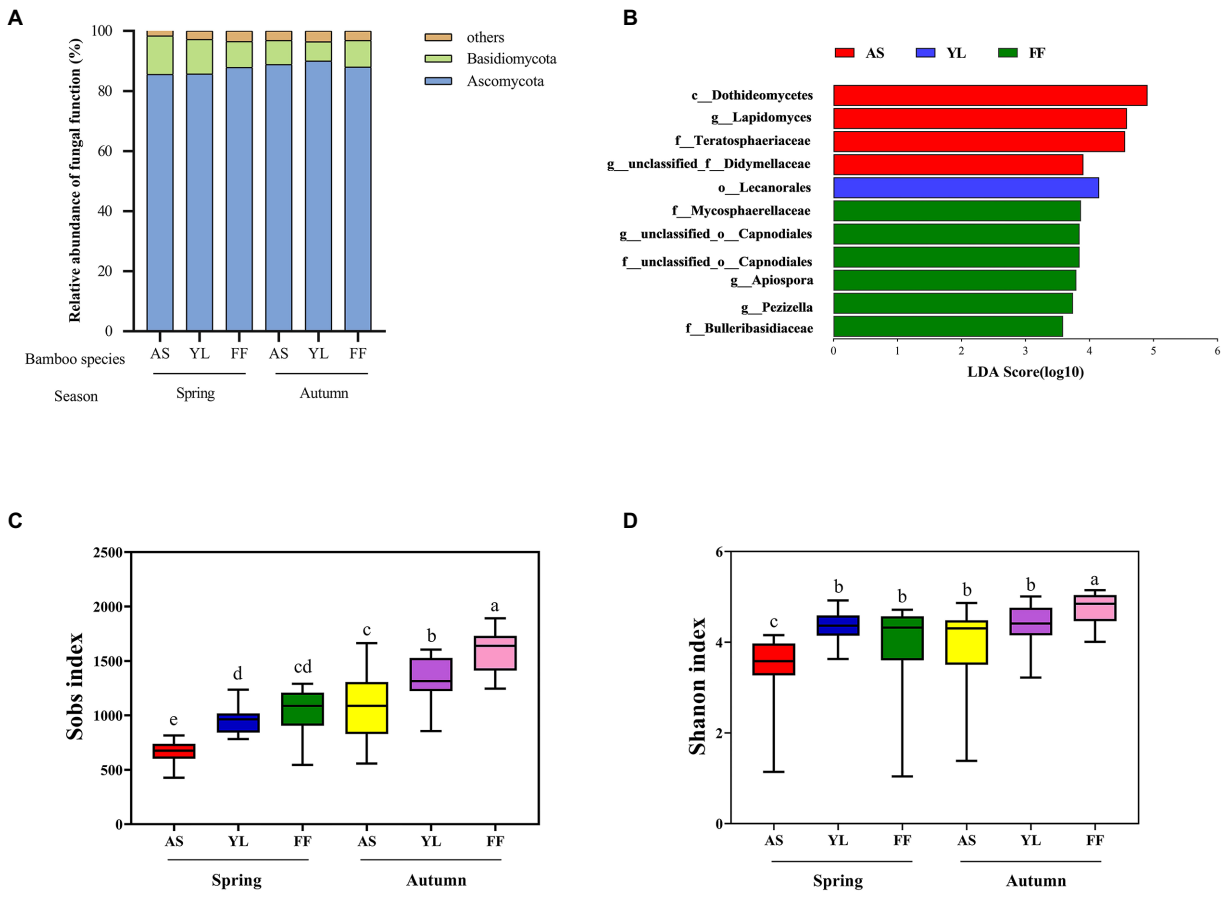

FIGURE 2
Taxonomic profiles of fungal communities among AS, YL, and FF phyllosphere. **(A)** Relative abundance of different phyllosphere fungi phyla among AS, YL, and FF in spring and autumn. **(B)** LEfSe analysis of phyllosphere fungi among AS, YL, and FF (LDA score≥3.5). The Sobs index **(C)** and Shannon index **(D)** of AS, YL, and FF phyllosphere fungi in spring and autumn.

spring, the relative abundance of unclassifed_p__Ascomycota was significantly higher in the YL phyllosphere (Supplementary Figure S4C; Supplementary Table S3). The relative abundance of *Trichomerium* was significantly lower in the AS phyllosphere, but a contrasting pattern was observed for *Lapidomyces* (Supplementary Figure S4C; Supplementary Table S3). In autumn, the relative abundance of unclassified_p__Ascomycota and unclassified_o__Helotiales were significantly higher in the YL phyllosphere (Supplementary Figure S4D; Supplementary Table S3). The highest relative abundance of unclassified_o__Pleosporales existed in the AS phyllosphere, but a contrasting pattern was observed for unclassified_c__Sordariomycetes (Supplementary Figure S4D; Supplementary Table S3). For all three bamboo species, the relative abundance of unclassified_c__Sordariomycetes in autumn was significantly lower than that in spring (Supplementary Table S3).

LEfSe analysis revealed that the LDA scores of microbial taxa differed significantly among AS, YL, and FF (Figure 2B). Four fungi groups (c__Dothideomycetes, g__Lapidomyces, f__Teratosphaeriaceae, and g__unclassified_f__Didymellaceae) in the AS phyllosphere were significantly enriched in comparison to the other two bamboo species. In YL phyllosphere, only o__Lecanorales was significantly enriched in comparison to the other two bamboo species. In FF phyllosphere, six groups of fungi (including f__Mycosphaerellaceae, g__unclassified_o__Capnodiales, f__unclassified_o__Capnodiales, g__Apiospora, g__Pezizella, and f__Bulleribasidiaceae) were significantly enriched in comparison to the other two bamboo species (Figure 2B).

The Sobs index and Shannon index were found to decrease from autumn to spring in all bamboo species (Figures 2C,D; Supplementary Table S4). AS phyllosphere fungal community had significantly lowest the Sobs index and Shannon index in spring (Figures 2C,D; Supplementary Table S4). In autumn, the Sobs index and Shannon index in FF phyllosphere were significantly higher than those of AS and YL (Figures 2C,D; Supplementary Table S4).

The PCoA results showed that the samples were clustered by season and species (Figure 3). Based on weighted UniFrac, three bamboo species clustered together in the same season, FF and YL clustered closer together. Based on unweighted UniFrac, the three bamboo species are easier to distinguish than the weighted

UniFrac (Figure 3). Further, the PERMANOVA based on the Bray-Curtis distance matrix showed that there were significant differences in the phyllosphere fungal community structure between the three bamboo species in spring and autumn (Table 1).

Ecological factors influencing bamboo phyllosphere fungal communities

Mantel analysis results showed that elevation had the greatest effect on the phyllosphere fungal community ($R = 0.617$, $p = 0.001$; Supplementary Table S5). Furthermore, CCA showed that elevation (E), mean base diameter of bamboo (MBDB), mean height of bamboo (MHB), bamboo deaths (BD), tree diameter at breast height (TDBH), shrub number (SN), total number of live bamboo (TNLB), number of trees (NT), shrub coverage (SC), slope (S), tree height (TH), canopy density (CD), and water source distance (DW) also influenced the fungal community (Figure 4; Supplementary Table S6).

The Spearman's correlation heatmap showed that Basidiomycota abundance was significantly negatively correlated to shrub number (SN; Figure 5A). Ascomycota abundance was significantly positively correlated to tree height (TH), tree diameter at breast height (TDBH), shrub coverage (SC), shrub number (SN), total number of live bamboo (TNLB), and bamboo deaths (BD; Figure 5A). At the genus level, *Lapidomyces* exhibited significantly positive correlations with elevation (E) and bamboo coverage (BC), but significantly negative correlations with tree height (TH), number of trees (NT), mean height of bamboo (MHB), and mean base diameter of bamboo (MBDB; Figure 5B). unclassified_o__Helotiales and unclassified_o__Pleosporales were both positively correlated with tree height (TH), tree diameter at breast height (TDBH), shrub coverage (SC), shrub number (SN), total number of live bamboo (TNLB), and bamboo deaths (BD). unclassified_c__Sordariomycetes exhibited significantly negative correlations with shrub coverage (SC), shrub number (SN), total number of live bamboo (TNLB), and bamboo deaths (BD), but significantly positive correlations with mean height of bamboo (MHB). *Trichomerium* exhibited significant positive correlations with bamboo coverage (BC), mean height of bamboo (MHB), and mean base diameter of bamboo (MBDB), but negative correlations with elevation (E), tree diameter at breast height (TDBH), and shrub coverage (SC). unclassified_p__Ascomycota was significantly positively correlated to water source distance (DW) and total number of live bamboo (TNLB), but negatively correlated to number of trees (NT; Figure 5B).

Further linear regression revealed that elevation had the highest effect for the Bray–Curtis distance, but the highest ecological influence factor on the Sobs index and Shannon index was mean base diameter of bamboo (Supplementary Table S7).

Co-occurrence patterns of fungal interactions with bacteria community

The results of co-occurrence network analysis showed that the network complexity, node connectivity, and average degree of the *F. ferax* microbial community were higher than those of *A. spanostachya* and *Y. lineolate* (Figure 6; Supplementary Table S8). In autumn, *A. spanostachya* communities employed a much more complex network than that in spring (Figures 5A,B), which was reflected in the node and edge number, as well as average degree, which was higher in autumn than in spring (Supplementary Table S8). However, the network complexity of *F. ferax and Y. lineolate* had no obvious differences in the two seasons (Figures 6C–F). *F. Ferax* microbial networks were mainly reflected in the interactions within the bacterial domain. *A. spanostach* and *Y. lineolate* microbial networks were mainly

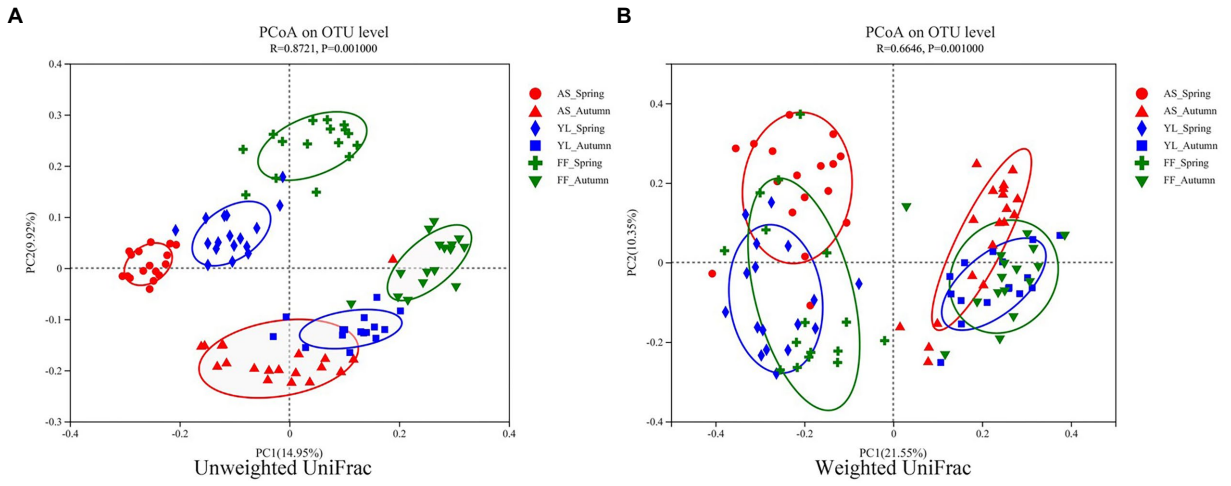

FIGURE 3
PCoA of phyllosphere fungal communities based on the (un)weighted UniFrac distance matrixes **(A,B)** among AS, YL, and FF in spring and autumn.

TABLE 1 PERMANOVA exploring the effects of three bamboo species and two seasons based on Bray–Curtis distance matrixes.

Grouping factors	Sums of Sqs	Mean Sqs	*F* model	R^2	Value of *p*
AS (Spring:Autumn)	2.298	2.298	11.947	0.260	0.001
YL (Spring:Autumn)	2.017	2.017	10.813	0.258	0.001
FF (Spring:Autumn)	1.611	1.611	7.132	0.192	0.001
Spring:Autumn	12.109	2.422	12.047	0.388	0.001
Autumn (AS:YL:FF)	3.568	1.784	8.845	0.269	0.001
Spring (AS:YL:FF)	4.843	2.422	12.087	0.340	0.001

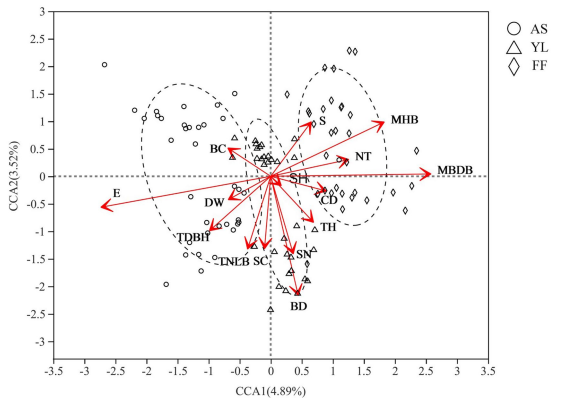

FIGURE 4
CCA analysis of environmental factors and phyllosphere fungal community among AS, YL, and FF. E, elevation; DW, water source distance; TDBH, trees diameter at breast height; TNLB, total number of live bamboo; SC, shrub coverage; BD, bamboo deaths; SN, shrubs numbers; TH, trees height; SH, Shrub height; CD, canopy density; MBDB, mean base diameter of bamboo; NT, number of trees; MHB, mean height of bamboo; S, Slope; BC, bamboo coverage.

reflected in the interactions within the fungal domain (Figure 6; Supplementary Table S8).

Function of the fungi (e.g., KEGG, CAZy, and ARGs)

The functional classification of fungi was performed based on the Fungi Functional Guild (Nguyen et al., 2016). Three ecological guilds – pathotroph, saprotroph, and symbiotroph – for ~40% of all fungal OTUs were detected (Supplementary Figure S5).

In order to further explore the variation of functional potential in AS phyllosphere, we screened 8 samples (spring: 4 samples; autumn: 4 samples) for whole-genome shotgun sequencing. A total of 3.91 million contigs and 2.47 Gb of assembly sequence were obtained, and the average contig N50 was 655 bp (Supplementary Table S9). The metagenomic analysis confirmed 402 KOs, including 46 KEGG Level 2 categories in fungi. In the KEGG level 2 categories, the relative abundance of global and overview maps (20%) was the highest in *A. spanostachya*, followed by neurodegenerative disease (7%), signal transduction (6%), and carbohydrate metabolism (4%; Supplementary Figure S6A). In spring, AS phyllosphere fungi showed high abundances in KEGG Level 2 categories of neurodegenerative disease, energy metabolism, environmental adaptation, and drug resistance: antimicrobial, endocrine, and metabolic disease (Figure 7A), and the functions of AS phyllosphere fungi are primarily a consequence of the fungi of the *Beauveria* and *Cladosporium* genus (Supplementary Table S10). In contrast, in autumn, translation; folding; sorting and degradation; endocrine systems; transcription; metabolism of cofactors and vitamins; immune systems; nucleotide metabolism; infectious diseases: bacterial, signaling molecules, and interactions; immune disease; cellular community-eukaryotes; metabolism of terpenoids and polyketides; and infectious diseases: parasitic, exhibited higher abundance (Figure 7A), and the functions of AS phyllosphere fungi are mainly a consequence of fungi of the *Beauveria* and *Lachnellula* genus (Supplementary Table S10).

The relative abundance of CAZy in glycoside hydrolases (43%) was the highest in AS phyllosphere fungi which contained some cellulases (GH5, GH6, GH7, GH28, etc.), followed by glycosyl transferases (27%), auxiliary activities (16%), and carbohydrate esterases (11%; Supplementary Figure S6B; Supplementary Table S10). According to the LEfse results for the CAZyme, three families had significantly higher relative abundances in spring, including glycoside hydrolases (GHs), glycosyl transferases (GTs), and carbohydrate esterases (CEs; Figure 7B), and the functions of AS phyllosphere fungi are mainly a consequence of fungi of the Chaetothyriales and Hypocreales order (Supplementary Table S11). In autumn, four families had significantly higher relative abundances: GHs, CEs, GTs, and AAs, and the functions of AS phyllosphere fungi are mainly a consequence of fungi of the Chaetothyriales and Pleosporales orders (Supplementary Table S10). Interestingly, GHs had the highest differences between spring and autumn (Figure 7B).

A total of 125 antibiotic resistance genes were detected in eight bamboo samples from the AS phyllosphere fungi based on metagenomic sequencing (Supplementary Table S11). At the genus level, g__unclassified_o__Helotiales, g__Epicoccum, g__Mortierella, and g__Paraphaeosphaeria had multidrug resistance genes; g__unclassified_k__Fungi had multidrug and tetracycline resistance genes; g__Periconia had aminocoumarin resistance genes; g__Lachnellula had multidrug, tetracycline, and rifamycin resistance genes; g__Beauveria had multidrug, tetracycline, sulfonamide, fluoroquinolone, pleuromutilin, and beta-lactam resistance genes (Supplementary Table S11). Multidrug (42%) and tetracycline (24%) resistance genes were the most common types of ARGs in all samples (Figure 8A). The abundance of multidrug, tetracycline, and glycopeptide resistance genes was high and

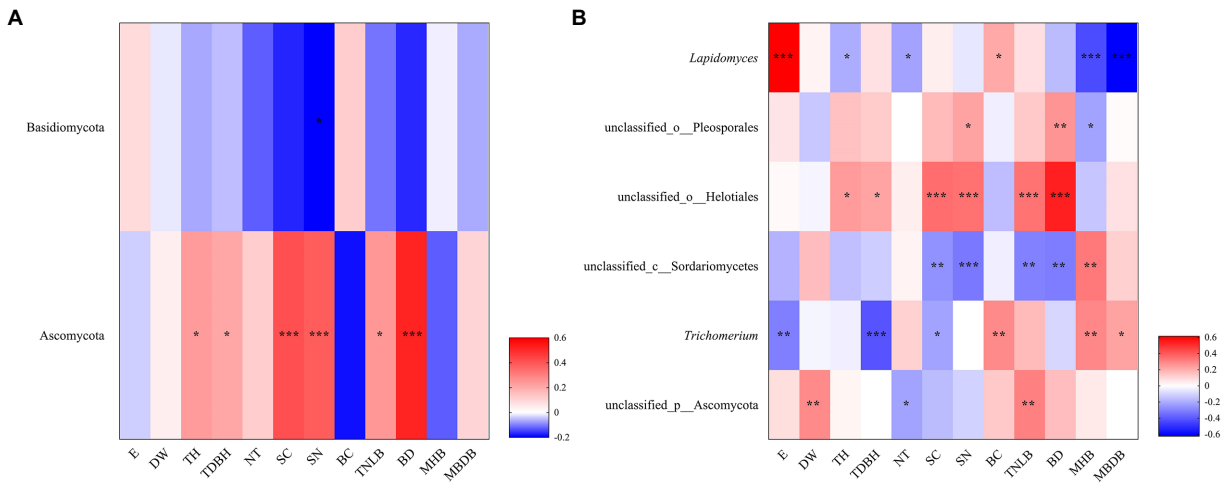

FIGURE 5
Correlation heatmap showed the relative abundance of fungi phylum **(A)** and genus **(B)** in relation to environmental factors. R is shown in different colors, and the legend on the right is the color range of different R values. E, elevation; DW, water source distance; TDBH, trees diameter at breast height; TNLB, total number of live bamboo; SC, shrub coverage; BD, bamboo deaths; SN, shrubs numbers; TH, trees height; SH, Shrub height; CD, canopy density; MBDB, mean base diameter of bamboo; NT, number of trees; MHB, mean height of bamboo; S, Slope; BC, bamboo coverage. (*0.01<p<0.05, **0.001<p<0.01, ***p<0.001).

closely correlated with other ARGs (Figure 9). Beta-lactam resistance gene had only one edge to multidrug resistance genes (Figure 9). The remaining ARGs were glycopeptide, aminocoumarin, mupirocin, fluoroquinolone, rifamycin, etc. According to the LEfse results of the CARD, in spring, AS fungi displayed higher abundances of beta-lactam and phenicol resistance genes than in autumn; however, glycopeptide and bicyclomycin resistance genes exhibited higher abundance in autumn (Figure 8B).

Discussion

In our study, Ascomycota and Basidiomycota were found to be the dominant phyla in AS, YL, and FF phyllosphere fungi microflora, which is similar to several previous studies (Helander et al., 2013; Xu et al., 2014; Jin et al., 2020). The fungal OTUs richness and diversity (e.g., Sobs index and Shannon index) of the three bamboo species in spring were significantly lower than those in autumn. This result matches that found by Zheng (2011), who determined that the diversity of *Pinus tabulaeformis* phyllosphere microbial communities exhibited the greatest diversity in autumn, followed by summer and spring. Further, Thompson et al. (1993) also found that the diversity of phyllosphere fungal communities of *Beta vulgaris* in spring was lower than that in autumn. The season is an important factor affecting phyllosphere microorganisms, whose diversity is higher in autumn than in spring because of higher temperature and humidity in autumn (Laforest et al., 2016).

The season is also a stronger driver of the phyllosphere fungal community structure than the host bamboo species based on the (un)weighted UniFrac distance matrix (Figure 3). However, this result differs from that of Laforest et al. (2016), who found that host species are a more important factor than the site or time in terms of determining the community of temperate tree phyllospheres.

The Mantel test found that elevation, water source distance, tree height, etc. significantly affected the phyllosphere fungal community, and effect of elevation is the most obvious (Supplementary Table S5), which is similar to the result of Long et al. (2021), who found that elevation, water source distance, etc. significantly impact the phyllosphere bacterial community. Similarly, Cordier et al. (2012) found that elevation affects the relative abundance and community composition of *Fagus sylvatica* fungal communities. Furthermore, CCA found that slope significantly affected the phyllosphere fungal community. Similarly, Bari et al. (2019) explored the effects of elevation, the field slope, site direction, and fungal spore fruit position height on trees, finding that the field slope was the main factor influencing the fungal spores abundance.

To explore the effect of the micro-ecological environment (biotic factor) on the bamboo phyllosphere fungal community, a co-occurrence network analysis was performed, which showed that FF had higher network complexity than AS and YL. This may be related to the higher Sobs and Shannon indices in FF phyllosphere microbial communities, because the higher Sobs and Shannon indices would increase the possibility of interactions between different microbial communities (Wang B. et al., 2022). This result was similar to those of Wang B. et al. (2022), who found that the high alpha diversity of microbial community in a given group provides

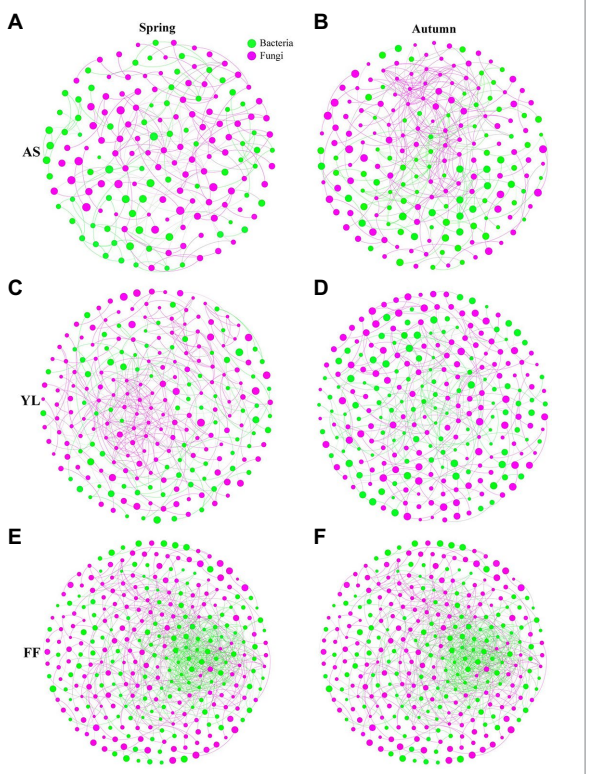

FIGURE 6
Co-occurrence network analysis showing the fungi interactions with bacteria among AS, YL, and FF phyllosphere in spring and autumn **(A–F)**.

additional potential interactions between groups. AS had higher network complexity in autumn than in spring. However, networks of FF and YL slightly differed in spring and autumn (Figure 6). It is possible that AS occurs at high altitudes and has more distinct climatic environments, in terms of both temperature and light. There are complex links between bacteria and fungi, bacteria and bacteria, and fungi and fungi in AS, YL, and FF. Bacteria and fungi usually have symbiotic relationships, with both positive and negative effects. For example, some bacteria stimulate mycorrhizal formation, and fungi and bacteria grow faster when they are present together (Bengtsson, 1992; Founoune et al., 2002; Mille-Lindblom et al., 2006); bacterial volatiles inhibit the growth of fungal spores, and fungal volatiles inhibit not only the growth of bacteria, but also the growth of other fungi (Schmidt et al., 2015).

In this study, the functions of AS phyllosphere fungi were mainly dominated by *Beauveria* (Supplementary Table S10), a genus of entomogenic fungi that plays an important role in pest control and has no pathogenic effect on humans and animals (Mascarin and Jaronski, 2016). *Beauveria* not only protects giant panda's food resources but can also kill giant panda's ectoparasites. Carbohydrate metabolism and GH enzymes are mainly a consequence of Eurotiomycetes and Dothideomycetes (Supplementary Table S10). It has been demonstrated that Eurotiomycetes and Dothideomycetes consume lignin when it is the only carbon source (Ferrari et al., 2021). The majority of vitamins come from an animal's diet. Both the giant panda and red panda forage on AS as a staple food in Liziping NR (Wei et al., 1999). The abundant metabolism of cofactors and vitamins may be beneficial to the physical and mental health of giant pandas and red pandas (Badawy, 2014; Rudzki et al., 2021). Further, the abundant energy metabolism and environmental adaptation in spring indicated that spring was not a suitable season for phyllosphere fungi (Laforest et al., 2016). More abundant translation, folding, sorting and degradation, transcription, nucleotide metabolism, signaling molecules and interactions, and cellular community-eukaryotes indicated fungal cell viability and cell proliferation in autumn.

The LEfse results of the CAZyme show that glycoside hydrolases (GHs) exhibit the main differences, which supports the fact that phyllosphere microorganisms rely primarily on carbohydrate metabolism for nutrients (Kawaguchi et al., 2011; Ryffel et al., 2016). GHs, which can decompose complex carbohydrates, are a highly diverse group of key enzymes related to gut microbiota and their metabolic function (Lee et al., 2013). Zhu et al. (2011) found that the gut microbiome of giant pandas contains a small number of cellulases, and giant pandas and red pandas may obtain cellulose-digesting flora from phyllosphere microorganisms by eating bamboo leaves in large quantities (Jin et al., 2020).

In the AS phyllosphere, multidrug and tetracycline resistance genes were the most dominant ARGs. ARG co-occurrence network analysis showed that beta-lactam resistance genes were rarely correlated with other ARGs (Figure 9), reducing the possibility of co-selection of other antimicrobials (Yang et al., 2022). Many phyllosphere fungi have multiple resistance genes, proving that phyllospheres have become reservoirs for resistance genes (Sun et al., 2021). Multidrug resistance genes have been shown to be the most abundant ARGs in some vegetables' phyllosphere (Blau et al., 2018; Yin et al., 2022). Multidrug resistance to many different classes of antibiotics, which impedes antibiotic treatment, is a huge risk to human life (Zhu et al., 2013; Ouyang et al., 2015), and is also a potential threat to giant panda health. Several recent studies have shown that diverse ARGs exist in the gut of wild and captive giant pandas, and multidrug resistance genes were the dominant type of ARGs in the giant panda gut (Guo et al., 2019; Hu et al., 2020; Mustafa et al., 2021; Zhu et al., 2021). A survey based on ARGs of human gut microbiota in three countries (China, Denmark, and Spain) found that tetracycline resistance genes were the most abundant group of ARGs, followed by MLSs and beta_lactam resistance genes (Hu et al., 2013). Given the abundance and diversity of ARGs in the bamboo phyllosphere, the diet has proven to be the main driving factor for ARG variation in the gut microbiome of giant pandas (Zhu et al., 2021).

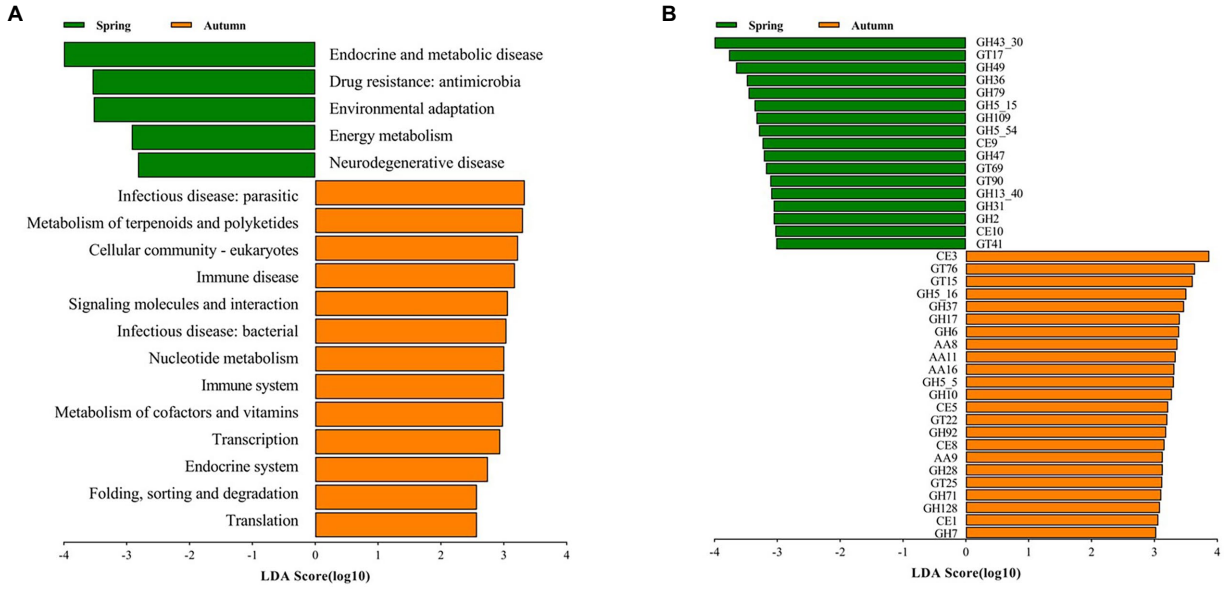

FIGURE 7
LEfSe analysis of KEGG level 2 **(A)** (LDA score≥2) and CAZy **(B)** (LDA score≥3) of AS phyllosphere fungal community between spring and autumn. GH, glycoside hydrolases; GT, glycosyl transferases; CE, carbohydrate esterases; AA, auxiliary activities.

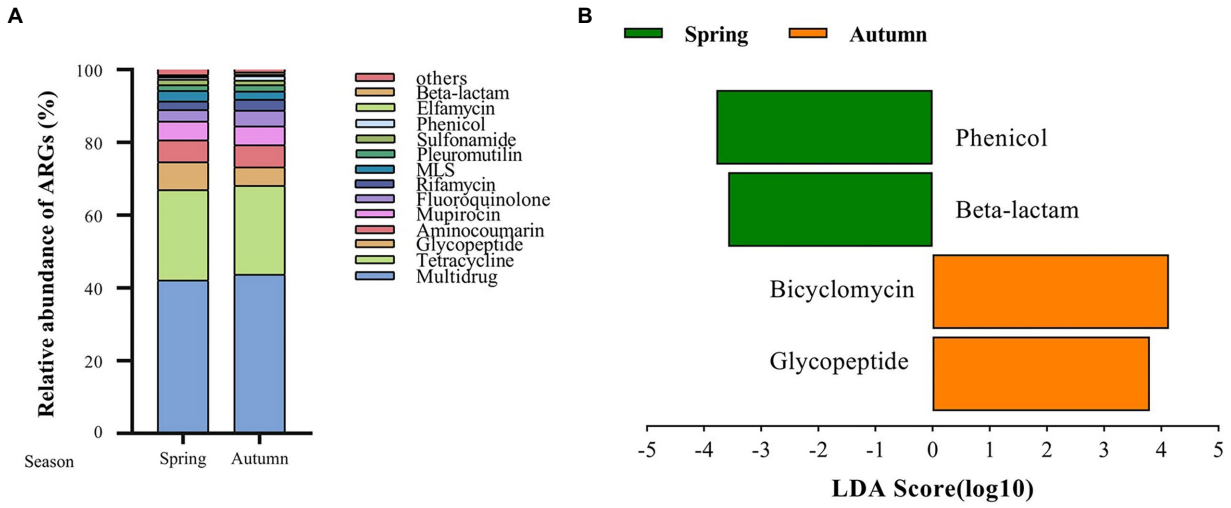

FIGURE 8
Fungal antibiotic resistance genes (ARGs) in AS phyllosphere. Relative abundance of fungal antibiotic resistance genes (ARGs) in AS phyllosphere between spring and autumn **(A)**. LEfSe analysis of fungal antibiotic resistance genes (ARGs) in AS phyllosphere between spring and autumn **(B)**. (LDA score≥3.5).

Conclusion

Overall, our results indicated that the fungal community's abundance, diversity, and community structure were mainly affected by the season, host species, and elevation. The season and host species are major factors affecting biological functions (KEGG and CAZyme), ARGs, and interactions between sympatric bacterial and fungal communities in bamboo phyllospheres. As the most important food resource for giant pandas in Liziping NR (Xiaoxiangling mountains; Hong et al., 2015; Sichuan Forestry Bureau, 2015; Xie and Hong, 2020), AS phyllosphere fungi contain many glycoside hydrolases (e.g., cellulases CH5, GH6, GH7, etc.), which may enter the giant pandas' intestine through ingestion, helping them to digest

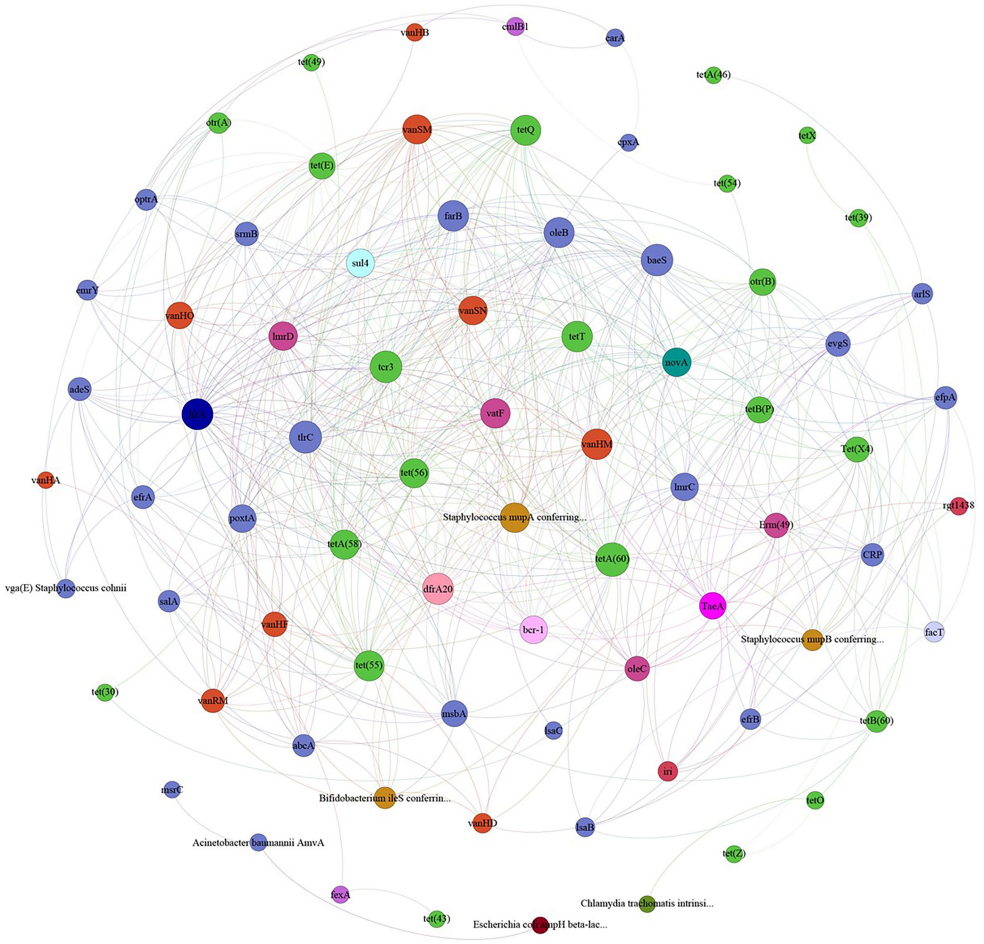

FIGURE 9
ARGs co-occurrence network of fungal resistance genes in AS phyllosphere. Nodes represent genes and are colored to indicate antimicrobial classes of ARGs.

cellulose and obtain nutrition. The functions of AS phyllosphere fungi were mainly dominated by *Beauveria*, which can be used for biological control of pests and diseases (Mascarin and Jaronski, 2016). Therefore, food microbes (bamboo phyllosphere microorganisms) may be an important technique for giant pandas' gut microbes to degrade cellulose and also protect food resources of giant pandas (Zhu et al., 2011). Although various ARGs were found in AS phyllosphere fungi flora, these ARGs could naturally occur in bamboo phyllosphere fungi flora (D'Costa et al., 2011; Sun et al., 2021). Therefore, numerous comparative studies on the effects of human activities on bamboo phyllosphere microorganism ARGs should be carried out in giant panda habitats to predict the risk of antibiotics for wild pandas.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

LK: conceptualization, methodology, investigation, software, resources, formal analysis, and writing original draft. WL, QD, HZ, WW, JT, HH, YY, and JL: investigation, validation and data curation. ZZ: conceptualization, supervision, and writing–review and editing. MH: conceptualization, supervision, funding acquisition, writing–original draft, and review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1009588/full#supplementary-material

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Effects of captive and primate-focused tourism on the gut microbiome of Tibetan macaques

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Documenting the effects of anthropogenic activities on the gut microbiome of wild animals is important to their conservation practices. Captivity and ecotourism are generally considered two common anthropogenic disturbances on the health of nonhuman primates. Here, we examined the divergences of gut microbiome in different environments of Tibetan macaques. Our results showed that there were no significant differences in the alpha diversity, predominant families and genera of gut microbiomes between wild and tourist groups. However, these indexes decreased significantly in the captive individuals. In addition, the significant differences of beta diversity and community compositions between wild and tourism groups also were detected. In particular, higher potential pathogenic and predicted KEGG pathway of drug resistance (antimicrobial) were detected in the gut microbiome of individuals in captive environment. Our results indicated that living in the wild are beneficial to maintaining gut microbial diversity of Tibetan macaques, while captivity environment is harmful to the health of this macaque. Exploring ways to restore the native gut microbiome and its diversity of captive individual should pay more attention to in the future studies.

KEYWORDS

gut microbiome, primate-focused tourism, captive, Tibetan macaque, wild

Introduction

Nonhuman primates (NHPs) are our closest living biological relatives, which can offer critical insights into the human evolution, behavior and biology, as well as the forest ecosystem health. Current information shows that the existing primates consist of 506 species in 79 genera. Unfortunately, above 60% of primate species are now threatened with extinction and above 75% have declining populations (Estrada et al., 2017). Habitat disturbance, caused by human activities, is considered the most important factor contributing to the decline in the wild primate populations (de la Torre et al., 2000). Assessing

the impacts of anthropogenic disturbance on the health of wild NHPs has become one of the important goals of wild living primate conservation (Junge et al., 2011; Luo et al., 2015; Stumpf et al., 2016; Cavada et al., 2019).

Recent studies highlight that habitat disturbance can result in the loss of gut microbial diversity in the wild NHPs. For example, research on wild black howler monkeys (*Alouatta pigra*) indicates that populations of degraded habitats risk 'double jeopardy' from both reduced resource availability and the diversity of gut microbiome (Amato et al., 2013). Similarly, wild populations living in fragmented habitats of Udzungwa red colobus (*Procolobus gordonorum*) have a lower gut microbial diversity compared to intact habitats, which is potentially linked to a decreased ability to digest toxic plant compounds (Barelli et al., 2015). Meanwhile, populations living in the disturbed habitat, both black howler monkeys and ring-tailed lemurs (*Lemur catta*), were more enriched by potentially pathogenic microorganism (Amato et al., 2016). The gut microbiome is known to play a crucial role in host nutrition, metabolic activity, immune homeostasis and behavioral patterns (Hooper and Gordon, 2001; Archie and Theis, 2011; Nicholson et al., 2012; Wheeler et al., 2016). Understanding how the gut microbiome of wild primates is influenced by habitat disturbance presents a new study area for conservation biologists (Stumpf et al., 2016; Clayton et al., 2018; Trevelline et al., 2019).

Captivity and ecotourism are generally considered two common anthropogenic disturbances on the health of nonhuman primates. For the primates that living in captive environments, individuals usually face changes or restrictions in diet, treatments with antibiotics, increased social pressure, limited in exposure to environmental microbes, as well as exposure to human-associated microbes (McKenzie et al., 2017). Many studies have shown that these changes or restrictions are associated with the dysbiosis of the animals' gut microecosystem, including the reduction of native gut microbes and the loss of microbial diversity (Amato et al., 2013; Clayton et al., 2016; McKenzie et al., 2017; Frankel et al., 2019), as well as increased in antibiotic resistance genes of gut microbiota (Tsukayama et al., 2018). Therefore, studies on the gut microbiome of captive individuals can inform captive management and conservation strategies for the protected animals (Stumpf et al., 2016; Trevelline et al., 2019; West et al., 2019).

Primate-focused tourism is considered as one effective strategy to achieve species conservation, financial and educational benefits for local communities (Johns, 1996; Berman and Li, 2002). As part of efforts to protect primates, invasive management methods such as translocation, provision and range restriction are often used to increase tourists' opportunities to encounter and/or see wild primates in many primate habitat countries (Struhsaker and Siex, 1998; Berman et al., 2007). Nevertheless, a number of previous studies have shown that tourism had many detrimental effects on the health, behavior and biology of wild NHPs, including changes of activity budgets and foraging patterns (Griffiths and Schaik, 1993; Hill, 1999; de la Torre et al., 2000), increased in individual stress and intra-group competition (Maréchal et al., 2016), as well as the potential for disease transmission (Woodford et al., 2002; Devaux et al., 2019). Given host diet and stress is closely related to the composition and metabolic functions of gut microbiome (Muegge et al., 2011; Foster and McVey Neufeld, 2013; Xu et al., 2020), the gut microbiome may offer valuable insight into the effects of the tourism on primates health, nutrition, disease, as well as the conservation decisions of wild primates (Stumpf et al., 2016). To date, little is known concerning the effects of primate-focused tourism on the gut microbiomes of wild NHPs.

As a species of genus *Macaca*, the Tibetan macaque (*Macaca thibetana*) is a Near Threatened primate species endemic to east central China, which habitat in subtropical, deciduous and evergreen broad-leaved forest (Sun et al., 2010). The free-ranging with semi-provisioned group of Tibetan macaques, habitat in Mt. Huangshan, Anhui province, presents a good opportunity to assess the effects of primate-focused tourism on the gut microbiomes of wild NHPs. In 1992, local government decided to drive the group named Yulinkeng A1 (YA1), ~1 km from their natural range, to an unoccupied area. Since then, to facilitate tourists' viewing opportunities, park staff has provided the group of ~6 kg of whole corn per day. In the present study, we compared the gut microbiomes of three groups living in different environmental settings. YA1 is a free-ranging with semi-provisioned group (Mt. Huangshan), which has long been used for primate-focused tourism. The study subjects also included a group lived in the captivity (Tong Ling City Zoo), and a wild group located some 10 km from Mt. Huangshan. We focus on the following three main questions. First, if the anthropogenic disturbance including primate-focused tourism and captivity can result in the loss of gut microbial diversity in Tibetan macaques? Second, are there significant differences in the gut microbial composition among primate-focused tourism, wild and captivity groups of Tibetan macaques? Third, what are the potential impacts of primate-focused tourism on the Tibetan macaque's health based on the current gut microbial data? The results of this study will improve our understanding of the potential effects of anthropogenic disturbance on the primate gut microbiome.

Materials and methods

Study objects and samples collection

This study was conducted at three sites in southern Anhui Province, China, including Mt. Tianhu (Wild group), Mt. Huangshan (Tourism group), and the Tong Ling City Zoo (Captive group). Individuals of the tourism group were supplied 3 times a day with a total of 6–8 kgs of corn. The amount of feeding was about one third of the group's daily food intake. Mt. Tianhu located 10 kilometers away from Mt. Huangshan. Individuals of this group get all their food from the wild. The habitats of Tibetan macaques at both Mt. Tianhu and Mt. Huangshan are evergreen broad-leaved forest and deciduous

broad-leaved forest respectively, with similar flora and fauna. Individuals of the captive population in Tong Ling City Zoo were migrated from Mt. Huangshan for about 1 year during the sampling period. This group's main diet was corn and sweet potatoes. All samples were collected from August, 2019, during a 2-week period. In total, 70 fresh fecal samples of macaques were sampled, including 26, 18 and 26 samples from the tourism, wild and captive group, respectively.

All fecal samples were stored in a sterilized tube with RNAlater (QIA-GEN, Valencia, CA, United States). Samples were transported to the laboratory of Anhui University in ice packs and stored at −80°C before DNA extraction. This research was approved by the Institutional Animal Care and Use Committee of the Anhui Zoological Society (permit number AHZS201711008). We performed all experiments in accordance with their approved guidelines and regulations, and complied with all principles of the China Animal Ethics Committee.

DNA extraction and sequencing

To avoid contamination, we extracted DNA from the inside of each fecal sample using a QIAamp® Fast DNA Stool Mini kit (Qiagen). The total DNA extracted from the fecal samples were sent to the Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China) for sequencing. The V3-V4 region of the 16S rRNA gene was amplified using primers 338F (5'-ACTCCTA CGGGAGGCAGCAG-3′) and 806R (5'-GGACTACHVG GGTWTCTAAT-3′) as previously described (Mori et al., 2014). PCR reaction mixtures contained 5–100 ng of DNA template, 1 × GoTaq Green master mix, 1 M $MgCl_2$, and 5 pmol of each primer. Reaction conditions include an initial 95°C for 2 min, followed by 35 cycles of 95°C for 30s, 55°C for 30s, and 72°C for 60s, and a final extension of 72°C for 5 min. After quantification step, amplicons were pooled in equal amounts, and pair-end 2 × 300 bp sequence was performed using the Illlumina Miseq platform (San Diego, CA, United States).

Bioinformatics and statistical analysis

We trimmed raw FASTQ sequencing data for the adaptor sequence and for quality control using the sliding window approach implemented in fastp v0.19.6 (Chen et al., 2018). Sequences containing N bases were removed. FLASH v1.2.7 was used to merge overlapping paired-end reads (Magoč and Salzberg, 2011). DADA2 within Qiime 2 was used to truncate forward and reverse reads, to denoise the data, and to detect and remove chimeras (Bolyen et al., 2019). Taxonomy was assigned to amplicon sequence variants (ASV) using classify-sklearn (Naive Bayes) with the database (v.132).[1] Qiime 2 was used to calculate Shannon diversity index, ASV richness, and unweighted and weighted UniFrac distance matrix. The sequence data has been stored in NCBI (project number is PRJNA871105).

Kolmogorov–Smirnov normality tests were used to evaluate the normal distribution of alpha diversity index and relative abundance of dominant phyla. Principal coordinates analysis (PCoA) was performed with the R packages Made4 and Vegan. Permutational multivariate ANOVA (PERMANOVA) was used to test variations in beta diversity (unweighted and weighted UniFrac distance) across the three different macaque groups using the Adonis functions in the vegan R package (Chen et al., 2012). We used Kruskal-Wallis ANOVA with Tukey's *post-hoc* tests to test the variation in different study groups. Values of p were adjusted using a false discovery rate (FDR) correction. Linear discriminant analysis effect size and default options were used to determine the phylum, class, order, family and genera enriched in each study group (Segata et al., 2011). BugBase tool was used to evaluate wide-scale phenotypic properties of the gut microbiome (Ward et al., 2017). In addition, to explore the functional profiles of our data set, KEGG pathways were predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (version 2; PICRUSt 2).

Results

General characteristics of gut microbiome profile

We acquired 2,063,887 high-qualities reads with 29,484 (ranging from 19,495 to 38,608 across all 70 samples) sequences per sample. Taxonomic assignment revealed 22 known bacterial phyla at 97% sequence identity. The dominant phyla were Firmicutes (x = mean ± SD, x = 58.91 ± 11.89%), Bacteroidetes (x = 25.79 ± 9.39%; **Figure 1**). The predominant families were Prevotellaceae (x = 17.13 ± 9.18%), Lachnospiraceae (x = 12.39 ± 9.05%) and Oscillospiraceae (x = 10.80 ± 4.10%). At the genus level, the fecal samples were dominated by *Prevotella* (x = 12.90 ± 7.89%), *UCG-005* (Oscillospiraceae; x = 5.29 ± 3.23%) and *Treponema* (x = 4.13 ± 3.81%).

Variation of gut microbial diversity and composition among different groups

Amplicon sequence variants richness (ASVs), Shannon diversity (Shannon) and Phylogenetic diversity (PD) index showed significant difference among the three study groups (Kruskal-Wallis, $F = 34.36$, df = 69, adjusted $p < 0.001$, $F = 16.65$, df = 69, adjusted $p < 0.001$ and $F = 13.93$, df = 69, adjusted $p < 0.001$). Pairwise comparison analysis showed that the ASV richness of the captive group was significantly lower which compared to the other two groups (Tukey–Kramer, Captive vs. Wild: adjusted $p < 0.001$; Captive vs. Tourism: adjusted $p < 0.001$).

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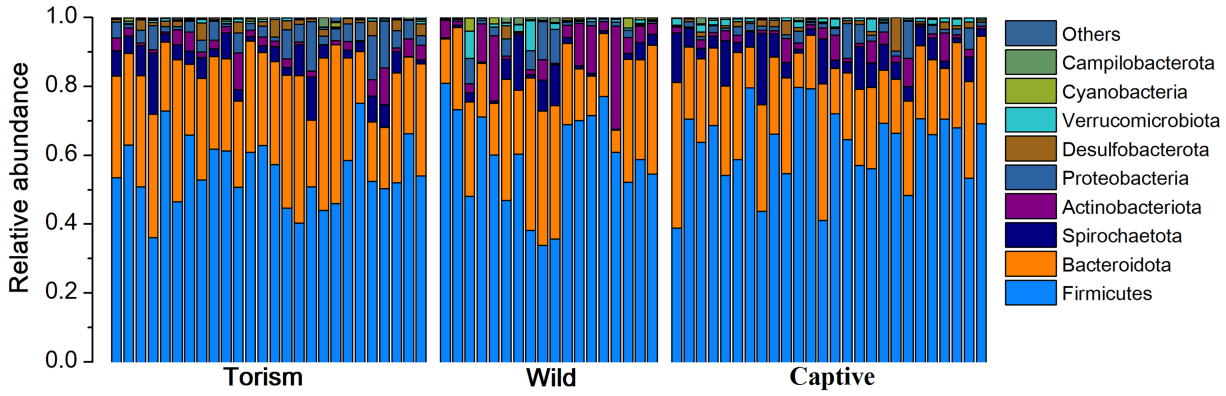

FIGURE 1
Relative abundance of fecal bacterial taxa at the phylum level. Stacked bar graphs illustrate the abundances of phyla and the x-axis represents the sample groups.

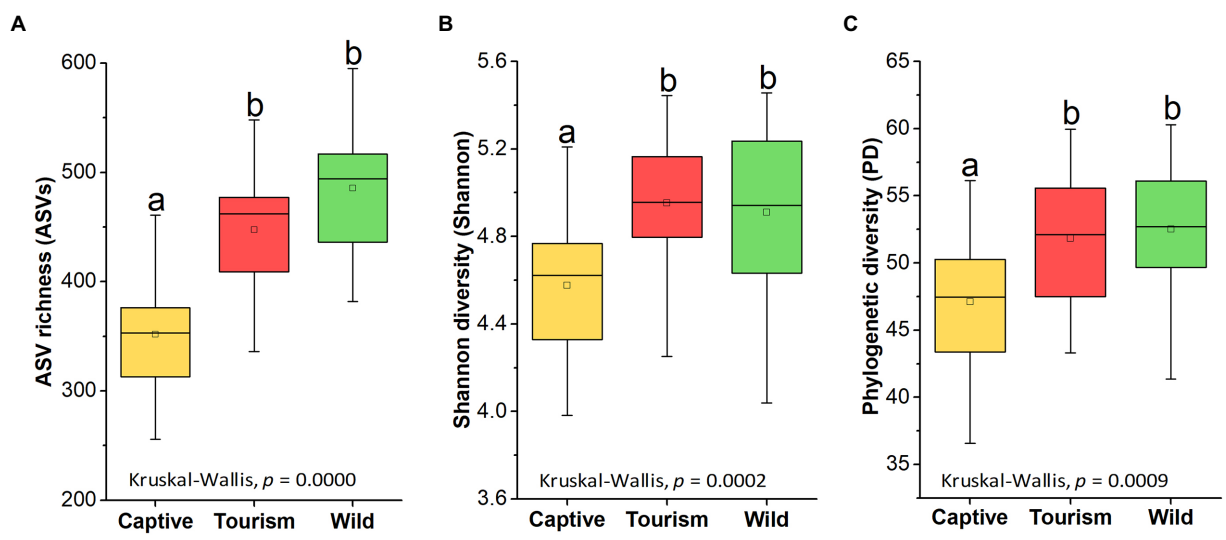

FIGURE 2
Variations on the alpha diversity in the gut microbiome among three study groups. **A**: Comparison of ASV richness. **B**: Comparison of Shannon diversity index. **C**: Comparison of PH diversity index. A Kruskal-Wallis test was used to evaluate the variation across three groups. Post-hoc tests (Tukey–Kramer test) for pairwise comparison tests (*p* values were adjusted by FDR). Letters in **(A)**, **(B)**and **(C)** represent significant differences.

The same results of the other two indices also were detected, including Shannon diversity (Tukey–Kramer, Captive vs. Wild: adjusted $p < 0.001$; Captive vs. Tourism: adjusted $p < 0.01$) and Phylogenetic diversity (Tukey–Kramer, Captive vs. Wild: adjusted $p < 0.01$; Captive vs. Tourism: adjusted $p < 0.01$; Figures 2 A–C). However, no significant differences were detected between individuals in the tourism and wild groups for the three indexes of alpha diversity.

PCoA revealed that individuals from the same group were possessed more similar microbial communities, whether based on unweighted UniFrac and weighted UniFrac dissimilarities. PERMANOVA showed the significant variation of the microbial community structures across samples from the three study groups (PERMANOVA, unweighted UniFrac, $R^2 = 0.3277$, $p = 0.001$; weighted UniFrac, $R^2 = 0.2415$, $p = 0.001$; Figures 3A,B). In detail, significant differences in beta diversity between same sample types were detected based on unweighted unifrac dissimilarities (Adonis, unweighted unifrac, Captive vs. Tourism, $R^2 = 0.292$, $p = 0.001$; Captive vs. Wild: $R^2 = 0.321$, $p = 0.001$; Tourism vs. Wild: $R^2 = 0.137$, $p = 0.001$; weighted unifrac, Captive vs. Tourism, $R^2 = 0.206$, $p = 0.001$; Captive vs. Wild: $R^2 = 0.240$, $p = 0.001$; Tourism vs. Wild: $R^2 = 0.115$, $p = 0.001$). We found that the dissimilarity in community structures of gut microbiomes between wild and tourism was significantly lower than that

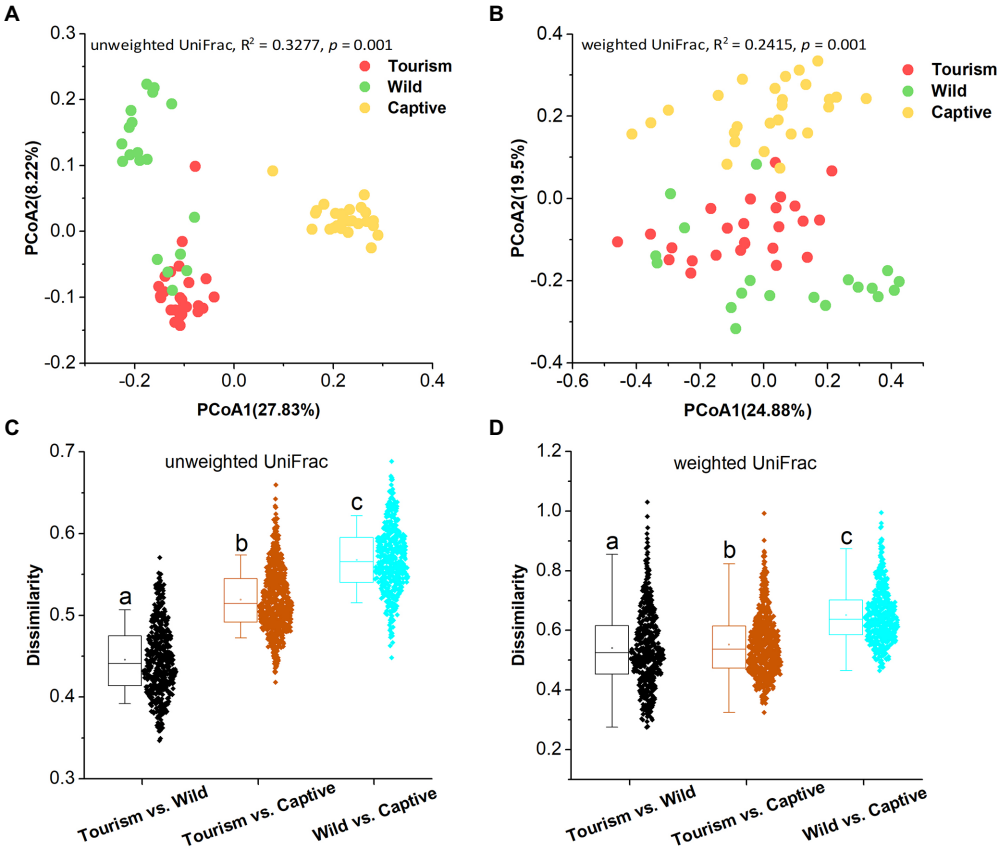

FIGURE 3
Differences in beta diversity of the gut microbiome across three study groups. **(A,B)** Differentiation of fecal microbiota structure. **A**: based on unweighted UniFrac distance, **B**: based on weighted UniFrac distance. PCoA was used to show patterns across three study groups. Adonis tests were performed on unweighted and weighted UniFrac, respectively. Significance was set at the 0.05 level. **(C,D)** Comparison of dissimilarity between Gut microbiome structures. **C**: based on unweighted UniFrac distance, **D**: based on weighted UniFrac distance. Significance was set at the 0.05 level. Letters in (C) and (D) represent significant differences.

between wild and captive (Wilcoxon signed-rank test, unweighted Unifrac and weighted Unifrac, $p < 0.001$; Figures 3C,D).

Variation of gut microbial composition among different groups

The top 10 families and genera were used to evaluate the variation of gut microbial composition among different groups. We found that nine known taxa of the 10 top families showed significant variation among the three study groups (Kruskal-Wallis, $p < 0.05$; Supplementary Figure S1). In addition, eight known taxa of the 10 top genera showed significant variation among the three study groups (Kruskal-Wallis, $p < 0.05$; Supplementary Figure S2). The predominant families Prevotellaceae and Lachnospiraceae showed significant variation among the three study groups (Kruskal-Wallis, Prevotellaceae, $F = 8.66$, df = 69, adjusted $p = 0.038$; Lachnospiraceae, $F = 30.68$, df = 69, adjusted $p < 0.001$; Figures 4 A,B). *Post hoc* tests indicated that the relative abundance of Prevotellaceae in tourism group was significantly higher than for individuals in captive group (Tukey–Kramer, Captive vs. Tourism, $p < 0.05$; Captive vs. Wild, $p > 0.05$; Tourism vs. Wild, $p > 0.05$). From wild to tourism group and then to captive group, the relative abundance of Prevotellaceae was decreased significantly (Tukey–Kramer, Wild vs. Tourism, $p < 0.01$; Wild vs. Captive, $p < 0.001$; Tourism vs. Captive, $p < 0.001$). We also found that the predominant genera *Prevotella* and *UCG-005* were significant differences among the three study groups (Kruskal-Wallis, *Prevotella*, $F = 17.24$, df = 69, adjusted $p < 0.001$; *UCG-005*, $F = 20.73$, df = 69, adjusted $p < 0.001$; Figures 4 C,D). Pairwise comparison analysis showed that the *Prevotella* (Tukey–Kramer, Wild vs. Tourism, $p > 0.05$; Wild vs. Captive, $p < 0.01$; Tourism vs. Captive, $p < 0.001$) and *UCG-005* (Tukey–Kramer, Wild vs. Tourism, $p > 0.05$; Wild vs. Captive, $p < 0.001$; Tourism vs. Captive, $p < 0.01$) in the captive group were significantly lower than those of the tourism and wild groups.

To explore the enriched indicators of each study group, we performed LEfSe (LDA Effect Size) analyses on the different levels of microbial taxa across samples (LDA > 4, $p < 0.05$). In total, 42 different known taxa (genus, family, order, class, and phylum

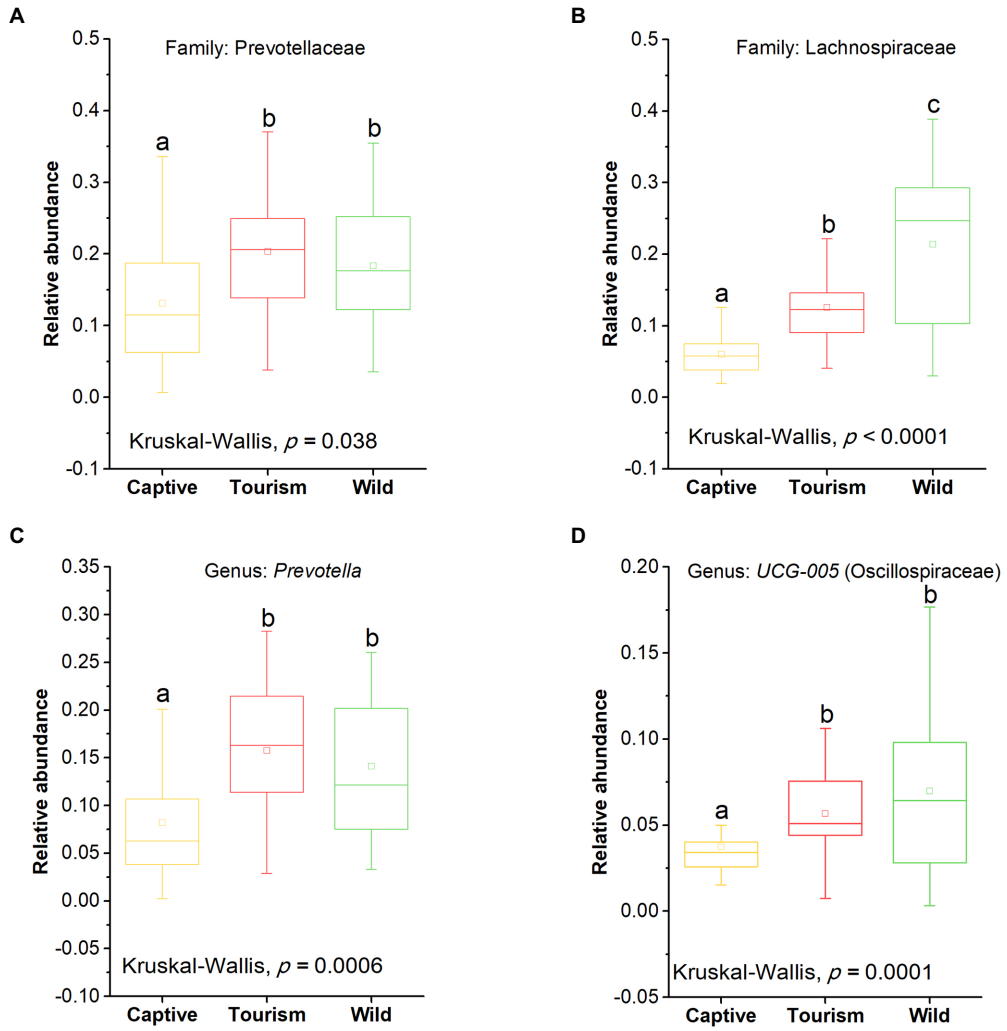

FIGURE 4
Variation in fecal bacterial taxonomy across three study groups. **(A)** and **(B)** Comparison of the predominant families. A: Prevotellaceae, B: Lachnospiraceae. **(C)** and **(D)** Comparison of the predominant genera. A: *Prevotella*, B: *UCG-005*. A Kruskal-Wallis test was used to evaluate the variation across three groups. Post-hoc tests (Tukey–Kramer test) for pairwise comparison tests (p values were adjusted by FDR). Letters in **(A)**, **(B)**, **(C)** and **(D)** represent significant differences.

levels) were significantly enriched in one of the three groups (Figure 5). Among these taxa, nine, 10, and 23 indicators were identified in wild, tourism, and captive groups, respectively. All of these known taxa were core set of the corresponding group (present in more than 90% and the average relative abundance >1% of the specific group samples). Three families (Lachnospiraceae, Ruminococcaceae and Eggerthellaceae) and two genera (*Subdoligranulum and UCG-005*) were significantly enriched in the wild group. Three families (Prevotellaceae, Succinivibrionaceae and Bacteroidales_RF16_group) and three genera (*Prevotella*, *Faecalibacterium* and *Succinivibrio*) were significantly enriched in the tourism group. Six families (Clostridiaceae, Lactobacillaceae, Christensenellaceae, Rikenellaceae, Spirochaetaceae and Peptostreptococcaceae) and eight genera (*Streptococcus*, *Sarcina*, *Clostridium_sensu_stricto_1*, *Christensenellaceae_R-7_group*, *Rikenellaceae_RC9_gut_group*, *Lactobacillus*, *CAG-873* and *Prevotellaceae_UCG-003*) were significantly enriched in the captive group.

Variation in phenotypic properties predicted KEGG pathways of gut microbiome among different groups

Based on the BugBase tool, nine of the wide-scale phenotypic properties of the gut microbiomes were detected, including Stress Tolerant, Gram Positive, Anaerobic, Potentially Pathogenic, Contains Mobile Elements, Gram Negative, Forms Biofilms, Aerobic and Facultatively Anaerobic. Kruskal-Wallis test revealed that six phenotypic properties (Stress Tolerant, Gram Positive, Anaerobic, Potentially Pathogenic, Gram Negative and Forms Biofilms) showed

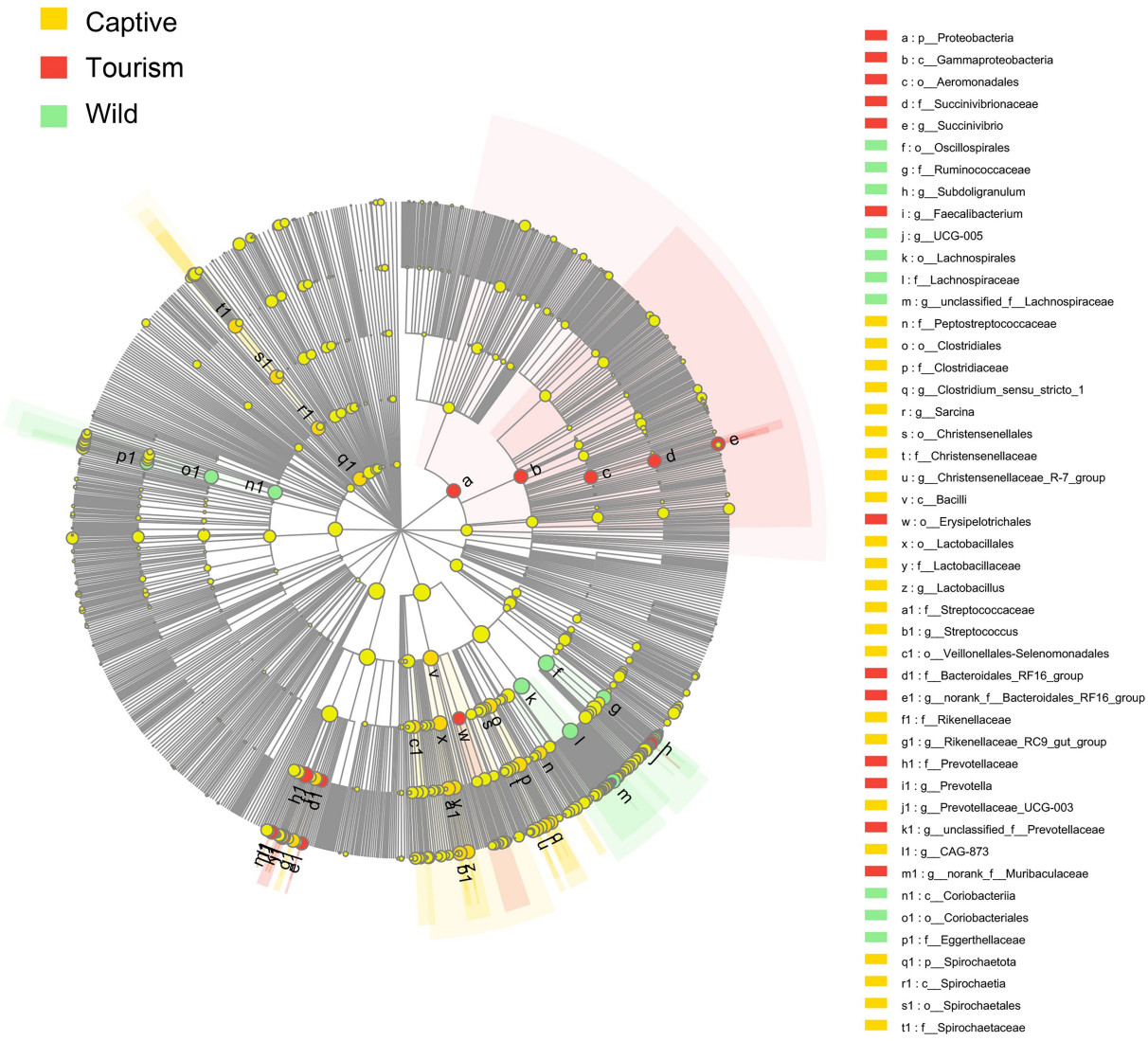

FIGURE 5
Indicators of known taxa in one of the three groups. At the genus, family, order, class, and phylum levels. Indicators identified by linear discriminant analysis effect size (LEfSe) analysis (LDA>3, p<0.05).

significant variation on the three study groups (all the adjusted $p < 0.001$). *Post hoc* tests indicated that the proportions of predicted phenotypic including Stress Tolerant (Tukey–Kramer, Captive vs. Tourism, $p < 0.001$; Captive vs. Wild, $p < 0.001$; Tourism vs. Wild, $p > 0.05$), Gram Positive (Tukey–Kramer, Captive vs. Tourism, $p < 0.001$; Captive vs. Wild, $p < 0.001$; Tourism vs. Wild, $p > 0.05$) and Anaerobic (Tukey–Kramer, Captive vs. Tourism, $p < 0.001$; Captive vs. Wild, $p < 0.01$; Tourism vs. Wild, $p > 0.05$) were significantly lower in captive individuals than in individuals of the other two groups (Figures 6 A–C). In contrast, the proportions of predicted phenotypic including Potentially Pathogenic (Tukey–Kramer, Captive vs. Tourism, $p < 0.001$; Captive vs. Wild, $p < 0.05$; Tourism vs. Wild, $p > 0.05$), Gram Negative (Tukey–Kramer, Captive vs. Tourism, $p < 0.001$; Captive vs. Wild, $p < 0.001$; Tourism vs. Wild, $p > 0.05$) and Forms Biofilms (Tukey–Kramer, Captive vs. Tourism, $p < 0.001$; Captive vs. Wild, $p < 0.001$; Tourism vs. Wild, $p > 0.05$) were significantly higher in captive individuals than the tourism and wild groups (Figures 6D–F).

In total, 45 KEGG pathways (level 2) were predicted by PICRUSt 2. The mean weighted nearest sequenced taxon index (NSTI) for all samples was 0.286 ± 0.056 (x = mean ± SD). We found that several predicted KEGG pathways of level 2 were enriched in one of the three study groups based on LEfSe results (LDA > 2, $p < 0.05$; Figure 6G). Four predicted KEGG pathways were overrepresented in the wild individuals, including Metabolism of terpenoids and polyketides, Immune system, Nervous system, Global and overview maps. Additionally, six predicted KEGG pathways Infectious disease_parasitic, Substance

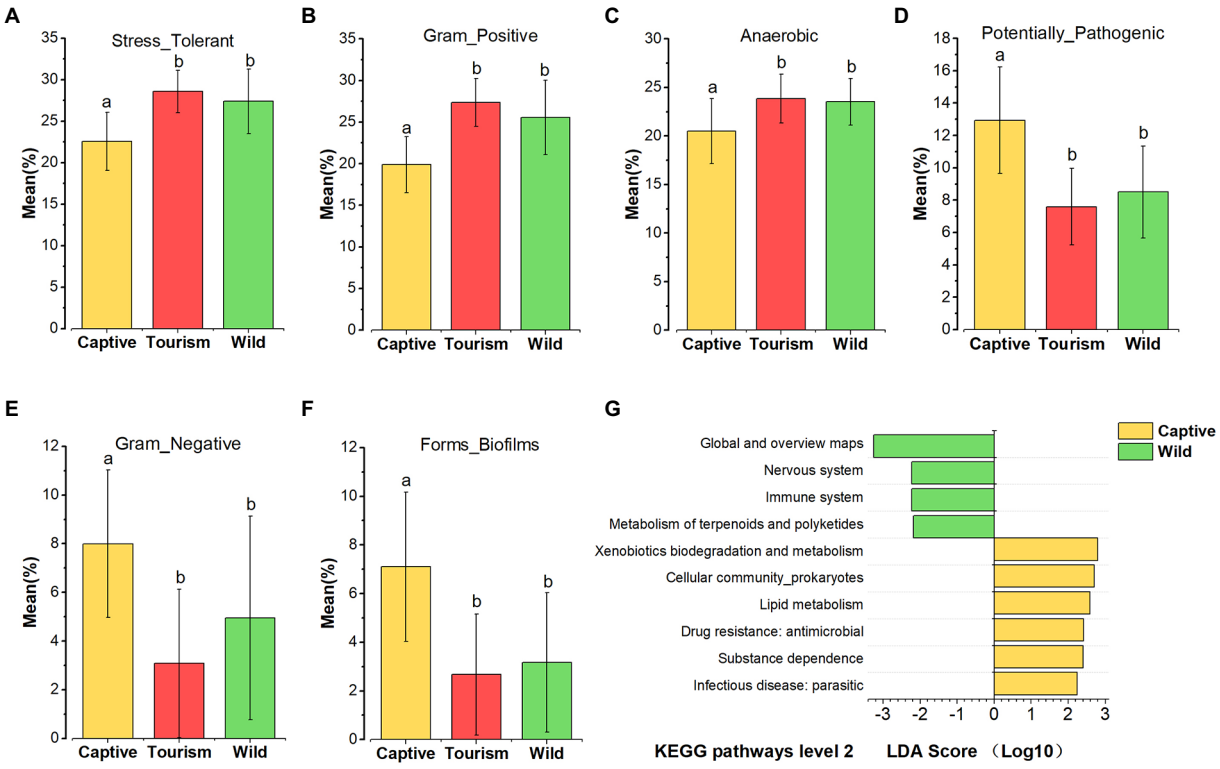

FIGURE 6
Variation in phenotypic properties and KEGG pathways of gut microbiome among different groups. **(A–F)**: Comparison of the phenotypic properties of Stress Tolerant, Gram Positive, Anaerobic, Potentially Pathogenic, Gram Negative and Forms Biofilms, respectively. A Kruskal-Wallis test was used to evaluate the variation across study groups. Post-hoc tests (Tukey–Kramer test) for pairwise comparison tests (*p* values were adjusted by Bonferroni). Significance was set at the 0.05 level. **G**: KEGG pathways at level 2. Indicators identified by linear discriminant analysis effect size (LEfSe) analysis, LDA>2, $p<0.05$.

dependence, Drug resistance (antimicrobial), Xenobiotics biodegradation and metabolism, Lipid metabolism, and Cellular community (prokaryotes) were overrepresented in captive individuals. However, we did not find any of the KEGG pathways overrepresented in the tourism group.

Discussion

In the current study, we found that the alpha diversity of gut microbiome in the tourism group still maintain the same level as wild living individuals, which is inconsistent with previous findings that habitat disturbance can result in the loss of gut microbial diversity in the wild NHPs (Amato et al., 2013). In addition, the similarity in community structures of gut microbiomes between wild and tourism was dissimilarity lower than that between wild and captive groups. For the tourism group of Tibetan macaque used in this study, a small amount of corn provided by staff each day, but feeding from tourists were forbidden strictly. Although the group has been a subject of over 30 years for behavioral research and primate-focused tourism (Li and Kappeler, 2020), individuals of this group still maintain a direct interface with natural environment, and getting their food and water from sources which contain complexity of microbial communities (Sun et al., 2021b). Our result demonstrated that living in the wild and consuming natural diet could maintain the gut bacterial diversity of tourism group, which is consistent with our previous study on gut mycobiome in Tibetan macaques (Sun et al., 2021a).

In contrast, even though the captive group of Tibetan macaques translocated from their natural habitat only 1 year ago, the alpha diversity of this group was reduced significantly compared to the wild and tourism groups, and lower similarity in community structure of gut microbiomes between wild and captive than that between wild and tourism groups. This result is consistent with recent studies on the gut bacterial microbiome of NHPs (Clayton et al., 2016; Frankel et al., 2019; Barelli et al., 2020). Previous studies have shown that beta diversity variation was strongly influenced by host diet type and living environment (Muegge et al., 2011; Clayton et al., 2016; McKenzie et al., 2017). In addition, recent studies have reported that natural diets or releasing captive animals back into their natural habitat can help restore their native gut bacteria diversity (Schmidt et al., 2019; Liddicoat et al., 2020; Martínez-Mota et al., 2020). From wild to the captive

environment, individuals of Tibetan macaques experienced a decrease in diet diversity and restricted in a direct contact with the microbes of natural environment, as well as antibiotic treatment. These changes may be responsible for the increased differentiation in community structure and decreased in diversity of captive Tibetan macaques' gut microbiome.

We also found that the gut bacterial microbiome of Tibetan macaques in all three study groups was dominated by two phyla Firmicutes and Bacteroidetes, which also been reported in previous studies of humans and NHPs (Yatsunenko et al., 2012; Amato et al., 2013; Grieneisen et al., 2019). The two families Lachnospiraceae and Ruminococcaceae, enrichment in wild group, are highly related to the decomposition and utilization of plant diet, as well as production of the short-chain fatty acids (SCFAs; Boeckaert et al., 2008; Huws et al., 2011; Byndloss et al., 2017). In particular, we found that the Prevotellaceae and *Prevotella* were significantly enriched in the individuals of wild and tourism groups. Species of Prevotellaceae and *Prevotella* are associated with digestion of hemicellulose, pectin, starch, carbohydrate and simple sugars (Wu et al., 2011; Amato et al., 2015). These results indicated that individuals of tourism group still maintained its ability to decompose and utilize the nature plant diet. In contrast, these abilities are significantly reduced in captive Tibetan macaques. It has been shown that providing captive animals with more natural diets can help restore their native gut bacteria (Schmidt et al., 2019; Liddicoat et al., 2020; Martínez-Mota et al., 2020). Our findings, in combination with previous studies, suggest that providing more natural food may help captive Tibetan macaques recover these declining gut microbes.

Furthermore, all the wide-scale phenotypic properties of the gut microbiome, in particularly the potential pathogens, did not variation significantly between tourism and wild group. However, the two genera, including *Streptococcus* and *Sarcina*, were significantly enriched in the captive individuals of Tibetan macaque. Most members of the *Streptococcus* and *Sarcina* are potential pathogenic bacteria in humans and animals (Wyder et al., 2011; Tintara et al., 2019). The prediction results of wide-scale phenotypic properties KEGG pathways also support that the captive breeding may cause the increase of potential pathogens in gut of these macaques (Amato et al., 2016; Balasubramaniam et al., 2022). In particular, the loss of diversity in gut microbiome will increase the risk of opportunistic infection (Arrieta et al., 2014; Malard et al., 2021). In addition, KEGG pathway of drug resistance (antimicrobial), overrepresented in captive Tibetan macaques indicated that the antibiotic resistance genes (ARGs) of gut microbiomes were enriched in this group. The ARGs of a microbial community could influence the function of native bacteria and increase pathogen morbidity (Howard et al., 2003; Lin et al., 2015; Kim et al., 2020). Therefore, lower alpha diversity, enrichment in drug resistance (antimicrobial) of KEGG pathways and higher potential pathogenic bacteria among the captive Tibetan macaques have important implications the negative health consequences of captive. In the future, exploring ways to restore native gut microbiome and its diversity of captive individuals are very important for primate conservation practice.

Conclusion

Our results provide evidence that different anthropogenic disturbances have different effects on the gut microbiome of Tibetan macaques. For the macaques used for primate ecotourism, living in the wild and consuming diverse natural diet could allow them maintain higher similarity with wild group in the alpha diversity and composition of gut microbiome including the predominant families and genera. It must be noted that the significant difference in beta diversity and community compositions between wild and tourism groups also was detected. A possible explanation is that corn provided by staff caused these changes. However, lower alpha diversity, higher KEGG pathway of drug resistance (antimicrobial) and higher potential pathogenic were detected in the gut microbiome of individuals in captive environment, which consuming a less varied diet, and limited exposure to soils and natural plants. Future studies should focus on investigating whether the changes in gut microbiome resulting from primate-focused tourism have negative effects on the health of Tibetan macaques, as well as exploring ways to restore the native gut microbiome and its diversity of captive individuals.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

BS and JL conceived and designed the experiments. BS, YX, XX, HC, RY, XW, and DX performed the experiments. BS, YX, XX, HC, and RY contributed to reagents, materials, and analysis tools. BS, YX, XX, and JL wrote the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1023898/full#supplementary-material

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Geographical patterns of *Fejervarya limnocharis* gut microbiota by latitude along mainland China's coastline

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The gut microbiota affects many aspects of host biology and plays key roles in the coevolutionary association with its host. Geographical gradients may play a certain role on gut microbiota variation in the natural environment. However, the distribution pattern of amphibian gut microbiota in the latitudinal gradient remains largely unexplored. Here, we sampled six natural populations of *Fejervarya limnocharis* along the eastern coastline of mainland China (spanning 20°–30° N=1,300km) using 16S rRNA amplicon sequencing to characterize the gut microbiota. First of all, a significant correlation between gut microbial diversity and latitude was observed in our research system. Second, we discovered that latitude influenced the composition of the gut microbiota of *F. limnocharis*. Finally, we detected that geographical distance could not determine gut microbiota composition in *F. limnocharis*. These results indicate that latitude can play an important role in shaping the gut microbial diversity of amphibian. Our study offers the first evidence that gut microbial diversity of amphibian presents a latitudinal pattern and highlights the need for increased numbers of individuals to be sampled during microbiome studies in wild populations along environmental gradients.

KEYWORDS

biogeographic patterns, latitude, amphibian, gut microbiota, 16S rRNA

Introduction

Understanding geographical distributions of gut microbiota and identifying which factors contribute to gut microbial diversity are critical steps for biodiversity conservation, because the gut microbiota affects many aspects of host health and key vital functions (such as immune function, metabolism, and inflammation) and plays key roles in the

coevolutionary association with its host (Ley et al., 2008; Huang et al., 2022). Numerous studies have established that microbial communities can exhibit biogeographic patterns, and in many cases these patterns are qualitatively similar to those of macro-organisms (Sullam et al., 2012; Linnenbrink et al., 2013; Suzuki et al., 2019, 2020). However, the biogeographical pattern of microbiota is largely unclear in relative to that in macro-organisms, and the underlying mechanisms are still largely unknown (Meyer et al., 2018).

The latitudinal gradient is one of the most striking geographic gradients (Schemske et al., 2009). It shows that species diversity is negatively correlated with latitude because latitudinal spatial variation in biotic and abiotic conditions leads to changes in spatial selective pressures (Gaston, 2007). Low-latitude hosts may make high gut microbial diversity increase metagenomic diversity to increase fitness (Liu et al., 2022). High-latitudes hosts may select low gut microbial diversity in order for specific microbes to buffer the abiotic pressures (Pugnaire et al., 2019). Researchers have only begun to understand how ecological forces that drive gut microbial biogeographic patterns at macroscales (Sudakaran et al., 2012; Sullam et al., 2012; Linnenbrink et al., 2013; Ren et al., 2017; Goertz et al., 2019; Suzuki et al., 2019, 2020), while the distribution pattern of gut microbiota in the latitudinal gradient remains unresolved. Furthermore, few studies have investigated geographical patterns and ecological and evolutionary drivers of gut microbial diversity in wildlife (Ge et al., 2021; Henry and Ayroles, 2022).

Amphibians are ideal for determining the effect of latitudinal increases as a result of climate change and habitat degradation because they heavily depend on specific environmental conditions since they are poikilotherms (Parmesan, 2007). Latitudinal gradient determines some characteristics of poikilotherms (such as body size, metabolism, diet, reproduction, and behavior), and they have a wide range of physiological adaptations that vary within members of clines when the same species lives in different latitudinal gradients (Gillooly et al., 2001; Ladyman et al., 2003; Angielczyk et al., 2015; Liu et al., 2018). Mainland China's coastline is located in Southeast Asia and spans eight different climatic zones and a distance of approximately 18,000 km (Cai et al., 2022), providing an ideal natural gradient of different environmental conditions (such as a stable climate and lack of gene flow; Wang et al., 2014). Amphibians on the coastline of mainland China allow for the study of the effect of environmental conditions on the gut microbial biota of poikilotherms along the latitudinal gradient.

Fejervarya limnocharis is an excellent model to investigate evolutionary responses in clines. *Fejervarya limnocharis* is found from 10°S to 40°N along the latitudinal gradient (Figure 1), and typically located in grass, crops and stones around rice fields, dry land, ponds, bogs and ditches (Wu et al., 2006). They emerge from hibernation at the end of March to early April. Males mature in less than 1 year and are characterized by a black pigment on the throat (Li et al., 2011). Females become mature after 1 year. The maximal life span is about 35 years for males and 4–5 years for females (Li et al., 2011). *Fejervarya limnocharis* breed between the end of April and late August. New juvenile frogs after metamorphosis can be found as early as July. There are no hunting activities carried out by humans on this frog in field (Wu et al., 2006). The diet of the *F. limnocharis* is mainly composed of arthropods with no significant difference among seasons and between males and females (Li et al., 2011). Seasonal foods and body mass were significantly correlated with gut microbial diversity of *F. limnocharis* (Chang et al., 2016; Huang et al., 2017; Xu et al., 2020; Huang and Liao, 2021).

To study whether the latitudinal gradient could affect the gut microbiota of amphibians, we sampled six natural populations of *F. limnocharis* near the eastern coastline of mainland China (spanning 20°–30° N = 1,300 km) using 16S rRNA amplicon sequencing to characterize the gut microbiota. We identified latitudinal patterns of the gut microbial diversity of *F. limnocharis* along the coastline of mainland China and evaluated the effects that climate factors had on the gut microbial diversity of *F. limnocharis*.

Materials and methods

Ethical approval

The animal study was reviewed and approved by Animal Care and Use Committee of Anhui Normal University.

Study sites and sampling

We conducted six line transects having a width of 2 m and a length of 100 m along the accessible edges of the rice fields, the shorelines of ponds, ditches, and dry land on each site. These were paralleled at an interval of about 15–20 m. We searched for *F. limnocharis* in transects with an LED lamp (Warsun W81, Warsun Optoelectronic Technology Company, Ningbo, China) between 19:00 and 22:30, and randomly captured 10 frogs (five adults of each sex) along transects on each site (Figure 1). A total of 60 adult's frogs were collected in a cool box with two ice bags and immediately transported to the laboratory. The gender of all individuals was confirmed by direct observation of secondary sexual characteristics. Body mass index was calculated as the weight to the nearest 0.1 mg using an electronic balance. We treated all individuals with the single pithing method. The gut contents were collected after pithing and emptied into a sterile vial and immediately stored at −80°C. Data on mean annual temperature and annual precipitation at each sampling site were obtained from the WorldClim at a resolution of 10′ (Hijmans et al., 2005).

DNA extraction, PCR amplification, and sequencing

The frozen aliquots of gut contents (200 mg per aliquot) were added to a 2 ml screw-cap and thawed on ice until 1.4 ml ASL

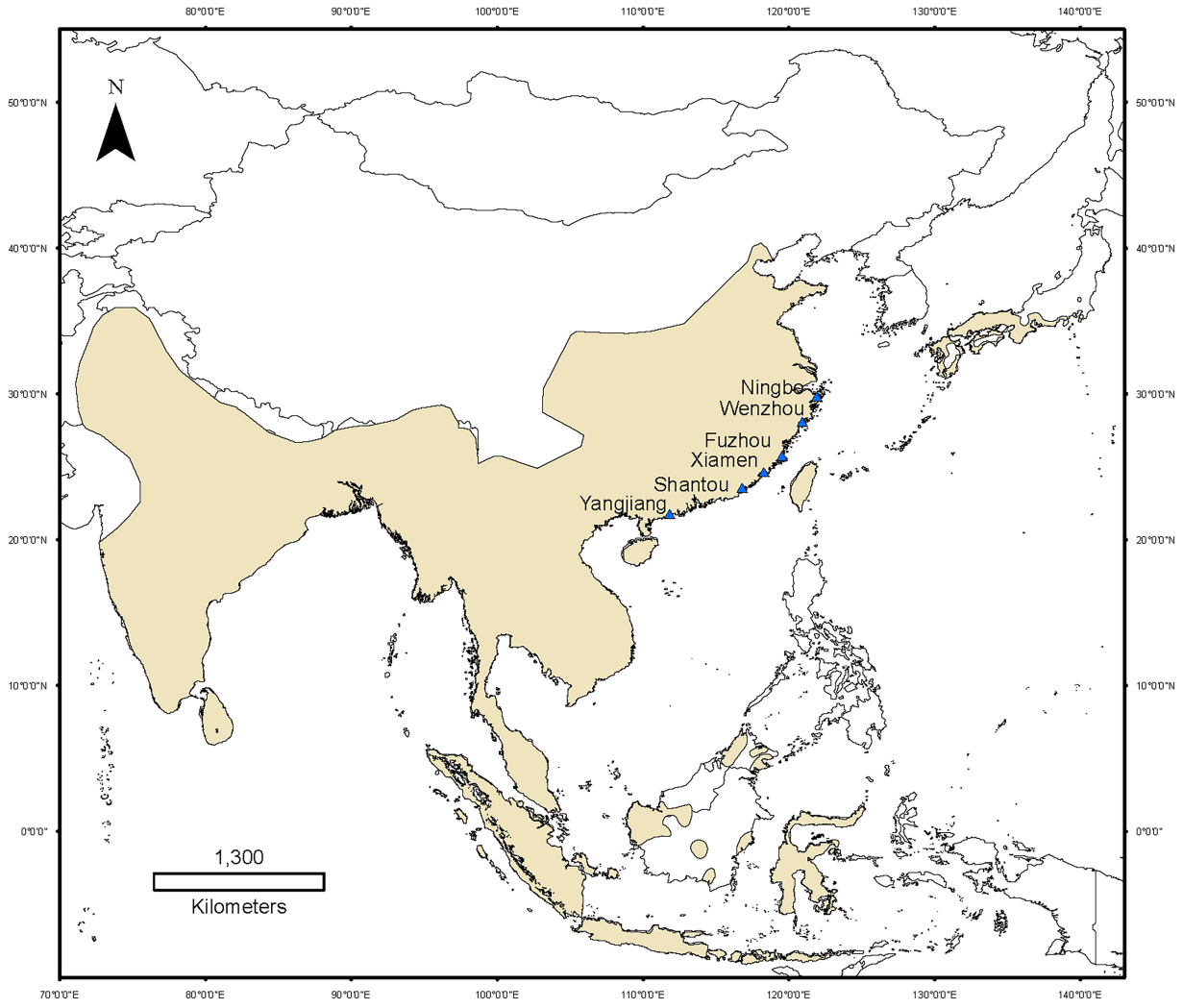

FIGURE 1
Map of *Fejervarya limnocharis* populations sampled along the eastern coast of mainland China. Yellow areas indicate the global distribution of *F. limnocharis*. Blue triangle denotes the sampling sites.

buffer from the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was added. The subsequent DNA extraction steps were conducted according to the QIAamp Kit protocol. The concentration of the DNA was measured using a NanoDrop™ 2000 (Thermo Scientific). Finally, the DNA was dissolved in 200 µl sterile ddH2O and stored at −20°C until use. We amplified the V3-V4 region of the 16S rRNA genes in triplicate using primers (341F: 5'-CCTAYGGGRBGCASCAG-3′, 806R: 5'-GGACTA CHVGGGTWTCTAAT-3′). The polymerase chain reaction (PCR) reactions were performed using the bio-Rad T100 gradient PCR instrument with 15 µl Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 µM forward and reverse primers and 10 ng template DNA per 30 µl reaction. The PCR cycle consisted of initial denaturation at 98°C for 1 min; 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s; and extension at 72°C for 5 min. The PCR products were mixed with the same volume of 1 × loading buffer and were detected using 2% agarose gels. PCR products were purified using the Omega Gel&PCR Clean Up Kit (Omega Bio-Tek, USA). Sequencing was operated by Shanghai Majorbio Bio-Pharm Technology Co. Ltd. (Majorbio, Shanghai, China) operated the sequencing (Liu et al., 2021).

Bioinformatic analyses

Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. The paired-end reads were merged using VSEARCH (v2.18.0; Rognes et al., 2016) and USEARCH (v10.0.240), and raw tags were successfully spliced. Quality filtering of the raw reads was performed to remove low-quality amplicon sequences by using USEARCH. The filtered reads were then processed as unique sequences using the "minuniquesize 10"

parameter in VSEARCH. Putative chimeras were discarded using silva data in VSEARCH. The UPARSE algorithm clusters the sequences with 97% similarity into operational taxonomic units (OTUs) after dereplication and discarding all singletons (Edgar, 2013). Finally, a feature OTU table can be obtained by quantifying the frequency of the feature sequences in each sample. Simultaneously, the feature sequences can be assigned taxonomy, typically at the kingdom, phylum, class, order, family, genus, and species levels, providing a dimensionality reduction perspective on the microbiota. Then, we performed a subsequent analysis of alpha and beta diversities based on this output normalized data.

Statistical analysis

Alpha diversity indexes, which are used to analyze the complexity of bacterial diversity for a sample, were calculated and displayed using R software (Version 4.2.0), such as the observed-species index, Chao1 index, Shannon-Wiener index, and Richness index (Vegan package). Rarefaction curves of OTU richness were calculated using Vegan (Oksanen et al., 2013). To evaluate the differences among samples in terms of bacterial community complexity, we calculated the beta diversity on Bray-Curtis, Euclidean distance, Jaccard matrix, weighted UniFrac and unweighted UniFrac using Vegan, Phyloseq, Ape, GUniFrac, and Amplicon packages in R.

The variation trends of a given gut microbiota across the latitudes used Kruskal-Wallis with *post hoc* tests, and the Wilcoxon rank-sum test. Principal coordinates analysis (PCoA) and linear discriminant analysis (LDA) with different distance matrixes was used to examine the separation of species across samples. Differences in relative abundance of the microbial features were determined by LDA effect size (LEfSe; Segata et al., 2011). Analysis of functional gene content using PICRUSt2 (Douglas et al., 2020) provides proportional contributions of KEGG categories for each sample. We determined the relationships between gut microbial diversity of *F. limnocharis* and latitude, mean annual temperature, and mean annual precipitation along the eastern coast of mainland China sites using a single-variable regression. The mantel test (Vegan package) was used to analyze the correlation between differences of gut microbiota and geographical distances in different samples.

Results

Characteristics of the 16S rRNA gene sequence data

Along six coastal mainland sites (Figure 1), The V3-V4 regions of the bacterial 16S rRNA gene in gut microbiota collected from 60 adult's frogs were sequenced to characterize the microbiotas of *F. limnocharis*. The rarefaction curves (Figure 2) approached a plateau, thereby suggesting that the number of OTUs was sufficient to reveal the authentic bacterial communities within each sample. All sequences could be classified into 18 phyla, 36 classes, 61 orders, 124 families, and 277 genera. At the phylum level (Figure 3A), Firmicutes, Proteobacteria and Bacteroidetes had the highest abundance and contributed 34.59%, 34.42%, and 11.73% of bacteria, respectively. The abundance of Actinobacteria phylum differed significantly among populations ($p < 0.05$).

At the genus level, *Desulfovibrio* (phylum: Proteobacteria), *Citrobacter* (phylum: Proteobacteria), *Peptoclostridium* (phylum: Firmicutes) and *Cetobacterium* (phylum: Fusobacteria), *Bacteroides* (phylum: Bacteroidetes) were the most abundant, and accounted for 15.46%, 6.95%, 6.39%, 6.01%, and 4.77% of bacteria, respectively (Figure 3B). The abundance of *Microbacterium*, *Nocardiopsis*, *Dorea*, *Rhizobium,* and *Rickettsiella* differed significantly among populations (wilcox test, $p < 0.01$), as did *Desulfovibrio*, *Faecalitalea*, *Bacillus,* and *Parabacteroides* (wilcox test, $p < 0.05$; Figure 3B; Supplementary Figure S1B).

Variation of bacterial diversity of the gut microbiota in different geographic populations

To further determine whether microbial communities differ among different geographic populations, we compared the diversity and richness indices among populations (Table 1). Gut microbial diversity ranged from 409.22 at Wenzhou to 665.47 at Shantou for the Chao1 index, 273 to 482.8 for the Richness index, and 3.08 to 4.58 for the Shannon index. Low latitudinal sites showed higher gut microbial diversity than high latitudinal sites ($p < 0.05$, ANOVA, Tukey-HSD test, Figure 4A). To better understand the differences in richness between different groups, the overlap of the core microbial communities between the groups was illustrated using a Venn diagram. This analysis showed that only 543 of the 1,288 OTUs accounting for the total richness were common to all of the samples (Figure 4B). These data demonstrated that approximately 57.84% of the OTUs were identified in the different geographic populations.

To measure the degree to which the gut microbiota differed in these populations, principal coordinates analysis (PCoA) was conducted based on these distance matrixes (Bray-Curtis, Jaccard, Euclidean distance, and weighted and unweighted UniFrac matrixes) between the gut samples. Although the PCoA analysis did not show a strong difference in the microbiota of individuals from different populations (Supplementary Figure S2A), the composition of the gut microbiota differed significantly from each other's populations (all $p < 0.01$, Kruskal-Wallis test, Supplementary Figure S2B). In this seemingly contradictory situation, a linear discriminant analysis (LDA) was conducted based on the Bray-Curtis distance matrix from the gut content samples. Despite significant inter-individual variation, the gut microbiota of different geographic populations could be clearly separated using LDA by Bray-Curtis distance (Adonis, $R = 0.105$, $p = 0.038$, Figure 4C).

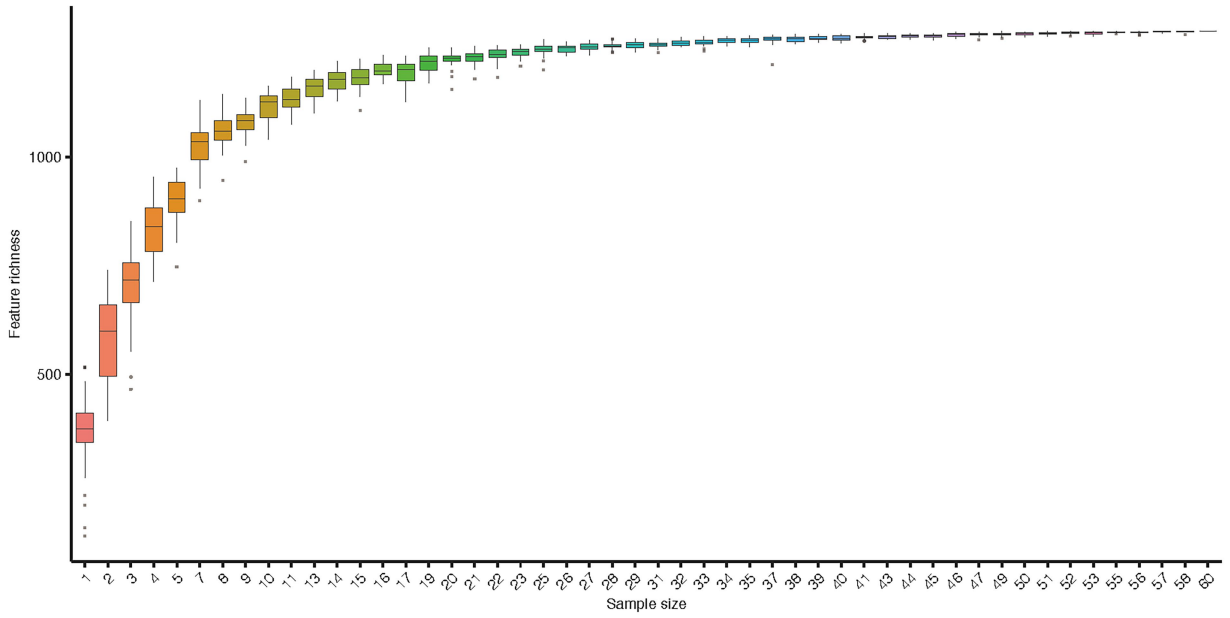

FIGURE 2
Rarefaction curve of gut microbiota. Accumulation curves for gut microbiota indices indicate that the sampling was comprehensive. Boxes denote the interquartile range (IQR) between the first and third quartiles (25th and 75th percentiles, respectively), and the line inside denotes the median. Whiskers denote the lowest and highest values within 1.5 times IQR from the first and third quartiles, respectively. Circles denote outliers beyond the whiskers.

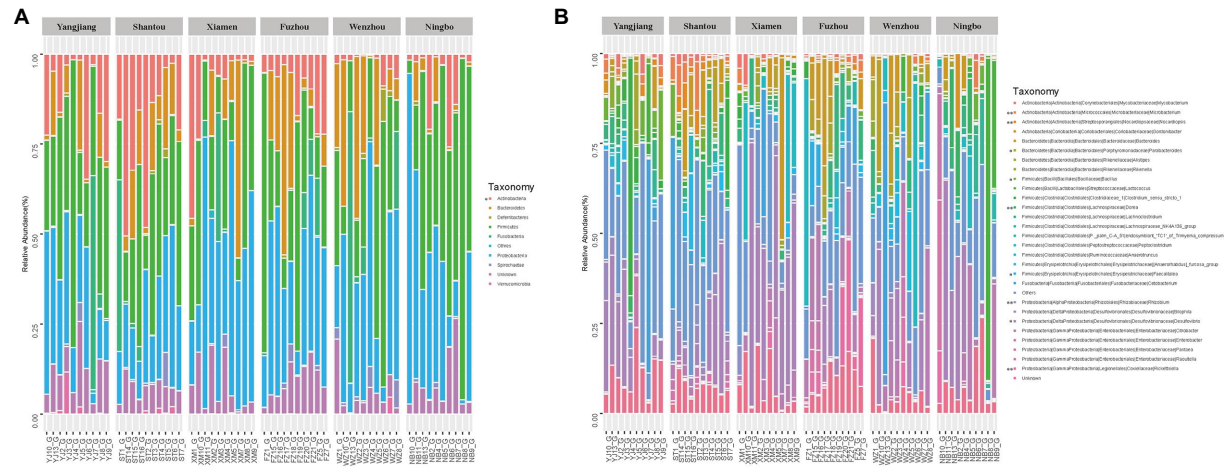

FIGURE 3
Relative contribution of gut microbiota in each sample. **(A)** Relative contribution in Phylum level. **(B)** Relative contribution in the top 30 dominant genera.

By using PICRUSt2, we predict the functional composition. Pathways level 1 includes metabolism, human diseases and cellular processes, and organismal systems. Pathways level 2 includes carbohydrate metabolism, drug resistance, endocrine, and infectious disease as the significant variation of predicted functional profiles of microbial communities among geographic populations ($p < 0.01$ or $p < 0.05$; see Table 2).

Latitude-associated alteration in gut microbiota

To identify the specific bacterial taxa associated with latitude, we compared the gut microbiota of high latitudinal individuals (Wenzhou and Ningbo) and low latitudinal individuals (Yangjiang and Shantou), using the LDA effect size (LEfSe)

TABLE 1 Alpha-diversity of gut microbiota in *Fejervarya limnocharis* along the eastern coast of mainland China.

Locations	Chao1	Richness	Shannon
Yangjiang	606.01 ± 91.51	433.80 ± 88.03	4.23 ± 1.16
Shantou	665.47 ± 86.95	482.80 ± 70.80	4.58 ± 0.62
Xiamen	465.39 ± 67.82	299.10 ± 63.24	3.38 ± 0.86
Fuzhou	542.80 ± 86.06	376.70 ± 83.96	3.92 ± 0.56
Wenzhou	409.22 ± 101.91	273.00 ± 85.08	3.08 ± 0.78
Ningbo	412.53 ± 71.12	297.90 ± 66.74	3.37 ± 1.21

method. A cladogram representative of the structure of the gut microbiota and the predominant bacteria is shown in Figures 5A,B, which also displays the greatest differences (LDA > 2) in taxa.

Moreover, we found a clear latitudinal pattern of gut microbial diversity in *F. limnocharis* populations on the eastern coast of mainland China (Figure 6, Supplementary Table S1). Gut microbial diversity of *F. limnocharis* decreased with increased latitude ($r = -0.829$, $p < 0.05$ for Chao1, Richness and Shannon). Furthermore, a positive correlation was found between the gut microbial diversity of *F. limnocharis* and the mean annual temperature ($r = 0.829$, $p < 0.05$ for Chao1, Richness and Shannon). Gut microbial diversity of *F. limnocharis* was not correlated to mean annual precipitation ($r = -0.086$, $p = 0.872$ for Chao1, Richness and Shannon) across sampling sites. Meanwhile, geographic distance had no significant influence on the gut bacterial beta-diversity of *F. limnocharis* (Mantel test: $p > 0.05$) (Supplementary Table S2).

Discussion

We studied variations in the *F. limnocharis* gut microbiota sampled across a large latitudinal range along the eastern coast of mainland China. Our results showed that the composition of the "important" gut microbiota of *F. limnocharis* had no significant difference between our study and studies in other parts of China. Importantly, we found several biogeographic general patterns. First, a significant correlation between gut microbial diversity (alpha-diversity) and latitude was observed in our research system. Second, we discovered that latitude influenced the composition of gut microbiota (beta-diversity). Finally, we detected that geographical distance could not determine the gut microbiota composition in *F. limnocharis*. In conclusion, we determined the most significant contributing factor to the microbial diversity of *F. limnocharis* among populations along the eastern coast of mainland China was their geographic latitude.

We observed significant negative correlations between alpha-diversity and latitudinal gradient. This result supports the hypothesis that species diversity is negatively correlated with latitude (Schemske et al., 2009). Host associated microbiomes display similar patterns was rarely found in natural populations (Meyer et al., 2018; Ge et al., 2021). For example, Ge et al. (2021) found that two honeybee species (*Apis cerana* and *Apis mellifera*) presented similar patterns that population of low latitudes harbored gut bacterial communities with a higher diversity in five geographically distant sites along a latitudinal gradient (Ge et al., 2021). However, the negative correlation between leaf fungal endophyte communities and several tree species was detected in a broad latitudinal gradient from the Canadian arctic to the lowland tropical forest of central Panama (Arnold and Lutzoni, 2007). The inconsistent pattern was also discovered between wild house mice and gut microbial diversity from three latitudinal transects across North and South America (Suzuki et al., 2020). Furthermore, the neutral dynamics dominated the gut microbial diversity of fly (*Drosophila melanogaster*) along a latitudinal cline (Henry and Ayroles, 2022). Compared with macro-organisms, microbial biogeographic patterns tend to be much weaker (Meyer et al., 2018; Henry and Ayroles, 2022). Longevity, or dispersal abilities of the microbiome are a possible explanation for this phenomenon (Meyer et al., 2018). However, *F. limnocharis* have the shortness of life (about 3–5 years for males and 4–5 years for females) and poor dispersal ability (Li et al., 2011). Our results clearly showed that geographic latitude has contributed to the gut microbial diversity pattern of *F. limnocharis* populations along the eastern coast of mainland China.

We found that latitude may explain significant differences in microbial variation in our research system. Latitude might be viewed as an approximation for the sum of environmental effects such as local weather patterns, which incorporates average annual temperatures and precipitation. In this study, we distinguish the effects of these ecological factors. We detected that gut microbial diversity of *F. limnocharis* was positively associated with mean annual temperature. This result is similar to that found in previous studies (Tsuchida et al., 2002; Huang and Zhang, 2013; Ge et al., 2021). However, we could not determine the relationship between gut microbial diversity with mean annual precipitation. One possible explanation for this is that there is too much rainfall along the eastern coast of mainland China (Shi et al., 2015).

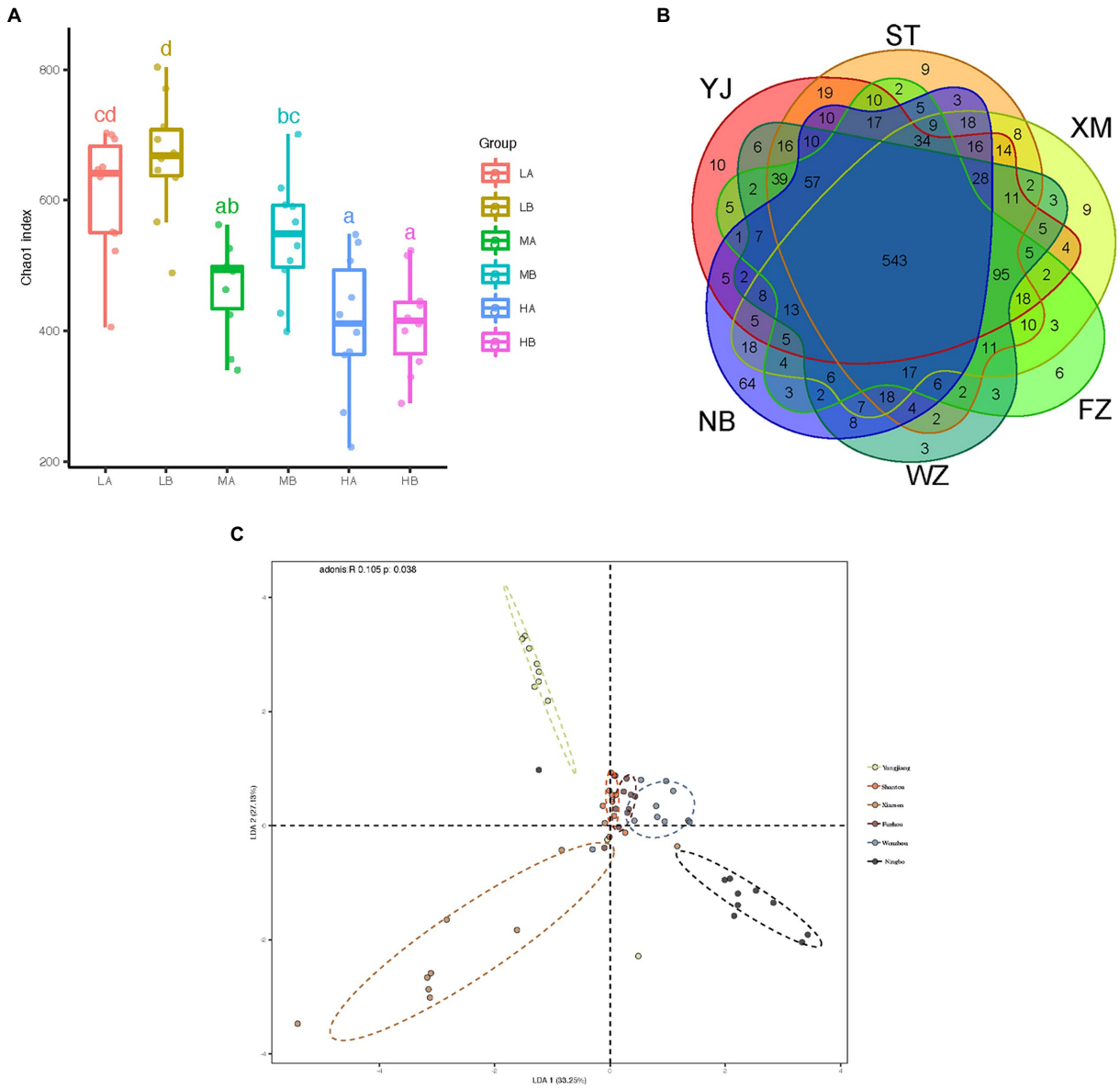

FIGURE 4
Comparison of the composition of fecal microbiota in different geographic populations. **(A)** Chao1 index of the microbiota of gut microbiota from different populations. The horizontal bars within boxes represent medians. The tops and bottoms of boxes represent the 75th and 25th percentiles, respectively. The upper and lower whiskers extend to data no more than 1.5× the interquartile range from the upper edges and lower edge of the box, respectively. **(B)** Venn diagram illustrating overlap of OTUs in gut microbiota between different groups. **(C)** The difference in the composition of the gut microbiota is illustrated by the linear discriminant analysis of the Bray-Curtis distance matrix among samples. All pairwise comparisons involved the first, second and third principal axes. The first, second and third principal axes explained most of the variances (33.25, 27.13, and 39.62%, respectively).

Several studies have suggested that host genetics impacts composition of ectotherm gut microbial communities (Yuan et al., 2015; Shu et al., 2019; Xu et al., 2020; Chen et al., 2022). They have reported various dietary effects on ectotherm gut microbiota across different environments (Kohl et al., 2014; Chang et al., 2016; Knutie et al., 2017; Tong et al., 2019; Montoya-Ciriaco et al., 2020; Tang et al., 2020; Wang et al., 2021). While we cannot rule out the possible contribution of host genetics or dietary availability to the gut microbial variation of *F. limnocharis* in the eastern coastline of mainland China, we sampled content microbial communities in a rigorous context. First, the collected samples were males and females of equal weight, in the same seasons, and from different sampling sites, respectively. This is due to fact that dietary variations and

TABLE 2 The proportion of predicted functional profiles in different populations (KEGG Pathway level 1 and level 2).

	Yangjiang		Shantou		Xiamen		Fuzhou		Wenzhou		Ningbo	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Pathway_Level _1												
Brite Hierarchies**	34.62%	1.23%	34.37%	0.92%	35.15%	1.31%	35.31%	0.96%	36.01%	0.49%	35.83%	0.59%
Metabolism**	35.62%	1.82%	36.18%	1.42%	34.62%	1.81%	35.17%	1.53%	33.65%	0.90%	33.87%	1.06%
Human Diseases**	3.27%	0.13%	3.20%	0.16%	3.52%	0.18%	3.39%	0.24%	3.38%	0.24%	3.36%	0.18%
Cellular Processes*	4.43%	0.38%	4.37%	0.33%	4.63%	0.44%	4.02%	0.25%	4.49%	0.34%	4.38%	0.56%
Organismal Systems*	1.85%	0.09%	1.88%	0.10%	1.78%	0.15%	1.80%	0.15%	1.70%	0.11%	1.67%	0.17%
Environmental Information Processing	6.06%	0.35%	6.16%	0.32%	6.24%	0.51%	6.23%	0.63%	6.35%	0.69%	6.77%	0.73%
Genetic Information Processing	6.80%	0.55%	6.55%	0.32%	6.55%	0.54%	6.47%	0.54%	6.55%	0.79%	6.32%	1.07%
Not Included in Pathway or Brite	7.37%	0.26%	7.29%	0.23%	7.52%	0.41%	7.61%	0.64%	7.86%	0.64%	7.79%	0.69%
Pathway_Level _2												
Carbohydrate metabolism**	9.38%	0.33%	9.64%	0.43%	8.97%	0.36%	9.66%	0.45%	9.10%	0.34%	9.48%	0.40%
Drug resistance: antimicrobial**	0.76%	0.09%	0.76%	0.08%	0.85%	0.11%	0.91%	0.14%	0.96%	0.13%	0.92%	0.17%
Endocrine system**	0.66%	0.04%	0.68%	0.05%	0.57%	0.09%	0.62%	0.08%	0.56%	0.07%	0.60%	0.11%
Infectious disease: bacterial**	0.68%	0.08%	0.65%	0.08%	0.79%	0.10%	0.64%	0.07%	0.75%	0.10%	0.72%	0.08%
Infectious disease: viral**	0.15%	0.04%	0.13%	0.04%	0.19%	0.04%	0.10%	0.03%	0.11%	0.05%	0.13%	0.07%
Protein families: metabolism**	5.83%	0.16%	5.72%	0.21%	6.01%	0.29%	5.96%	0.22%	6.04%	0.15%	5.86%	0.10%
Signaling molecules and interaction**	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Transport and catabolism**	0.23%	0.05%	0.27%	0.04%	0.20%	0.05%	0.25%	0.08%	0.19%	0.05%	0.20%	0.04%
Cellular community – eukaryotes**	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Substance dependence**	0.02%	0.01%	0.02%	0.01%	0.01%	0.02%	0.01%	0.01%	0.00%	0.00%	0.01%	0.01%
Metabolism of cofactors and vitamins*	4.05%	0.11%	3.94%	0.14%	4.06%	0.22%	3.84%	0.23%	3.93%	0.25%	3.65%	0.37%
Amino acid metabolism*	6.66%	0.73%	6.81%	0.55%	6.28%	0.76%	6.30%	0.48%	5.91%	0.36%	5.99%	0.59%
Lipid metabolism*	1.97%	0.26%	1.99%	0.20%	1.80%	0.31%	1.92%	0.15%	1.73%	0.09%	1.84%	0.20%
Development and regeneration*	0.02%	0.01%	0.02%	0.01%	0.02%	0.01%	0.02%	0.01%	0.02%	0.01%	0.01%	0.01%
Excretory system*	0.03%	0.00%	0.03%	0.01%	0.03%	0.01%	0.03%	0.01%	0.03%	0.01%	0.02%	0.01%
Metabolism of terpenoids and polyketides*	0.92%	0.12%	0.92%	0.10%	0.83%	0.15%	0.87%	0.08%	0.78%	0.07%	0.81%	0.11%
Unclassified: genetic information processing*	0.75%	0.07%	0.72%	0.05%	0.80%	0.07%	0.81%	0.13%	0.85%	0.15%	0.90%	0.19%

*Significant values as $p < 0.05$.
**Significant values as $p < 0.01$ by using non-parametric Kruskal–Wallis test.

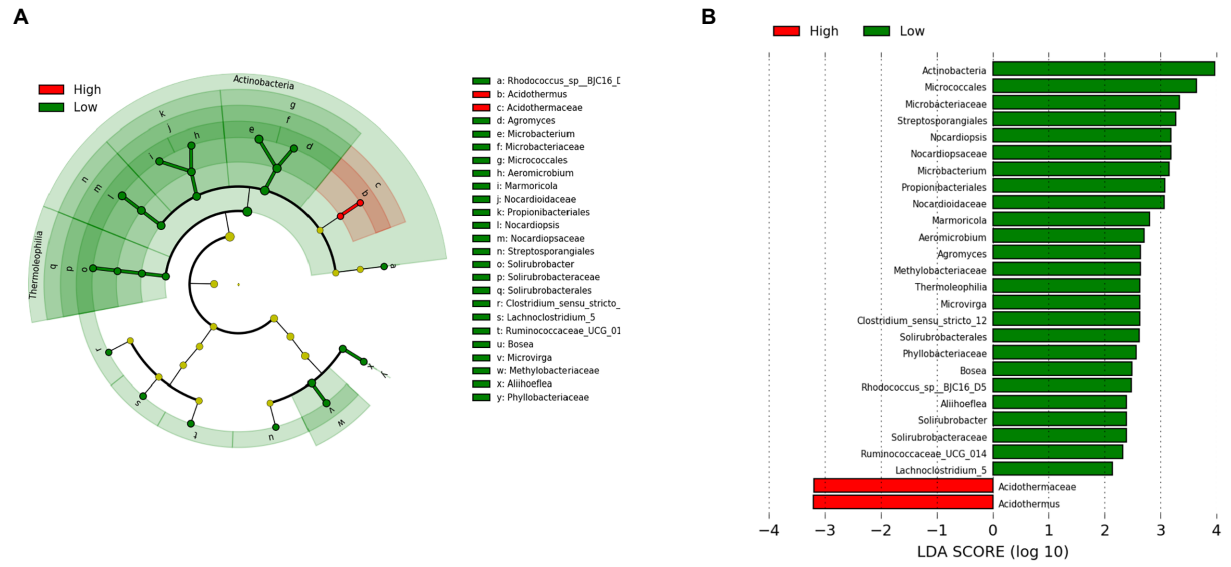

FIGURE 5
The taxa whose abundance differed between high latitudinal individuals (Wenzhou and Ningbo) and low latitudinal individuals (Yangjiang and Shantou) were identified by LDA effect size (LEfSe). **(A)** Taxonomic cladogram obtained from LEfSe analysis of sequences (relative abundance ≥0.5%). Biomarker taxa are highlighted by colored circles and shaded areas (high latitudinal samples are shown in red and low latitudinal samples are shown in green). Each circle's diameter reflects the abundance of that taxa in the community. **(B)** The taxa whose abundance differed between the high latitudinal samples (red bars) and the low latitudinal samples (green bars). The cutoff value of ≥2.0 used for the LDA is shown.

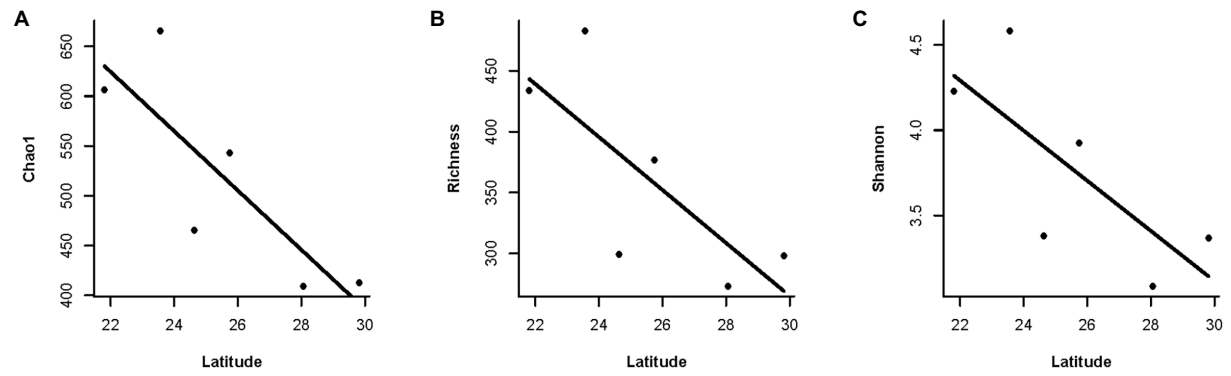

FIGURE 6
The relationship between latitude and gut microbial diversity along the eastern coast of mainland China. **(A)** Chao1 index. **(B)** Richness index. **(C)** Shannon index.

the body mass of *F. limnocharis* were significantly correlated with gut microbial composition, and the composition of gut microbiota of *F. limnocharis* vary seasonally in response to diet variations (Chang et al., 2016; Huang and Liao, 2021). Second, similar bacteria were found as in other studies of natural *F. limnocharis* populations (Chang et al., 2016; Xu et al., 2020).

In summary, we found that there was a significant variation in the gut microbiota diversity of *F. limnocharis* along the eastern coast of mainland China, and latitude had a significant effect on the gut microbial composition of natural populations of *F. limnocharis*. However, our understanding of the importance of latitude for the gut microbial communities of animals was limited because samples were only collected from shoreline frog populations. Thus, further studies (such as longitudinal sampling or controlled transplant experiments of microbes) are needed in order to comprehensively understand the composition, structure, and function of the gut microbiome of *F. limnocharis* in natural populations (Suzuki et al., 2020; Henry and Ayroles, 2022). Our study provided the first

evidence that latitude might shape the gut microbiota of frog along the eastern coast of mainland China.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by Anhui Normal University.

Author contributions

SW, SL and NZ designed the study and wrote manuscript. NZ, ZM, YJ, YS, YX, and YW collected samples. NZ, ZM, YJ, YS, SW, YX, and YW performed experiments. NZ and SW analyzed data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1062302/full#supplementary-material

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The divergent effects of moderate climate warming on the gut microbiota and energetic state of cold-climate lizards from open and semi-closed microhabitats

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Introduction: Understanding the physiological responses to warming temperatures is critical for evaluating the vulnerabilities of animals to climate warming. The physiological responses are increasingly affected by gut microbiota. However, the interactions between physiological responses and the gut microbiota of sympatric animals from various microhabitats in the face of climate change remain largely unknown.

Methods: To evaluate the effects of warming temperatures on animals from different microhabitats, we compared locomotor performance, metabolic rate, growth, survival, and gut microbiota of two sympatric ectothermic species (*Eremias argus* and *Takydromus amurensis*) from open and semi-closed microhabitats under present and moderate warming climate conditions, respectively.

Results and discussion: We found that locomotor performance and growth rates of snout-vent length (SVL) were enhanced in both lizard species by warming climate. Interestingly, warming temperatures enhanced resting metabolic rates (RMR) in the open-habitat lizard, *E. argus*, but depressed them in the semi-closed habitat lizard, *T. amurensis*. Reversely, the metabolism-related gut microbiota was not affected by warming in *E. argus*, whereas it was significantly enhanced by warming in *T. amurensis*, indicating a plausible compensatory effect of the gut microbiota on the metabolic regulation of *T. amurensis*. Furthermore, warming likely improved immunity in both lizard species by significantly reducing pathogenic bacteria while increasing probiotics. This study found that high-latitude sympatric lizards from both

open and semi-closed habitats were beneficial to warming temperatures by physiological modification and regulation of the gut microbiota and highlighted the importance of integrating the physiology and gut microbiota in evaluating the vulnerability of animals to climate warming.

KEYWORDS

climate warming, physiology, gut microbiota, fitness-related traits, metabolism, sympatric lizards

Introduction

Warming temperatures caused by climate change have threatened animals universally (Foden et al., 2008; Leung et al., 2020). The manner in which animals respond to warming temperatures determines their vulnerability to climate warming (Williams et al., 2008; Huey and Tewksbury, 2009; Kearney et al., 2009). Animals can respond to increasing temperatures induced by climate warming by modifying their behavioral, physiological, and life-history metrics (Huey et al., 2012; Logan et al., 2018; Maebe et al., 2021; Dematteis et al., 2022; Gomez Ales et al., 2022; Li et al., 2022; Zhang and Wang, 2022). Among various metrics, physiological responses such as metabolic rate might be the most important since it determines energetic allocation and other biological rates, which are the foundations for other responding metrics (e.g., Brown et al., 2004; Chown and Gaston, 2008; Logan et al., 2014; Bestion and Cote, 2018; Logan and Cox, 2020). For example, *Takydromus* lizards from low latitudes exhibit limited metabolic acclimation capacities at multiple biological hierarchies and in embryonic acute heat tolerance (EAHT); thus, they are predicted to be more vulnerable to climate warming (Sun et al., 2021, 2022). Adjustment of metabolic rates can regulate energy expenditure, and thus may buffer the negative effects of warming temperatures on animals (Marshall and Mcquaid, 2011; Ma et al., 2018b), or even benefit cold-climate ectotherms (e.g., Liu et al., 2022). In addition, locomotion can determine the ability of an animal in food and resources acquiring. Therefore, ectotherms will be vulnerable to climate change if their locomotion are depressed at extremely high body temperatures as thermal performance curve predicts (Gunderson and Leal, 2016).

As a regulator in physiology, immunity, and fitness of animals (Ramakrishna, 2013; Gould et al., 2018; Zhang et al., 2018; Rastelli et al., 2019), the gut microbiota is also depressed by warming temperatures. For instance, a 2–3°C increase in ambient temperatures induced a 34% loss in diversity of gut microbiota in common lizard, *Zootoca vivipara* (Bestion et al., 2017). Similarly, a severe warming environment (6.7°C on average) decreased the diversity of gut microbiota but increased pathogenic bacteria in race-runner *Eremias argus* (Zhang et al., 2022b). In contrast, the gut microbiota has been shown to benefit the host thermoregulation, immunity, metabolism, growth, development, and even social behaviors (e.g., Macke et al., 2017; Zhang and Wang, 2022), all of which can in turn regulate responses to warming temperatures (e.g., Kearney et al., 2010; Triggs and Knell, 2012; Montoya-Ciriaco et al., 2020). For example, the gut microbiota of tadpoles that experienced 3-week high temperatures (28°C) could modify the metabolism and enhance the heat tolerance of tadpoles (Fontaine et al., 2022). Therefore, how gut microbiota affects the physiological responses of the host to climate warming is complex and still largely controversial (Bestion et al., 2017; Bestion and Cote, 2018). More research is needed to comprehensively understand the relationship between physiology and gut-microbiota responses to warming temperatures, which is critical for evaluating the vulnerabilities of animals and interpreting their mechanisms.

Recently, the beneficial effects of climate warming on high-latitude animals have been increasingly demonstrated (e.g., Cui et al., 2022; Liu et al., 2022). However, the differences in the effects of warming temperatures on the interaction between physiological metrics and gut microbiota in different microhabitats are unknown (e.g., open habitats, semi-closed habitats, and shaded habitats). Animals from different microhabitats might be differently affected by a warming climate. For example, ectotherms in open habitats have higher thermal preferences and tolerances than congeners in semi-closed habitats, and thus they are therefore predicted to be less vulnerable to climate warming (Li et al., 2017; Wang et al., 2019; Gomez Ales et al., 2022). Similarly, the abundance of anaerobic gut bacteria is significantly lower in flying insects than in siblings underground (Yun et al., 2014). Therefore, sympatric animals constitute an ideal research system for investigating the different effects of climate warming on animals from different microhabitats.

In this study, we selected two high-latitude and oviparous sympatric lizards from different microhabitats (*E. argus*: open habitat; *Takydromus amurensis*: semi-closed habitat) to address the physiological and gut-microbiota, as well as their potentially interactive responses to climate warming. We first mimicked the present climate and moderate warming conditions based on natural ambient temperatures to rear the lizards; we then tested the physiological metrics of locomotion and metabolic rates;

we also determined the fitness-related metrics of growth and survival. Furthermore, we applied high-throughput sequencing on 16S rRNA gene amplicons from the feces of *E. argus* and *T. amurensis* under present and warming climates to determine the diversity, composition, and functions of the gut microbiota. By relating the physiological responses and gut microbiota, we further aimed to determine the underlying mechanisms at the microbiological level that affect physiological responses to warming temperatures of high-latitude lizards from different microhabitats.

Materials and methods

Study system and lizard collection

The Mongolian racerunner, *E. argus*, is a small lizard [average snout-vent length (SVL) = 56.30 mm] that originated in Central Asia. It inhabits plains, hills, and other warm areas with dry sands and open environments (**Supplementary Figures 1**, **3**). The Heilongjiang grass lizard, *T. amurensis*, is a small lacertid (average SVL = 55.30 mm) that distributes in northeastern China, near the boundary of Russia and the Korean Peninsula. It mostly inhabits a mixture of shrubland and grasslands, which are cool and semi-closed environments (**Supplementary Figures 2**, **3**). Both *E. argus* and *T. amurensis* are from the Lacertidae, with the primary food being the larvae and adults of insects (Zhao and Adler, 1993; Zhao et al., 1999). The thermal biology and distribution of *E. argus* and *T. amurensis* indicate that they are cold-climate species (Zhao et al., 2008; Park et al., 2014; Li et al., 2017; Hao et al., 2020).

In late May 21, 2021 adult *E. argus* individuals (10 females and 11 males) (45.8°N, 126.5°E) and 40 adult *T. amurensis* individuals (32 females and 8 males) (45.2°N, 127.9°E) were captured by hand and noose in Harbin, China. During collection, three data loggers (iButton, DS1921; MAXIM Integrated Products Ltd., San Jose, CA, USA) were set in the field to collect the hourly ambient temperatures for each sample site. The collected lizards were then transferred to the laboratory and reared in semi-natural enclosures.

Experimental design and lizard husbandry

After being transferred to the laboratory, the lizards were individually marked, measured (SVL ± 1 mm), and weighed (±0.001 g) after 1 day recovery. Then, all lizards were released into semi-natural enclosures for husbandry under different thermal treatment. Semi-natural enclosures were built at Harbin Normal University within the natural distribution range of *E. argus* and *T. amurensis*.

The enclosures for *E. argus* were using their natural substrate of sand, while the enclosures for *T. amurensis* were using the soil substrate. The vegetation in the enclosures were transferred from the site they were collected. Following established method (Liu et al., 2022), we mimicked the thermal environments under the present and warming conditions for each species. We set the temperatures in the present climate similar to the field temperatures (**Supplementary Figure 3**). As the moderate warming scenario are universally accepted in experimental design of global change simulation (e.g., Bestion et al., 2015, 2017; Sun et al., 2018b, 2021; Liu et al., 2022), we set the temperatures in the warming climate mimicked a moderate climate warming scenario (Shared Socioeconomic Paths, SSP1-2.6, 1.3–2.4°C) (IPCC, 2021). We manipulated the thermal environments in the enclosures using a shaded net to simulate the present climate and plastic cover to simulate a warming climate, according to published methods (Sun et al., 2018b; Liu et al., 2022). We used four enclosures for each treatment of each species. The lizards from each species were released evenly and randomly into both present and warming climate conditions (*E. argus*: present climate vs. warming climate = 5♀ 5♂ vs. 5♀ 6♂; *T. amurensis*: present climate vs. warming climate = 16♀ 4♂ vs. 16♀ 4♂). Husbandry was conducted during the active season (i.e., summer) and lasted from June to August 2021. During husbandry, we monitored the operative temperatures (T_e) hourly in the enclosures using iButtons (DS1921; MAXIM Integrated Products Ltd., San Jose, CA, USA) sealed in copper tube models. The models were evenly and randomly placed in enclosures (Liu et al., 2022). Supplementary food (larval *Tenebrio molitor* and crickets dusted with vitamins) was provided twice a week.

Locomotor performance

After 2-months of husbandry, sprint speed was determined as locomotor performance. Based on ambient temperatures under the present and warming climate conditions in the semi-natural enclosures, locomotor performance was measured at two test temperatures for each species in a randomized sequence (i.e., *E. argus*: 24, 32°C; *T. amurensis*: 22, 30°C; see details in **Figure 1** and **Supplementary Figure 3**). The lizards were acclimated in an incubator at the test temperature for approximately 2 h before the test. To check whether the body temperatures of lizards matched the test temperatures, we measured the body temperatures of a subset of lizards before the test (Sun et al., 2014). Locomotor performance was tested by stimulating the lizard to run through a racetrack; that was recorded using an HD video camera (Sony, DCRSR220E, Japan). The racetrack was 1,500 × 100 × 150 mm, with intervals marked every 200 mm. Each lizard was stimulated using a paintbrush to run twice at each temperature, with an interval of 1 h for rest. The videos were analyzed using AVS Video Editor. For

each lizard, the fastest speed through 200 mm was recorded for each running, and the fastest record in two running was used as the sprint speed for each lizard (Taylor et al., 2020). After sprint speed determining, the lizards were released back into the enclosures for 3 days, and their resting metabolic rates (RMR) were tested.

The resting metabolic rates

The resting metabolic rates of the lizards were determined using a "FOXBOX" Respirometry System (Sable Systems International, Henderson, GA, USA) at two test temperatures for each species in a randomized sequence (*E. argus*: 24 and 32°C; *T. amurensis*: 22 and 30°C), based on ambient temperatures under the present and warming climate conditions in the semi-natural enclosures (see details in **Figure 1** and **Supplementary Figure 3**).

We used a respiratory gas exchange method to evaluate the CO_2 production rate (VCO_2) as an index for the RMR following established methods (Ma et al., 2018a; Sun et al., 2020). In summary, individuals were fasted for 24 h in the laboratory before testing. At the beginning of the test, the lizards were acclimated to the test temperatures for 2 h in a temperature-controlled incubator (Blue Pard MGC-100, China). Subsequently, the test was conducted in a closed-circuit system (~300 mL volume), with the chamber housed in an incubator. The circuit system was first opened to air and the air was scrubbed with water and CO_2 using a tube with a flow rate of 240 mL/min to stabilize the baseline. After 5 min, the system was transferred to a closed cycle by connecting the output and the input to the tube. The carbon dioxide production rate (slope of CO_2 volume increase) in the closed-circuit system was continuously recorded for at least 15 min, and the slope was used to calculate the RMR of the individuals. After metabolic rates determination, we measured the body mass of the lizards. Then mass-specific respiratory gas exchange (VCO_2) was used to indicate metabolic rates, expressed as mL/g/h (Ma et al., 2018a; Sun et al., 2020). During the test, the lizards were kept in a chamber with dark surroundings. To minimize the effect of circadian rhythms on RMRs, tests were performed from 8 a.m. to 16 p.m.

Growth rates

After the RMR test, the lizards were re-captured, re-measured (SVL ± 0.01 mm), and re-weighed [body mass (BM) ± 0.001 g]. No individuals died during the experiment. Therefore, the survival rate for the 2-month husbandry period was 100% for each species under both the present and warming climate conditions. Using established methods (e.g., Sun et al., 2011; Hao et al., 2021), the growth rates of SVL and BM were calculated as the daily change in SVL (mm/day) and BM (g/day).

Feces collection and gut microbiota analysis

After the physiological metrics were tested, the feces of the lizards were started to be collected for gut microbiota analysis. To avoid inter-experiment effects, the analysis was started after putting the lizards back into the enclosure for a few days. In brief, we collected the lizards on sunny days, and transferred the lizards to the laboratory. To avoid contamination, the entire process was performed on a clean bench on the day when the lizards were collected. Because the feces of individuals were limited in volume, the feces from 3 to 6 adult individuals from the same treatment were pooled to compose one biological sample. Therefore, the sample size for each group (i.e., each species under each treatment) was evenly three. We totally pooled the feces into three biological samples for each species under each treatment. Each biological sample was placed in a sterile tube and stored at −80°C until DNA extraction.

Deoxyribonucleic acid extraction, amplification, and sequencing were conducted by PersonalBio Biotechnology Co., Ltd. (Shanghai, China). In brief, complete DNA samples were extracted using the E.Z.N.A^{TM} Mag-Bind Soil DNA Kit (M5635, OMEGA, USA), following the manufacturer's protocol. The quantity and quality of the extracted DNA were assessed using a fluorescence spectrophotometer (Quantifluor-ST fluorometer, Promega, E6090; Quant-iT PicoGreen dsDNA Assay Kit, Invitrogen, P7589) at 260 and 280 nm, respectively, and was also detected *via* 1.2% agarose gel electrophoresis. PCR amplification of the bacterial 16S rRNA gene V3-V4 hypervariable region was performed using a forward primer (338F:5′-ACTCCTACGGGAGGCAGCA-3′) and reverse primer (806R:5′-GGACTACHVGGGTWTCTAAT-3′). The PCR reaction (25 μL) was prepared as follows: template DNA 1 μL, amplicon PCR forward primer (10 μM) 1 μL, amplicon PCR reverse primer (10 μM) 1 μL, dNTP (2.5 mM) 2 μL, Fast Pfu DNA Polymerase (0.25 μL), 2 × buffer (5 μL), and ddH_2O (14.75 μL). PCR was performed using the following program: denaturation at 98°C for 5 min followed by 25 cycles consisting of denaturation at 98°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 45 s, with a final extension of 5 min at 72°C. PE250 paired-end sequencing was performed according to the Illumina MiSeq (Illumina San Diego, CA, USA) instrument manual after the DNA libraries were mixed. Raw Illumina amplicon reads were processed using the QIIME2 Core 2019.7 distribution (Bolyen et al., 2019). The Divisive Amplicon Denoising Algorithm (DADA2) pipeline that is implemented in QIIME 2 platform was used to conduct the sequence quality control, including filtering reads for quality, denoising reads, merging forward and reverse reads, removing chimeric reads, and assigning reads to amplicon sequence variants (ASVs) (Callahan et al., 2016). The ASVs were taxonomically classified using the GreenGenes

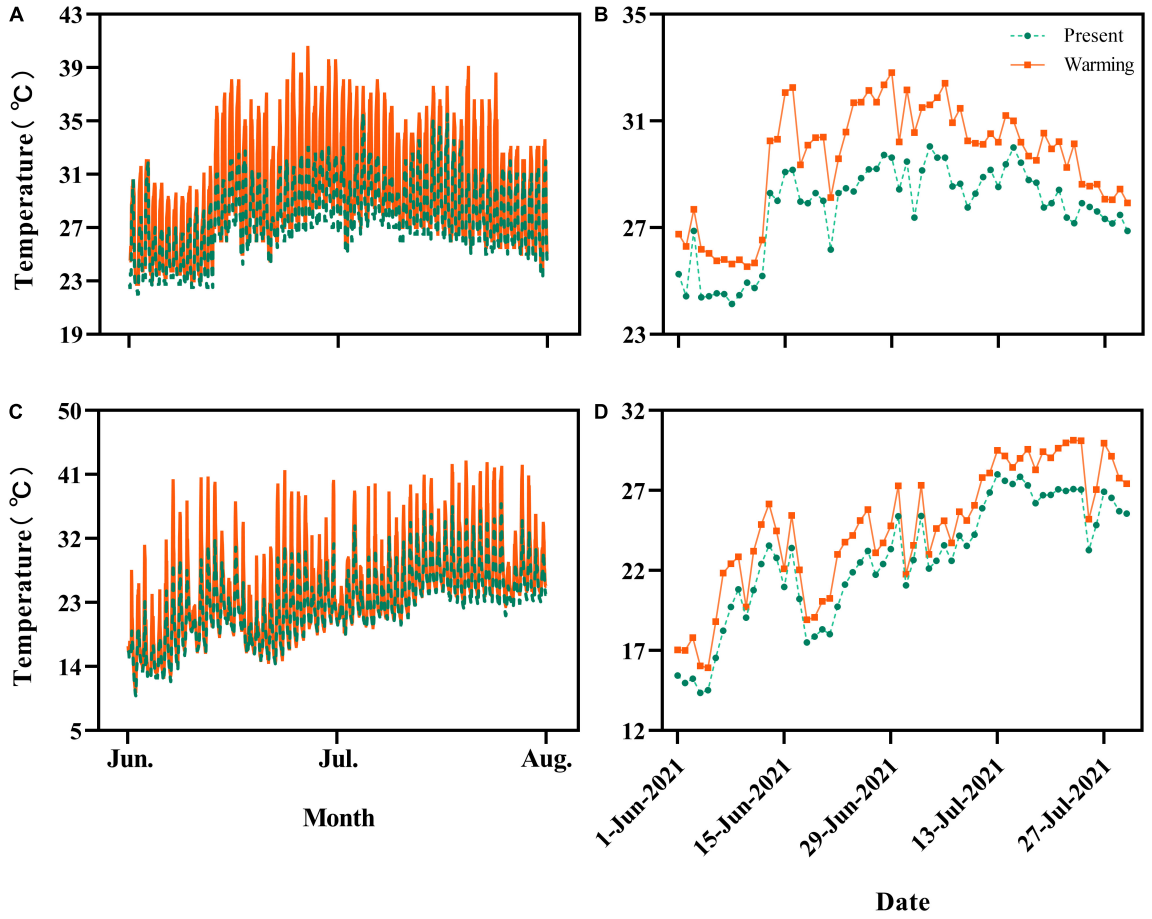

FIGURE 1
Hourly and daily average temperatures experienced by *Eremias argus* **(A,B)** and *Takydromus amurensis* **(C,D)** in the semi-natural enclosures, respectively. Green and orange lines indicate the temperatures of the present climate and warming climate conditions, respectively.

database classifier v13.8, as it is universally employed and its comprehensive range of microbiota (Desantis et al., 2006). The classifier was trained to differentiate taxa present in the 99% Greengenes (v13.8) full-length reference set. Finally, a total of 388,885 and 393,639 valid sequences of the hypervariable V3-V4 region of the 16S rRNA gene were obtained from fecal samples for *E. argus* and *T. amurensis*, respectively.

Statistical analysis

Statistical analyses were conducted using the SPSS software (version 21.0; SPSS, Inc., Chicago, IL, USA). Normality and homogeneity were evaluated using the Kolmogorov–Smirnov and *Levene's* tests, respectively. The differences in the daily average temperatures between the present and warming climate conditions during husbandry were analyzed using a dependent *t*-test. Sprint speed and RMRs were analyzed using repeated measures ANOVAs, with thermal treatments as the main factors and test temperatures as repeated-measure factors. The growth rates in SVL and BM were analyzed using general linear models with thermal treatments as factors.

Alpha diversity indices (ACE, Shannon index) and beta diversity metrics (Bray-Curtis dissimilarity and weighted UniFrac distance) were calculated using QIIME2, with the ASVs table rarefied to 32,335 reads per sample. Principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) based on the Bray-Curtis distance were used to determine the variations in community diversity among different samples (Ramette, 2007). Using the Mann–Whitney U test, we compared the changes in the relative abundance of the gut microbiota composition between the present and warming climate conditions in *E. argus* and *T. amurensis*.

The unique and shared ASVs between the groups were plotted using a Venn diagram. The linear discriminant analysis (LDA) effect size (LEfSe) method was employed to identify variations in microbial communities based on LDA sources (Segata et al., 2011). The statistical significance level was set at $\alpha = 0.05$. The function of gut microbiota was predicted using PICRUSt2 (Phylogenetic Investigation of Communities

by Reconstruction of Unobserved States) based on the KEGG (Kyoto Encyclopedia of Genes and Genomes) database according to 16S rRNA sequencing data, which was based on the ASV tree from the Greengene database (Langille et al., 2013). Welch's *t*-test and false discovery rate (FDR) adjusted *p*-values were used to test the differences in genes, and KEGG pathways[1] were compared between groups to predict their function. All data were analyzed using the Personalbio Gene Cloud.[2]

Results

Thermal environments experienced by lizards

The daily average temperature for *E. argus* in the warming climate conditions (29.56 ± 0.27°C, 25.53–32.81°C) was 1.84°C higher than those of the present climate conditions (27.72 ± 0.22°C, 24.14–30.04°C) (t = 18.436, df = 59, $P < 0.0001$; **Figures 1A,B**). Similarly, the daily average temperature for *T. amurensis* in the warming climate conditions (24.63 ± 0.51°C, 15.92–30.15°C) was 1.93°C higher than those of the present climate conditions (22.67 ± 0.49°C, 14.36–28.02°C) (t = 22.687, df = 59, $P < 0.0001$; **Figures 1C,D**).

Locomotor performance

The sprint speed of *E. argus* was significantly enhanced by test temperatures from 24 to 32°C [$F(1,38) = 32.686$, $P < 0.0001$]. Lizards in the warming climate had higher sprint speeds than those in the present climate [$F(1,38) = 5.167$, $P = 0.029$; **Figure 2A**]. Similarly, the sprint speed of *T. amurensis* was significantly enhanced by test temperature from 22 to 30°C [$F(1,78) = 93.203$, $P < 0.0001$]. Lizards from the warming climate had a higher sprint speed than those from the present climate [$F(1,78) = 4.964$, $P = 0.029$; **Figure 2B**].

Resting metabolic rates

Resting metabolic rates was significantly enhanced by the increase in test temperatures in both species [*E. argus*: $F(1,14) = 77.721$, $P < 0.001$; *T. amurensis*: $F(1,9) = 17.449$, $P = 0.002$]. The RMR of *E. argus* was significantly enhanced in the lizards from the warming climate [$F(1,14) = 5.171$, $P = 0.039$, **Figure 2C**], whereas the RMR of *T. amurensis* was not affected by climate treatment [$F(1,9) = 0.109$, $P = 0.749$]. Notably, the interaction between test temperature and thermal treatment significantly influenced the RMR of *T. amurensis* [$F(1,9) = 8.163$, $P = 0.019$]. For *T. amurensis*, at a low-test temperature (22°C), RMR was higher in the warming treatment, whereas at a high-test temperature (30°C), the RMR in the warming treatment was lower than those in the present treatment (**Figure 2D**).

Growth rates

Warming climate conditions significantly enhanced the growth rate (GR) in SVL for both lizard species [*E. argus*: $F(1,19) = 5.920$, $P = 0.025$, **Figure 3A**; *T. amurensis*: $F(1,34) = 4.571$, $P = 0.040$, **Figure 3B**], whereas GR in BM was not affected in either species [*E. argus*: $F(1,19) = 0.331$, $P = 0.572$, **Figure 3C**; *T. amurensis*: $F(1,34) = 0.380$, $P = 0.542$, **Figure 3D**].

Bacterial diversity and community structure of gut microbiota

Rank abundance and rarefaction were constructed based on ASVs and showed that the depth of the sequencing results was sufficient (**Supplementary Figures 4**, **5**). In total, we obtained 5,361 and 5,466 ASVs from the present and warming climate conditions of *E. argus* and 1,090 and 2,047 ASVs from the present and warming climate conditions of *T. amurensis*, respectively. The lizards under present and warming climate conditions shared 1,729 ASVs (**Supplementary Figure 6A**); however, 3,632 and 3,737 ASVs were unique to the present and warming climate conditions of *E. argus*, respectively. In addition, present and warming climate conditions shared 382 ASVs (**Supplementary Figure 6B**), but 708 and 1,665 ASVs were unique to the present and warming climate conditions of *T. amurensis*, respectively. Our results showed that warming did not change the bacterial community of either species (**Supplementary Figure 7** and **Supplementary Tables 1**, **2**).

Taxonomic composition of gut microbiota

The ASVs of *E. argus* obtained from the samples consisted of 10 phyla, 20 classes, 28 orders, 42 families, and 59 genera (**Figure 4**). The most dominant phyla of *E. argus* were *Bacteroidetes* (present vs. warming = 41.47% vs. 46.51%), *Proteobacteria* (present vs. warming = 29.26% vs. 32.38%), *Firmicutes* (present vs. warming = 24.07% vs. 16.87%), and *Verrucomicrobia* (present vs. warming = 3.77% vs. 2.34%) (**Figure 4A**). The warming climate condition significantly increased the relative abundance of *Bacteroides fragilis* and *Bacteroidales nordii* but depressed the relative abundance of the *Enterobacteriales* order and *Enterobacteriaceae* family significantly in *E. argus* (all $P < 0.05$; **Figures 4E,G,K**).

1 http://picrust.github.io/picrust/

2 https://www.genescloud.cn

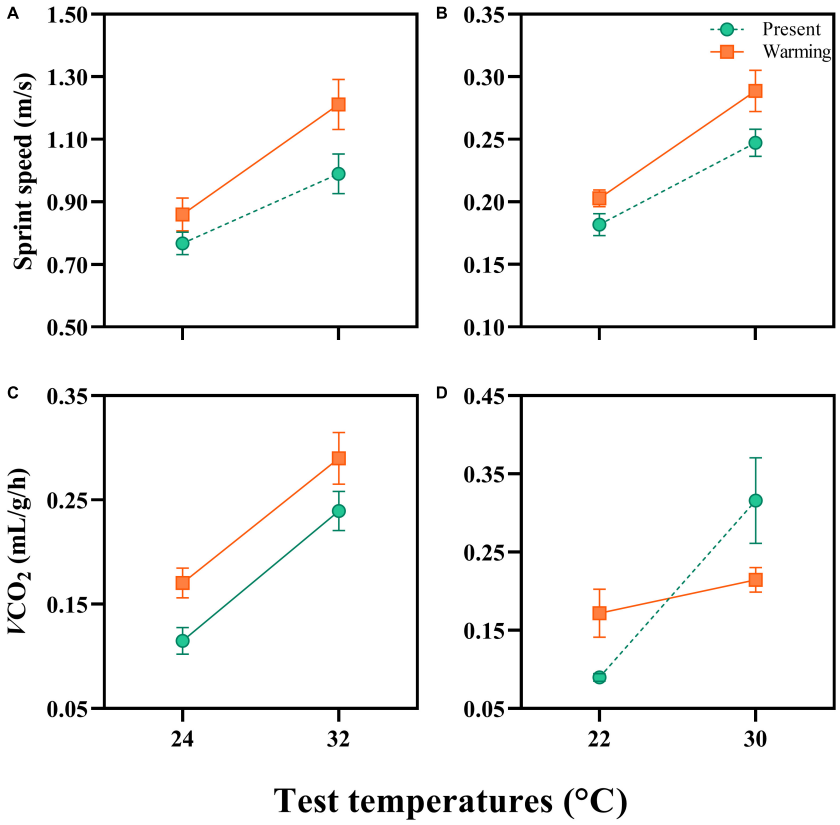

FIGURE 2
Sprint speed and resting metabolic rates (RMR) of *Eremias argus* **(A,C)** and *Takydromus amurensis* **(B,D)** from the present and warming climate conditions. The test temperatures for *E. argus* were 24 and 32°C, and for *T. amurensis* were 22 and 30°C, respectively. Green and Orange dots and lines indicate the present and warming climate conditions, respectively. Data are shown as mean ± standard error (SE).

The ASVs of *T. amurensis* obtained from the samples consisted of 8 phyla, 16 classes, 25 orders, 42 families, and 51 genera (Figure 4). The most dominant phyla of *T. amurensis* were *Proteobacteria* (present vs. warming = 70.88% vs. 41.30%), *Bacteroidetes* (present vs. warming = 12.69% vs. 33.02%), *Firmicutes* (present vs. warming = 3.29% vs. 23.73%), and *Fusobacteria* (present vs. warming = 10.68% vs. 1.48%) (Figure 4B). The warming climate condition significantly increased the relative abundance of bacteria belonging to the phyla *Bacteroidetes*, *Firmicutes,* and *TM 7*, but significantly depressed the relative abundance of the bacteria belonging to the phyla *Fusobacteria*, *Proteobacteria,* and *Tenericutes* in *T. amurensis* (all $P < 0.05$; Figure 4B).

Microbial community similarities and differences

Linear discriminative analysis of effect size analysis was used to detect variations in the relative abundance of microbiota at different hierarchies to further identify shifts in composition of gut microbes in different lizards. The results showed that five gut microbiota taxa differed in abundance in *E. argus*, with three taxa (1 order, 1 family, and 1 species) being more abundant in the present climate condition and two species being more abundant in the warming climate condition. The *Bacteroides* genus was the major taxon contributing to these differences (all LDA scores > 2, $P < 0.05$) (Figure 5A). In *T. amurensis,* 37 gut microbiota taxa differed in abundance, among which 15 taxa (3 phyla, 3 classes, 3 orders, 3 families, 2 genera, and 1 species) were more abundant in the present climate condition and 22 (3 phyla, 3 classes, 3 orders, 7 families, 5 genera, and 1 species) were more abundant in the warming climate condition. Three phyla, *Bacteroidetes*, *Firmicutes,* and *TM7*, were the major taxa contributing to these differences (all LDA scores > 2, $P < 0.05$) (Figure 5B).

Prediction of bacterial functions

Principal coordinate analysis analysis revealed that the function of the gut microbiota was highly aggregated in the different groups (Supplementary Figure 8A: *E. argus*; Supplementary Figure 8B: *T. amurensis*)

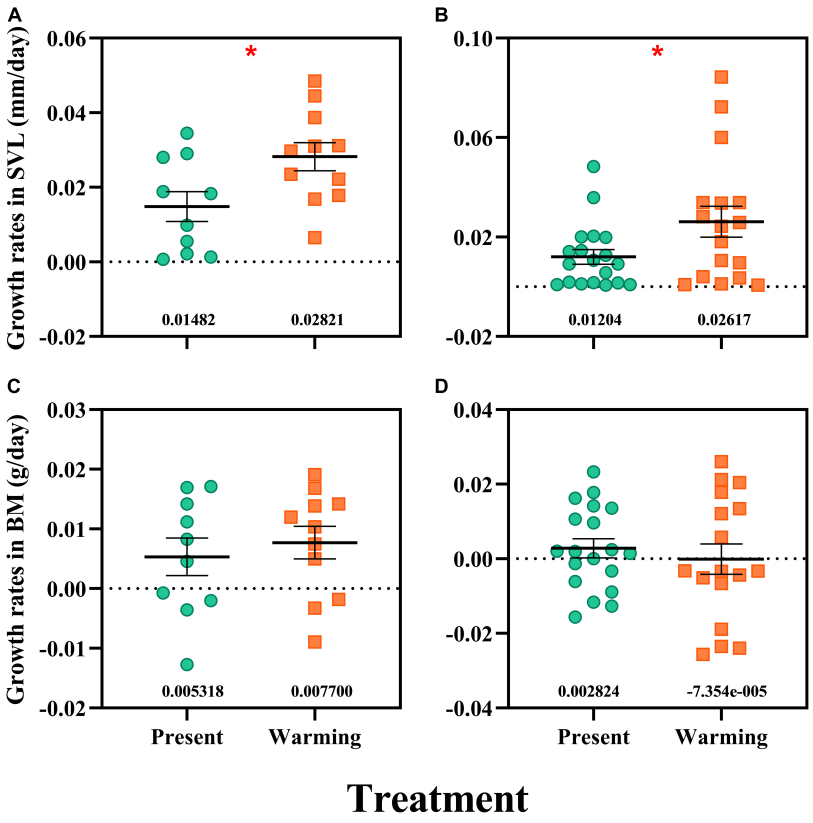

FIGURE 3
Growth rates in snout-vent length (SVL) and body mass (BM) of *Eremias argus* **(A,C)** and *Takydromus amurensis* **(B,D)**. Each dot indicates an individual; the green and orange dots indicate the present and warming climate conditions, respectively. Data are shown as mean ± standard error (SE). *Asterisk indicates significant differences between present and warming climate conditions.

(**Supplementary Table 2**). In the first-level functional classification of KEGG pathways, only the metabolic function of *T. amurensis* was significantly upregulated (*Z*-value > 0, $P < 0.0001$) (**Figure 6**). Upon further analysis of the differences in specific pathways, *E. argus* showed up-regulation of ko00523 (Polyketide sugar unit biosynthesis) *via Bacteroides* genus enrichment ($P < 0.0001$), and down-regulation of ko05100 (bacterial invasion of epithelial cells) through a reduction in the *unclassified-Rikenellaceae* family under warming climate conditions ($P = 0.015$) (**Figures 7A,C**). *T. amurensis* showed up-regulation of three pathways (ko05130-Pathogenic *Escherichia coli* infection, ko00906-Carotenoid biosynthesis, and ko00511-Other glycan degradation) caused by the reduction of the unclassified-*Enterobacteriaceae* family under warming climate conditions ($P < 0.0001$) (**Figures 7B,D**).

Discussion

In this study, we mimicked thermal environments under moderate warming scenarios (SSP1-2.6,1.3–2.4°C) (IPCC, 2021) to rear cold-climate lizards (*E. argus* and *T. amurensis*) from different microhabitats and found that the effects of this moderate warming enhanced growth rate and locomotor performance but did not affect survival rates in both lizard species. These positive effects support the increasing claims that ectotherms from high latitudes would benefit from moderate climate warming (e.g., Hao et al., 2021; Cui et al., 2022; Liu et al., 2022). Interestingly, the effects of warming climate on gut microbiota differed between *E. argus* and *T. amurensis*, which are from open and semi-closed microhabitats, respectively. Although the alpha and beta diversities did not differ between the present and warming treatments in either species, the composition of gut microbiota differed between the present and warming treatments in both lizard species. According to further functional analysis, the microbiota in *T. amurensis* might be adaptive in metabolism, assisting the lizards in adapting to warming climate conditions. In addition, warming climate conditions likely improved immunity by significantly reducing pathogenic bacteria and increasing probiotics in both lizard species.

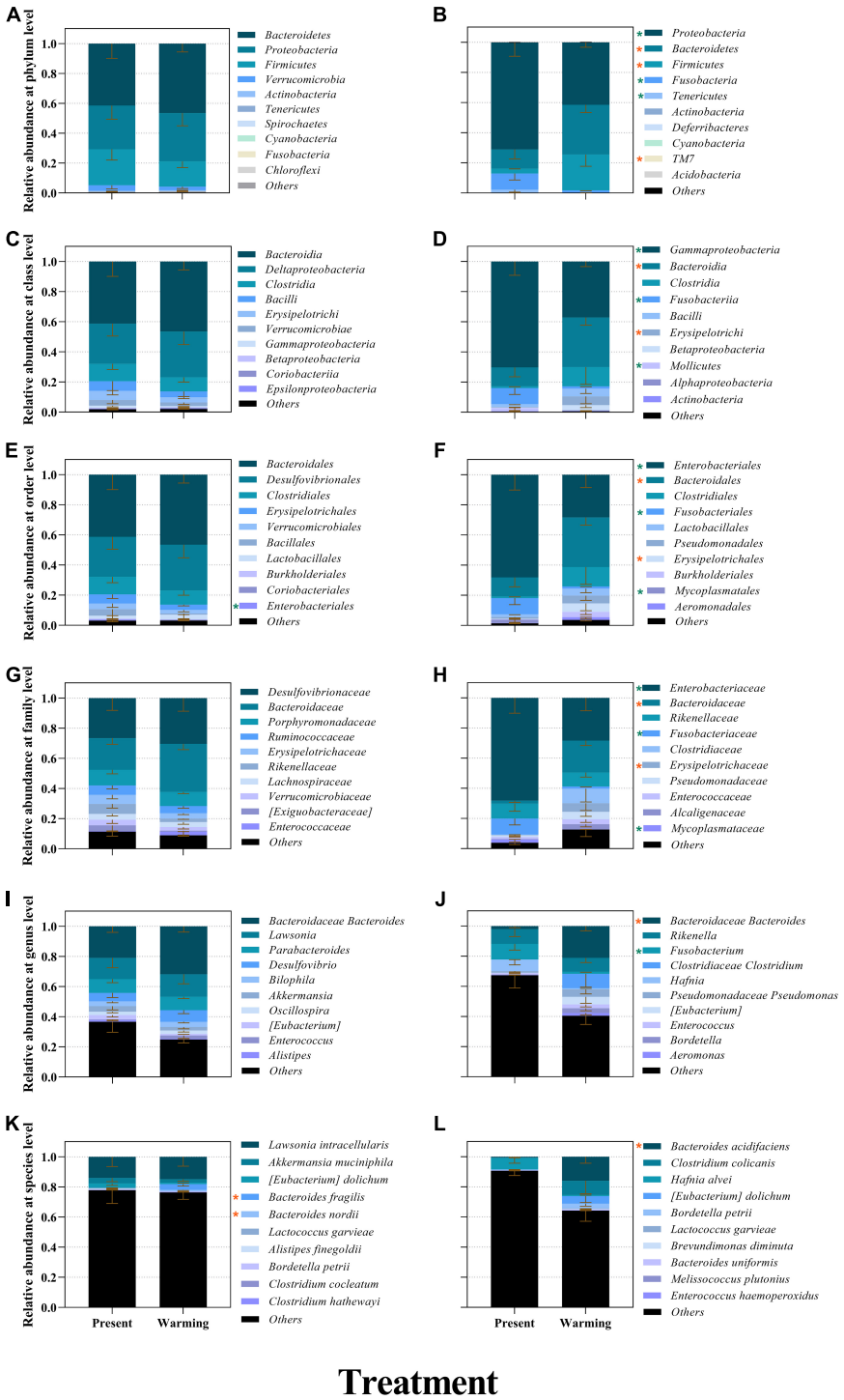

FIGURE 4

The relative abundance of bacterial compositions of *Eremias argus* **(A,C,E,G,I,K)** and *Takydromus amurensis* **(B,D,F,H,J,L)** at different levels (**A,B**: phylum; **C,D**: class; **E,F**: order; **G,H**: family; **I,J**: genus; **K,L**: species). Different colors in the figures indicate the different groups, and details are shown on the right sides of each figure, respectively. Present and warming indicate present and warming climate conditions, respectively. Data are shown as mean ± standard error (SE). Orange and green asterisks indicate significant upregulation and downregulation of warming climate conditions against the present climate conditions, respectively ($P < 0.05$).

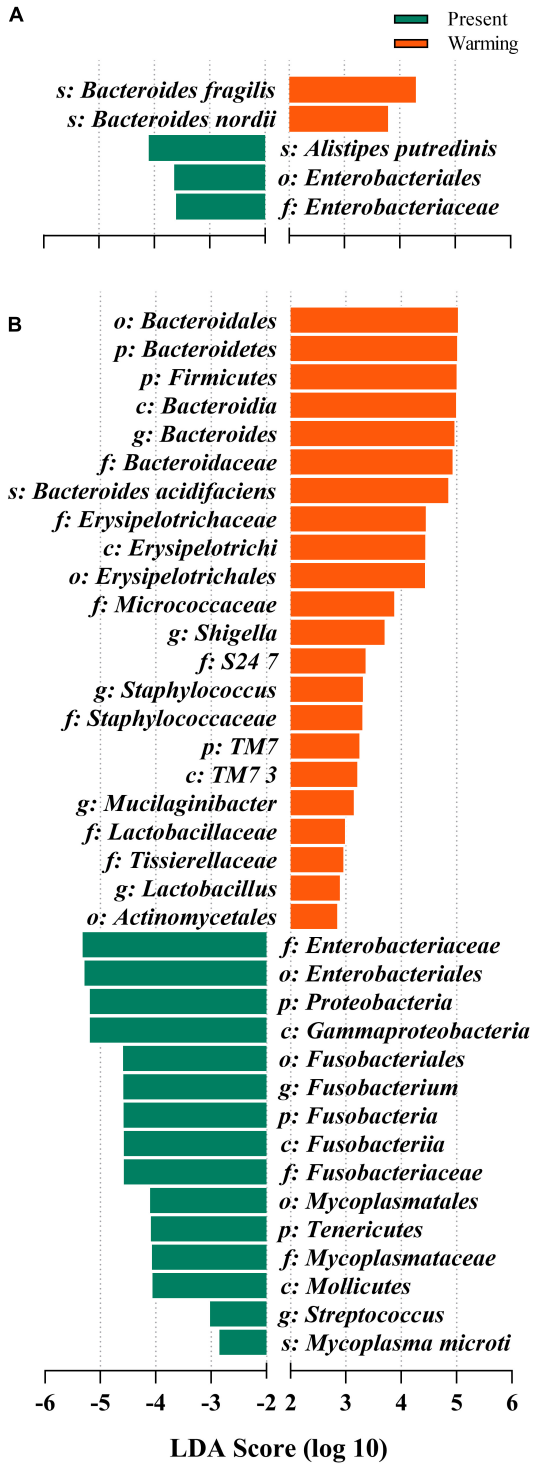

FIGURE 5
Differences in bacterial taxa determined by linear discriminative analysis of effect size (LEfSe) of **(A)** *Eremias argus* and **(B)** *Takydromus amurensis*. The highlighted taxa were significantly enriched in the group that corresponds to each color ($P < 0.05$). Linear discriminatory analysis **(**LDA**)** scores can be interpreted as the degree of difference in relative abundance. Green- and orange-colored bars indicate the present and warming groups.

Warming climate benefits in the physiological responses and fitness of both lizard species

Darwin fitness metrics, such as survival, growth, locomotor performance, and metabolic rates, are used universally in evaluating individual conditions and thus vulnerabilities to climate warming (e.g., Seebacher et al., 2014; Sun et al., 2014, 2018a, 2022). For example, warming temperatures have decreased the survival rate of common lizard juveniles, thus the population was predicted to crash in the near future (Bestion et al., 2015). Similarly, spawning migration survival of Fraser River sockeye salmon (*Oncorhynchus nerka*) decreased significantly in Quesnel and Adams, British Columbia, Canada, as temperature increased, which depressed the population recruitment (Martins et al., 2011). In this study, both lizard species exhibited enhanced growth rates in the warming climate (**Figure 3**), but their survival rates were not affected. With the same survival, warming temperatures enhanced the growth rate, which is considered to have a beneficial effect on individuals and populations (e.g., Buckley et al., 2017; Hao et al., 2021; Liu et al., 2022). A higher growth rate allows for a larger body size before winter and enhances the survival rate over winter in ectotherms, especially for cold-climate species (e.g., Steiger, 2013; Kallis and Marschall, 2014; Lu et al., 2019). At the same time, we found enhancements in the sprint speed under a warming climate in both lizard species in this study (**Figures 2A,B**). Higher speeds could give lizards an advantage in activities such as predation, evading predators, and reproduction (Shu et al., 2010; Dematteis et al., 2022). In addition, metabolic rates are considered as pace regulators of life and a significant increase in the metabolic rate at high temperatures would pose a threat to tropical species (Mcnab, 2002; Dillon et al., 2010). In this study, integrating the regulation of metabolic rates in *E. argus* and *T. amurensis*, sufficient food supply, and enhancement of growth rates, we predict that the metabolic responses in both lizard species can also be beneficial. Similarly, high-latitude lizards would respond to warming temperatures by enhancing metabolic rates (i.e., *E. argus* in this study) to produce more energy with sufficient food or decreasing the metabolic rates (i.e., *T. amurensis* in this study) at high temperatures avoid excessive expenditure of energy (e.g., Sun et al., 2022). Therefore, the physiological and fitness-related responses of lizards in a warming climate revealed an advantage for lizards from both open and semi-closed microhabitats in cold climates.

Warming climate benefit on gut microbiota in both lizard species

Alpha and beta diversities are widely used to evaluate the effects of various environmental factors on the gut microbiota of animals (e.g., Bestion et al., 2017; Macke et al., 2017;

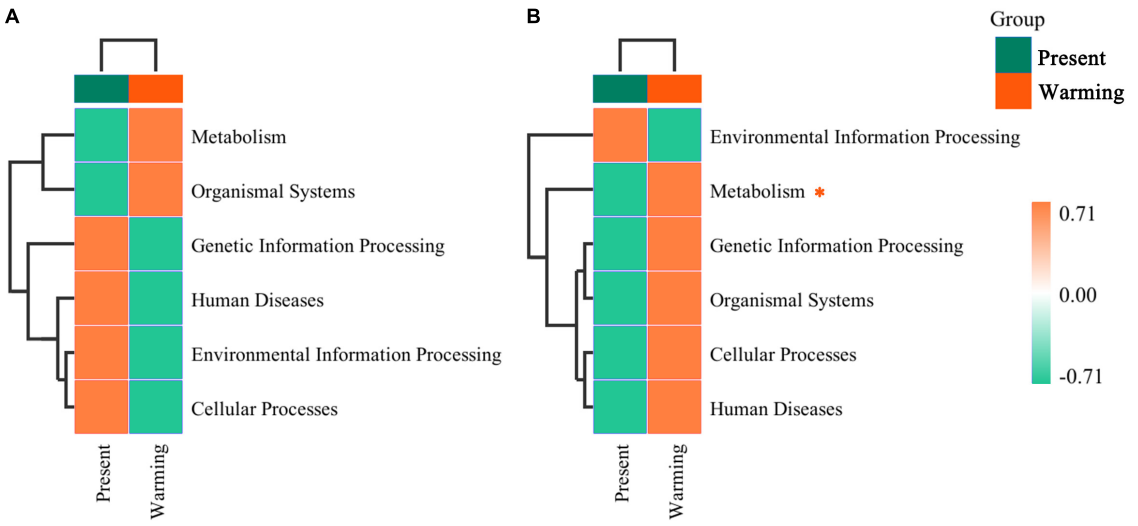

FIGURE 6
The hierarchical clustering of functional classifications in microbiota at top level between present and warming of *Eremias argus* **(A)** and *Takydromus amurensis* **(B)**. The horizontal ordinate represents the sample information and species annotation information; the cluster trees on the left and the top are species clustering and sample clustering, respectively. Gradient colors indicate the regulation of the warming climate against the present climate. *Asterisk indicates a significant difference between present and warming climate conditions ($P < 0.05$).

Tang et al., 2020). In this study, we found that the diversity of gut microbiota did not change significantly between the present and warming climate conditions. A similar diversity of gut microbiota across climate conditions implied that warming had a neutral effect on gut microbiota. However, the composition of the gut microbiota differed between the present and warming climate conditions for both *E. argus* and *T. amurensis*. In this study, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were the dominant microflora in both *E. argus* and *T. amurensis* (**Figures 4A,B**), which was consistent with previous findings in other reptile species/populations (e.g., Ren et al., 2016; Kohl et al., 2017; Tang et al., 2020; Ibanez et al., 2021; Zhang et al., 2022b). Under warming climate conditions, the relative abundance of *Bacteroidetes* was significantly increased in *E. argus*, whereas the relative abundance of *Bacteroidetes* and *Firmicutes* was increased in *T. amurensis* (**Figures 4**, **5**). *Bacteroidetes* and *Firmicutes* participate in the metabolism of carbohydrates, fats, and vitamins (e.g., glucose metabolism, SCFA production, and vitamin production), and play a positive role in regulating host metabolism (e.g., Colston and Jackson, 2016; Costantini et al., 2017; Lapébie et al., 2019). The regulation of the proportion of *Bacteroidetes* and *Firmicutes* may reflect the energy reserve of the body and regulate the energy balance of the body through changes in the bacterial community (Turnbaugh et al., 2006). In addition, the up-regulation of the "ko00511-Other glycan degradation" pathway in *T. amurensis* may also suggest that the warming climate has a positive effect on the metabolic function of the gut microbiota (**Figures 6B**, **7B,D**; Martens et al., 2009; Eilam et al., 2014).

Notably, the gut microbiota of lizards in warming climate conditions also showed changes related to immunity, similar to other lizard species (e.g., Zhang et al., 2022a). In warming climate conditions, both *E. argus* and *T. amurensis* could probably enhance immunity by increasing the number of probiotics and their mediating pathways. *Bacteroides*, the main taxa that produce SFCAs (Short-chain fatty acids), have attracted much attention in recent years. In addition, it performs some immune functions (e.g., Wexler, 2007; Mazmanian et al., 2008). These functions may be related to the synthesis of antibiotics, which were related to the up-regulation of "ko00523-Polyketide sugar unit biosynthesis" of *E. argus* in the warming climate conditions (**Figures 7A,C**). In terms of the composition of the gut microbiota of *T. amurensis*, the warming climate conditions enhanced the relative abundance of *TM7* significantly (**Figures 4B**, **5B**), which usually plays an active role in reducing the pathogenicity of other bacteria (e.g., Bor et al., 2019; Chipashvili et al., 2021). Meanwhile, upregulation of carotenoid biosynthesis (ko00906), which is involved in innate immunity, may also contribute to host longevity and health (Blount et al., 2003; **Figure 7B**). In addition, both *E. argus* and *T. amurensis* could enhance their immunity by reducing pathogenic bacteria and downregulating metabolic pathways associated with disease. We found that *Enterobacteriales* was significantly reduced in both lizard species under warming climate conditions (**Figures 4E,F**, **7D**). As *Enterobacteriaceae* contains most of the pathogenic bacteria, its reduction can be beneficial to the health of the host under warming climate conditions (e.g., Schwab et al., 2014). The increase in carotenoid content in *T. amurensis* might have been caused by

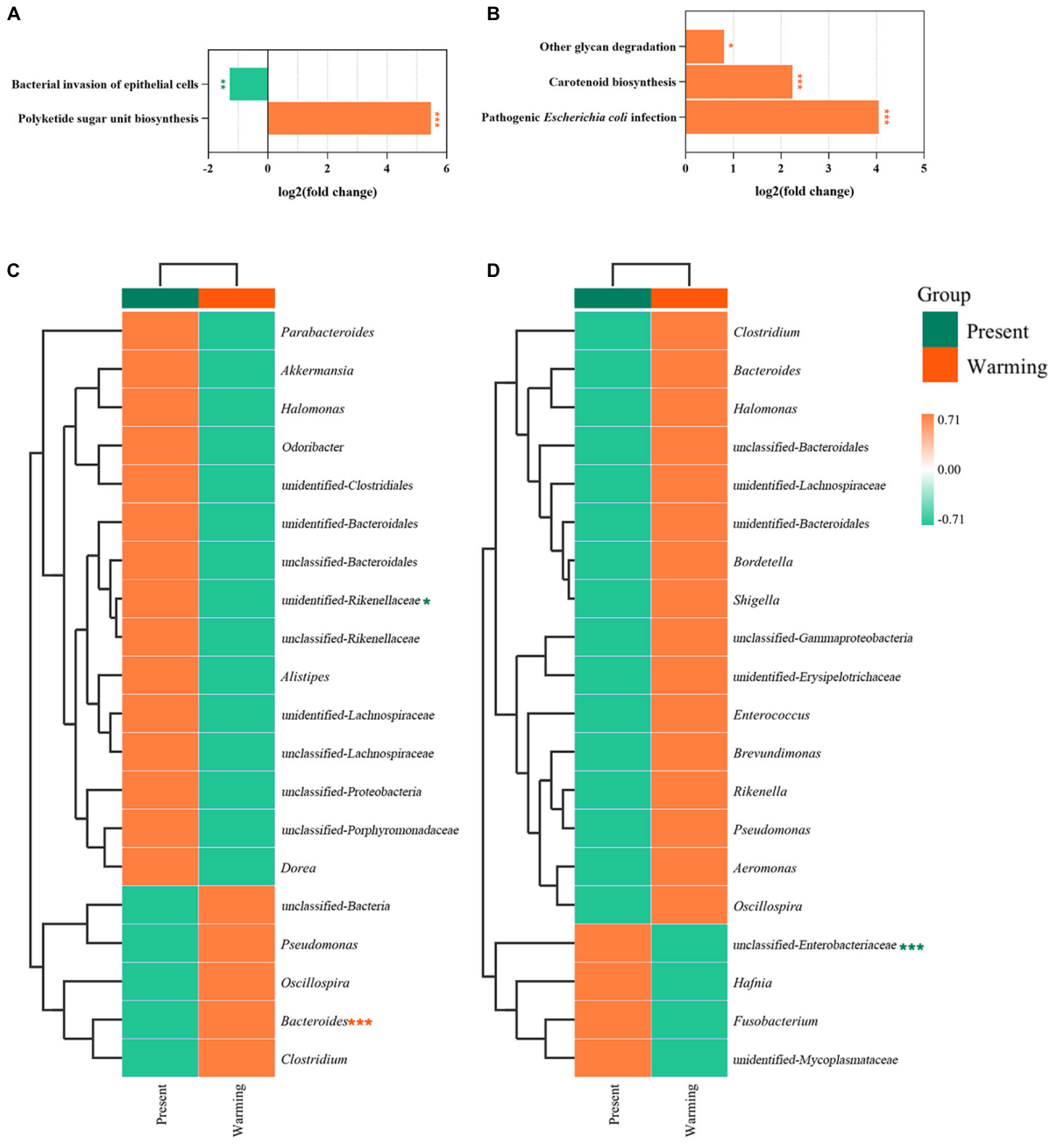

FIGURE 7
Functionally predicted KEGG pathways and the hierarchical clustering heatmap of species contribution composition of differential pathways between present and warming in *Eremias argus* **(A,C)** and *Takydromus amurensis* **(B,D)**, respectively. The horizontal ordinate represents the sample information and species annotation information; the cluster trees on the left and the top are species clustering and sample clustering, respectively. Present and warming indicate present and warming climate conditions, respectively. The highlighted pathways were significantly up-regulation (orange color) or down-regulation (green color) of warming climate against the present climate, respectively (*$P < 0.05$; **$P < 0.01$; and ***$P < 0.0001$).

a decrease in *Enterobacteriaceae* (**Figures 7B,D**). *Rikenellaceae*, an inflammatory indicator, is significantly enriched when the body is under stress. Its reduction may contribute to the body's health by interacting with the down-regulated "ko05100-Bacterial invasion of epithelial cells" of *E. argus* under warming climate conditions (e.g., Litvak et al., 2017; Brennan and Garrett, 2019; Wang et al., 2020; **Figures 7A,C**). Similarly, the relative abundance of pathogenic bacteria (e.g., *Fusobacteria*, *Proteobacteria*, and *Tenericutes*) in the gut microbiota of *T. amurensis* under warming climate conditions may also

have a positive impact on the body's immunity (Brennan and Garrett, 2019; **Figures 4B,D,F,H,J,L**). However, "ko05130-Pathogenic *Escherichia coli* infection" was also upregulated in *T. amurensis* under warming climate conditions (**Figure 7B**). The upregulation of conditioned pathogen-related pathways may complicate the immune response of *T. amurensis* to warming climate conditions. Therefore, we suggest that the gut microbiota of *E. argus* and *T. amurensis* responded to warming climate conditions due to changes in composition rather than diversity. Such changes are likely beneficial to the metabolism, individual health, and immunity of lizards.

Interaction between physiology and gut microbiota facilitating the metabolism of *Takydromus amurensis* under warming climate

Interestingly, we found a plausible interaction between the metabolic rates of lizards and metabolism-related gut microbiota. When faced with a warming climate, the lizards *E. argus* from open microhabitats up-regulated their own RMRs (**Figure 2C**), without any regulation of metabolism-related gut microbiota (**Figures 6A, 7A,C**). In contrast, the lizard *T. amurensis* from semi-closed microhabitats did not regulate the metabolic rates significantly, but the metabolism-related gut microbiota was up-regulated. At high body temperatures, the RMR of *T. amurensis* was depressed due to the warming climate (**Figure 2D**), but the metabolism-related gut microbiota might compensate for the metabolic depression of the host, indicating a plausible complementary effect between the host and gut microbiota in the metabolic regulation of *T. amurensis* in warming climates (**Figures 6B, 7B,D**; O'connor et al., 2017; Gould et al., 2018; Rastelli et al., 2019).

The occurrence of this condition may be related to sufficient food and ambient temperature used in this study. As a cold-climate lizard from open habitats, *E. argus* prefers a hot environment, and sufficient food improves its metabolism under heating without negative effects (e.g., Wang et al., 2019; Sun et al., 2022). However, food is not always plentiful in natural habitats; therefore, warming may negatively affect the fitness of individuals and even populations while maintaining high metabolic rates (e.g., Bestion et al., 2015; Hao et al., 2021; Sun et al., 2022). In contrast, although the metabolism of *T. amurensis* in the semi-closed habitat was inhibited when the ambient temperature increased (**Figure 2D**), gut microbiota could assist energic regulation *via* composition change, which was probably beneficial for the proliferation of microbiota, which in turn assisted the thermal responses of the host (e.g., Seebacher et al., 2014; Fontaine et al., 2022). However, to comprehensively understand this complementary effect, further experimental manipulations are required to confirm the detailed regulation (e.g., Fontaine et al., 2022).

Conclusion

In summary, we found that moderate warming climates were plausibly beneficial to lizards from both open and semi-closed microhabitats at high latitudes improving their growth rate, locomotion, and allowing for a high survival rate. Notably, we found a likely complementary effect between the host metabolic rates and the metabolism regulation of gut microbiota: *E. argus* from open microhabitats only responded *via* physiology, whereas *T. amurensis* from a semi-closed microhabitat showed an interactive effect of physiology and gut microbiota responses in addition to physiological response. Furthermore, the compositional changes in gut microbiota without any diversity change might enhance the immunity of lizards under warming climates. We encourage future experimental manipulations to determine the potential interactive and complementary effects of metabolism regulation by the physiological response of the host and the contribution of the gut microbiota. In addition, experimental verification of the enhancement of immunity by the gut microbiota is required. These manipulations are helpful in evaluating the vulnerabilities of animals to climate warming and reveal the underlying mechanisms at the microbiological level.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary material**.

Ethics statement

The animal study was reviewed and approved by the Animal Ethics Committees at the Institute of Zoology, Chinese Academy of Sciences (IOZ14001).

Author contributions

PL, BS, WL, TL, and YM designed the study. WL, JY, YM, DW, and LC data collected and analyzed. JY, BS, TL, WL, and PL wrote the manuscript. All authors contributed critically to the drafts and revisions and gave final approval for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1050750/full#supplementary-material

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Reintroduction modifies the intraspecific variations of symbiotic microbes in captive bred Chinese giant salamander

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Microorganisms play as fundamental contributors to maintain hosts' fitness, which can be shaped by external environment. Moreover, symbiotic microbiome also varied within species (e.g., between sexes and developmental stages). However, we still need more studies to quantify whether the intraspecific variation patterns of symbiotic microbes can be modified with the change of environment. The Chinese giant salamander (CGS; *Andrias davidianus*) is a Critically Endangered species. Despite quantitative captive bred individuals were released to rebuild wild populations, the effectiveness is limited. More importantly, no studies have revealed the adaptation of released CGSs to the complex field conditions. In the present study, we explored whether reintroduction can reshape the intraspecific variations of symbiotic microbiota in captive bred CGSs using high-throughput amplicon sequencing of the16S rRNA gene. We found no significant difference of symbiotic microbiome in captive bred males and females, but released males and females differed significantly in skin microbiome. Juveniles had higher diversity of microbial symbiont than adults in hatchery, but lower diversity in field. Moreover, dominant bacterial taxa differed between juveniles and adults in both hatchery and field. Importantly, this symbiotic microbiome variations within species can be modified (alpha and beta diversity, and community composition) when captive bred individuals were released to the field. Overall, we observed a lower alpha diversity and higher relative abundance of *Chryseobacterium*, *Plesiomonas*, and *Acinetobacter* in the bacterial community of captive bred individuals. Instead, higher alpha diversity of symbiotic microbiota and higher relative abundance of S24-7 and *Lactobacillus* was detected in released individuals. These modifications may associate with the change of living environment, as well as the specific behavior within CGSs (e.g., movement patterns and foraging activities). Future studies can incorporate other approaches (e.g., blood physiology) to better evaluate the growth and health of reintroduced CGSs.

KEYWORDS

alpha and beta diversity, reintroduction, microbiome reorganization, amphibian conservation, *Andrias davidianus*

Introduction

Microorganisms widely exist throughout the whole body sites of animals. They co-evolve with hosts over time, and play as fundamental contributors to maintain hosts' fitness (Suzuki, 2017; Gould et al., 2018). Specifically, oral cavity acts as a channel connecting the living environment and the digestive tract of host. Accordingly, oral microbial symbionts can protect host against the disturbance of external pathogenic bacteria when feeding (Takahashi, 2015; Gao et al., 2018). Moreover, skin microbiome is considered to be an integral component of hosts immune system, acting as a barrier to improve the disease resistance (Naik et al., 2012; Rebollar et al., 2016). Gut microbes display key functional roles, regulating hosts important physiological processes such as digestion, energy metabolism, pathogen defense, and immunomodulation (Chung et al., 2012; Hu et al., 2018; Raymann and Moran, 2018; Levin et al., 2021; Xia et al., 2022). Therefore, investigating the dynamics of symbiotic bacterial diversity and composition can help us better understand hosts immunity and health.

The composition of host-associated microbial symbiont can be shaped by external environmental conditions (Bletz et al., 2016; Muletz Wolz et al., 2018; Moeller et al., 2020). Therefore, one can expect that the host-associated microbiota should be distinct when organisms occupy different habitats. Accordingly, the symbiotic microbes of captive bred animals should be changed when they were released to the field. For instance, the captive and wild Beal's eyed turtle (*Sacalia bealei*) displayed different alpha diversity, beta diversity, and community composition of gut microbiota due to different diets and habitat conditions (Fong et al., 2020). Moreover, hosts symbiotic bacteria can be also different between developmental stages and sexes within species (Kueneman et al., 2014; Bennett et al., 2016; Hernandez-Gomez et al., 2018). For instance, the alpha and beta diversity of brown frogs (*Rana dybowskii*) gut bacterial varied significantly between tadpoles and adults, which can be attributed to the shift of diet from plants to arthropod species during metamorphosis (Tong et al., 2020). Similar situation can be detected between male and female Chinese concave-eared frogs (*Odorrana tormota*), which exhibited distinct community composition of gut microbiota. This may be because females have a wider trophic niche size than males (Shu et al., 2019). However, few studies have been conducted to test whether the transition of captive bred individuals from hatchery to the wild will induce the change of intraspecific variations of their symbiotic microbes. Captive bred individuals live in the similar external environment, thus there should be no significant difference of symbiotic microbes between juveniles, males, and females in the hatchery. In contrast, since the field condition is more complex, and released juveniles, males, and females can move freely to select the preferred habitat, their symbiotic microbes should be significantly different.

Chinese giant salamander (CGS; *Andrias davidianus*) is the largest extant amphibian species in the world (Murphy et al., 2000), attracting quantitative attentions of ecologists and biologists for decades (Hou et al., 2004). However, the natural populations of CGS have dramatically declined since 1950s due to habitat loss, environmental contamination, and overexploitation (Wang et al., 2004; Zhao et al., 2020). Accordingly, this species has been listed as a protected species in China since 1988 (Cunningham et al., 2015), as well as Critically Endangered in China (Jiang et al., 2021) and in the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species (IUCN, 2022). To rebuild wild populations, a total of 287,840 captive bred CGS individuals have been released to the wild by the end of October 2019 in China (Shu et al., 2021). However, it is still hard to detect CGS in the wild (except several natural researves; Shu et al., 2021). Therefore, it is urgent to evaluate the health of released CGS, and their microbiota was considered to be a good predictor because of its important functional contribution to host (Gould et al., 2018; Raymann and Moran, 2018). Previous studies have shown that the gastrointestinal microbiota of captive bred CGS can be significantly different in terms of composition and diversity between age groups (Zhang et al., 2018), as well as between groups under different acclimation temperatures (Zhu et al., 2021). However, we still need empirical evidences to evaluate the reorganization of captive bred CGS's symbiotic bacterial communities when they were released to the wild.

In the present study, the captive bred and the released CGS individuals with different developmental stages and sexes were selected to explore: 1) the sex bias in symbiotic microbiota (skin, oral, stomach, small intestine, and rectum microbiome), 2) the stages bias in symbiotic microbiota, and 3) the differences in symbiotic microbiota between captive bred and released individuals.

Materials and methods

Study area

A montane stream located at Dujiangyan, Sichuan Province, China (103°38′22″–103°39′03″E, 31°02′18″–31°02′32″N) was selected as the study site to conduct the reintroduction activity. This stream has been proved to be suitable for the living of released CGS (Liu, 2021; Zhao et al., 2022). Specifically, this area belongs to a subtropical climate, with the annual precipitation of 1,244 mm, and the average annual temperature of 15.2°C (Liang et al., 2004). The altitude of this stream is approximately 950 m, with deciduous broad-leaved forests and bamboo forests distributing along the riverbank. Chemical oxygen demand and biochemical oxygen demand in the water are extremely low, with little pollution and weak alkaline pH (Liu, 2021). Deep pools with quantitative fish, crabs, and shrimp can be observed in this stream, providing sufficient food resources for reintroduced CGS individuals. More importantly, this area belongs to a private bamboo planting company, ensuring no fishing pressure.

Study animals

A total of 42 captive bred CGSs belonging to different developmental stages (ages) and sex groups were collected from a

hatchery in Hongya County of Sichuan Province, China (103°09′15″E, 29°52′23″N). Based on the molecular analyses, they belonged to the Shaanxi clade. These individuals were the offspring of the same parents and were bred in hatchery in 2016 and 2018, respectively. Specifically, 12 individuals including 4 males (age 4), 4 females (age 4), and 4 juveniles (age 2) were considered as the captive bred group. The rest 10 males (age 4), 10 females (age 4), and 10 juveniles (age 2) were used for reintroduction, which were considered as the released group. Before release, these individuals were injected with passive integrated transponder devices (HONGTENG, GuangZhou, China) for individual identification following Gibbons and Andrews (2004). Finally, these individuals were released at six sites (i.e., two sites for upstream, midstream, and downstream, respectively) of the study stream on 22th May, 2020, with each site containing several males, females, and juveniles (Supplementary Figure S1). The released individuals can move freely in this stream to look for their preferred habitats.

Salamanders recapture and sample collection

We used a combination of visual encounter approach and wire-mesh baited traps (length × width × height = 110 × 60 × 30 cm, mesh size = 8 mm) to recapture the released individuals from 24th June to 19th July, 2020. The visual encounter approach consisted of wading, nocturnal spotlighting, turning substrate, and netting in the stream (Liu et al., 2021). We searched for CGSs in the whole stream after sunset (from 20:00 to 24:00), and individuals encountered were captured by hand nets. Wire-mesh baited traps have been proved to be effective for surveying Cryptobranchids species (Browne et al., 2011). Fresh chicken giblets or frozen hairtail were selected as the baits in a hanging net bag, ensuring CGSs cannot swallow the baits. Traps were placed at the deep pools of the stream at 18:00 every day, and were checked at 6:30 am the next morning. The captured CGSs were carefully transferred on a piece of white nylon cloth one by one by hands wearing sterile gloves. Before microbiota sampling, each individual was rinsed three times by ultra-pure water to remove the potential environmental transient bacteria (Lauer et al., 2007). After that, sterile swabs were immediately used to collect the skin microbes by wiping the dorsal, ventral, and lateral sides of the salamanders (Xu et al., 2020). New sterile swabs were used to gently swab salamanders oral cavity for sampling oral microbiota. The above swabs were preserved into 2 ml sterile centrifuge tubes with 95% alcohol, separately. After that, individuals were euthanized with MS-222 (Webb et al., 2005). They were subsequently dissected to collect the stomach, small intestine, and rectum contents, which were preserved into 2 ml sterile centrifuge tubes, respectively (Zhang et al., 2018). We recaptured 8 juveniles, 6 females and 5 males in total, and all of their microbe samples were used for further analyses. Environmental microbiota were sampled as follows: 5 L of the water in each release site was collected and filtered through 0.45-um micropore membrane (Shi et al., 2013), which were preserved into 2 ml sterile centrifuge tubes with 95% alcohol. Three repetitions were conducted in each release site. All the above samples were preserved into liquid nitrogen in the field, and were then transferred to the laboratory immediately for further analyses. The same sampling processes were conducted for captive bred individuals in the hatchery directly.

DNA extraction and 16SrRNA amplicon sequencing

All microbiota samples were thawed on ice, and genomic DNA was extracted using a QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol, and included a negative extraction control. The quality and quantity of the DNA was verified using 1.0% agarose gel electrophoresis and a NanoDrop spectrophotometer, separately. The V4-V5 region of the 16S rRNA gene was amplified from genomic DNA using 515F (5'-GTGCCAGCMGCCGCGGTAA-3′) and 907R (5'-CCGTCAATTCMTTTGAGTTT-3′) primers (Biddle et al., 2008). The PCR amplification conditions were as follows: initial denaturation 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, with a final extension step at 72°C for 10 min. High-throughput sequencing of barcoded amplicons was performed using the Illumina MiSeq platform by Mingke Biotechnology Co., Ltd. (Hangzhou, China).

Microbiota sequence analyses

We used QIIME 1.9 to process the raw reads and to obtain clean sequences (Caporaso et al., 2010). The *search*, *flash*, and *trimmomatic* function were used for removing low-quality sequences, splicing and quality control, respectively (Edgar, 2010). Operational taxonomic units (OTUs) were defined as the identity sharing >97% sequences, and representative sequences (the largest number of sequences in each OTU) were classified against the SILVA132 database (Quast et al., 2013). Finally, we obtained OTU abundance tables containing taxon information. To standardize the number of reads across samples for our main analyses, all samples were rarefied to 26,484 sequences (the lowest number of sequences of all samples in this study).

Statistical analyses

Alpha diversity (i.e., observed OTUs and Shannon index) was calculated for each sample in QIIME 1.9 according to the relative abundance-based OTU table (Caporaso et al., 2010). All alpha diversity values were assessed for normality using Shapiro–Wilk tests (Varona et al., 2005). Alpha diversity indices with normal distribution were compared using Student's t-test, while Mann–Whitney U test was conducted to compare indices with non-normal distribution. Beta diversity was calculated with Bray-Curtis, unweighted UniFrac, and weighted UniFrac dissimilarity metrics by QIIME pipeline, respectively. PERMANOVA was performed to

determine the difference of microbiome composition between groups at OTU level, and principal coordinates analysis (PCoA, based on dissimilarity matrices) was used to visualize the dissimilarity of beta diversity. Benjamini–Hochberg (BH) correction was used to obtain the corrected *p*-values. We evaluated the variation of symbiotic microbial composition between captive bred and released individuals using the top 10 families and genera based on the Mann–Whitney U analysis. The linear discriminant analysis effect size (LEfSe) was performed to explore the differentially-abundant bacterial taxa between groups (Segata et al., 2011). Source-Tracker 0.9.5 was used to calculate the relative contribution of habitat and original symbiotic microbiota to the reorganization of released individuals microbiome (Knights et al., 2011). LEfSe analyses were performed on the Galaxy web-based platform.[1] Other analyses were conducted in R (R Core Team, 2020). Specifically, the Shapiro–Wilk test was performed using the *stats* package (R Core Team, 2020). Student's t-test and Mann–Whitney U test were conducted using *ggpubr* package (Whitehead et al., 2019). PERMANOVA was performed using the *vegan* package (Dixon, 2003). PCoA was conducted using *ape* package (Paradis et al., 2004). And figures were created using *ggplot2* package (Ginestet, 2011).

Result

Sex bias in symbiotic microbiota

No significant differences of alpha ($p > 0.05$, Supplementary Table S1) and beta diversity ($p > 0.05$, PERMANOVA and BH correction) were detected in skin, oral, stomach, small intestine, and rectum microbiome between captive bred males and females. However, released males exhibited higher Shannon diversity (t test, $p = 0.019$, Figure 1B) than females in skin bacterial communities. Additionally, beta diversity of the skin bacterial communities differed between released males and females (PERMANOVA and BH correction: $R^2 = 0.18$, $p = 0.048$ for Bray-Curtis; $R^2 = 0.15$, $p = 0.039$ for unweighted Unifrac; $R^2 = 0.19$, $p = 0.048$ for weighted Unifrac metrics; Figure 1C). A LEfSe analysis revealed 19 divergent taxa of skin bacterial communities between sexes (5 for females and 14 for males; $\alpha = 0.05$, LDA = 3.2, Supplementary Figure S2A). Specifically, released females had higher relative abundance of Bacilli (Females: 45.7%, Males: 34.4%), Lactobacillales (females: 45.4%, males: 33.2%), Lactobacillaceae (females: 44.8%, males: 32.3%) and *Lactobacillus* (females: 44.8%, males: 32.3%) than released males at class, order, family, and genus levels, respectively (Supplementary Figure S2B).

Stage bias in symbiotic microbes

Captive bred juveniles had higher OTU richness (observed OTU: Mann–Whitney U test, $p = 0.004$) and Shannon diversity (*t* test, $p = 0.010$) in the rectum microbiome, and higher OTU richness (observed OTU: Mann–Whitney U test, $p = 0.008$) in the skin microbiome compared to captive bred adults (Supplementary Figures S3A,B). Moreover, the captive bred juveniles and adults differed in beta diversity of skin and rectum microbiome (rectum: $R^2 = 0.62$, $p = 0.002$ for Bray-Curtis; $R^2 = 0.29$, $p = 0.002$ for unweighted Unifrac; $R^2 = 0.58$, $p = 0.002$ for weighted Unifrac metrics; PERMANOVA and BH correction; skin: $R^2 = 0.74$, $p = 0.002$ for Bray-Curtis; $R^2 = 0.39$, $p = 0.002$ for unweighted Unifrac; $R^2 = 0.79$, $p = 0.002$ for weighted Unifrac metrics; PERMANOVA and BH correction; Supplementary Figure S3C). In contrast, released adults have higher Shannon diversity (t test, $p = 0.002$) in the small intestine microbiota compared to released juveniles (Figures 2A,B). In addition, released adults and juveniles also differed significantly in beta diversity of the small intestine microbiota ($R^2 = 0.11$, $p < 0.001$ for Bray-Curtis; $R^2 = 0.04$, $p = 0.009$ for unweighted Unifrac; $R^2 = 0.10$, $p < 0.001$ for weighted Unifrac metrics; PERMANOVA and BH correction). The bacterial communities of small intestine can be clustered into two groups by the PCoA plot according to the developmental stages (i.e., juveniles and adults; Figure 2C). Based on the LEfSe analyses, released juveniles had higher relative abundance of Firmicutes at phylum level, while Bacteroidetes, Bacteroidia, Bacteroidales, S24-7, and unclassified genus of S24-7 were more abundant in released adults at phylum, class, order, family, and genus levels, respectively (Supplementary Figures S4A,B).

Differences of microbial symbionts between captive bred and released individuals

No significant difference of alpha diversity and beta diversity was detected in skin, oral, stomach, small intestine, and rectum microbiome between captive bred males and females ($p > 0.050$). And marginal difference was only observed in skin microbiota composition between released females and males ($p = 0.048$ for Bray-Curtis and weighted Unifrac, $p = 0.039$ for unweighted Unifrac metrics). Therefore, females and males were pooled together (i.e., released adults) to explore the differences in microbial symbionts between captive bred and released adults.

Released adults had significant higher alpha diversity (observed OTU: Mann–Whitney U test, $p = 0.021$; Shannon: t test, $p = 0.002$) in the skin microbiome and more diverse OTU richness (observed OTU: Mann–Whitney U test, $p = 0.048$) in the small intestine bacterial community than captive bred adults (Figures 3A,B). And released juveniles had significant higher oral bacteria OTU richness (observed OTU: Mann–Whitney U test, $p = 0.048$, Figure 3A) compared to captive bred juveniles. There was a significant difference in the skin, small intestine, and rectum bacterial composition between captive bred and released juveniles, as well as that between captive bred and released adults ($p < 0.050$, PEMANOVA and BH correction). These microbiota samples

1 http://huttenhower.sph.harvard.edu/galaxy/

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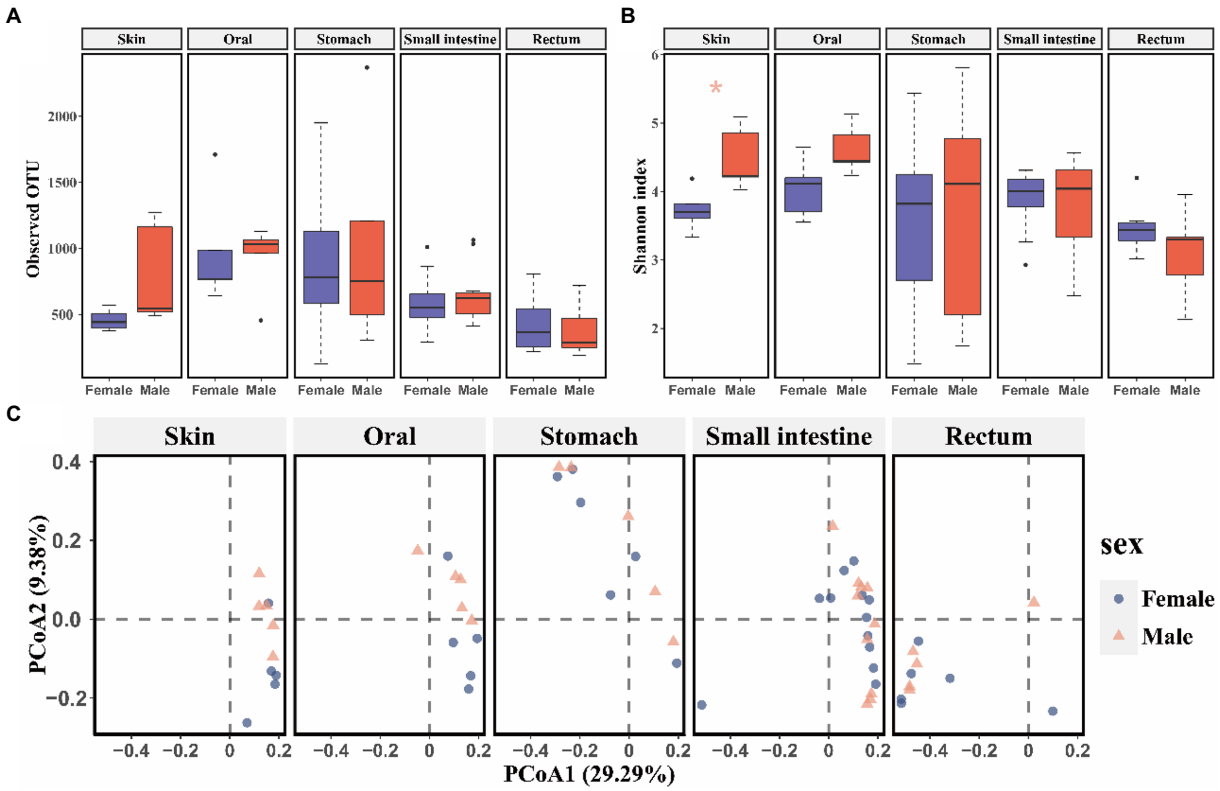

FIGURE 1
The comparison of alpha and beta diversity of symbiotic microbiota between released female and male CGSs. **(A)** Boxplot of observed OTU values; **(B)** Boxplot of Shannon diversity values. Data are presented as means±SE, and significant differences are marked with an asterisk; **(C)** PCoA scatter plots present the dissimilarity of microbiomes at the OTU level based on Bray-Curtis distance.

could be divided into captive bred and released groups by PCoA plots (Figure 3C).

The community composition of symbiotic microbiome was showed in Figures 4A–C at phylum, family, and genus level, respectively. Seven known taxa of the top 10 families and six known taxa of the top 10 genera in skin microbiome showed significant variation between captive bred and released juveniles (Mann–Whitney U test, $p < 0.05$; Figures 5A,B). Specifically, the predominant family S24-7 and unclassified genus of S24-7 were more abundant in released juveniles, whereas the predominant family Flavobacteriaceae and genus *Chryseobacterium* were more abundant in captive bred juveniles. Nine known taxa of the top 10 families and 10 known taxa of the top 10 genera were significantly different between captive bred and released adults (Mann–Whitney U test, $p < 0.05$; Supplementary Tables S2 and S3). Specifically, the predominant family Lactobacillaceae and genus *Lactobacillus* were more abundant in released adults, whereas the predominant family Moraxellaceae and genus *Acinetobacter* were more abundant in captive bred adults.

In the small intestine microbiome, we found that four known taxa of the top 10 families and seven known taxa of the top 10 genera showed significant variation between captive bred and released juveniles (Mann–Whitney U test, $p < 0.05$; Supplementary Tables S4 and S5). Specifically, the predominant family Lactobacillaceae and genus *Lactobacillus* were more abundant in released juveniles, whereas the predominant genus *Plesiomonas* were more abundant in captive bred juveniles. Six known taxa of the top 10 families and seven known taxa of the top 10 genera were significantly different between captive bred and released adults (Mann–Whitney U test, $p < 0.05$; Supplementary Tables S6 and S7). Specifically, the predominant family S24-7 and unclassified genus of S24-7, genus Bacteroides were more abundant in released adults.

In the rectum microbiome, we found that four known taxa of the top 10 families and three known taxa of the top 10 genera showed significant variation between captive bred and released juveniles (Mann–Whitney U test, $p < 0.05$; Supplementary Tables S8 and S9). Specifically, the predominant family Lactobacillaceae and genus *Lactobacillus* were more abundant in captive bred juveniles. Only one known taxa of the top 10 families and one known taxa of the top 10genera were significantly different between captive bred and released adults (Mann–Whitney U test, $p < 0.05$; Supplementary Tables S10 and S11). Specifically, the predominant family Fusobacteriaceae and genus *Cetobacterium* were more abundant in captive bred adults.

In terms of the skin microbiome, LEfSe analysis indicated that Bacteroidia, Bacteroidales, S24-7, and unclassified genus of S24-7 were more abundant in released juveniles, whereas

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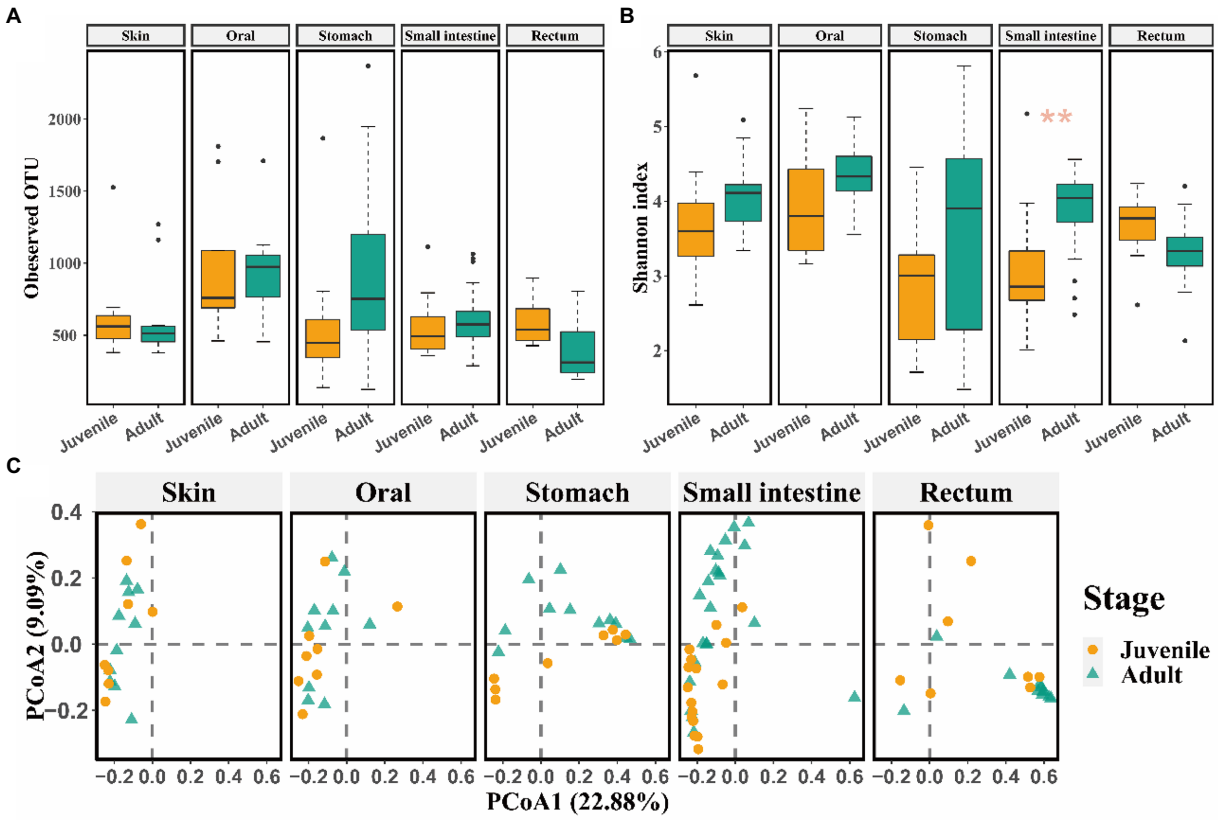

FIGURE 2
The comparison of alpha and beta diversity of symbiotic microbiota between released juvenile and adult CGSs. **(A)** Boxplot of observed OTU values; **(B)** Boxplot of Shannon diversity values. Data are presented as means±SE, and significant differences are marked with an asterisk; **(C)** PCoA scatter plots present the dissimilarity of microbiomes at the OTU level based on Bray-Curtis distance.

Flavobacteriia, Flavobacteriales, Flavobacteriaceae, and *Chryseobacterium* were more abundant in captive bred juveniles at class, order, family, and genus levels, respectively (Figures 6A,B). Released adults had higher relative abundance of Firmicutes, Bacilli, Lactobacillales, Lactobacillaceae, and *Lactobacillus*, while captive bred adults had higher relative abundance of Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae, and *Acinetobacter* at phylum, class, order, family, and genus levels, respectively (Supplementary Figures S5A,B). In the small intestine microbiome, released juveniles had higher relative abundance of Firmicutes, Bacilli, Lactobacillales, Lactobacillaceae, and *Lactobacillus*, while captive bred juveniles had higher relative abundance of Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, and *Plesiomonas* at phylum, class, order, family, and genus levels, respectively (Supplementary Figures S6A,B). Bacteroidetes, Bacteroidia, Bacteroidales, S24-7, and unclassified genus of S24-7 were more abundant in released adults compared to captive bred adults at phylum, class, order, family, and genus levels, respectively (Supplementary Figures S7A,B). In the rectum microbiome, Clostridia, Clostridiales, Clostridiaceae_1, and *Clostridium_sensu_stricto_1* were more abundant in released juveniles, whereas Bacilli, Lactobacillales, Lactobacillaceae, and *Lactobacillus* were more abundant in captive bred juveniles at class, order, family, and genus levels, respectively (Supplementary Figures S8A,B). In addition, released adults had higher relative abundance of Firmicutes at phylum level, while captive bred adults had higher relative abundance of Fusobacteriia, Fusobacteriales, Fusobacteriaceae, and *Cetobacterium* at class, order, family, and genus levels, respectively (Supplementary Figures S9A,B).

Differences of water environment microbiome were also observed between hatchery and the released sites (Supplementary Figures S10A,B). At the phylum level, Proteobacteria were more abundant in captive bred environment, whereas Firmicutes were more abundant in released environment (Supplementary Figure S10A). At the genus level, *Chryseobacterium* and unclassified genus of Comamonadaceae were more abundant in captive bred environment, while *Lactobacillus* and unclassified genus of S24-7 were more abundant in released environment (Supplementary Figure S10B). In addition, source-track analyses indicated that environmental microbiome were the major sources of skin microbiome for both released females (67.69%) and males (57.84%; Supplementary Figure S11).

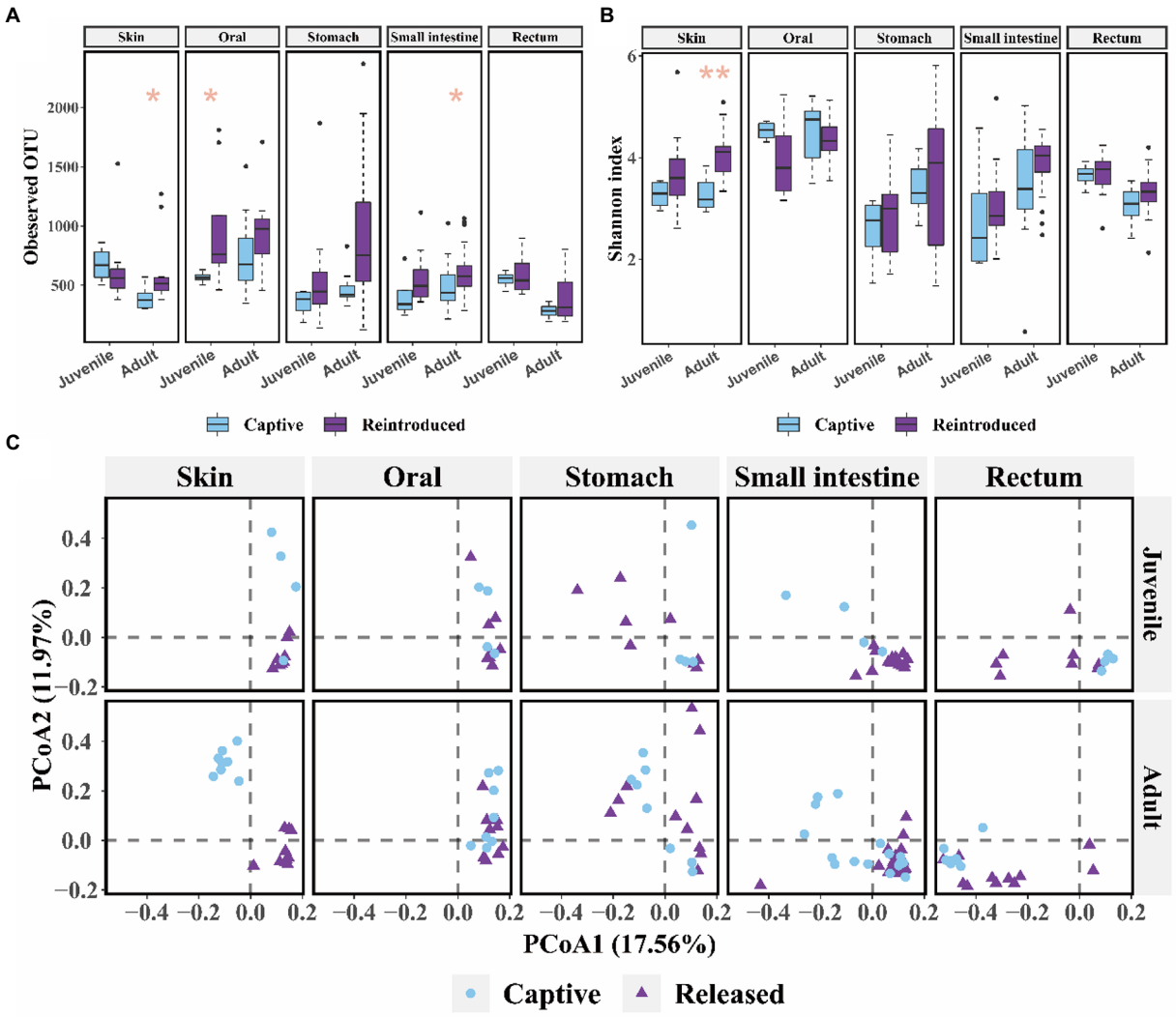

FIGURE 3
The comparison of alpha and beta diversity between captive bred and released CGSs symbiotic microbiota. **(A)** Boxplot of observed OTU values; **(B)** Boxplot of Shannon diversity values. Data are presented as means±SE, and significant differences are marked with an asterisk; **(C)** PCoA scatter plots present the dissimilarity of microbiome at the OTU level, based on weighted Unifrac distance.

Discussion

Sex bias in symbiotic microbiota

No significant differences of alpha and beta diversity were observed in all the symbiotic microbiome between captive bred males and females. This probably because all captive bred adults were raised under similar external environmental conditions (e.g., the water supply, food, and temperature) in the hatchery. However, this situation has been strongly changed in the skin microbial community between released females and males, as they exhibited significant difference in alpha and beta diversity. A previous study demonstrated that the linear home range and daily movement of released male CGSs were significantly higher (i.e., more diverse habitat utilization) than those of released females (Zhao et al., 2022). Therefore, such skin microbial community differences between sexes should be attributed to males and females different microhabitat utilization in the field, as amphibian skin microorganisms are susceptible to environmental factors (Loudon et al., 2014; Muletz Wolz et al., 2018; Varela et al., 2018; Hernandez-Gomez et al., 2019). This can be also supported by our results from the source-track analyses showing that environmental microbiota was the main sources of released individuals skin microbiome. More importantly, amphibian skin is an important immune organ (Varga et al., 2018), the higher diversity of skin bacterial communities may better protect amphibians against pathogens (Piovia-Scott et al., 2017). Therefore, this could be one of the reasons that the survival rate of released male CGSs was higher than released females in the studied stream (Liu, 2021).

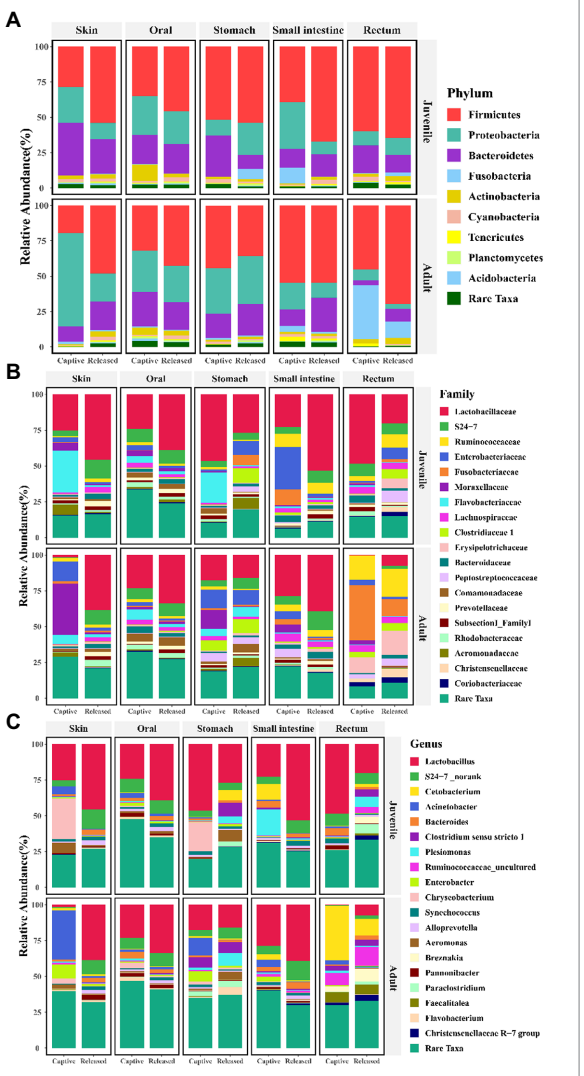

FIGURE 4
Bar plots show the symbiotic microbiota composition between captive bred and released individuals at phylum **(A)**, family **(B)**, and genus **(C)** level.

Stage bias in symbiotic microbes

The skin and rectum bacterial community between captive bred juveniles and adults were strongly divergent. Specifically, captive bred juveniles had higher alpha diversity in the rectum microbiome, and higher OTU richness in the skin microbiome compared to captive bred adults. This is in contrast with a previous study that indicated the alpha diversity of gastrointestinal microbial community in captive bred adults was higher than captive bred juveniles (Zhang et al., 2018). This is because CGSs were raised under different environment in different hatcheries (e.g., temperature, water condition, and food resources). In the present study, captive bred juveniles and adults are raised under similar environmental conditions except food supply (red worms *Chironomus* sp. and frozen fish *Hemiculter leucisculus* for juveniles while only frozen fish for adults). Previous studies indicated that more diverse food resources can induce higher diversity of skin and gut microbiome in the same amphibian species, such as the red-eyed tree frog (*Agalychnis callidryas*) in Belize (Antwis et al., 2014). Therefore, more diverse diet may contribute to a higher alpha diversity of skin and rectum microbiome in captive bred juveniles than adults. Furthermore, captive bred juveniles had higher relative abundance of *Lactobacillus* in the skin and rectum microbiome than adults. Since their living environment is similar, food items could be the potential important contributors to the colonization of *Lactobacillus*. Source-tracking analyses can be used in future studies to verify this inference.

In terms of the released group, adults and juveniles differed in Shannon and beta diversity of small intestine microbiome. Released adults should be the top predators in the stream ecosystem, consuming a wide range of food items such as fish, craps, and shrimps (Fei et al., 2006). In contrast, released juveniles may concentrated in preying limited food resources as they occupied smaller habitat niche in the stream (Zhao et al., 2022). Accordingly, higher Shannon diversity of small intestine microbiome in released adults may contribute to digest more kinds of food than released juveniles. This can be also supported by the higher relative abundance of S24-7 in released adults, as S24-7 microbes were associated with diverse complex carbohydrate degradation and the breakdown of proteins (Ormerod et al., 2016; Lagkouvardos et al., 2019).

Differences in microbial symbionts between captive bred and released individuals

Overall, released juveniles and adults had higher alpha diversity in microbiome than captive bred juveniles and adults, respectively. This pattern was consistent with previous studies showing that wild individuals of *Lissotriton vulgaris*, *Triturus cristatus*, and *Cynops pyrrhogaster* exhibited higher alpha diversity of cutaneous bacteria than captive bred individuals (Sabino-Pinto et al., 2016; Bates et al., 2019). Because lower diversity of symbiotic microbiome may lead to a higher susceptibility of hosts to diseases (Becker and Harris, 2010), our results may explain why captive bred CGSs were easily infected by bacteria, fungi, and parasites (Zhou et al., 2012). Accordingly, increasing the alpha diversity of symbiotic microbiome (e.g., the application of microbial inoculum) may be helpful to promote the survival of CGSs. Moreover, the increase of diversity of symbiotic microbiome in released individuals can be attributed to a shift of a simple to complex living environment, which promote their ability to defense against pathogenic bacteria (Antwis et al., 2014; Bataille et al., 2016). This can explain the observations that few infected CGSs were detected in the field (Liu et al., 2021). Accordingly, pre-exposure to the field water conditions of CGSs before reintroduction can be an effective approach to help the colonization of diverse microbes, and thus enhance the survival of

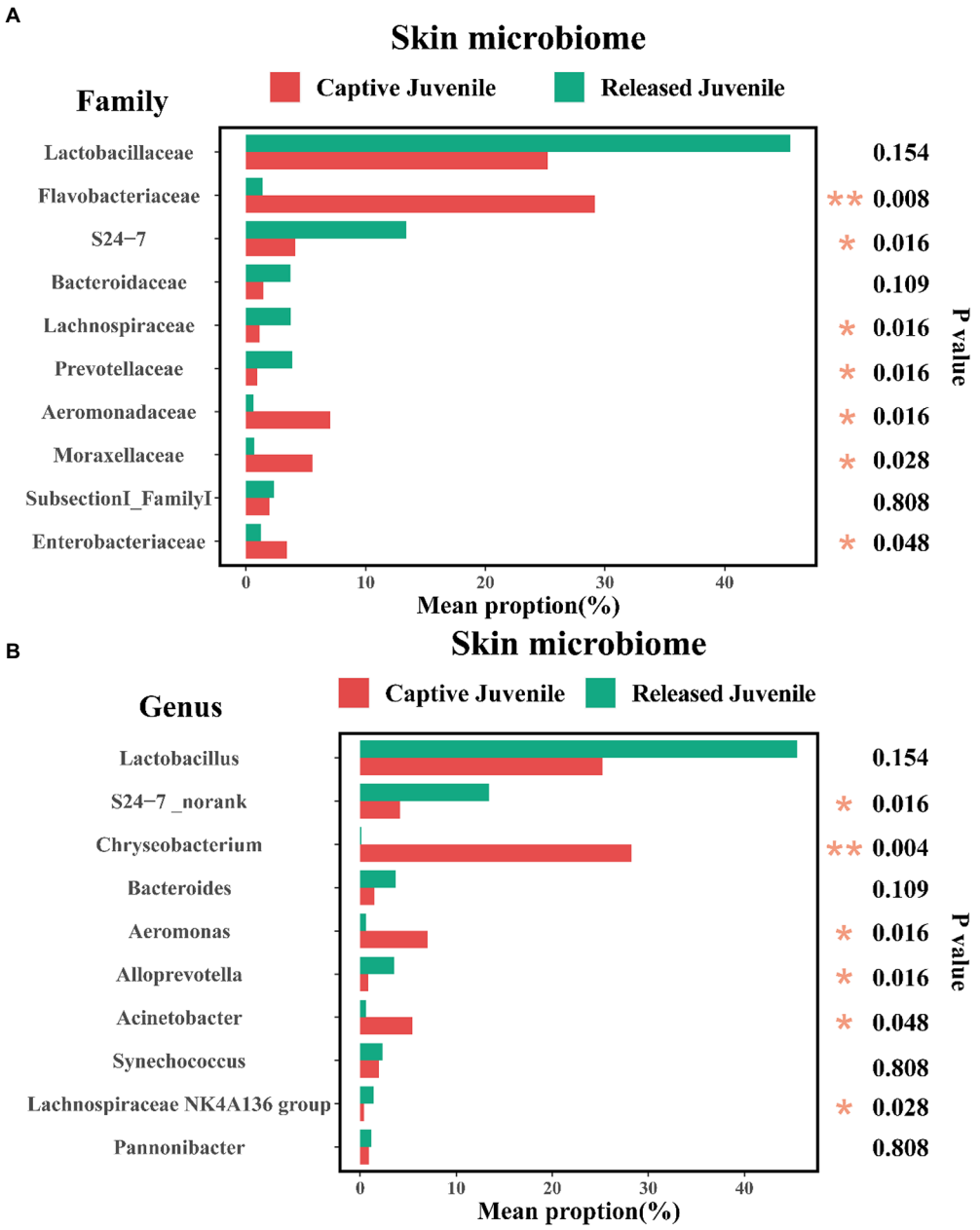

FIGURE 5
Variations of top 10 families **(A)**, and genera **(B)**, of skin microbiome between captive bred and released juveniles. A Mann–Whitney U test was used to evaluate the variation across two groups. Significance was set at the 0.05 level.

released individuals (Zhu et al., 2022). This is especially true for the juveniles, as we found that released juveniles exhibited lower alpha diversity of microbiome than adults. However, more evidences are still needed in future studies.

Microbiome composition was also significantly changed when comparing captive bred individuals with released individuals. Genus *Chryseobacterium* and *Plesiomonas* were more abundant in captive bred juveniles, while genus *Acinetobacter* were more abundant in captive bred adults. Since *Chryseobacterium* was the abundant genus in captive bred environment, our results supported the previous findings that hosts symbiotic microbes were associated with environmental microbes (Muletz Wolz et al., 2018; Moeller et al., 2020). However, symbiotic microbes were also affected by food resources (Antwis et al., 2014; Chang et al., 2016; Edwards et al., 2017). This may be the reason that abundant genus were different between captive bred juveniles and adults, which were provided different food in the hatchery. In contrast, the abundant genera were changed into *Lactobacillus* and unclassified genus of S24-7 in both released juveniles and

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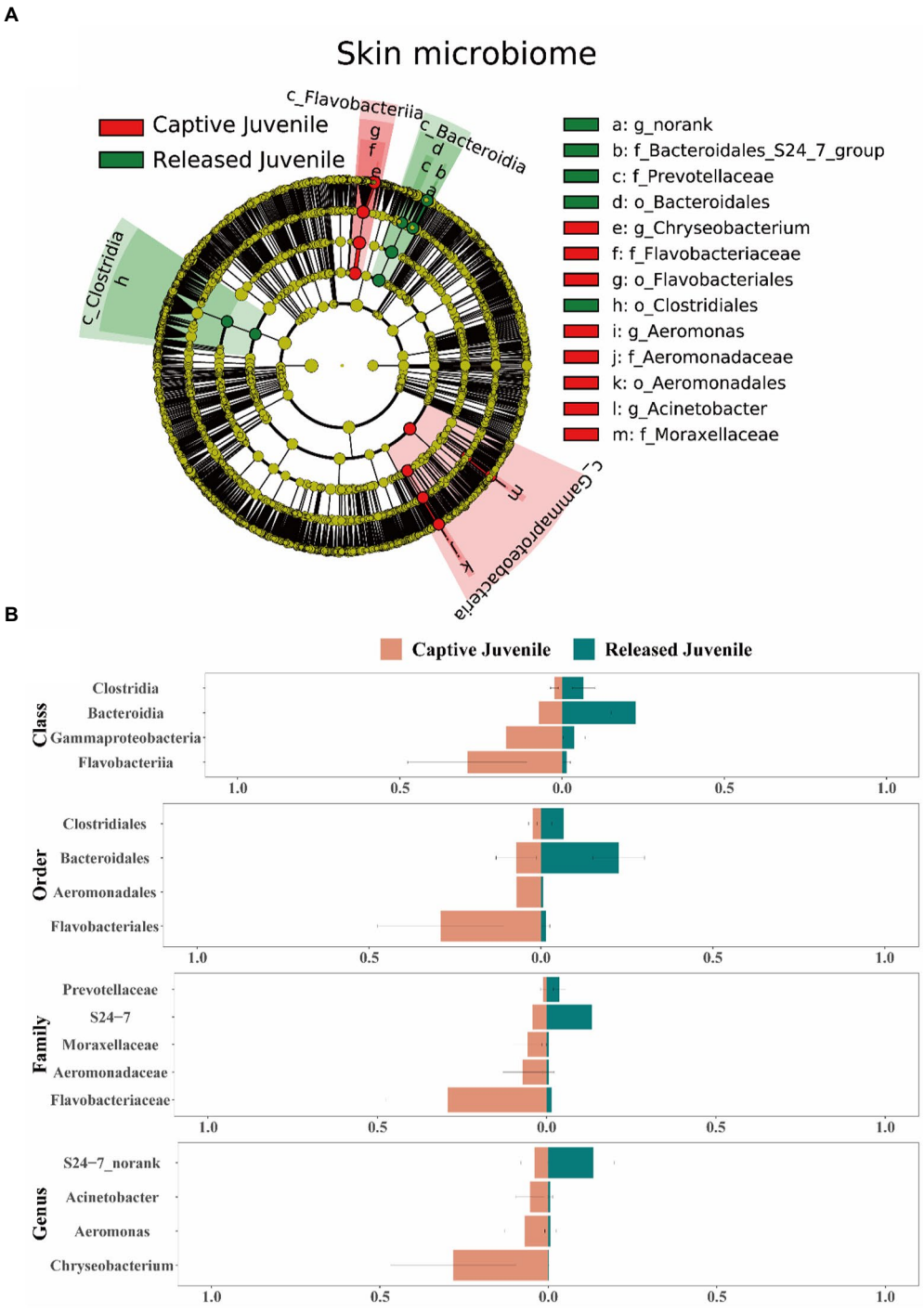

FIGURE 6
Difference of the skin microbiota between captive bred and released juveniles. **(A)** A LEfSe analysis identifies the different abundant skin bacterial taxa between captive bred and released juveniles. **(B)** Side-by-side comparison of the mean relative abundance of different abundant skin bacterial taxa between captive bred and released juveniles at class, order, family, and genus levels. Data are presented as means±SE in bar graphs.

adults. These genera contributed a lot to the released environmental microbes, which play more important roles to determine released individuals symbiotic microbes. Future studies can investigate using environmental microbes to infer hosts health in the field.

Conclusion

Our results indicated no sex but stage bias of symbiotic microbiome in captive bred CGSs. However, this intraspecific variation patterns of symbiotic microbiome can be modified when

captive bred individuals were released to the field. Overall, we observed a lower alpha diversity and higher relative abundance of *Chryseobacterium*, *Plesiomonas*, and *Acinetobacter* in the bacterial community of captive bred individuals. Instead, higher alpha diversity of symbiotic microbiota and higher relative abundance of S24-7 and *Lactobacillus* was detected in released individuals. Whether these modifications are related to specific functions for the adaptation of released CGSs could be tested. Moreover, since the effectiveness of CGS reintroduction is limited in most of the freshwater ecosystems, future studies can incorporate other approaches (e.g., blood physiology) to better evaluate the growth and health of reintroduced CGSs.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://ngdc.cncb.ac.cn/gsub/submit/gsa/subSAM102547, PRJCA012924.

Ethics statement

The animal study was reviewed and approved by the Animal Ethical and Welfare Committee of Chengdu Institute of Biology, Chinese Academy of Sciences.

Author contributions

TZ, WZ, and JF conceived this study. JF, CZ, TZ, and ZS collected the samples and performed the experiments. JF and WZ analyzed the data. JF and TZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1062604/full#supplementary-material

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Urbanization increases stochasticity and reduces the ecological stability of microbial communities in amphibian hosts

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Urbanization not only profoundly alters landscape profiles, ecosystems and vertebrate faunal diversity but also disturbs microbial communities by increasing stochasticity, vulnerability, biotic homogenization, etc. However, because of the buffering effect of host species, microbial communities are expected to be influenced by both host species and urbanization stresses. Therefore, the impacts of urbanization on animals' microbial symbionts could be more complex and uncertain. In this study, we quantified the urbanization degree of sampling sites and surveyed the gut and skin microbes of three amphibian host species in different sites in urban parks and nearby villages of Chengdu, Southwest China. Furthermore, a co-occurrence network analysis, the phylogenetic normalized stochasticity ratio and Sloan neutral community models were applied to infer the impact of urbanization on symbiotic microbial communities. For the three host species, urbanization increased the diversity of symbiotic microbes and the number of keystone microbial taxa. However, the negative effects of such increased diversification were evident, as the community stochasticity and co-occurrence network structure vulnerability also increased, while the network structure complexity and stability were reduced. Finally, the community stochasticity had positive associations with the network vulnerability, implying that the existence of many transient symbiotic rare microbial taxa in urban parks makes the symbiotic microbial community structure more fragile. Conclusively, urbanization increased the symbiotic microbial diversity at the cost of community stability; the results provide a new perspective for better understanding the complex triangulated environment–host–microbe relationship.

KEYWORDS

biotic homogenization, disturbance, abiotic gradient, community patterns, ecological niches

1. Introduction

Urbanization is one of the most direct manifestations of human activities (Rothwell et al., 2015; Zhai et al., 2020) and has been a major cause of the biodiversity crisis, which has remarkably reduced the suitable habitats available for wild animals to survive and breed (Grimm et al., 2008; McDonald et al., 2008; Fenoglio et al., 2020; Li et al., 2022). Various studies have reported the effects of urbanization on macroecological biodiversity patterns (Kondratyeva et al., 2020; Li et al., 2022; Pan et al., 2022). However, few studies (Lokmer et al., 2020; Barnes et al., 2021) have reported the impacts of urbanization on the biological diversity and composition of microorganisms associated with the organs of animal hosts.

Cities are expected to expand due to increase in the population through immigration from neighboring wild areas and the aggregation of supporting infrastructure, particularly in developing countries (Henderson, 2002; Xu et al., 2014; Zhai et al., 2020). Therefore, urbanization is inevitable and will be intensified. Over the past decade, Chengdu has been the fastest-growing city in Southwest China, with a total population of over 20 million. Consequently, suitable habitats for wild animals to inhabit within city boundaries have been greatly reduced (Kong et al., 2010). Furthermore, as a result of increased urbanization, these 'green spaces' are continuously fragmented and isolated, leaving wild animals vulnerable to human disturbance (Gaston, 2010) (e.g., pollution, roadkill, and illegal injury) and making it less likely to avoid population decline or local extinction through migration (Marzluff and Rodewald, 2012; Bendik et al., 2014). This equally applies to microorganisms of host species. Because of the difficulty of finding suitable habitats for animal host species in cities, microbial communities of the hosts are also greatly selected and modulated by urban environments in comparison to those in rural and wild habitats (Lokmer et al., 2020; Barnes et al., 2021).

Over one-third of amphibian species are threatened by urbanization (Hamer and McDonnell, 2008), which may be because amphibians are the most sensitive taxa in response to climate and urban environments compared with others such as birds and mammals (Hamer and McDonnell, 2008; Magle et al., 2012; Callaghan et al., 2021). Small patches and fragmented habitats caused by urbanization can remarkably affect the home range of animals and, as a result, their reproduction and survival, particularly for amphibians (Hinam and Clair, 2008; Decout et al., 2012; Amburgey et al., 2021). However, as amphibians are small-bodied animals, they can survive in small patches of forested and humid areas of 'green spaces', provided that these areas are not heavily polluted or disturbed (Hamer and McDonnell, 2008). The impact of urbanization on the biodiversity (Callaghan et al., 2021; Yang, L. et al., 2022) and genetic structure (Wei et al., 2021) of amphibian populations has been extensively studied, but microbial data records associated with amphibian hosts in urban settings are lacking.

Symbiotic microorganisms are critical for amphibian survival (Becker et al., 2015) and adaptation to rapid environmental changes (Xu et al., 2020b; Barnes et al., 2021). Consequently, it is crucial to elucidate the response of the amphibian symbiotic microbial community to rapid urbanization, which is valuable for wildlife conservation in urban settings (Wei et al., 2021). However, urban ecosystems present distinctive microhabitat and microclimate conditions in comparison to wild regions (Zhang et al., 2022). Therefore, it is expected that the microorganism community structure and diversity of amphibian hosts must also alter and adapt to the urban environment. However, to the best of our knowledge, few studies have reported on the potential distinctive features (e.g., stochasticity and stability) of the microbial community structure of animal hosts in urban settings (see concept Figure 1A), especially for amphibians, which are indicator species for assessing urbanization's ecological impact (Guzy et al., 2012).

The process of community assembly is generally used to uncover the formation mechanisms of microbial diversity and composition differences, which include deterministic processes based on niche theory and stochastic processes based on neutral theory (Stegen et al., 2013, 2015; Ning et al., 2020). Theories based on niches contend that species distribution is primarily controlled by the deterministic processes involve with abiotic factors (e.g., environmental factors) and biotic factors (e.g., biological interactions) (Stegen et al., 2013; Ning et al., 2020). In contrast, neutral theories view all species as ecologically equivalent, with stochastic processes largely determining species dynamics and patterns, such as birth/death, speciation/extinction, and migration (Stegen et al., 2013; Ning et al., 2020). Furthermore, ecological stability is vital for microbial communities to adapt to rapid environmental changes (Yuan et al., 2021; Liu et al., 2022). Generally, microbial network analysis is used to evaluate the interspecific interactions and ecological stability or complexity of microbial communities (Yuan et al., 2021; Liu et al., 2022).

In summary, we aimed to answer the following questions: (i) Does urbanization affect the diversity and community structure of symbiotic microbes? (ii) What is the actual influence of urbanization on the stochasticity and stability of amphibian symbiotic microbial communities? (iii) What is the relationship between the stochasticity and stability of symbiotic microbial communities under urbanization gradient? To this end, we chose three amphibian host species (*Pelophylax nigromaculatus*, abbr: PN; *Fejervarya multistriata*, abbr: FM; *Bufo gargarizans*, abbr: BG) that are widely distributed in the city parks and surrounding wild areas of Chengdu, southwest China, to elucidate the community assembly and diversity patterns of microbial taxa in the skins and guts of the host species. We compared the microbial differences between amphibians in urban and wild environments, between host species and between different organs of the host species. This work explores the urbanization-host animal-microbial relationship with the aim of providing new perspectives on bioenvironmental interactions in the context of rapid global land use change.

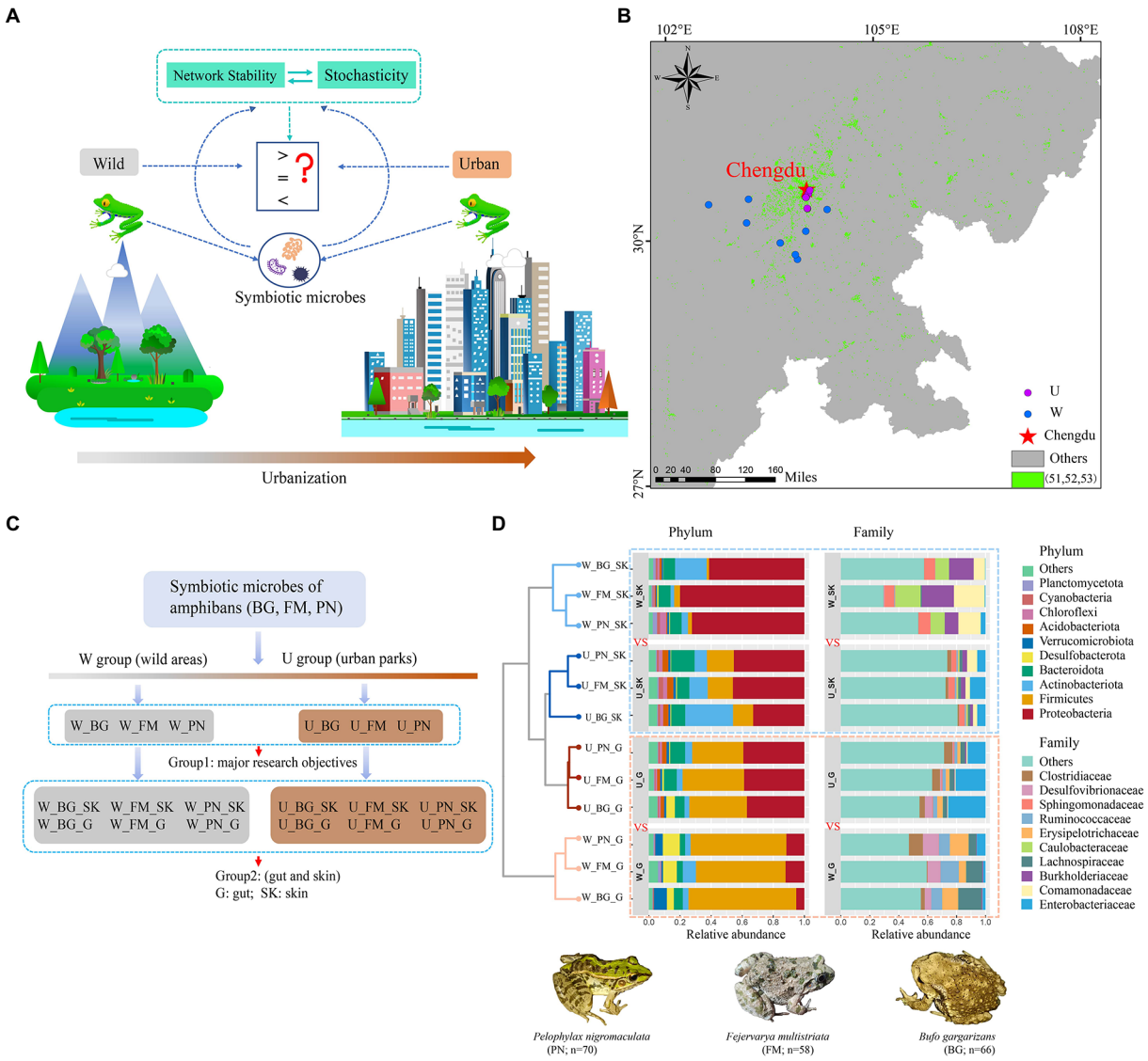

FIGURE 1
Community composition of microbes of amphibians in the urban parks and wild area habitats. **(A)** Schematic illustration of the effects of urbanization on the community network stability and assembly mechanisms of amphibian symbiotic microorganisms. **(B)** Diagram of grouping. **(C)** Map of sampling sites in city parks and wild areas of Chengdu city in China based on the land use types of the dataset. The green points represent urban land, including the 51 (urban land), 52 (rural residential areas), and 53 (other construction land) land use types. The purple and blue points represent the U (urban park samples) and W (wild area samples) groups, respectively. **(D)** Comparison of the taxonomic composition of the top 10 phyla and family between the U and W groups. BG, *Bufo gargarizans*; FM, *Fejervarya multistriata*; PN, *Pelophylax nigromaculatus*. G, gut; SK, skin; W_G, gut microbiota of amphibians in wild areas; W_SK, skin microbiota of amphibians in wild areas; U_G, gut microbiota of amphibians in urban parks; U_SK, skin microbiota of amphibians in urban parks.

2. Materials and methods

2.1. Sample collection and sequencing

From April to August 2021, we collected 194 microbial samples from three frog species in downtown Chengdu city and surrounding wild areas, which included four city park sampling sites and eight wild sampling sites (that is, exurban regions) (Figure 1B; Supplementary Table S1). For each sampling site, we searched for frog species alongside ditches, brooks or ponds. We captured adult individuals by wearing sterile gloves and took the microbial samples from the skin by using cotton swabs after rinsing three times with sterile water to remove the potential transient bacteria (Lauer et al., 2007). A 2-ml aseptic centrifuge tube was used to collect gut microbial content after the frog was euthanized and dissected. After returning to the laboratory, all gut and skin samples were immediately stored at −80°C. Experimental approval was obtained from the Animal Ethical and Welfare Committee of Chengdu Institute of Biology, Chinese Academy of Sciences (permit no. CIBDWLL2022008).

Microbial DNA was extracted using the MN NucleoSpin 96 Soil kit (MACHEREY-NAGEL) according to the manufacturer's instructions. The V3-V4 regions of the 16S rRNA gene were amplified using the universal primer pair 338F/341F and 806R/805R (Yao et al., 2019; Gutierrez-Villagomez et al., 2021). Briefly, PCR thermocycling was carried out under the following conditions: 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 50°C for 30 s, 72°C for 60 s and a final extension at 72°C for 5 min. The PCR products were further purified with e.Z.N.A. TM Cycle-Pure Kit (Omega).

The raw sequence data were processed using the QIIME2 software package (version QIIME2-2021.2) (Bolyen et al., 2019). The paired reads (2 × 250 bp paired-end mode) generated by the Illumina platforms of MiSeq and Nova6000 were demultiplexed and filtered by vsearch and quality filtered with the following quality-control criteria: sequences of length < 200 bp and average quality score < 25 were removed; no ambiguous bases were allowed. High-quality sequence reads with >97% identity were defined as operational taxonomic units (OTUs) with the vsearch cluster-features-de-novo (Rognes et al., 2016). Then, the samples were rarefied to the same sequence depth (33,187 bacterial sequences per sample). To minimize inclusion of sequencing errors, the clustered feature tables were further filtered using QIIME2 feature-table filter-features (Revise: p-min-samples 2 --p-min-frequency 10) (Zhang et al., 2020). Taxonomy assignment to the OTUs was performed using the Silva v138 database and the Naive Bayes classifier (Quast et al., 2012). Subsequently, the sequences identified as chloroplasts or mitochondria were removed. The final dataset retained 19,042 OTUs for the downstream analyses. In our study, we classified the collection of microbial samples from urban park samples and wild area samples into U and W groups, respectively (Figure 1B). We also separated the microbial samples into two groups for three host species in the analyses. Group 1 contained all the microbes from all the organs (gut and skin) of the three host species, including W_BG, U_BG, W_FM, U_FM, W_PN, and U_PN (Figure 1C). Group 2 distinguished the microbial communities from the skin (abbr: SK) and gut (abbr: G) ecosystems, including W_BG_G, W_BG_SK, U_BG_G, U_BG_G _SK, W_FM_G, W_FM_SK, U_FM_G, U_FM_SK, W_PN_G, W_PN_SK, U_PN_G, and U_PN_SK (Figure 1B). In this study, Group 1 was the primary research target. The analyses were conducted and compared at the OTU, family and phylum levels.

2.2. Measures of diversity and community structure

All result analysis and visualization were performed in R v4.1.3.[1] For the alpha diversity, we computed the richness, Shannon index and phylogenetic diversity for each community and used the Wilcoxon rank-sum test and the false discovery rate (FDR) corrected method to identify significant differences between the U and W groups.

For the beta diversity, we compared the difference in beta diversity built on the Bray–Curtis distance among the six groups by using Kruskal–Wallis tests with multiple testing correction Behrens–Fisher using the *nmpc* function in the 'nmpc' R package (Munzel and Hothorn, 2001). Additionally, PERMANOVA and ANOSIM tests (number of permutations: 999) based on the Bray–Curtis distance were used to analyze the differences in gut and skin microbes between the Group U and Group W amphibians. Nonmetric multidimensional scaling (NMDS) was used to visualize the results using the 'vegan' R package (Oksanen et al., 2002).

We assessed whether neutrality is the principal driver of the microbial community structure in urban and wild environments. To determine the strength of the neutral stochasticity, we utilized the Sloan neutral community model (NCM) (Sloan et al., 2006; Chen et al., 2019). This method evaluates the relationship between occupancy and species abundance, in which R^2 indicates the overall fit to the neutral model (Sloan et al., 2006; Chen et al., 2019). In this model, the parameters Nm and *m* represent the metacommunity size times migration and migration rate, respectively, where higher m values indicate less dispersal limitation (Burns et al., 2016; Chen et al., 2019). To further assess the relative importance of stochastic versus deterministic processes in the community assembly, the phylogenetic normalized stochasticity ratio (pNST, ranging from 0 to 1) index was calculated using the *pNST* function in the 'NST' package in R (Ning et al., 2019, 2020). The pNST was used with 50% as the boundary point between stochastic assembly (>50%) and deterministic assembly (<50%). The total differences in the pNST between the U and W groups were tested by Kruskal–Wallis tests with multiple testing correction (Behrens–Fisher) using the *nmpc* function in the 'nmpc' R package (Munzel and Hothorn, 2001). In addition, the relationships between the pNST and urbanization, robustness and vulnerability were estimated with Spearman's correlation analyses.

To evaluate the levels of OTU species co-occurrence patterns, the co-occurrence networks were constructed based on robust correlations with Spearman's correlation coefficients (r) of >0.6 or <−0.6 and false discovery rate-corrected p-values of <0.01 using the 'WGCNA' R package (Langfelder and Horvath, 2008). To minimize the impacts of rare OTUs in the dataset, only OTUs with relative abundance >0.01% and the bacterial taxa that were detected in at least 10% of all samples per group were included in the analysis (Jiao et al., 2021). Subsequently, visualization of networks and the calculation of network properties were performed using the 'igraph' R package (Csardi and Nepusz, 2006). Additionally, we extracted the subnetwork topological characteristics of each sample by using the *subgraph* functions in the 'igraph' R package (Csardi and Nepusz, 2006; Ma et al., 2016). In this study, the network characterizations of the node numbers, edge numbers, average degree, degree centralization, graph density, average path length, clustering coefficient, graph

1 http://www.r-project.org/

modularity, and betweenness centralization were calculated to estimate the network structure differences in different groups. In addition, the robustness based on the method of 50% of nodes was randomly removed with 100 repetitions of the simulation, and the vulnerability was also calculated to assess the network stability (or ecological stability) for the symbiotic microbiota of amphibians from urban park and wild area habitats (Yuan et al., 2021; Liu et al., 2022). The total differences in the network characterization in different groups were tested by using Kruskal–Wallis tests with multiple testing correction (Behrens–Fisher) using the *nmpc* function in the 'nmpc' R package (Munzel and Hothorn, 2001). Furthermore, the relationships between the urbanization and robustness and vulnerability were assessed with Spearman's correlation analysis.

To decipher changes in keystone taxa between Group 1 levels, we identified the putative role of each node based on two network topological characteristics of the within-module connectivity (*Zi*) and the among-module connectivity (*Pi*) following the previous classification: peripherals ($Zi < 2.5$ and $Pi < 0.62$), connectors ($Zi < 2.5$ and $Pi > 0.62$), module hubs ($Zi > 2.5$ and $Pi < 0.62$) and network hubs ($Zi > 2.5$ and $Pi > 0.62$) (Olesen et al., 2007). The connectors, module hubs and network hubs are generally defined as keystone taxa, which represent key species that may play crucial roles in maintaining the stability of the microbial community structure (Röttjers and Faust, 2019; Yuan et al., 2021; Liu et al., 2022).

2.3. Impacts of abiotic factors

We tested the impacts of abiotic variables on the microbial community structure and diversity. To do so, we utilized a set of variables, including 19 bioclimatic variables such as the latitude, longitude, urban_degree (degree of urbanization), elevation, PH, AT (air temperature) and AH (air humidity) (Supplementary Table S2). Bioclimatic variables were extracted from the WorldClim database.[2] PH was measured using a portable HANNA HI 9913001/1 instrument, and AT and AH were detected every 10 min by using an automatic temperature and humidity recorder (Elitech GSP-8A) at each sampling site. In addition, we quantified the degree of urbanization by calculating the total proportion of three land use types, i.e., urban land (code 51), rural residential areas (52) and other construction land (53) to all land use classes within a 10-km radius buffer around each sampling site. Land use class data from 2020 (approximate to our sampling time) were obtained from the Resource and Environment Science and Data Center.[3]

Because the utilization of all variables could lead to multicollinearity, to reduce the risk of overfitting, we first utilized random forest (RF) to identify potentially highly correlated and important variables, which can indicate the importance of variables by the percentage increase in the MSE (mean squared error) (Jiao et al., 2021). In the RF models, the environmental variables are referred to as predictors for symbiotic microbial richness. Then, we selected the top 10 most important environmental variables for collinearity analysis using the *check_collinearity* function in the 'performance' R package (Lüdecke et al., 2021). Finally, we retained five environmental variables, the PH, AT, urban_degree, bio_3 (isothermality) and bio_18 (precipitation of warmest quarter) (Supplementary Table S2), for subsequent analysis. In this work, the relative abundance of the top 10 phyla and families and network characteristic correlations with environmental variables were calculated using the *corrplot* function in the 'corrplot' package (Wei et al., 2017). Moreover, redundancy analysis (RDA) was applied to disentangle the relationship between the symbiotic microbes of amphibians and environmental variables with the *rda* function of the 'vegan' package (Oksanen et al., 2002).

3. Results

3.1. Overview of the amphibian symbiotic bacterial composition and diversity

In terms of the community composition of host microbes, at the phylum level, for the skin and gut microbiota of amphibians, the bacterial composition was primarily dominated by Proteobacteria, Firmicutes, Actinobacteriota, and Bacteroidota. Specifically, for the skin microbiota of the three amphibians, the relative abundance of Proteobacteria, Firmicutes, and Actinobacteriota in wild areas (W_SK) was significantly higher than that in urban parks (U_SK) (Figure 1D). Conversely, for the gut microbiota, the relative abundance of Proteobacteria in wild areas (W_G) was significantly lower than that in urban parks (U_G), while the relative abundance of Firmicutes in the W_G group was significantly higher than that in the U_G group (Figure 1D). Furthermore, in contrast to the skin microbiota, Desulfobacterota and Verrucomicrobiota were also major phyla in the gut microbiota. Analogously, the relative abundance of Desulfobacterota in the W_G group was significantly higher than that in the U_G group, in addition to *Bufo gargarizans* (Figure 1D).

Interestingly, at the family level, the relative abundance of families in different groups presented remarkable microbial community composition differences (Figure 1D). For skin microbiota, Enterobacteriaceae, Comamonadaceae, and Burkholderiaceae were major dominant families. Additionally, in addition to Comamonadaceae and Burkholderiaceae, the relative abundance of Caulobacteraceae and Sphingomonadaceae of the three amphibians in the wild areas (W_SK) was also significantly higher than that in the urban parks (U_SK), whereas Enterobacteriacea had a higher relative abundance in the U_SK group (Figure 1D). For gut microbiota, Enterobacteriaceae was the dominant family in the urban parks (U_G), while

2 https://www.worldclim.org/

3 https://www.resdc.cn/data.aspx?DATAID=335

Lachnospiraceae, Erysipelotrichaceae, Ruminococcaceae, and Desulfovibrionaceae were the major dominant families in the wild areas (W_G; Figure 1D).

In regard to the biological diversity of the microbes, at the alpha diversity level, the overall microbial diversity was consistently high in the U groups for the species-level richness (Figure 2A), Shannon index (Figure 2B), and phylogenetic diversity (Figure 2C). However, when comparing the alpha diversity difference for the skin and gut of host species, in general, skin microbes presented higher diversity (Supplementary Figure S2). This might be attributed to the fact that skin microbes are jointly influenced by host species and external abiotic environments. However, at the beta diversity level, the beta diversity built on the Bray-Curtis distance of the three amphibians was significantly higher in the W group than in the U group (Kruskal–Wallis tests, $p < 0.001$; Figure 2D; Supplementary Figure S3). Non-metric multidimensional scaling (NMDS) analysis was used to further disentangle this dissimilarity. The results show that there was clear segregation between the U and W groups, which presented significant differences (PERMANOVA, $p < 0.001$; ANOSIM, $R = 0.12$, $p < 0.001$; Figure 2E).

3.2. Urbanization influences the symbiotic bacterial community assembly of amphibians

Here, the NCM model was used to effectively evaluate the relationship between the occurrence frequency and relative abundance of OTUs. The results show that the explained proportion of variation (R^2) of the symbiotic microbial community in the urban parks (U group: U_BG, $R^2 = 0.626$; U_FM, $R^2 = 0.602$; U_PN, $R^2 = 0.679$) versus the wild areas (W group: W_BG, $R^2 = -0.027$; W_FM, $R^2 = 0.357$; W_PN, $R^2 = 0.214$) present a remarkable difference. The greater R^2 values of the U group indicate that stochastic processes played a more important role in the U group than in the W group (Figure 3A). This further suggests that urbanization enhanced the ecological stochasticity of the microbial community assembly. Furthermore, the Nm and m values were higher for microbial taxa in the U group (U_BG, Nm = 2,477, $m = 0.078$; U_FM, Nm = 1931, $m = 0.061$; U_PN, Nm = 1,583, $m = 0.05$) than in the W group (W_BG, Nm = 702, $m = 0.023$; W_FM, Nm = 919, $m = 0.03$; W_PN, Nm = 693, $m = 0.023$) (Figure 3A). These results illustrate that species dispersal of symbiotic microbes was higher in the U group than in the W group, indicating that the microbial taxa of the W group had a higher chance of dispersal limitation. Additionally, similar results were found in the gut and skin symbiotic microbe taxa between the U and W groups (Group 2 level) (Supplementary Figure S4).

The pNST model was further used to quantify the relative importance of stochastic and deterministic processes of the symbiotic microbe community assembly. The results show that the pNST value of the W group was <0.5, while that of the U group was >0.5, which indicates that stochastic processes and deterministic processes were the predominant processes for the W and U groups, respectively (Figure 3B). Moreover, significant differences in the pNST values were found between the U and W groups (Kruskal–Wallis tests, $p < 0.001$; Figure 3B). Importantly, the pNST was significantly positively correlated with urban_degree (Figure 3C), which further identifies the effect of urbanization on microbial community assembly processes. These results indicate that urbanization significantly alters microbial community assembly processes and increases stochastic processes in amphibian symbiotic microbes. These results are in line with previous NCM findings.

3.3. Urbanization influences the network structure of the amphibian symbiotic bacterial community

Using network analyses, we further identified important evidence for community simplicity, with a well-fitted power-law model ($R^2 = 0.995$) for the degree distribution of the total network, which indicates that the network was scale-free and nonrandom (Barberan et al., 2012). The edge numbers were higher in the U groups than in the W group, whereas the node numbers were lower (Figure 4A). Importantly, the co-occurrence instances (edge/node ratio) in the U group (U_BG, edge/node = 19.32; U_FM, edge/node = 3.26; U_PN, edge/node = 5.97) were higher than those in the W group (W_BG, edge/node = 86.70; W_FM, edge/node = 27.11; W_PN, edge/node = 36.18), which indicates that the microbial taxa of the W group tend to co-occur more tightly and frequently (Figure 4A; Shu et al., 2021). The average degree and graph density, representing the network complexity (Wu et al., 2016), decrease from the W group to the U group, indicating that the W group had higher network complexity than the U group (Figure 5). The degree centralization shows similar patterns, suggesting that the network structures of the U group are more 'even' networks (Wu et al., 2016). Compared with the U group, the W group had a higher clustering coefficient and shorter average path length, indicating that the small-world properties of the W group were typical (Figure 5). Additionally, the betweenness centralization, which reflects the role of a node as a bridge between components of network (Banerjee et al., 2019), was higher in the U group than in the W group, excluding the FM amphibian (Figure 5).

As indicators of the network stability, the robustness and vulnerability (Yuan et al., 2021) were calculated. Our results show that the robustness was significantly higher (all $p < 0.001$) in the W group than in the U group (Figure 4B), while the vulnerability had the opposite trend (Figure 4C), suggesting that the U group had lower network stability than the W group. More importantly, the correlation results show that urbanization

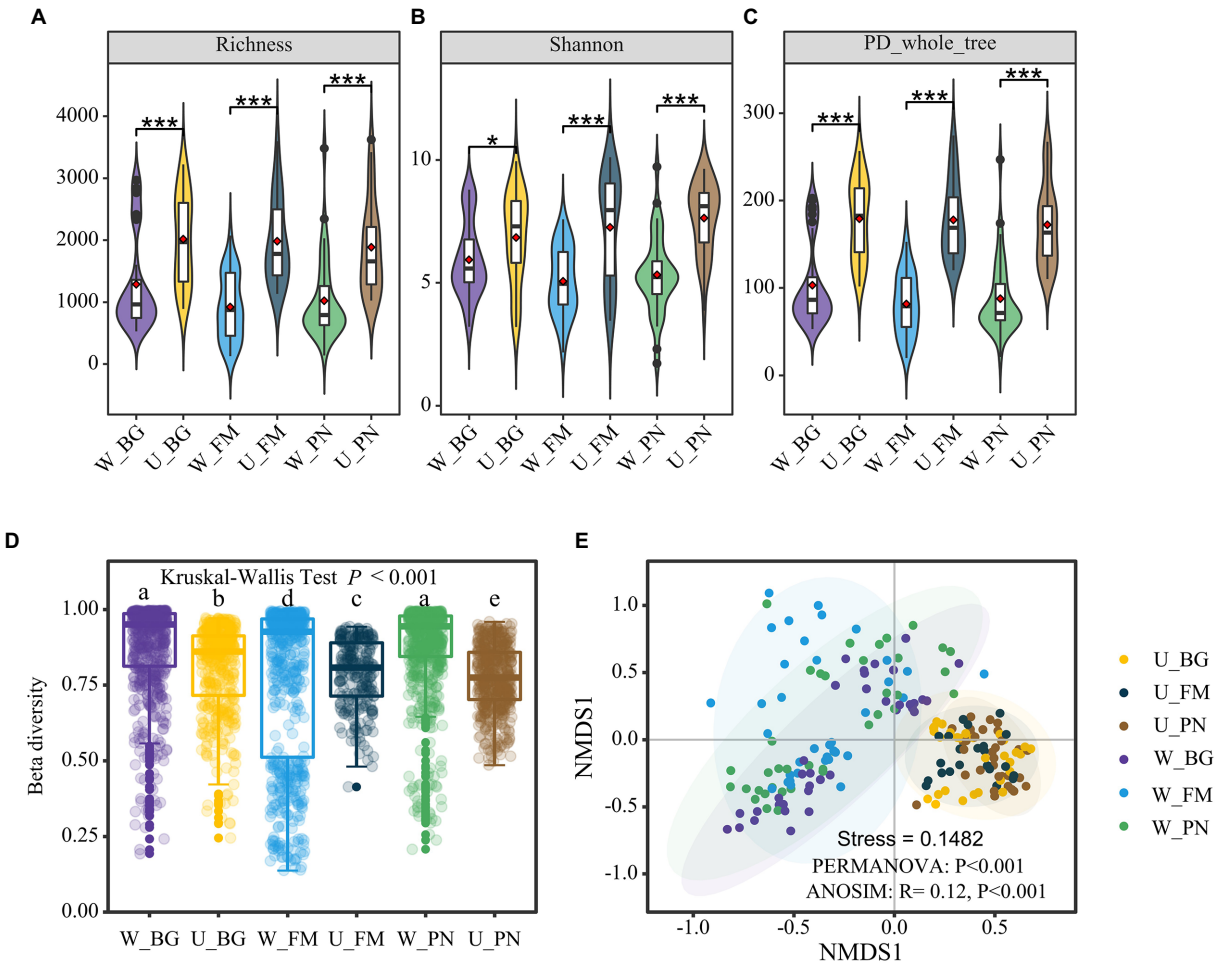

FIGURE 2
Microbial diversity of amphibians in the urban park and wild area habitats. Comparison of the alpha diversity between Group U (urban park samples) and Group W (wild area samples) amphibian symbiotic microorganisms, including the richness **(A)**, Shannon index **(B),** and PD phylogenetic diversity index **(C)**. The difference in the alpha diversity was tested using the Wilcoxon rank-sum test and false discovery rate (FDR) at the corrected level of $p<0.05$. **(D)** Differences in the beta diversity between the U and W groups based on the Bray–Curtis distances. The different superscripts indicate significant differences between groups ($p<0.05$, Kruskal–Wallis tests and Behrens–Fisher *post hoc* tests). **(E)** Nonmetric multidimensional scaling (NMDS) analysis based on the Bray–Curtis distance of the relative abundance of operational taxonomic units (OTUs). Differences are denoted as follows: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$. W_BG, W_FM, and W_PN represent the symbiotic microbiota of *Bufo gargarizans*, *Fejervarya multistriata,* and *Pelophylax nigromaculatus* in the wild areas, respectively. U_BG, U_FM, and U_PN indicate the symbiotic microbiota of *Bufo gargarizans*, *Fejervarya multistriata,* and *Pelophylax nigromaculatus* in the urban parks, respectively.

(urban_degree) was remarkably negatively correlated with the robustness ($R=-0.82$, $p<0.01$), whereas vulnerability presented the opposite trend ($R=0.94$, $p=0.017$) (Figure 4D), which further suggests that urbanization significantly altered the robustness and vulnerability of the network. The above findings strongly indicate that urbanization profoundly affects microbial associations, significantly reduces complexity and stability, and enhances the vulnerability of the symbiotic microbial community.

The keystone taxa were identified to further estimate the effect of urbanization on the network structure of symbiotic microbes by using Zi–Pi relationships, with the connectors, module hubs and network hubs regarded as keystone taxa. The results reveal that the periphery had the most nodes in all groups, and the numbers of keystone taxa were higher in the U group (U_BG, $n=713$; U_FM, $n=729$; U_PN, $n=1{,}088$) than in the W group (W_BG, $n=99$; W_FM, $n=178$; W_PN, $n=124$) (Figures 6A–C), which demonstrates that urbanization notably increased the numbers of keystone taxa. Additionally, we found that the Acidobacteriota, Bacteroidota, Firmicutes, Actinobacteriota, Proteobacteria, and Planctomycetota play critical roles in maintaining the ecological stability of commensal microbes (Figures 6A,B). Dechloromonas at the genus level plays a dominant role in symbiotic microbes under urban settings (Figure 6A). Importantly, the degree of keystone taxa in group U was significantly lower than in group W, which may imply that many rare taxa play a more important role in regulating the ecological stability of symbiotic microbes in the urban setting (Figures 6A,B).

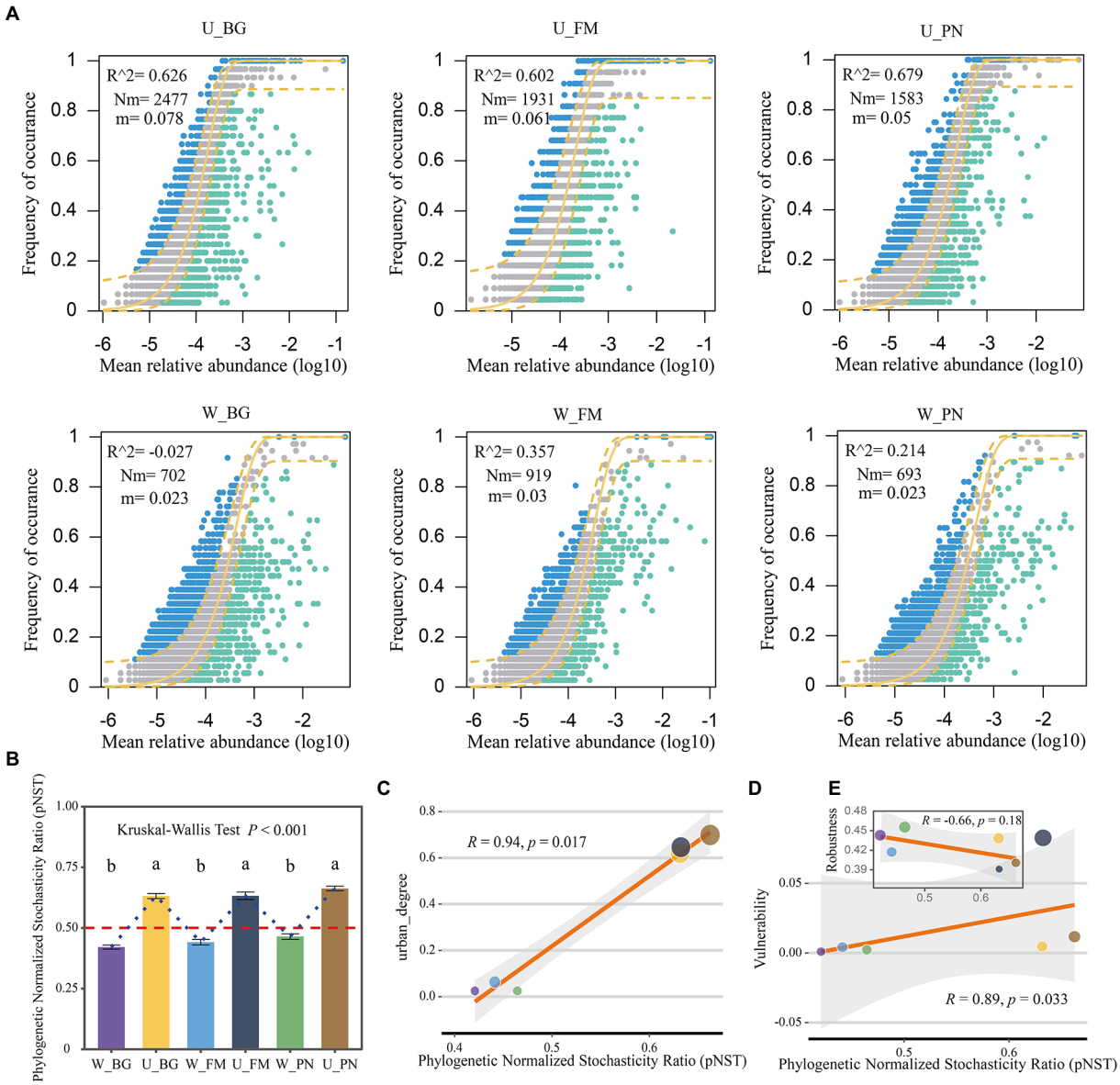

FIGURE 3
Microbial assembly mechanisms of amphibians in urban park and wild area habitats. **(A)** Neutral community model (NCM) applied to estimate the influence of random dispersal and ecological drift on the community assembly of the symbiotic microbiota of amphibians from urban parks and wild areas. Nm is the metacommunity size times immigration, R^2 is the fit to the neutral model, and *m* indicates the migration rate. The solid yellow line indicates the best fit to the neutral model, while a dashed yellow line indicates 95% confidence intervals around the prediction. The different colors are used for OTUs that occur more or less frequently than predicted by the NCM. **(B)** The ecological stochasticity in the assembly of the amphibian symbiotic microbiota community evaluated by the phylogenetic normalized stochasticity ratio (pNST). The value of 0.5 is regarded as the boundary between a stochastic (>0.5) and deterministic (<0.5) assembly. The different superscripts indicate significant differences between groups (p<0.05, Kruskal–Wallis tests and Behrens–Fisher *post hoc* tests). **(C)** Regression relationships between the pNST and urban_degree (urban degree of sampling sites). **(D,E)** Regression relationships between the pNST and the robustness and vulnerability, respectively. W_BG, W_FM, and W_PN represent the symbiotic microbiota of *Bufo gargarizans*, *Fejervarya multistriata,* and *Pelophylax nigromaculatus* in the wild areas, respectively. U_BG, U_FM and U_PN indicate the symbiotic microbiota of *Bufo gargarizans*, *Fejervarya multistriata,* and *Pelophylax nigromaculatus* in the urban parks, respectively.

3.4. Impacts of urbanization and environmental variables on the symbiotic bacterial community of amphibians

The RF analysis show that bio_3, AT, urban_degree and PH were the four most important variables for symbiotic microbial richness (Supplementary Figure S1A). Specifically, correlations between the symbiotic bacterial composition and urbanization (urban_degree) and environmental factors were detected. At the phylum level (Figure 7A), the urban_degree, AT, bio_18, bio_3 and pH were significantly correlated (all $p < 0.05$) with the relative abundance of Proteobacteria, Firmicutes, and Desulfobacterota,

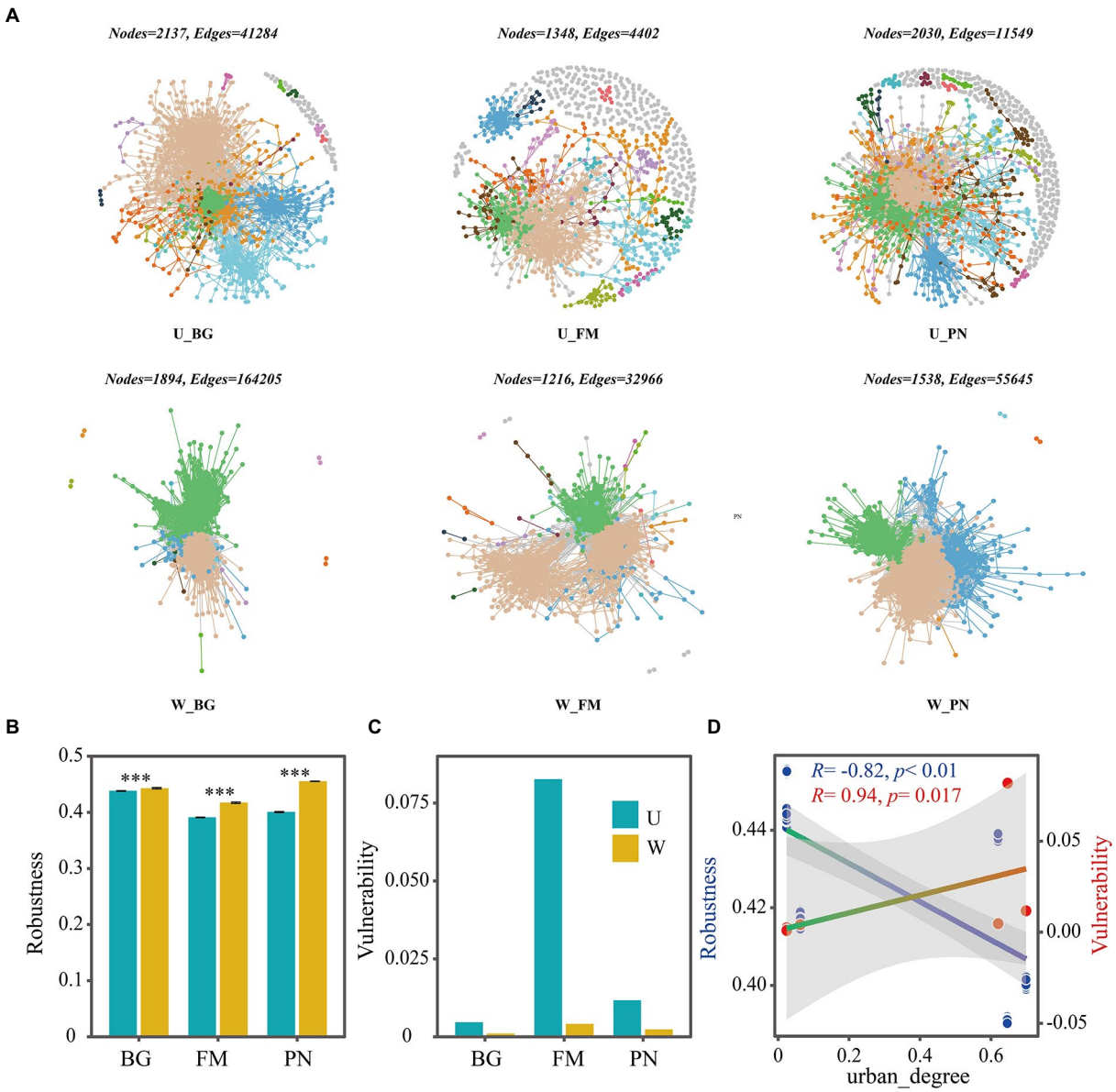

FIGURE 4
Co-occurrence networks of symbiotic microbial taxa of amphibians in the urban park and wild area habitats. **(A)** Network diagram of the symbiotic microbiota of amphibians in urban parks and wild areas. The robustness **(B)** was calculated as the proportion of the remaining species in the community after random node 50% removal, and the vulnerability **(C)** was determined by the maximum node vulnerability in each community. **(D)** The regression relationships between urban_degree (urban degree of sampling sites) and robustness and vulnerability. The difference in robustness was tested using the Wilcoxon rank-sum test and false discovery rate (FDR) at the level of corrected $p<0.05$. Differences are denoted as follows: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$. W_BG, W_FM, and W_PN represent the symbiotic microbiota of *Bufo gargarizans*, *Fejervarya multistriata,* and *Pelophylax nigromaculatus* in the wild areas, respectively. U_BG, U_FM, and U_PN indicate the symbiotic microbiota of *Bufo gargarizans*, *Fejervarya multistriata,* and *Pelophylax nigromaculatus* in the urban parks, respectively.

respectively. At the family level (Figure 7B), the urban_degree and all variables presented significant correlations with the relative abundance of the top 10 families, excluding *Enterobacter australis*, for which urban_degree presented a remarkable positive correlation.

Additionally, we examined the correlations between the network topological features and environmental variables (Figure 7C). The results show that AT and bio_18 were significantly positively correlated with nodes_num, the average path length and graph modularity, and the correlations with the degree, graph density and degree centralization presented the opposite trend. Moreover, PH was negatively correlated with nodes_num, the average path length and graph modularity, while it was positively correlated with the degree, graph density and degree centralization. Importantly, significant positive correlations between the degree and graph density and urban_degree were

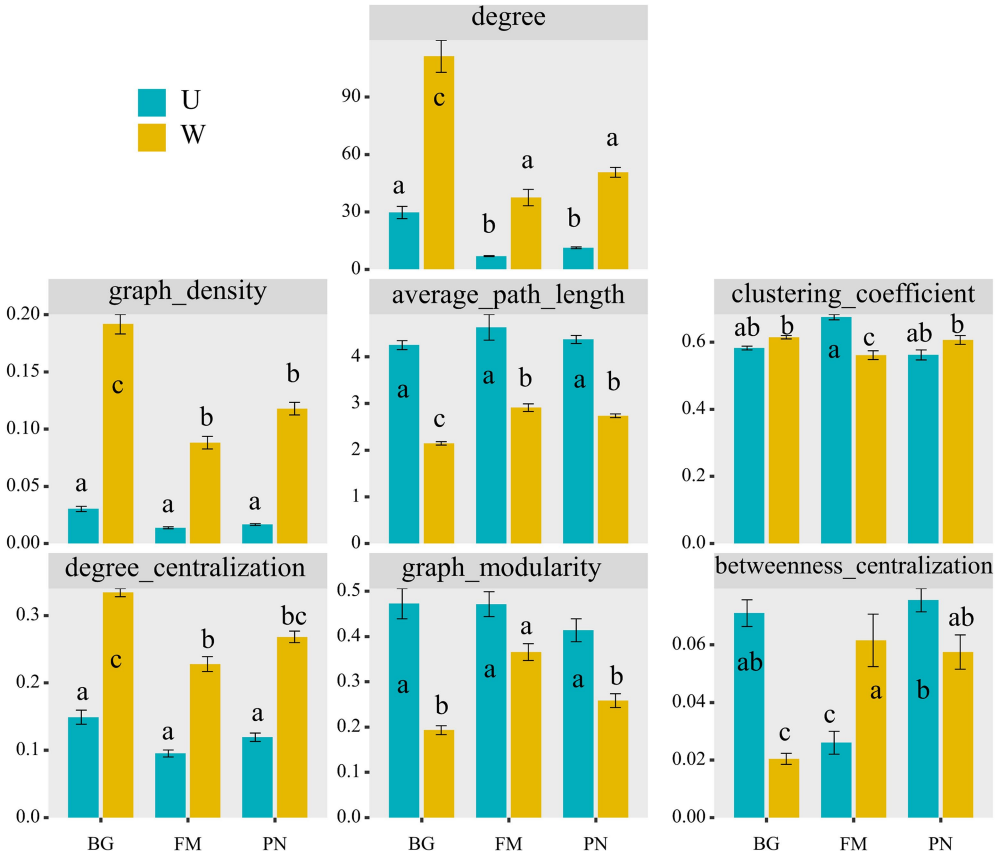

FIGURE 5
Comparison of different network characterizations between the U and W groups, including the node number (nodes_num), edge number (edges_num), average degree (degree), degree centralization, graph density, average path length, clustering coefficient, graph modularity, and betweenness centralization. The different superscripts indicate significant differences between groups ($p<0.05$, Kruskal–Wallis tests and Behrens–Fisher *post hoc* tests). W_BG, W_FM, and W_PN represent the symbiotic microbiota of *Bufo gargarizans*, *Fejervarya multistriata*, and *Pelophylax nigromaculatus* in the wild areas, respectively. U_BG, U_FM, and U_PN indicate the symbiotic microbiota of *Bufo gargarizans*, *Fejervarya multistriata*, and *Pelophylax nigromaculatus* in the urban parks, respectively.

observed, indicating that urbanization remarkably affects the complexity of microbial communities. Furthermore, correlations between urban_degree and edges_num and the degree centralization presented similar patterns, while urban_degree was significantly negatively correlated with nodes_num, the average path length, graph modularity and betweenness centralization.

The RDA further revealed the effect of urbanization and environmental variables on the symbiotic bacterial community (Figure 7D). The results show that five variables were correlated with the microbial community, especially PH, AT and urban_degree, with the RDA axis accounting for the total variance and RDA axis 2 accounting for 20.64%. The PH, urban_degree and bio_3 were positively correlated with the microbial community in the U group, while bio_18 and AT were negatively correlated. Moreover, the W group showed opposite correlations.

Finally, the relationship between the pNST and robustness was further identified. The results show that the pNST was remarkably positively correlated with the network vulnerability ($R=0.89$, $p=0.033$; Figure 3D), yet the robustness was slightly negatively correlated with the pNST ($R=-0.66$, $p=0.18$; Figure 3E), which could indicate a certain degree of correlation between the stochasticity and network stability.

4. Discussion

The environmental disturbances, high spatial heterogeneity, and habitat fragmentation caused by urbanization (Gaston, 2010; Kondratyeva et al., 2020) can remarkably affect the composition and diversity of an animal's symbiotic microbiome and adaption to complex urban settings (Lokmer et al., 2020; Barnes et al., 2021). In this study, we found that urbanization increased the richness and homogenization of microbes in amphibian hosts. First, in the alpha diversity analyses, we found that the microbial richness, abundance-based diversity (Shannon) and phylogenetic diversity tended to be higher in urban green spaces than in wild habitats (Figure 2A). This suggests that urbanization significantly increases the diversity of amphibian commensal microbes, while

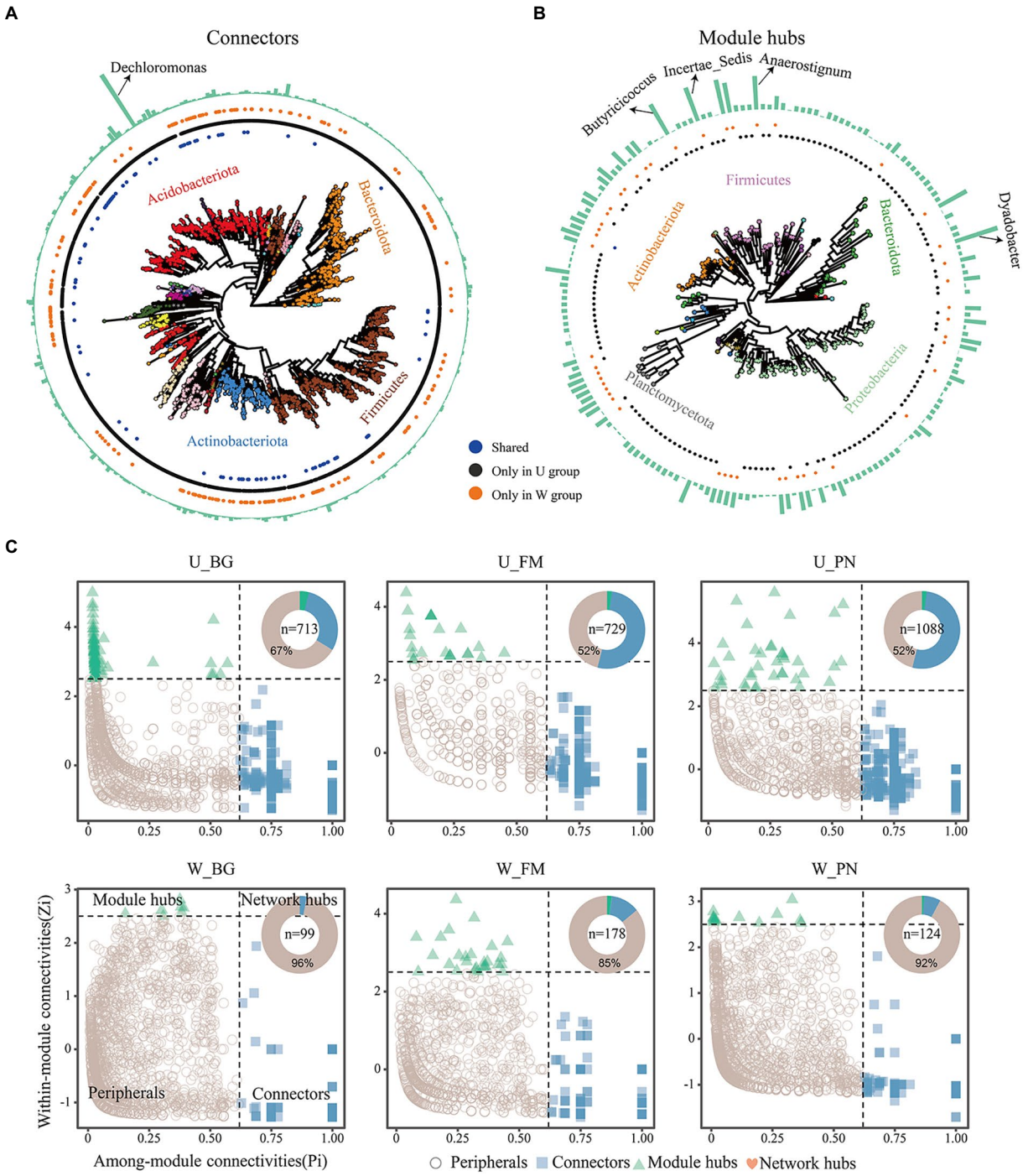

FIGURE 6
The keystone taxa distribution of amphibian symbiotic microbiota in urban parks and wild area habitats. The phylogenetic tree of keystone species of connectors **(A)** and module hubs **(B)**. Colours of the tip point indicate the phyla distribution. Blue, black, and orange dots indicate the keystone taxa that occurred in both urban and wild settings, only in urban setting (U group), and only in wild areas (W group), respectively. The outermost bar plot represents the degree of keystone taxa, and the annotations correspond to genus-level species. **(C)** Identifying keystone taxa across habitats based on their topological roles in networks. The pie chart shows the proportion of each keystone taxa. *n* denotes the number of keystone taxa, including the module hubs, network hubs, and connectors. W_BG, W_FM, and W_PN represent the symbiotic microbiota of *Bufo gargarizans*, *Fejervarya multistriata,* and *Pelophylax nigromaculatus* in the wild areas, respectively. U_BG, U_FM, and U_PN indicate the symbiotic microbiota of *Bufo gargarizans*, *Fejervarya multistriata,* and *Pelophylax nigromaculatus* in the urban parks, respectively.

altering their kinship and phylogenetic diversity. Importantly, these results align with previous ecological theory that more diverse communities are resistant to invasion and further protect the host (Costello et al., 2012) as urban wildlife has more enhanced immune function, pathogen prevalence and stress than wildlife in wild areas (Murray et al., 2019). However, in our beta analyses,

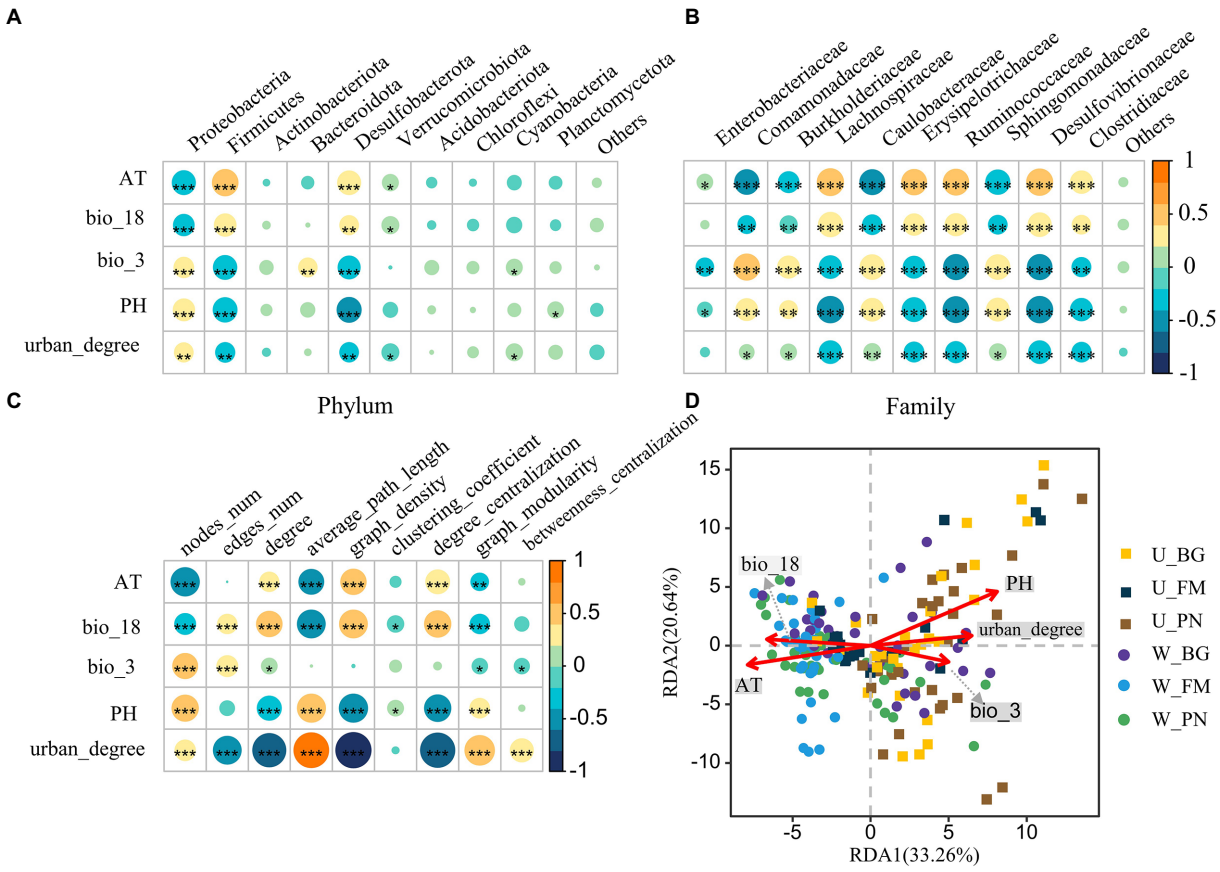

FIGURE 7
The relationship between the symbiotic microbes of amphibians and environmental variables. Correlations between environmental variables and the top 10 phyla **(A)** and families **(B)**. **(C)** Correlations between network characterizations and environmental variables. The size and color of the circles show the strength and sign of the correlation, respectively. Differences are denoted as follows: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$. **(D)** Redundancy analysis (RDA) of the community structure of symbiotic microbiota amphibians and environmental variables, including bio_18 (precipitation of warmest quarter), bio_3 (isothermality), PH, AT (air temperature), and urban_degree (degree of urbanization). W_BG, W_FM, and W_PN represent the symbiotic microbiota of *Bufo gargarizans*, *Fejervarya multistriata,* and *Pelophylax nigromaculatus* in the wild areas, respectively. U_BG, U_FM, and U_PN indicate the symbiotic microbiota of *Bufo gargarizans*, *Fejervarya multistriata,* and *Pelophylax nigromaculatus* in the urban parks, respectively. The full names of the environmental factors are given in Supplementary Table S2.

we found that the beta diversity of microbial taxa on the skin, in the gut or both organs generally presented a marked decrease in urban host specimens. Furthermore, ordination analyses (NMDS) showed that the microbial composition from urban samples tended to group together, but the microbial taxa from wild samples tended to be disperse in the ordination space. Therefore, it can be concluded that the microbial taxa of amphibian hosts presented high heterogeneity in wild areas and high homogeneity in urban areas. One explanation for this could be that the urban environments have similar local species pools and high dispersal rates, which drive the homogenization of symbiotic microorganisms (Figure 3A). This may suggest that there is remarkable habitat variation between sample sites in the wild, which leads to significant differentiation of symbiotic microorganisms. In contrast, urban habitats tend to have more similar habitats, which results in similar environmental pressures and symbiotic microbial convergence. More importantly, our results showed that a high alpha diversity does not necessarily imply high community compositional differences or high biotic heterogeneity for host-specific microorganisms.

Furthermore, we revealed the sensitivity and importance of amphibian skin as a first line of defense (Cramp et al., 2014; Xu et al., 2020a) in terms of community structural differences and community assembly. For instance, our results showed that the relative abundance of the remaining families (Figure 1D) and the three alpha indices (Supplementary Figure S2) of the skin microbes differed more between urban settings and wild areas than the gut microbes. At the community assembly (Supplementary Figure S4), we found that urbanization notably shifts differential patterns of stochasticity (R^2) and dispersal rates (m) on the skin and gut symbiotic microbes. These results suggest that the environmental stressors imposed by urbanization markedly alter the conditions and niche spaces of the skin and gut, with the skin microbes becoming more sensitive (Xu et al., 2020a) and having a higher rate of dispersal and stochasticity versus the gut (Xu et al., 2022). Importantly, these findings will enhance our understanding of the mechanisms underlying the

effects of urbanization on amphibians and contribute to the conservation of sensitive taxa.

Previous studies have revealed that stochastic processes dominate the assembly of skin microbial communities of amphibians along land use gradients, especially drift (Barnes et al., 2021). The stochasticity includes three assembly processes: homogeneous dispersal (as high dispersal rates can homogenize communities and cause high taxonomic turnover), dispersal limitation (as low dispersal rates could enhance community taxonomic alters) and ecological drift (Stegen et al., 2015; Ning et al., 2020). In this study, we found that urbanization increased the microbial stochasticity of amphibian hosts by transforming the dominant deterministic processes in wild areas into dominant stochastic processes in urban settings (Figure 3B). This change could be attributed to higher heterogeneity of conditions and the environmental filtering ability of different sampling sites in wild areas versus urban parks, while the sampling sites in urban parks have similar environmental stress and more numbers of rare or keystone taxa (Figure 6C). Interestingly, our results also verified that the amphibians in urban settings had lower abundance of existence of transient unique or rare symbiotic microbial taxa, such as more network nodes (Figures 4A, 5) or keystone taxa (Figures 6A,B) with no or low degree and a higher proportion of the remaining other families with lower abundance (Figure 1D).

The urban habitat is a separate local species pool, containing many new or unique microbial taxa, which provides a greater diversity of symbiotic taxa for the animals that live there (Barnes et al., 2021; Dillard et al., 2022). Previous studies show that rare taxa are susceptible to local exclusion by drift or competition with other dominant taxa (Melbourne and Hastings, 2008). However, in this study, the passive dispersal could play crucial roles in community assembly (Ai et al., 2013; Jia et al., 2018), as the diverse and distinctive urban local species spool may bring more low-abundance rare or unique taxa to the amphibians, thus causing high turnover and increasing the stochasticity (Stegen et al., 2015). These results align with the previous studies in which the assembly of rare species is mediated by stochastic processes (Jia et al., 2018). More importantly, this is further corroborated by our results that the symbiotic microbiome in urban parks has higher dispersal rates, indicating that homogeneous dispersal can play a profound role in the community assembly in urban settings. This may be due to frequent disturbances (such as human activities) and highly connected river network systems (Fan et al., 2022), which increase the exposure of amphibians to environmental microbes (especially water microbes) and could further increase dispersal rates. However, this may also introduce more new microbial taxa, thereby increasing the speciation, diversification, priority effects and colonization of new species, which may increase stochasticity (such as drift or homogeneous dispersal) (Vellend, 2010; Zhou and Ning, 2017).

In this study, stochastic features were also indicated by the random combination of occasional unique or rare microbial taxa at the OTU level in different locations and host individuals in urban samples, which is indicated by the phylum-and family-level compositional analyses of OTUs and the network analyses (Figures 4A, 6A,B). These microbial taxa increased the node number in the networks in urban areas, but they had few edges connecting other taxa in the networks (Figures 6A,B). Furthermore, although the average degree and graph density representing the network complexity over the urbanization gradient were markedly reduced, the network modularity, representing functional units (Luo et al., 2006) or niches (Eiler et al., 2012), and keystone taxa numbers exhibited an increasing trend (Figure 6C). This suggests that although urbanization reduces the complexity of the network, it increases the functional units to cope with various risks or pressures brought by the urban environment.

Urbanization can significantly change the ecological stability and complexity of environmental microorganisms (Yuan et al., 2021), but the impact on the ecological stability of symbiotic microorganisms has not been studied, which may be because it is a more complex triangular relationship involving the host, symbiotic microorganisms and environment. Here, we examine the network stability and complexity of a symbiotic community by calculating the robustness, vulnerability and topological properties. We found that urbanization reduces the network complexity and ecological stability, which may be one of the reasons for the increased randomness, since the network structure of microorganisms generally represents a nonrandom association (Barberan et al., 2012).

The key is that urban ecosystems are always disturbed and manipulated by human activities and pollution (Zhai et al., 2020; Yang, Y. et al., 2022). For the host species, they are completely different from wild habitats. For example, water bodies in city rivers, ditches or ponds are usually polluted by sewage emissions. Moreover, they are typically managed by local administrative units for the purpose of improving water quality. As such, microhabitat conditions are easily disturbed or altered because of human interference. Two typical pieces of evidence are the remarkable abiotic factor differences in water bodies and air features of urban settings: PH and AT showed significant correlations with symbiotic microbial composition and network topological properties. Additionally, we found that bio_18 and bio_3 also presented remarkable correlations. In summary, these results suggest the important roles of current and historical environmental factors in driving the dissimilarity of symbiotic communities between urban and wild settings.

More importantly, we quantified the degree of urbanization and revealed that it is significantly positively correlated with the stochasticity and vulnerability and significantly negatively correlated with the complexity and robustness. At the same time, we verified that the stochasticity has a significant negative correlation with the ecological stability, which is an interesting and important result. Consequently, our findings provide valuable and new insight into understanding the complex triangular relationship involving the host, environment, and symbiotic microbes.

It must be noted that there are some uncertainties (discussed in Supplementary Text S1 and Figure S5) in the analysis. For future research, it would be interesting to explore the impacts

of urbanization on the host species community structure and how the impacts affect symbiotic microbiota. We argue that such analyses would clarify the triangular relationship between hosts, microbes and abiotic environments. In addition, it is important to test the transmission processes of microbial communities hosted within species at various trophic levels between food webs, which may have potential excavation value for understanding interspecific relationships in the context of global change. Furthermore, rare taxa play a pivotal role in regulating the community assembly and ecological stability of environmental microorganisms (Liang et al., 2020; Shu et al., 2021), but their specific regulatory mechanisms in amphibian symbiotic microbes along an urbanization gradient have not been quantitatively and deeply studied, especially the functional mechanisms and assembly processes of each taxon.

Data availability statement

The data presented in the study are deposited in the National Center of Biotechnology Information (NCBI: https://www.ncbi.nlm.nih.gov/) repository, accession number PRJNA889361.

Ethics statement

The animal study was reviewed and approved by the Animal Ethical and Welfare Committee of Chengdu Institute of Biology, Chinese Academy of Sciences.

Author contributions

JZ analyzed the data, wrote the manuscript, performed the research, investigation, and methodology. ZiL wrote the manuscript. ZhL, XG, and WZ: investigation. YC: wrote the manuscript, methodology, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Different living environments drive deterministic microbial community assemblages in the gut of Alpine musk deer (*Moschus chrysogaster*)

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Substantial variation in the environment directly causes remodeling of the colonized gut microbiota, controlling community diversity, and functions in the host to tune-up their adaptive states. However, the mechanisms of microbial community assembly in response to environmental changes remain unclear, especially in endangered ruminants. In this study, we analyzed the microbial communities of 37 fecal samples collected from captive and wild Alpine musk deer (*Moschus chrysogaster*) to characterize the complexity and assembly processes using 16S rRNA gene sequencing. We found significantly different diversities and compositions of gut microbiota among both groups associated with different living environments. Heterogeneous selection was the predominant factor regulating the gut microbiota community under similar climatic conditions, indicating that microbial community assembly was largely driven by deterministic mechanisms. The species co-occurrence network showed complex and tight connections with a higher positive correlation in the wild environment. Moreover, the captive group exhibited significant differences in chemoheterotrophy and fermentation compared with the wild group, but the opposite was observed in animal parasites or symbionts, which might be closely related to diet, energy supply, and healthcare of animals. This study provides a framework basis and new insights into understanding gut microbiota in different environments.

KEYWORDS

ruminants, microbial diversity, assembly mechanism, environmental variation, function

1. Introduction

Microbial communities that colonize the gastrointestinal tract at birth are critical in maintaining the health of the host (Ben Shabat et al., 2016; Zeevi et al., 2019; Deng et al., 2022) and can be shaped with diet (David et al., 2014; Schnorr et al., 2014; Friedman et al., 2017), phylogenic development (Turnbaugh et al., 2009; Jiang et al., 2021), and environment modifications (Wang et al., 2019; Zhang et al., 2022). Changing and shaping of gut microbiota are particularly apparent in captive and translocated populations (Li et al., 2017; Sun et al., 2020), indicating the manifestation of co-evolution and adaptation to the external environment between a host and its microbial communities. This process provides an optimal opportunity to understand microbial diversity, assembly patterns, and complex connection(s) to function.

Understanding the microbial community assembly mechanism is a key topic in microbial ecology and is an objective method that can facilitate obtaining health data and preventing potential disease in animals (Nemergut et al., 2013). It is commonly debated that deterministic processes, including biotic (interspecies interactions such as competition, predation, and mutualism) and abiotic factors (environmental filtering such as pH, temperature, and salinity), and stochastic processes (e.g., dispersal, immigration, speciation, and drift) are affected by the assembly of microbial communities (Chesson, 2000; Fargione et al., 2003; Chave, 2004; Wang et al., 2013; Zhou and Ning, 2017). Previous studies on microbial community assembly have predominantly focused on environment-related factors (Jiao et al., 2019; Chen et al., 2022) and hydrobionts (Kokou et al., 2019; Mo et al., 2021; Liu et al., 2022), and there has been a lack of studies in mammals, especially rare and endangered species.

Musk deer (*Moschus* spp.), a small and timid ruminant, is widely known for its musk secreted by males (Meng et al., 2011). For this reason, this population has historically suffered severe disasters (poaching and illegal trade), resulting in a dramatic decline in the population size, which has remained at a low level. Alpine musk deer (*Moschus chrysogaster*), the largest musk deer in terms of body size, lives in a narrow area bordering Bhutan, India, Nepal, and China (Harris, 2016). In addition, local isolated populations of Alpine musk deer exist in the Helan Mountains, separated by the Yellow River, the desert, and the city, on the edge of the Qinghai–Tibet Plateau in China; however their population sizes are not promising. Moreover, the population is far less competitive than other sympatric ruminants [e.g., blue sheep (*Pseudois nayaur alashanicus*) and red deer (*Cervus elaphus alashanicus*)] and is very sensitive to disturbances. Alpine musk deer is listed as an endangered species on the Red List by the International Union for Conservation of Nature (IUCN) and is a national key protected wildlife of class I in China (Jiang et al., 2020). Small populations of Alpine musk deer are present in captivity in China. Musk deer are susceptible to gastrointestinal diseases in captivity owing to frequent changes in diet, unclean water, transmission of germs carried by keepers, changeable weather, and so on. However, limited food with disparate sources is the major factor between captive and wild musk deer that directly affects and shapes the gut microbiota associated with relevant functions to adapt to change (Li et al., 2017; Sun et al., 2020). Clarification of the assembly processes of the microbial community of musk deer under different feeding conditions is needed to facilitate population conservation and restoration.

In our previous study investigating the gut microbiota of captive (C) and wild Alpine musk deer (W), different microbiota were enriched (C: Firmicutes; W: Proteobacteria, and Euryarchaeota) to link with different functions and metabolic pathways (C: methanogenesis; W: digesting cellulose, and generating short-chain fatty acids) (Sun et al., 2020). However, gut microbiota assembly processes related to the aforementioned differences are still unclear. In this study, metadata of gut microbiota were collected, and null modeling and network analysis were conducted to estimate the relative importance of ecological processes and reveal complex connections and functional predictions between captive and wild Alpine musk deer. The objectives of the study were to (a) compare the diversity and composition of gut microbiota of C and W groups, (b) elucidate the mechanisms of microbial community assembly processes in this context, and (c) analyze the interaction(s) among indicators and predict potential functions. This study provides a framework basis and new insights into understanding gut microbiota in different environments.

2. Material and methods

2.1. Sample collection and sequencing

In total, 14 fecal samples were collected from the Xinglongshan Alpine musk deer farm in Lanzhou city, Gansu Province (104°6′E, 35°48′N). Prior to the collection of the samples, the animals were healthy, no diseases were observed, and they had been domesticated and bred for over 30 years. A total of 23 fecal samples were collected from wild Alpine musk deer at the Helanshan National Nature Reserve in the Inner Mongolia Autonomous Region, China (105°48′-57′E, 38°39′-51′N). All samples were collected around February 2015 as they were less susceptible to deterioration at the low temperatures of this time of year. All feces were pellet-like without diarrhea and were immediately stored at −80°C. Total DNA from fecal samples was extracted using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. DNA was diluted to 1 ng/μL. The V3–V4 hypervariable region of the 16S rRNA gene was amplified with specific primers (515F: 5′-GTGCCAGCMGCCGCGGTAA-3′; 806R: 5′-GACTACHVGGGTWTCTAAT-3′). The PCR system contained 1.5 μL of DNA template, 10 μL of Phusion High-Fidelity buffer (5×), 0.5 μL of Phusion DNA polymerase, 1 μL (10 mM) of dNTPs, 0.5 μM of forward and reverse primers, and ddH_2O of 50 μL. The PCR conditions comprised an initial denaturation at 98°C for 30 s, denaturation at 98°C for 5–10 s, annealing at 55°C for 30 s, 35 cycles of elongation at 72°C for 3 min, and a final elongation at 72°C for 20 min. Next, 16S rRNA libraries were constructed using a TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, United States) and sequenced on an Illumina HiSeq platform (HiSeq 2500, PE250). Sequence data were mainly analyzed on the QIIME platform (v1.9.1), and chimeras were filtered and removed by using FLASH (v1.2.11). Sequences with an identity threshold of 0.97 were defined as the same operational taxonomic units (OTUs) using UPARSE (v7.0.1090). Taxonomy of the OTUs was performed by annotation using Ribosomal Database Project (RDP) (v11.5) against the Silva 138 16S rRNA database at a confidence threshold of 70%.

2.2. Environment-related microbial community structure analysis

Alpha diversity (including the Sobs, Ace, Simpson, Chao1, and Smith–Wilson indices) was calculated by mothur (v1.30.2). Non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarities was performed for each microbiome by calculating compositional similarity and mapping using the online Majorbio Cloud platform.[1]

Partial least squares–discriminant analysis (PLS-DA), a supervised discriminant analysis method, was conducted to cluster microbial data based on their sources (Metwaly et al., 2020). To assess the relative importance of deterministic and stochastic processes

1 https://cloud.majorbio.com/page/tools/

on microbial community assembly, the null model analysis was first applied, and 999 randomizations were used to generate model expectations. The β-nearest taxon index (βNTI) and the Bray–Curtis-based Raup–Crick (RCBray) index were used to measure the variation in both phylogenetic and taxonomic diversities (Stegen et al., 2013). Then, Levins' niche width index was applied to estimate the patterns of determinism and stochasticity and their influence on microbial communities (Levins, 1968; Finn et al., 2020). Meanwhile, according to the community structure distribution of samples among groups, source tracking, which predicts the composition proportion, was performed based on Bayesian algorithms (Knights et al., 2011).

Random forest, a tree-based machine-learning model, was applied to examine the clustering of each sample and calculate variable importance using mean decrease accuracy (Boulesteix et al., 2012; Pawlik and Harrison, 2022; Zhao et al., 2022). The area under the curve (AUC) was used to evaluate the accuracy of the model, and data were normalized using Z-score. To identify the characteristics and categories of the gut microbiota between all groups, the Indval function in the "labdsv" R package was used to seek indicators showing significant differences (Ren et al., 2022). All indicators with a significant difference were classified into three levels based on the relative abundance, with thresholds of 0.75 (high), 0.50 (medium), and 0.25 (low), and indicators with a low relative abundance that cannot be ignored were defined as rare species. A network analysis based on Spearman's correlations ($|r| > 0.5$, $p < 0.05$) was performed to fully describe the covariation between captive and wild groups. The top 100 abundant OTUs, which are considered to play important roles and show complex positive and negative interactions in the microbial community, were identified based on the topological roles of their module-based co-occurrence networks (Deng et al., 2012).

To predict the differences in microbial phenotypic information between the two groups, the Wilcoxon rank-sum test was conducted on the BugBase cloud platform[2] (Zhang et al., 2019). Functional annotation of prokaryotic taxa (FAPROTAX) was used to predict microbial functions. FAPROTAX predictions were based on the normalized contig-based 16S rRNA OTU table annotated in the database of prokaryotic environmental functions (Louca et al., 2016).

2.3. Statistical analysis

All reported values are expressed as mean ± standard deviation (SD). Differences in microbial diversity, niche width, phenotype, and predicted function between groups were analyzed by using the Wilcoxon rank-sum test and visualized using Origin (v2023). Annotations with a corresponding sign in the text were considered statistically significant.

3. Results

3.1. Gut microbiota of Alpine musk deer are environment-specific

Environment specificity of Alpine musk deer microbes was assessed by comparing the similarities of microbial communities of hosts in captivity ($n = 14$) and those in the wild ($n = 23$). Alpha diversity (Sobs, Ace, Simpson, Chao1, and Smith–Wilson indices) and beta diversity were used to evaluate the structure of gut microbiota in captive (C) and wild (W) Alpine musk deer. The Sobs Index values in C and W Alpine musk deer were 1,217.20 ± 102.18 and 1,063.20 ± 127.24, respectively (Figure 1A); the Ace Index values in the C and W groups were 1,519.00 ±142.59 and 1,303.40 ±121.43, respectively (Figure 1B); the Simpson Index values in the C and W groups were 0.017 ± 0.003 and 0.025 ± 0.011, respectively (Figure 1C); the Smith–Wilson values in the C and W groups were 0.473 ± 0.008 and 0.459 ± 0.033, respectively (Figure 1D); and the Chao1 Index values in the C and W groups were 1,531.40 ±142.59 and 1,311.50 ± 123.33, respectively (Supplementary Figure 1). All of these indices were significantly higher in the captive Alpine musk deer group than in the wild group ($p < 0.01$), which indicated that there was a significant difference associated with the living environments of Alpine musk deer. Furthermore, NMDS on OTU level based on Bray–Curtis dissimilarities revealed a clear cluster of the gut microbial communities from the captive group and the wild group, respectively (Supplementary Figure 2A), and a cluster between females (C–F) and males (C–M) in the captive group (Supplementary Figure 2B).

3.2. Microbial community assembly processes are relatively deterministic

The PLS-DA score plot on the OTU level further showed that samples clustered together according to different living environments and confirmed the gender-related clustering phenomenon in captive Alpine musk deer by applying this supervised cluster analysis method (Figures 2A, B). The null model analysis also showed a higher relative contribution of deterministic processes ($|\beta NTI| \geq 2$) in Alpine musk deer gut microbiome assembly was largely affected by different living environments and sexes (Figures 2C, D). Furthermore, the niche width calculated based on the OTU level was estimated to display the contributions of species classification and dispersal limitation to microbial community construction. The niche width values in the C and W groups were 55.40 ± 15.17 and 54.20 ± 35.43, respectively, and no significant difference was observed between the two groups ($p > 0.05$, Figure 3A), while there was a significant difference in the niche width between C–F (74.29 ± 14.84) and C–M groups (58.30 ± 10.79; $p < 0.05$; Figure 3B). Moreover, 77% of the gut microbiome of the C group was homologous to that of the W group, as found using SourceTracker analysis. For the C–F and C–M groups, the values of shared flora were as high as 96% (Figures 2E, F).

3.3. Indicators and species (OTU) interactions

The samples exhibited significant groupings based on the random forest at OTU and genus levels, indicating habitat specificity between both groups (Figures 4A, C). The top 30 taxa of variable importance from random forest-based prediction at OTU and genus levels are shown in Figures 4B, D. Mean decrease accuracy was used as a measure of variable importance. OTU1350, OTU1012, and OTU1388 exhibited the top three taxa of variable importance in the

2 http://bugbase.cs.umn.edu

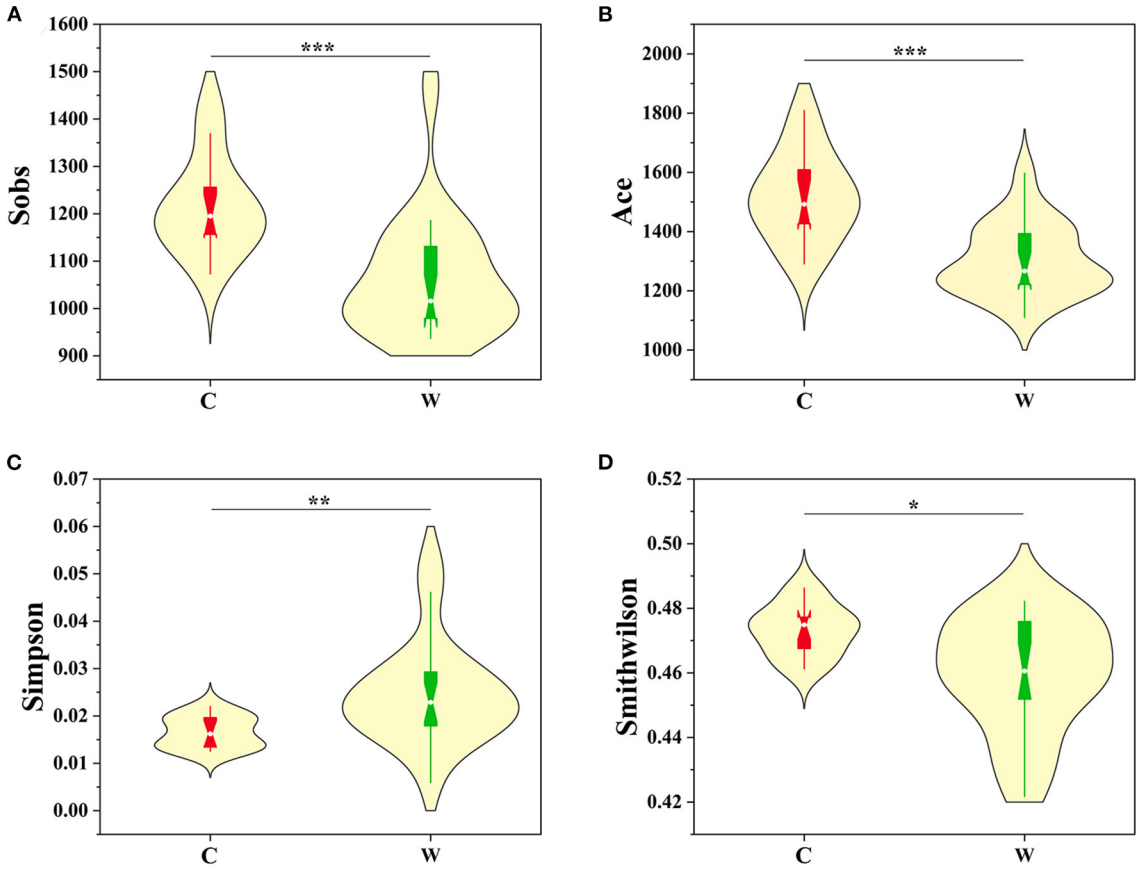

FIGURE 1
Comparison of alpha diversity indices [**(A)** Sobs, **(B)** Ace, **(C)** Simpson, and **(D)** Smithwilson] of gut microbiota of the captive (C) and wild (W) Alpine musk deer (significance *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

random forest model, and these belonged to the phyla Firmicutes and Proteobacteria, to which the top three families also belong (Supplementary Figure 3). However, the top three genera in the ranking are from the phyla Firmicutes and Bacteroidetes.

To identify the characteristics and categories of the gut microbiota between all groups, the R package function Indval was used to seek indicators showing significant differences ($p < 0.05$). Here, indicator species (OTUs) can signal changes in the gut microbiota of captive and wild Alpine musk deer and, therefore, can serve as a proxy to characterize these changes. There were 166 indicators with higher richness (threshold value: 0.75–1.0) in the C group, mainly belonging to Firmicutes (31.93%), Bacteroidetes (18.67%), Proteobacteria (27.11%), Actinobacteriota (7.23%), and Verrucomicrobiota (4.22%). However, there were only 53 indicators in the W group, which were classified as Firmicutes (60.38%), Actinobacteriota (18.87%), Proteobacteria (13.21%), Desulfobacterota (3.77%), and Bacteroidetes (3.77%) (Supplementary Table 1). The C and W groups shared the same category and number (33) of indicators with a medium abundance (threshold value: 0.25–0.75) in significant differences (Supplementary Table 2). By contrast, the rare species indicators with a low relative abundance (threshold value: 0–0.25) exhibited extreme differences between the W and C groups, and no indicators were shared by the two groups. The number of rare species indicators (indicators with a significant difference and low relative abundance) in the W group (69) was much higher than that in the C group (43) and mostly belonged to the phyla Firmicutes and Bacteroidetes (Supplementary Table 3). For the captive sex group, the unique indicators in the C–F group contained *Gordonibacter*, norank_f_norank_o_Microtrichales, ynechococcus_CC9902, Lachnospiraceae_UCG-008, and *Acidibacter*, which belong to the phyla Actinobacteriota, Firmicutes, and Proteobacteria (Supplementary Figure 4).

OTU-level co-occurrences were also represented by Spearman's correlations ($|r| > 0.5$, $p < 0.05$), and the networks among species (OTUs) only incorporated taxa under different environments and sexes. The networks in the W group contained 858 edges with positive and negative correlation proportions of 75.29 and 24.71%, respectively, which was considerably more complex than the 624 edges in the C group with positive and negative correlation proportions of 53.85 and 46.15%, respectively (Figures 5A, B, Supplementary Table 4). Modules V and VI were lacking in the W group (modularity = 0.326), and hub OTUs (top 10) with higher connectivity were predominantly scattered in module I, while it is module IV in group C (modularity = 0.327). All hub OTUs (top 10) between C and W groups are in the phylum Firmicutes. The layout of the functional modules varies between sexes in the captive group. Hub OTUs (top 10) are reflected in modules III and IV, respectively (Figures 5C, D). Most hub OTUs in both groups (C–F and C–M) belonged to Firmicutes, but OTU445 (in the C–F group),

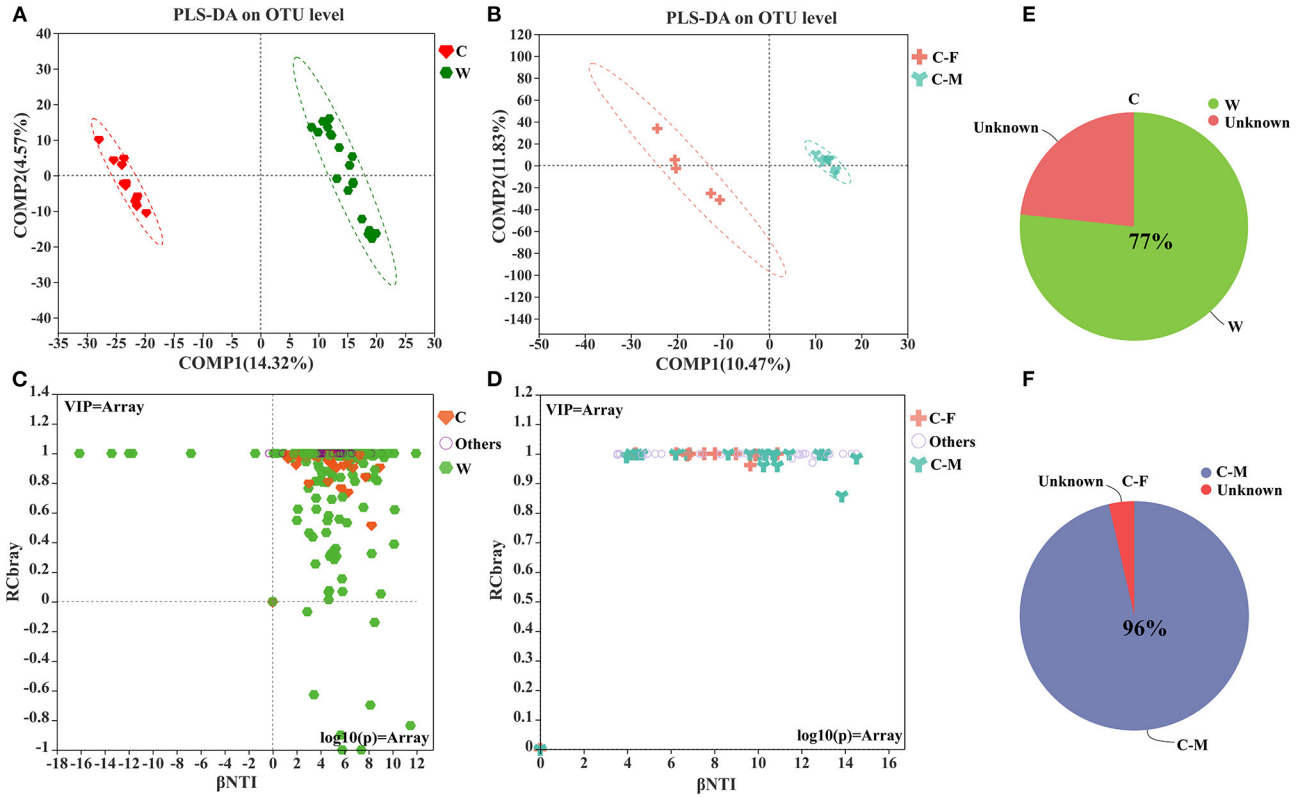

FIGURE 2
(A, B) Partial least squares-discriminant analysis (PLS-DA) of microbial communities for each group; **(C, D)** Microbial community assembly or community structure by βNTI/RCbray; **(E, F)** Pie charts of the mean SourceTracker proportion estimates for 100 draws from Gibbs sampling in captive vs. wild groups and captivity between female and male groups (W: wild, C: captive, C-F: captive female, and C-M: captive male).

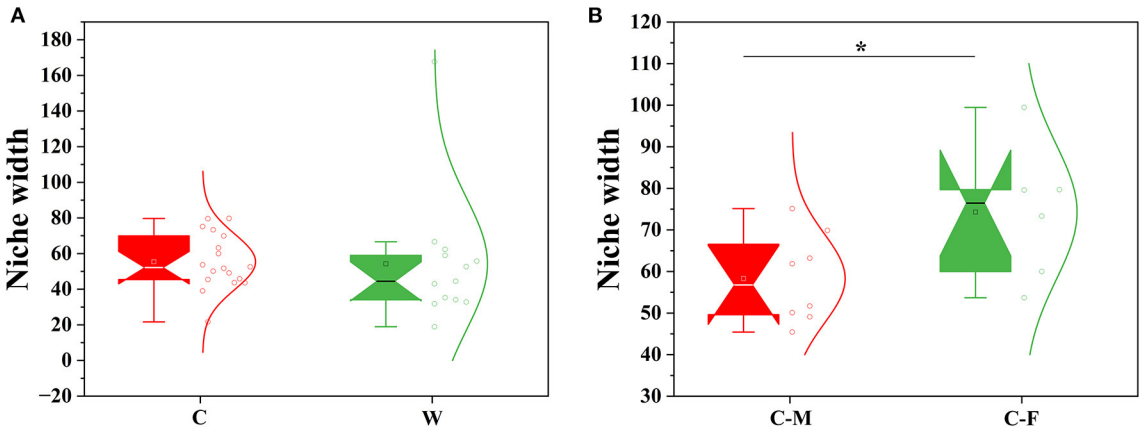

FIGURE 3
Comparison of mean habitat niche width for all groups. **(A)** Wild vs. captive groups. **(B)** Captive female vs. captive male groups (W: wild, C: captive, C-F: captive female, and C-M: captive male; Significance *$p < 0.5$).

OTU1537, and OTU579 (in the C–M group) were derived from the phylum Bacteroidetes. Meanwhile, hub OTUs 1445 (in the C–F group) and 579 and 555 (in the C–M group) belong to Alistipes, Rikenellaceae_RC9_gut_group, and Atopostipes, respectively, which is consistent with the results obtained using random forest. Some of the top 30 OTUs were derived from random forest results, which were distributed in different modules, and exhibited a certain network relationship (Figure 5).

3.4. Functional prediction of gut microbiota

A Wilcoxon rank-sum test on the phenotype in both groups revealed a significant difference between proportions based on BugBase (Figure 6). The phenotypes with a relative contribution of more than 10% were stress-tolerant, Gram-positive, anaerobic, potentially pathogenic, and contain mobile elements, thus playing an important role in the adaptation of the microbial community to their

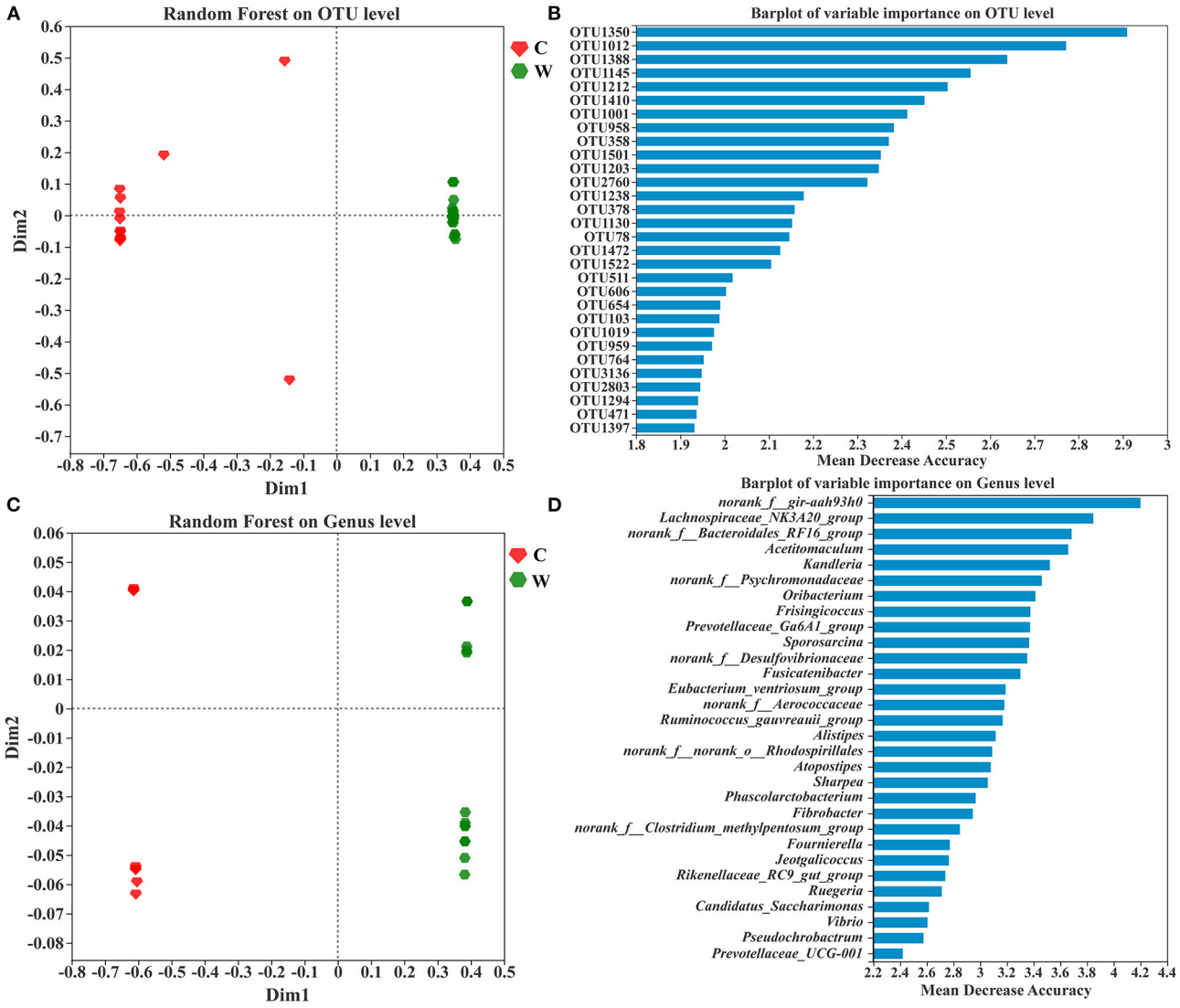

FIGURE 4
Random forest analysis of sample clustering at OTU **(A)** and genus levels **(C)**, and the top 30 OTUs **(B)** and genus **(D)** with variable importance based on their mean decrease in accuracy.

respective environmental state. Stress tolerance was dominant in both groups, and the W group had a higher relative contribution from the families Lachnospiraceae, Oscillospiraceae, and UCG-010 belonging to the phylum Firmicutes, whereas the family Rikenellaceae in the phylum Bacteroidetes had a higher relative contribution of this phenotype in the C group (Supplementary Figure 5).

FAPROTAX and the Wilcoxon rank-sum test were employed to predict the functions of the identified microbial community. As shown in Figure 7A, a relatively higher proportion of chemoheterotrophy and fermentation was observed in the C group than in the W group, which might relate to the diet and energy supply of the deer. In addition, the functions with a relatively higher proportion and significant difference in the W group compared with the C group were animal parasites or symbionts, mammal gut, and human gut (Table 1). This indirectly reflects the fact that captive animals experience less stress and disease encounters owing to proactive health testing by managers, while wild animals are more resistant to disease. Moreover, in this study, no significant differences in enterotype and ascending functions were observed between captive male and female Alpine musk deer (Figure 7B).

4. Discussion

Gut microbiota, an important "acquired organism," have a marked impact on the survival and fitness of the host and are involved in many important physiological processes. The survival status of animals under various conditions is easily obtained without harm to the animals through feces sampling and 16S rRNA high-throughput sequencing (Aguirre and Venema, 2015; Rounge et al., 2018; Antwis et al., 2019). Therefore, this approach is widely applied to maintain and restore rare and endangered wildlife populations (Wang et al., 2016; Thitaram and Brown, 2018; Ning et al., 2020). In this study, a series of statistical tests and machine learning approaches were employed to obtain new data on the gut microbiota of captive and

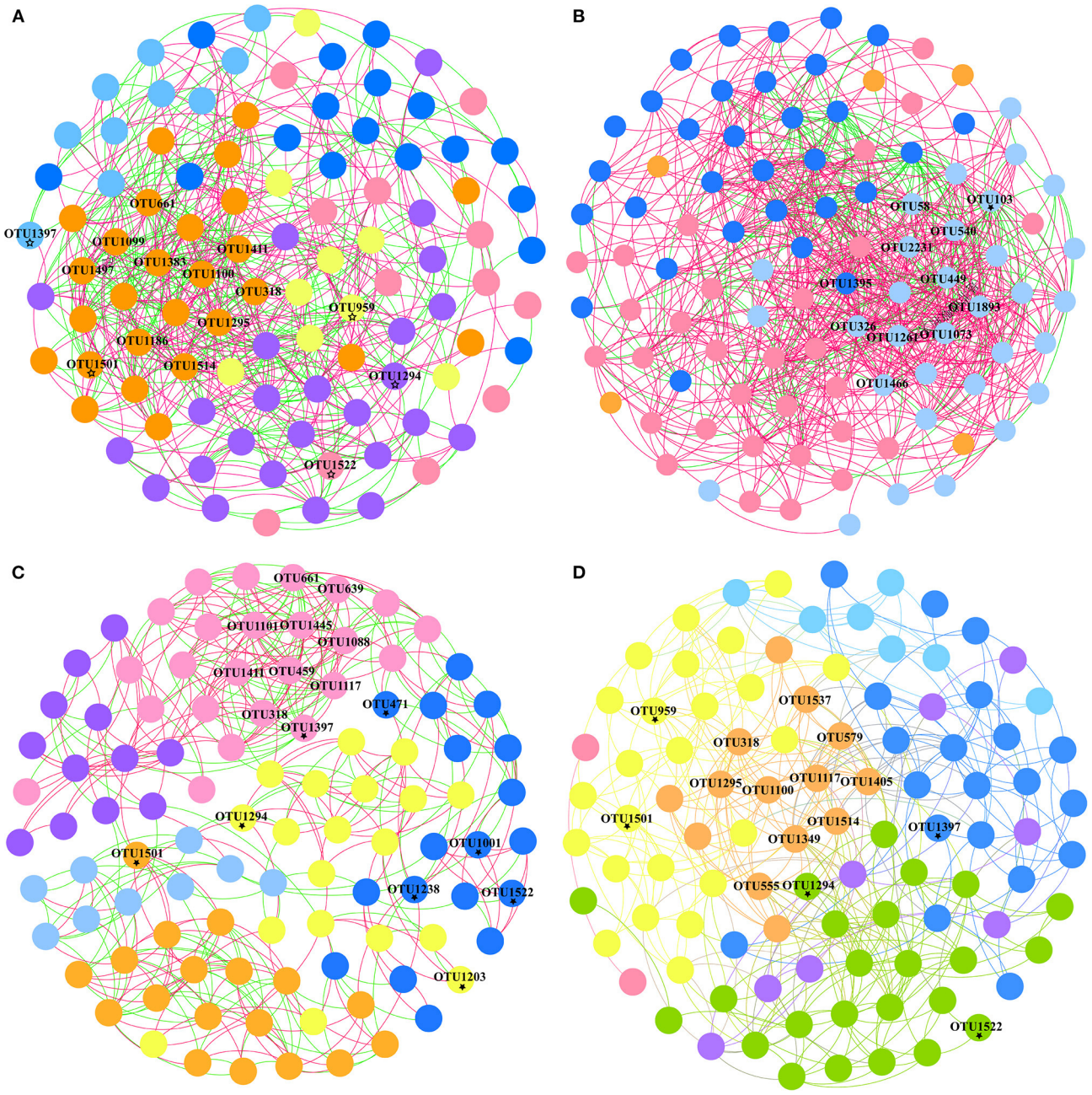

FIGURE 5
Network modules and connectivity of OTUs in different groups. Co-occurrence networks of OTUs present different modules in captive **(A)**, wild **(B)**, captive female **(C)**, and captive male groups **(D)** with positive and negative correlations (top 30 OTUs derived from random forest results indicated by ⋆).

wild Alpine musk deer based on fecal samples. Alpine musk deer of captive populations were found to have higher gut microbial diversity (alpha and beta diversities) than wild populations, suggesting that captive populations have complex and stable gut microbiota and have adapted well to local habitats. These results are different from those of a previous study on gut microbiota between captive and wild forest musk deer, where there was only a significant difference in beta diversity and no significant difference in alpha diversity (Li et al., 2017). Various factors affect the gut microbiota in mammals, including diet, which is the main and most important source of energy and nutrition for both the host and the gut microbiota (Ley et al., 2008; Wu et al., 2011; Navarrete et al., 2012; Hasebe et al., 2022). Numerous studies have shown decreasing community similarity with increasing differences between habitats and species (Liu et al., 2021; Barrionuevo et al., 2022; Yang et al., 2022), and that animal sex is also a predominant factor affecting the gut microbiota (Zhang et al., 2022). Although the direct analysis method did not reflect the differences in gut microbiota by sex (Supplementary Figure 2B), significant differences were revealed by using a supervised analysis method (PLS-DA) (Figure 2). In general, captive animals are limited by the freedom of living space and food, but their food sources are adequate and balanced. In this study, both local isolated populations (captive and wild populations) survive in the same latitudinal zone and have similar climatic conditions. The diet of the captive Alpine

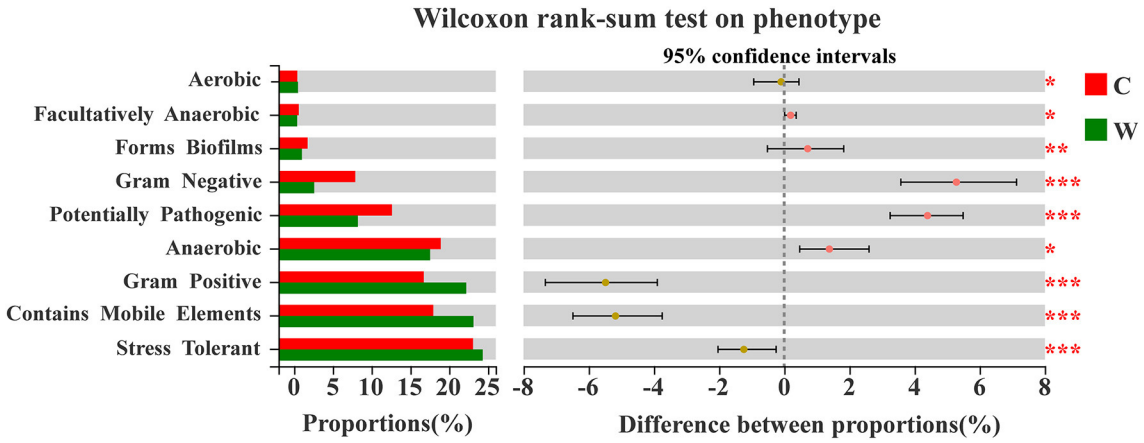

FIGURE 6
Enterotype by Wilcoxon rank-sum test based on Bug Base in both groups (W: wild, C: captive; significance *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

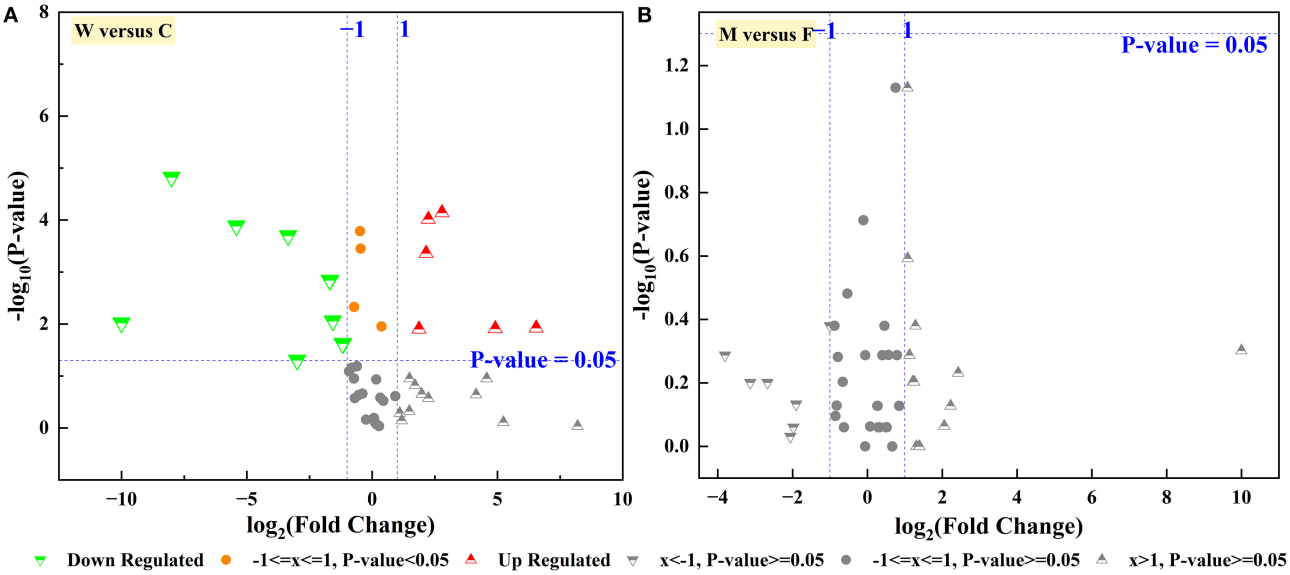

FIGURE 7
Volcano plot gut microbiota function of Alpine mush deer. **(A)** Captive group (C) vs. wild group (W), **(B)** female group (F) vs. male group (M) in captivity.

musk deer mainly consisted of fresh leaves combined with foods having high protein and carbohydrate contents, while the wild Alpine musk deer predominantly fed on wild plant leaves and fungi with high crude fiber and ether extract. Thus, differences in gut microbiota between captive and wild Alpine musk deer might be related to the difference in the environment and access to food.

Community assembly mechanisms—an important ecological aspect driving forces that shape the community—are determined by a combination of deterministic and stochastic ecological processes (Stegen et al., 2012; Dini-Andreote et al., 2015). Our results showed that the assembly processes of gut microbial communities in captive and wild Alpine musk deer were mostly deterministic. Heterogeneous selection ($\beta NTI \geq +2$) was the predominant deterministic process, which contains ecological selection by abiotic environmental factors (environmental filtering) and mutual antagonism and synergism between species (Vellend, 2010). For captive and wild Alpine musk deer, significant differences in foods result in different habitat preferences and adaptations of gut microbiota under similar climatic conditions, contributing to this assembly process, which can be explained through a weak influent immigration influence and strong environmental filtering. Sex also influences the community assembly process, which is consistent with the effect of sex on gut microbial diversity. Network analysis and co-occurrence patterns revealed strong and complex species (OTUs) interactions and indicators (Toju et al., 2016), highlighting the importance of interspecific interactions in the community assembly process. The indicators with higher richness in both groups predominantly belong to the phylum Firmicutes, which is commonly found in the gut microbiota in ruminants (Gruninger et al., 2014; Ishaq and Wright, 2014; Li et al., 2017). Indicators with low richness in both groups also demonstrated significant differences, and their low abundance but large numbers, which were mostly derived from the phyla Firmicutes

TABLE 1 Predicted composition of functional groups by FAPROTAX and Wilcoxon rank-sum test in captive (C) and wild (W) groups.

Functional groups	Log_2 (fold change)	*p*-value	Significance
Reductive acetogenesis	−8.0046	1.500×10^{-5}	Down
Mammal gut	2.7863	7.220×10^{-5}	Up
Human gut	2.7863	7.220×10^{-5}	Up
Animal parasites or symbionts	2.2436	9.640×10^{-5}	Up
Manganese oxidation	−5.4184	1.289×10^{-4}	Down
Chemoheterotrophy	−0.4792	1.630×10^{-4}	Down
Cellulolysis	−3.3564	1.998×10^{-4}	Down
Fermentation	−0.4614	3.545×10^{-4}	Down
Dark thiosulfate oxidation	2.1542	4.359×10^{-4}	Up
Nitrite denitrification	−1.6761	1.434×10^{-3}	Down
Nitrous oxide denitrification	−1.6761	1.434×10^{-3}	Down
Nitrate denitrification	−1.6761	1.434×10^{-3}	Down
Denitrification	−1.6929	1.434×10^{-3}	Down
Aerobic chemoheterotrophy	−0.7193	4.693×10^{-3}	Down
Iron respiration	−1.5674	8.723×10^{-3}	Down
Manganese respiration	−10	9.497×10^{-3}	Down
Intracellular parasites	0.3716	1.113×10^{-2}	Up
Human pathogens meningitis	6.5452	1.198×10^{-2}	Up
Ligninolysis	4.9141	1.227×10^{-2}	Up
Human pathogens all	1.8693	1.254×10^{-2}	Up
Nitrate respiration	−1.1565	2.367×10^{-2}	Down
Hydrocarbon degradation	−1.1811	2.367×10^{-2}	Down
Thiosulfate respiration	−2.9937	4.940×10^{-2}	Down

and Bacteroidetes, illustrates their subtle role in the host gut. Bacteroidetes are another component of the gut microbiota present in ruminants (Sundset et al., 2007; Gruninger et al., 2014; Li et al., 2017).

Although the correlation network of the wild Alpine musk deer had two fewer functional modules than the captive group at the OTU level, it showed a stronger positive and negative correlation to evaluate intestinal health. The hub OTUs (top 10) with higher connectivity are presented inside each group. For instance, in the captive group, OTU661 and OTU1295 belonged to the family Lachnospiraceae, which participates in fermentation to produce acetic acid and butyric acid, the main source of energy for the host, and aids in the prevention of colon cancer (Dahiya et al., 2019). OTUs 1411, 1100, 1186, and 1514 were from the family Eubacterium_coprostanoligenes_group, which has beneficial effects on dyslipidemia (Wei et al., 2021). OTU1497 belonged to the family Christensenellaceae, the members of which are associated with immune regulation and healthy homeostasis and are widely found in human and animal intestines and mucous membranes (Kong et al., 2016). For the wild group, OTUs 2231, 1073, and 1466 were from the family Lachnospiraceae, while OTU540 belonged to the family Eubacterium_coprostanoligenes_group. UCG-010 is a family shared between the captive (OTU1383) and wild (OTUs 58, 1893, 1262, and 326) groups. However, all hub OTUs in both groups belonged to the phylum Firmicutes, which can degrade cellulose into volatile fatty acids for the host to use. The network analysis showed that both populations of Alpine musk deer had relatively good health, and although the intestinal microbes of the deer differed in different living environments, their complex relationships indicated participation in the energy supply process of the host. The correlation network of the captive male Alpine musk deer had one more functional module than that of the female group, but the correlations within the groups were essentially similar, and all hub OTUs belonged to the phyla Firmicutes and Bacteroidetes. Bacteroidetes also degrade carbohydrates and proteins and are vital for improving host immunity and maintaining the balance of gut microbiota (Hooper et al., 2001; Bäckhed et al., 2004; Hooper, 2004; Sears, 2005).

Functional predictions in our study were performed in parallel using BugBase and FAPROTAX, which have powerful algorithms and novel databases (Louca et al., 2016; Zhang et al., 2019). The phenotypes of stress tolerance and contains mobile elements were two relatively important players, and the wild group was significantly higher than the captive group. Meanwhile, the microbial families between these two phenotypes and network interactives described earlier were consistent. We speculate that Alpine musk deer require more diverse intestinal phenotypes to aid or supplement metabolic needs in the extreme survival environment in the wild. Owing to concentrated captive breeding, captive populations also exhibit a significantly higher proportion of phenotypes of potentially pathogenic bacteria, despite health management. The relatively higher abundance of the function of animal parasites or symbionts in the wild population may favor host gut homeostasis and response to environmental changes. It was previously reported that the energy intake capacity of a wild group was better than that of a captive group, referring to a higher Firmicutes/Bacteroidetes (F/B) ratio that may be associated with the increased energy harvest from colonic fermentation (Sun et al., 2020). However, function prediction of the gut microbiota showed that fermentation of the captive group was relatively higher than that of the wild group in our study, which does not indicate a contradiction with our previous findings. The function of gut microbiota in the wild group may be more tilted or involved in other aspects such as the human gut and mammal gut.

5. Conclusion

The results obtained in this study enhance the understanding of microbial ecology in ruminants derived from captive and wild environments. This study was focused on the gut microbial community assembly process of Alpine musk deer in different environments, with the aim of revealing the state of adaptation under the presence of direct environmental changes. There were significant differences in gut microbial diversity and structure among both groups, and the microbial community assembly was largely driven by deterministic mechanisms. The heterogeneous selection was the main deterministic process, which might be closely related to food obtained under similar climatic conditions. Although the relative abundance

of indicators showed significant differences at each level, their origin was ultimately attributed to the phyla Firmicutes, Bacteroidetes, and Proteobacteria. There was a higher positive correlation in the wild group, and the captive group showed significant differences in the function of chemoheterotrophy and fermentation compared with the wild group, but the opposite was observed for the functions of animal parasites or symbionts, which might be linked to the diet, energy supply, and healthcare of the animals. Our study also suggested sex was an important factor in the deterministic process of microbial community assembly, but the quantitative and correlated relationships between influential factors (e.g., environment, food, and sex) and gut microbiota warrant further investigation in future.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found at: NCBI/BioProject/PRJNA380002 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA380002).

Ethics statement

The animal study was reviewed and approved by the Board of Ethical Committee for Experimental Animals, Northeast Forestry University.

Author contributions

ZZ: conceptualization, methodology, visualization, sample collection, supervision, and writing the original draft. MD: conceptualization, methodology, visualization, and data curation. YS: investigation and sample collection. RHK: investigation and writing, reviewing, and editing. JC: visualization. LT: supervision, investigation, funding acquisition, writing, reviewing, and editing. ZL: conceptualization, supervision, investigation, and funding acquisition. All authors contributed to the manuscript and approved the submission.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Does diet or macronutrients intake drive the structure and function of gut microbiota?

Yuhang Li[1], Yujie Yan[1], Hengguang Fu[1], Shiyu Jin[1], Shujun He[1], Zi Wang[1], Guixin Dong[2,3], Baoguo Li[1,4] and Songtao Guo[1,5]*

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Shift of ingestive behavior is an important strategy for animals to adapt to change of the environment. We knew that shifts in animal dietary habits lead to changes in the structure of the gut microbiota, but we are not sure about if changes in the composition and function of the gut microbiota respond to changes in the nutrient intake or food items. To investigate how animal feeding strategies affect nutrient intakes and thus alter the composition and digestion function of gut microbiota, we selected a group of wild primate group for the study. We quantified their diet and macronutrients intake in four seasons of a year, and instant fecal samples were analyzed by high-throughput sequencing of 16S rRNA and metagenomics. These results demonstrated that the main reason that causes seasonal shifts of gut microbiota is the macronutrient variation induced by seasonal dietary differences. Gut microbes can help to compensate for insufficient macronutrients intake of the host through microbial metabolic functions. This study contributes to a deeper understanding of the causes of seasonal variation in host-microbial variation in wild primates.

KEYWORDS

16S rRNA gene sequencing, metagenomic, metabolic functions, gut microbiota, wild golden snub-nosed monkey, seasonal variations

1. Introduction

What factors cause or have interaction with gut microbiota is a key and hot issue in animal evolutionary adaptation and original of human diet health. It is reported that the composition and structure of animal gut microbiota changes with host diet, which is shown in macroscopic indicators such as diversity of the microbes (Hooper et al., 2012; Morgan et al., 2012; Markle et al., 2013; Amato et al., 2019). Moreover, changes of gut microbiota show seasonal fluctuation in wildlife and humans, and literatures also widely indicated that seasonal dietary changes lead to the reconfiguration of gut microbiota of hosts, or at least both aspects have strong interaction. For example, seasonal cycling in the gut microbes following the dietary fluctuation has been reported in the Hadza hunter-gatherers in Tanzania (Smits et al., 2017), the western lowland gorillas and chimpanzees (Hicks et al., 2018), and red squirrels (Ren et al., 2017).

While, recent studies have shown that diets of invertebrates and vertebrates may be determined by the nutrient components in foods (Ruohonen et al., 2007; Simpson and Raubenheimer, 2012). The intake of each macronutrient should be stable no matter whether an animal is a vegetarian, carnivore, or omnivore and how complex its food composition is (Raubenheimer et al., 2009, 2015; Machovsky-Capuska et al., 2016). Non-human primates can take in stable proportions of

macronutrients from foods with complex and diverse components, which suggests that they have stable requirements for three macronutrients. The nutrient intakes of non-human primates are influenced by both the type and the amount of food consumed. Studies on folivorous primates including species of the subfamily Colobus (Chapman et al., 2003) and the subfamily Indriidae (*Indri*) (Junge et al., 2009; Fleming and John Kress, 2011) show huge variation in macronutrient composition in their foods. Meanwhile, some species such as Mountain gorillas (*Gorilla Beringei*) can maintain a constant non-protein intake during the period when non-protein nutrients from fruits are scarce by consuming an excessive amount of leaves (Rothman et al., 2011).

As the diet may co-evolve with gut microbiota among different animals, the notion that diet variation can influence gut microbiome composition and structure has been confirmed at the taxonomic level of family (Ley et al., 2008). However, within genera, or ranks below genera, species from the same taxa may vary greatly in diet (Guo et al., 2007). For example, chimpanzees (*Pan troglodytes*) are considered frugivorous primates, but they feed heavily on leaves and even prey on other animals during the fruitless season (Rothman et al., 2007). The dietary variability makes it difficult to explain the changes in the composition and structure of gut microbes. So far, it has been found that such changes are closely related to the macronutrients consumed on the study of captive animals (Grześkowiak et al., 2015). However, due to the difficulty in quantifying the nutrient intakes in the field and the scarcity of such studies on wild animals, especially endangered wild mammals, we are hampered to figure out the mechanisms of gut microbiota-host co-evolution in many wild species. Therefore, this study aims to investigate how whether diet or nutrients intake affect the structure and composition of gut microbiota and the interaction among these three aspects. This will reveal the mechanisms behind the seasonal shifts in diet in animals that rely heavily on gut microbiota for digestion.

Golden snub-nosed monkeys (*Rhinopithecus roxellana*) belong to the genus *Rhinopithecus* in the subfamily Colobinae, and their habitats vary seasonally (Hou et al., 2018). Our previous research on their diet based on time ratio reveals that wild golden snub-nosed monkeys have seasonal diet variation (Guo et al., 2007). Recent studies show that their feeding strategy stabilizes protein intakes and balances energy requirements by regulating carbohydrate and lipid intakes (Hou et al., 2021). Since golden snub-nosed monkeys live in an environment with complex foods and are capable of maintaining a stable amount of macronutrient *via* various feeding strategies, they are an excellent model for studying the interactions between food consumed, nutrient intakes, and gut microbiota. In view of the reasons above, we propose the following research questions. (1) Do seasonal changes in food types lead to changes in the composition and structure of gut microbiota? and (2) Do seasonal variations in nutrient intakes lead to seasonal variations in gut microbiota composition? (3) Are there any seasonal variations in gut microbial gene function? Does the gene function correlate with seasonal variations in dietary and nutrient intakes?

2. Materials and methods

2.1. Data collection

Our observation site was in Guanyin Mountain National Nature Reserve (107°51′-108°01′E, 33°35′–33°45′N,135.34 km^2) on the southern slope of Qinling Mountains, which locates at the northwest of Fuping County, Shaanxi Province, China (Supplementary Figure S1). This region experiences the classic and distinct four seasons throughout the year. The seasons are divided according to climate: spring is from March to May, summer is from June to August, autumn is from September to November, and winter is from December to February (Guo et al., 2007, 2018). We collected feeding data of four season groups (i.e., spring, summer, autumn, and winter). For each season, we chose a month with typical phenological characters, that is, March (spring), June (summer), October (autumn), and December (winter) for data collection.

Our study group of golden snub-nosed monkeys had 78 individuals, all haven been habituated to the presence of researchers. Adult and juvenile individuals haven been identified by us. Because we needed to collect quantitative observation data, the natural feeding space of the target animals was narrowed. To prevent that their total energy intakes being reduced due to this condition and thus impacting their health, we referred to our previous experience to provision foods (Hou et al., 2021). We provisioned 5 kg of maize grains twice daily at 10 a.m. and 3 p.m. as supplementary food for the group. Maize grains were spread evenly in the feeding grounds.

We randomly chose one individual per day and conducted continuous observations of the focal animal from dawn to dark to record its feeding data. During the observation session, the distance between the observer and the subject was less than 5 m. We recorded the type, quantity, and predefined units of the food and the amount of time feeding (Hou et al., 2018). After the focal individual completed feeding, leftover foods were collected as food samples. All samples were labeled with the information of the collection time, type, and size. Then, they were immediately frozen in liquid nitrogen and sent to the laboratory for storage before the analysis of their nutrient components. We also collected same-day fecal samples for high-throughput sequencing. After the focal individual defecated, we immediately collected the feces with sterile cotton swabs and sterile toothpicks. The sample was then stored in 2 mL centrifuge tubes and frozen in liquid nitrogen before being delivered for testing.

2.2. Nutrient analysis

We used the standard techniques to collect the food samples (Rothman et al., 2012), analyzed the foods nutrients, and calculated the energy values (Jung, 1995) with the same methods used in our previous studies (Guo et al., 2018; Hou et al., 2018). The macronutrients of each food were analyzed for lipid, water-soluble carbohydrate (WSC), starch, neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), available protein (AP), and ash content. Available proteins are determined by the standard Kjeldahl method (using BUCHI, K-360).

To calculate the daily nutrient intake (DNI), we multiplied the nutrient content of each food item by the recorded amount of that item consumed, then summed these values for all items consumed by that individual on that day. We also calculated the rate of nutrient ingestion per hour (NIH) for each individual by dividing the amount of nutrient ingested by the amount of hours the focal animal was observed. The rate was multiplied by the sunshine duration to estimate the total daily intake (TDNI; Rothman et al., 2008). We lured each monkey with a small portion of food and led it onto a platform scale (accuracy, 0.02 kg; EM-60KAL, A&D, Japan) to record their weight when the readings were stable (Hou et al., 2021). To standardize weight differences between

individuals, the calculation was divided by the individual's estimated metabolic body mass (MBM = $M^{0.762}$, where M is the body weight in kg).

2.3. DNA extraction and 16S rRNA gene sequencing

The microbial DNA (with a total mass of 1.2–10.0 ng) was isolated from each fecal sample using the MOBIO Pow erSoil DNA Isolation Kit and was quantified with NanoDrop One (Thermo Fisher Scientific, Waltham, MA, United States). The V4 regions of the DNA genes were amplified by using the specific primer 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTW TCTAAT-3′) with 12 bp barcode. Primers were synthesized by Invitrogen (Invitrogen, Carlsbad, CA, United States). The PCR instrument was Bio-Rad S1000 (Bio-Rad Laboratory, CA, United States). The length and concentration of the PCR product were detected by 1% agarose gel electrophoresis. PCR products with bright main strip between were mixed in equidensity ratios according to the GeneTools Analysis Software (Version 4.03.05.0, SynGene). Then, mixture of PCR products was purified with E.Z.N.A. Gel Extraction Kit (Omega, United States). Sequencing libraries were generated using NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (New England Biolabs, MA, United States) following the manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Fisher Scientific, MA, United States). At last, the library was sequenced on an Illumina Nova6000 platform and 250 bp paired-end reads were generated (Guangdong Magigene Biotechnology Co., Ltd. Guangzhou, China).

2.4. Metagenomic sequencing and gene catalog construction

The sequencing library was created using NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Beverly, MA, United States) and indexes were added to attribute sequences to each sample. The DNA sample was fragmented by sonication to a size of 300 bp. DNA fragments were polished at the extremities and were attached to the full-length adapter for Illumina sequencing with further PCR amplification. The library was analyzed for size distribution by Agilent2100 Bioanalyzer (Agilent, United States), and then was sequenced by Illumina HiSeq 2500 platform in Magigene Co., Ltd. (Shenzhen, China).

Quality control was conducted by Trimmomatic (Version 0.38). The reads aligned to the NCBI non-redundant (NR) database were removed with MEGAHIT (Version 1.05). The remaining high-quality reads were used for further analysis. The assembly of reads was conducted using MEGAHIT *de novo*. For each sample, a series of k-mer (substrings of length k) values (49–87) were used and the optimal one with the longest N50 value was chosen for the remaining scaffolds. The clean data were mapped against scaffolds using MEGAHIT. Unused reads from each sample were assembled using the same parameters.

Genes (minimum length of 100 nucleotides) were predicted on scaftigs longer than 500 bp using Prodigal (Version 2.6.3). Then, a non-redundant gene catalog was constructed with Linclust (Version 2.0) using a sequence identity cut-off of 0.9. To determine the abundance of genes, reads were realigned to the gene catalog with BBMap (Version 37.68). Only genes with 2 mapped reads no less than 2 were considered exist in a sample. The abundance of genes was calculated by counting the number of reads and normalizing by gene length. Genes were then searched in Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and Goto, 2000) for annotation.

2.5. Statistical analysis

Kruskal-Wallis test with Bonferroni correction for multiple post-hoc pairwise comparison was used to compare the difference of available protein, fat, and carbohydrate in four seasons. Permutational Multivariate Analysis of Variance (PERMANOVA) was used to compare the difference of Shannon index and Chao1 index. The Analysis of Similarities (ANOSM) was used to compare the result of Principal co-ordinate analysis (PCoA). The Mantel test was used to compare the correlation between food groups and the gut microbial composition in each season. Pearson's correlation coefficients were calculated to analyze correlations between weighted gene co-expression network analysis (WGCNA) module groupings and traits. Data visualization was performed by R4.1.3 and Cytoscape 3.8.2.

3. Results

3.1. Seasonal diets

In this study, feeding data of 96 days across 4 months (25 days in spring, 24 days in summer, 24 days in autumn, and 23 days in winter) were collected from the target group. It was observed that the natural foods consumed by wild golden snub-nosed monkeys consisted of 24 plant species belonging to 16 families. Six items of plant including barks, seeds, buds, brunches, leaves, and stems have been observed to be consumed. Throughout the year, the proportion of each plant item consumed by wild snub-nosed monkeys was 33.43% for bark, 3.09% for seed, 1.33% for bud, 3.25% for brunch, 0.17% for stem, and 58.72% for leaf. However, there were huge differences in the amount of the plant items being consumed across four seasons. Herbaceous stems were only taken in spring with a small quantity. Seeds were taken mainly in spring and autumn. Leaves were taken throughout the year. Buds, barks, and brunches were the main food in autumn and winter when leaves become scarce, especially in winter (Supplementary Figure S2A).

In addition, differences have also been found in the plant species consumed between seasons—*Photinia beauverdiana*, *Acer davidii*, *Dendrobenthamia japonic*a, *Kerria japonica*, *Ulmus macrocarpa*, *Quercus aliena*, *Acer mono Maxim*, and *Lonicera japonica* were mainly taken in spring; *Cerasus clarofolia*, *Ailanthus altissima*, *Juglans mandshurica*, and *Spiraea blumei* were mainly taken in summer; *Rubus pungens*, *Arachis hypogaea*, *Quercus mongolica*, *Pinus bungeana*, *Lonicera hispida*, and *Carpinus cordata* were mainly taken in autumn; and *Litsea pungens*, *Quercus dolicholepis*, *Fargesia qinlingensis*, *Bothrocaryum controversum*, *Glechoma longituba*, *Litchi chinensis*, and *Callicarpa nudiflora* were mainly taken in winter (Supplementary Figure S2B). The results of PCA on seasonal percentage data of food mass for 24 plant species and 6 plant items showed that there were significant differences in the plant species consumed by golden snub-nosed monkeys between any two seasons (PERMANOVA, $p < 0.05$, Table 1). Also, there were significant differences in plant items consumed between any two seasons except for spring and summer (PERMANOVA, $p < 0.05$, Table 2).

TABLE 1 Statistical analysis (PERMANOVA) of food mass seasonal difference of plant species consumed by wild golden snub-nosed monkeys.

Group	*F*-Model	R^2	Value of *p*	*p*-adjusted
Spring vs. Summer	6.979	0.145	0.001	0.0012
Spring vs. Autumn	7.155	0.143	0.001	0.0012
Spring vs. Winter	10.429	0.192	0.001	0.0012
Summer vs. Autumn	10.373	0.206	0.001	0.0012
Summer vs. Winter	15.486	0.274	0.001	0.0012
Autumn vs. Winter	3.787	0.081	0.003	0.0030

TABLE 2 Statistical analysis (PERMANOVA) of food mass seasonal difference of plant items consumed by wild golden snub-nosed monkeys (PERMANOVA).

Group	*F*-Model	R^2	Value of *p*	*p*-adjusted
Spring vs. Summer	1.548	0.036	0.184	0.1840
Spring vs. Autumn	8.362	0.163	0.001	0.0015
Spring vs. Winter	14.559	0.249	0.001	0.0015
Summer vs. Autumn	8.275	0.171	0.001	0.0015
Summer vs. Winter	12.679	0.236	0.001	0.0015
Autumn vs. Winter	3.451	0.074	0.005	0.0060

3.2. Nutritional properties

We collected 55 types of food from 24 plant species and evaluated their nutritional properties. We measured the energy of metabolic body mass (KJ/MBM) provided by macronutrients, which showed that spring food intake provides 25.93 ± 11.04 kJ/MBM (M ± SE) of available protein, 22.41 ± 10.55 kJ/MBM of fats, and 245.83 ± 106.47 kJ/MBM of carbohydrates; summer food intake provides 43.80 ± 9.72 kJ/MBM of available protein, 36.88 ± 9.21 kJ/MBM of fats, and 411.19 ± 96.55 kJ/MBM of carbohydrates; autumn food intake provides 39.83 ± 22.26 kJ/MBM of available protein, 37.17 kJ/MBM of fats, and 394.68 ± 219.21 kJ/MBM of carbohydrates; winter food intake provides 28.17 ± 10.56 kJ/MBM of available protein, 24.76 ± 9.68 kJ/MBM of fats provides, and carbohydrates provided 278.15 ± 100.48 kJ/MBM. Statistical analysis of available proteins, carbohydrates, and fats provided by foods in four seasons found that they were all significantly different between spring vs. summer and summer vs. winter groups, and that fats also differed in spring vs. autumn group (Figure 1).

We also divided the sources of macronutrients into natural foods and artificial foods. For natural foods, the energy provided by available proteins decreases in the sequence of summer, winter, autumn, and spring, while the energy provided by carbohydrates and fats increases in the sequence of spring, summer, autumn, and winter. For artificial foods, available proteins, carbohydrates, and fats presented a uniform seasonal pattern throughout the year with energy values decreasing from summer to autumn to winter and to spring (Supplementary Figure S3).

3.3. Microbial compositions

The 16S rRNA gene sequencing of fecal samples revealed that the observed species and the two alpha-diversity indexes reflecting species richness and diversity (Chao1 index and Shannon index, respectively) showed a decreasing in the order of spring, winter, autumn, and summer. That is, the richness of gut microbiota and the diversity of community in golden snub-nosed monkeys were highest in spring and lowest in summer during all seasons. The Chao1 index was significantly different between spring vs. summer, summer vs. autumn, summer vs. winter, and autumn vs. winter (Kruskal-Wallis test, $p < 0.05$, Figure 2A), while the Shannon index was only significantly different between spring vs. summer (Kruskal-Wallis test, $p < 0.05$, Figure 2B). Principal co-ordinate analysis (PCoA) based on weighted UniFrac distances (Figure 3A) and unweighted UniFrac distances (Figure 3B) of OTUs showed divergence between the groups of different seasons along the first and second principal components. Analysis of Similarities (ANOSM) showed that *p* values of all groups were less than 0.05, indicating a significant difference between the gut microbiota of four seasons.

To further investigate seasonal differences in gut microbiota, species composition was analyzed. Species annotation of the 16S rRNA sequencing showed that most OTUs could be taxonomically assigned to the phylum (96%) and order (92%) level, but assignments decreased substantially at the genus (38%) level. A total of 38 phyla were annotated, of which the top 10 identifiable dominant phylum including *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Spirochaetes*, *Tenericutes*, *Verrucomicrobia*, *Planctomycetes*, *Epsilonbacteraeota*, *Fibrobacteres*, and *Euryarchaeota* accounted for 99% of the total abundance ratio. These formed the core gut microbiota of golden snub-nosed monkeys. When considering them in different seasons, they showed seasonal differences in abundance, with *Firmicutes* and *Euryarchaeota* being most abundant in spring and *Proteobacteria* being least abundant in spring compared to other seasons (Figure 4A). The ratio of *Firmicutes* to *Bacteroidetes* (F/B) was highest in spring and lowest in autumn, suggesting a seasonal variation in the capacity of energy absorption by gut microbiota (Supplementary Figure S4).

A total of 140 orders were annotated. The dominant identifiable bacteria were *Clostridiales*, *Bacteroidales*, *Aeromonadales*, *Methanobacteriales*, *Mollicutes_RF39*, *Spirochaetales*, *Verrucomicrobiales*, *Pirellulales*, *Erysipelotrichales*, and *Selenomonadales* (Figure 4B). It is noteworthy that the abundance of the *Aeromonadales* was much lower in spring than in other seasons. Meanwhile, *Methanobacteriales*, which are associated with methane production, were observed to have abundance much higher in spring and summer than in autumn and winter. They had a particular high abundance in spring. Metagenomic analysis showed that several bacterial taxa with high abundance at the phylum and order levels were consistent with the 16S rRNA study (Figures 4C,D).

At the genus level, there were 352 taxa annotated and the top 100 genera covered nearly 99.9% of the total abundance. The study of these

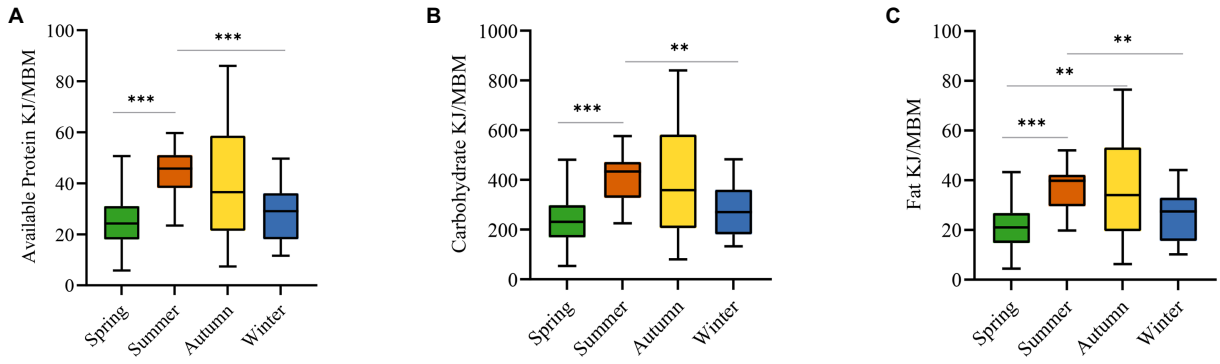

FIGURE 1
Comparison of macronutrients among four seasons (we measured the energy of metabolic body mass (KJ/MBM) provided by macronutrients). **(A)** The comparison of available protein intake. **(B)** The comparison of carbohydrate intake. **(C)** The comparison of fat intake. **p<0.01, ***p<0.001. (Kruskal-Wallis test).

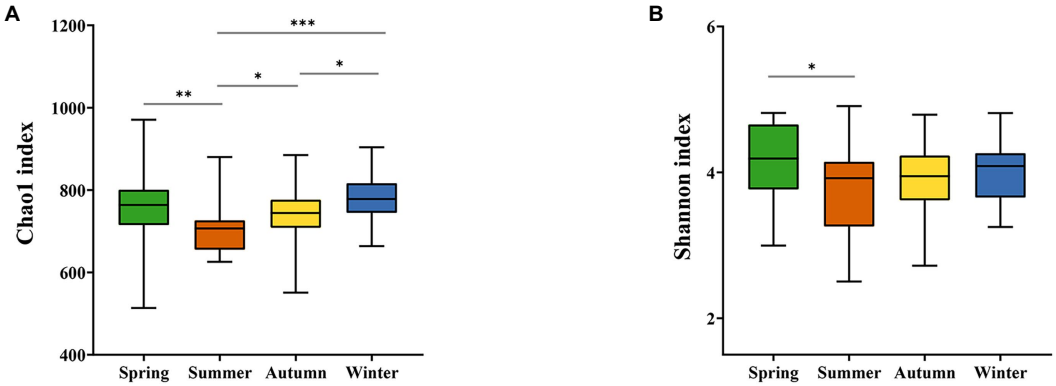

FIGURE 2
Alpha-diversity of gut microbiota. The Chao1 indexes fluctuate significantly between seasons **(A)**, while the Shannon indexes vary only in spring and summer and are stable for the rest **(B)**. *p<0.05, **p<0.01, ***p<0.001 (Kruskal-Wallis test).

100 genera found that the gut microbes of golden snub-nosed monkeys were mostly related to hindgut fermentation in ruminant animals. These genera include those that can degrade complex polysaccharides such as *Methanobrevibacter*, *Methanosphaera*, *prevotella_7*, *Roseburia*, *Ruminococcaceae_UGG-014*, *Treponema_2*, *Clostridium*, those that can produce hydrogen efficiently such as *christensenellaceae_R-7_group*, and those play roles in lipid metabolism such as *[Eubacterium]_coprostanoligenes_group*, *Blautia*, *Dorea*, *lactobacillus*, *Dialister*, and *Phascolarctobacterium* (Figure 4E).

3.4. Gene function prediction of gut microbiota

We performed KEGG annotation using metagenome data to find out the main functions of the gut microbiota in golden snub-nosed monkeys. According to the function prediction based on the KEGG database, we identified 395 metabolic pathways. Among these pathways, gut microbes were mainly involved in the nucleotide metabolism, carbohydrates metabolism, glycans metabolism and biosynthesis, amino acids metabolism, energy metabolism, lipids metabolism, terpenoids and polyketides metabolism, as well as cofactors and vitamins metabolism. Moreover, some functions annotated concerning macronutrients showed relatively high abundance such as glycolysis/gluconeogenesis, pyruvate metabolism, starch and sucrose metabolism, pentose phosphate pathway in carbohydrate metabolism and glycerophospholipid metabolism, glycerolipid metabolism, and fatty acid synthesis in lipid metabolism.

Inter-seasonal KEGG enrichment analysis demonstrated that there were 288, 210, 237, 98, 71, and 78 differentially expressed genes and were enriched in 20, 21, 20, 17, 16, and 14 KEGG pathways in spring vs. summer, spring vs. autumn, spring vs. winter, summer vs. autumn, summer vs. winter, and autumn vs. winter groups, respectively. The seasonal function variation of these differentially expressed genes can be presented in terms of metabolism, organic systems, and environmental information processing. Specifically, the enriched pathways were similar in all season groups. These pathways included biosynthesis of siderophore group nonribosomal peptides (ko01053); protein digestion and absorption (ko04974); flavonoid biosynthesis (ko00941); stilbenoid, diarylheptanoid, and gingerol biosynthesis (ko00945); cardiac muscle contraction (ko04260), phenylpropanoid biosynthesis (ko00940); arachidonic acid metabolism (ko00590); atrazine degradation (ko00791); flavone and flavonol biosynthesis (ko00944); linoleic acid metabolism (ko00591), fatty acid elongation (ko00062); alpha-Linolenic acid metabolism (ko00592); and bile secretion (ko04976) (Figure 5).

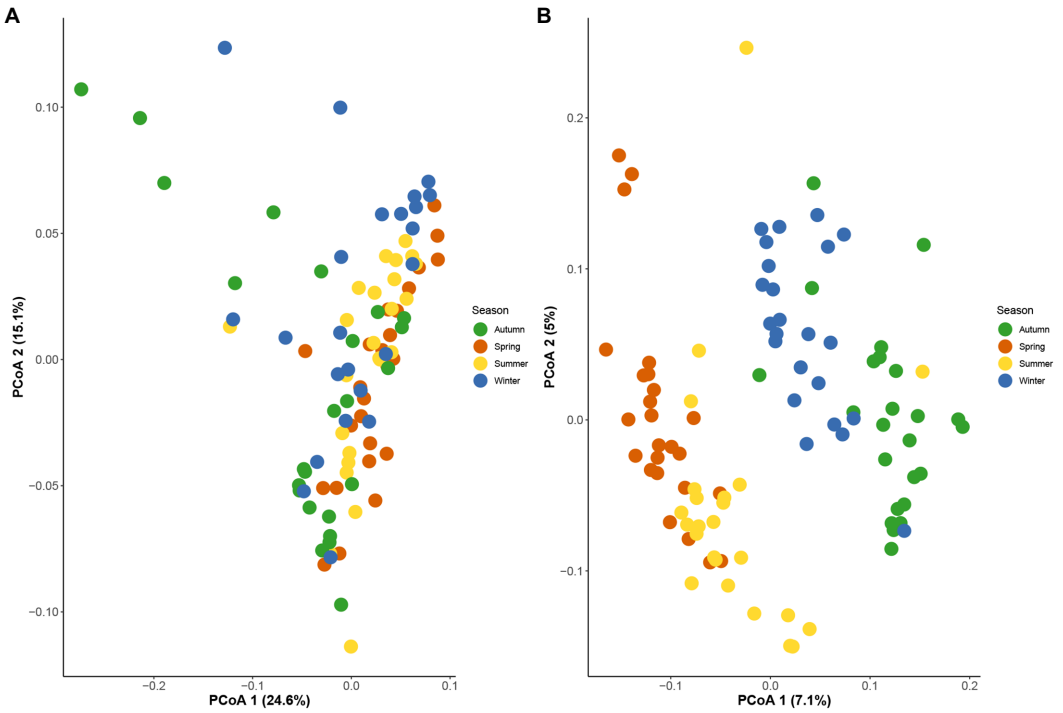

FIGURE 3
Principal co-ordinate analysis based on weighted UniFrac distances **(A)** and unweighted UniFrac distances **(B)** between all seasons showed significant difference in structure of seasonal gut microbial composition.

3.5. Correlation between gut microbiota and food types

Based on the significant seasonal differences of natural food types and gut microbiota in golden snub-nosed monkeys, we conducted a correlation analysis between food types and OTUs in four seasons. The results demonstrated that *Dendrobenthamia japonica* and *Zea mays* were correlated with gut microbiota in spring (Mantel test, $p < 0.05$, Figure 6A), and *Callicarpa nudiflora* was correlated with gut microbiota in summer (mantel test, $p < 0.05$, Figure 6B). In autumn and winter, there was no correlation between food types and gut microbiota (Figures 6C,D).

3.6. WGCNA on the hub OTUs

The present study used 3,638 OTUs for weighted gene co-expression network analysis (WGCNA). OTUs that exist over half of the sample in each season were selected and the network was constructed in one step. To define the adjacency matrix based on the criterion of approximate scale-free topology (Supplementary Figure S5), the network type was set as sign and the soft threshold parameter set to 10 with a minimum module size of 30 and the module detection sensitivity DeepSplit of 3. Modules that are correlated above 0.75 would be merged (Supplementary Figure S6). The clustering results showed that a total of 814 OTUs were parsed into 5 different modules. The gray module refers to ones that were not classified. The correlation between module eigenvalue and trait was calculated. The module-trait relationship heatmap demonstrated the correlation coefficient between module eigenvalues and traits. The green module refers to OTUs that were significantly correlated with three macronutrients (fat, carbohydrate, and available protein) at the same time, and the blue module was significant correlation with fat ($p < 0.05$, Figure 7A).

In addition, OTUs with betweenness centrality at top 10% in WGCNA were selected and the network graph was constructed in Cytoscape. The results showed that green module took the largest proportion and had richer network relationships (Figure 7B). Therefore, the green and blue modules were selected for hub gene analysis. We calculated the correlation between the module membership (MM) and the genes significance (GS) and nutritional traits. It was found that the relationship between MM and GS for these modules was relatively strong, particularly for those in the green module ($r > 0.4$, $p < 0.05$, Supplementary Figure S7). We also identified 22 candidate hub OTUs that are correlated with fat, carbohydrate and available protein in the green module, and 9 candidate hub OTUs that are correlated with fat in the blue module (threshold values of $MM > 0.6$ and $GS < 0.1$, respectively). The network graphs were constructed accordingly (Figures 7C,D). OTU_472, OTU_2009, OTU_226, OTU_81, OTU_67, and OTU_349 in the green module and OTU_44 in the blue module were found to be the most important hub OTUs. They belonged to the family *Ruminococcaceae* (OTU_472 and OTU_81), *Lachnospiraceae* (OTU_2009, OTU_226, and OTU_349) in the order *Clostridiales* and family *Muribaculaceae* (OTU_67), and *Prevotellaceae* (OTU_44) in the order *Bacteroidales*, respectively.

4. Discussion

Our results reveal that there are remarkable seasonal differences in both food items and macronutrients intake from food for wild golden snub-nosed monkeys. However, seasonal feeding strategies cannot fully

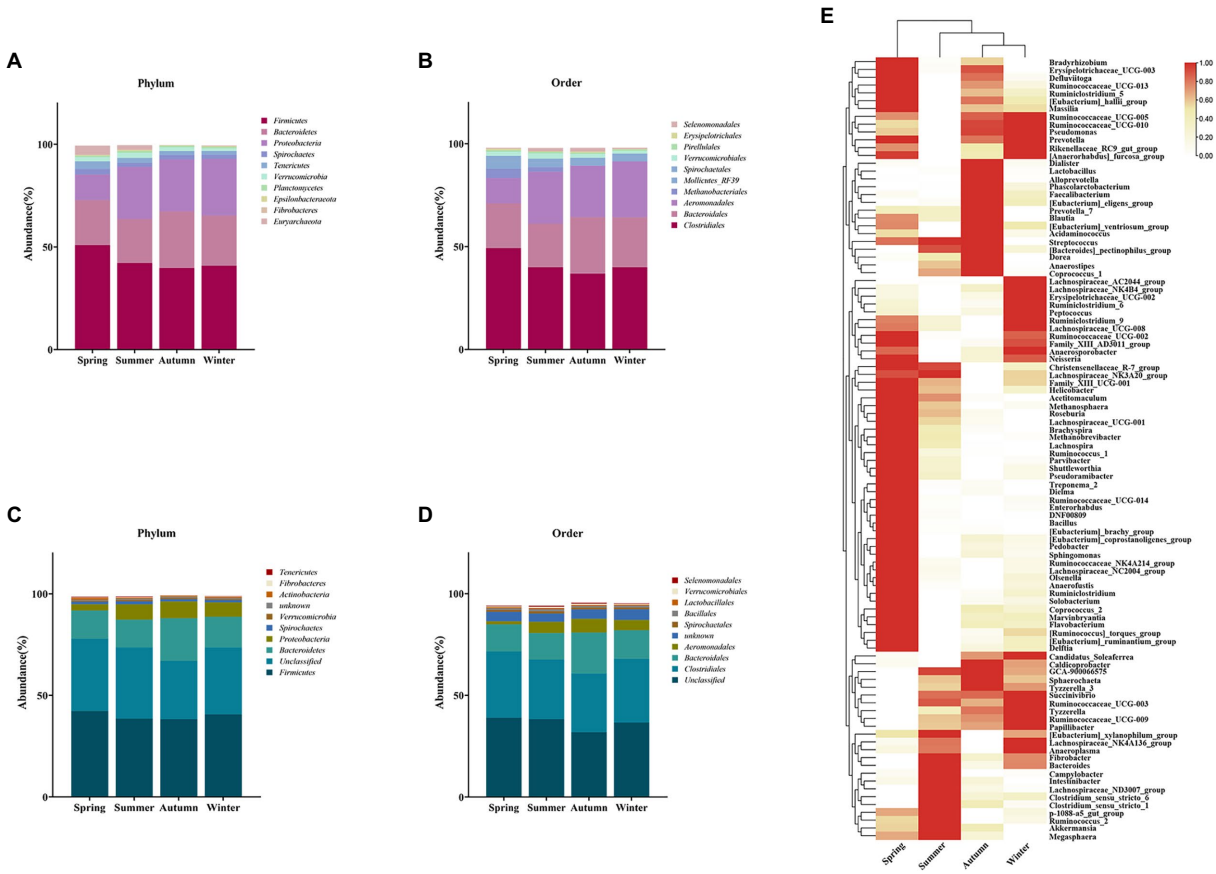

FIGURE 4
Analysis of seasonal differences in dominant bacterial populations. Relative abundance of dominant phylum **(A)** and order **(B)** in four seasons based on 16S rRNA gene pools. In contrast, macrogenome annotation of dominant bacteria at the level of phylum **(C)** and order **(D)**. Heat map of proportions at the genus level **(E)**, the darker the color, the higher the proportion of this genus present in the season compared to other seasons.

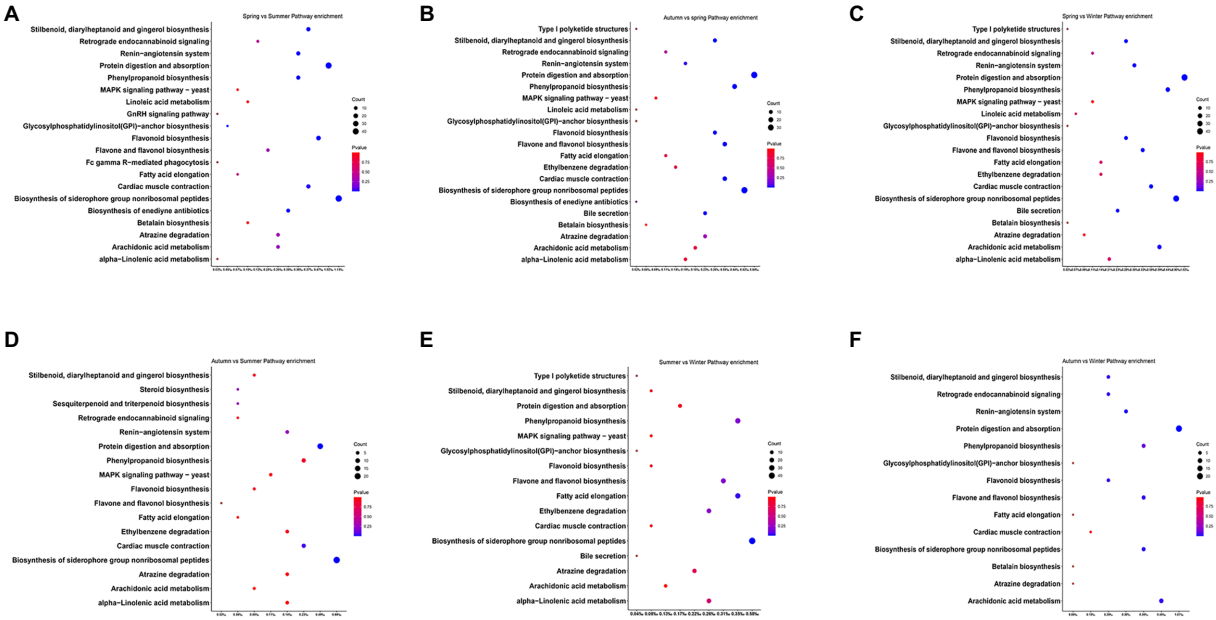

FIGURE 5
Analysis of KEGG annotation in groups with significant differences in macronutrient intakes to predict the function of gut microbial, including spring vs. summer group **(A)**, autumn vs. spring group **(B)**, spring vs. winter **(C)**, autumn vs. summer **(D)**, summer vs. winter group **(E)**, and autumn vs. winter **(F)**.

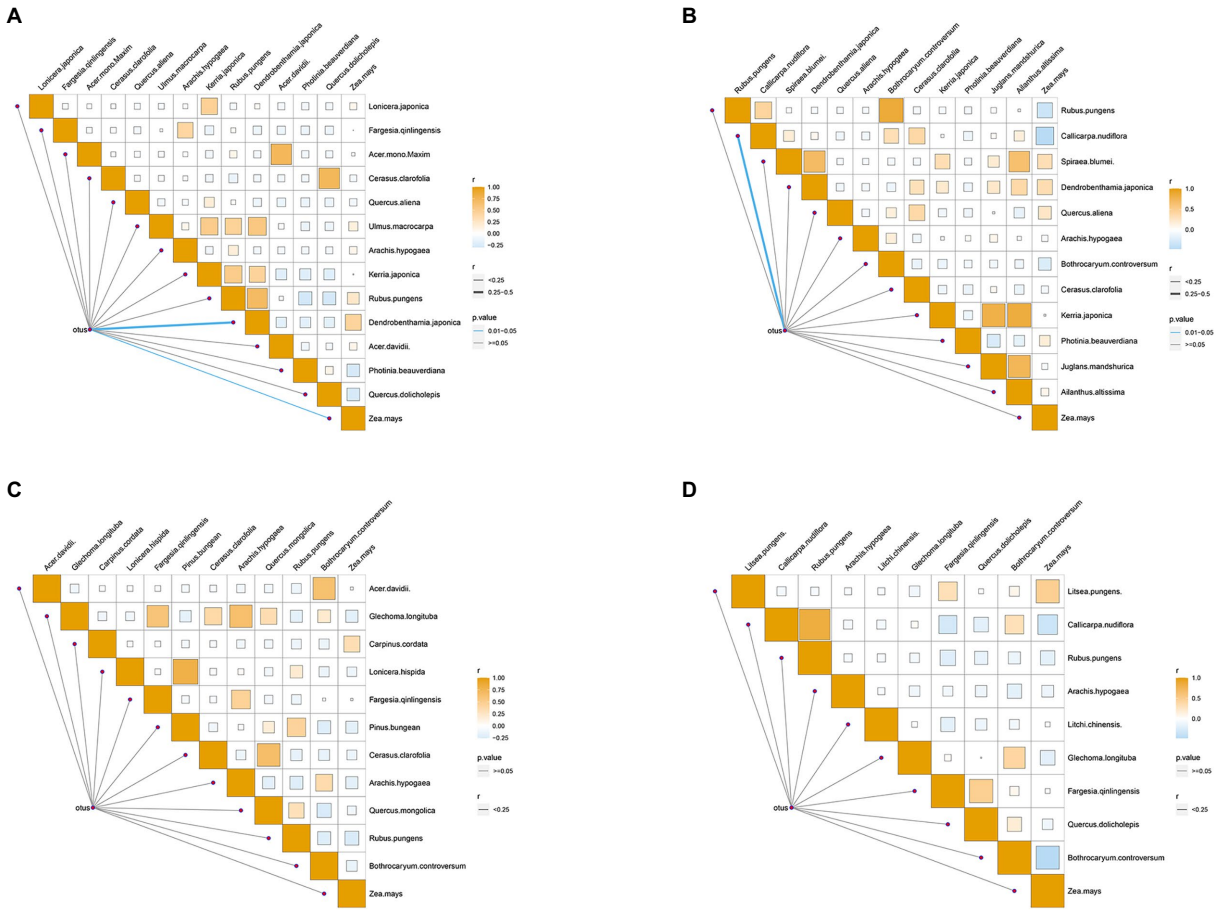

FIGURE 6
Heat map of the correlation between food groups and gut microbes of spring **(A)**, summer **(B)**, autumn **(C)**, winter **(D)**. Darker color refers to that r value is closer to 1; thicker line refers to higher *r* value between food groups and gut microbes. The color of the line segment shows the *p* value between food group and gut microorganism (Blue indicates *p* value <0.05, grey indicates *p* value >0.05).

explain the composition and fluctuation of their gut microbiota. On the contrary, the changes in carbohydrates, fats, and available proteins present similar trends with the changes of gut microbiota. Our findings suggest that the key factor that shapes the composition and function of wild golden snub-nosed monkeys' gut microbiota is macronutrient intakes rather than food types. This differs from the view that diverse gut microbiotas in wild mammals are the result of the seasonal changes in dietary habits or food types in the previous studies.

4.1. The effects of nutrients on gut microbial composition

Previous studies suggest that seasonal changes in the composition and function of mammalian gut microbiota may be related to seasonal changes in the host's diet. A study on wild blue sheep (*Pseudois nayaur*) found that changes in the composition of the animals' gut microbiota is due to seasonal shifts in dietary habits (Zhu et al., 2020). Giant panda (*Ailuropoda melanoleuca*) gut microbes would produce more single-chain fatty acids during the shoot-eating season compared to the leaf-eating season (Huang et al., 2022). For wild geladas (*Theropithecus gelada*), the gut microbes in the rainier periods are mainly cellulolytic or fermentative bacterial that specialized in digestion grass, while during dry periods the gut is dominated by bacteria that break down starches found in underground plant parts (Baniel et al., 2021).

Meanwhile, many studies also suggest that the community structure and function of gut microbiota are influenced by the macronutrients consumed by host. In human guts, *Bacteroides* will be the dominant microbes when diets are rich in proteins and fats, while *Prevotella* is central to the diet rich in carbohydrates (Devkota et al., 2012; Henao-Mejia et al., 2012). Shifts in nutrient intakes have also been found to lead to changes of gut microbial composition in captive mammals. For example, high-fat diet with a high cholesterol intake resulted in dysbiosis of gut microbes and downregulation of microbial tryptophan metabolism in mice (Zhang et al., 2021a). Increasing different types of carbohydrates in the feed could lead to changes in the abundance of gut microbes in pigs (Lyu et al., 2020; Wang et al., 2021). Gut microbes in dogs and cats regulated their growth, reproduction, and homeostasis *per se* by breaking down nutrients that were not digestible by host digestive enzymes (Oh et al., 2021).

The analysis of the composition of gut microbiota in this study shows that both alpha-diversity and beta-diversity exhibited differences in richness and diversity in different seasons. *Firmicutes* was the most dominant phylum in all seasons, followed by *Bacteroidetes* and *Proteobacteria*. The results also indicate that *Firmicutes* can interact with other gut microbes to influence the absorption of nutrients. The previous

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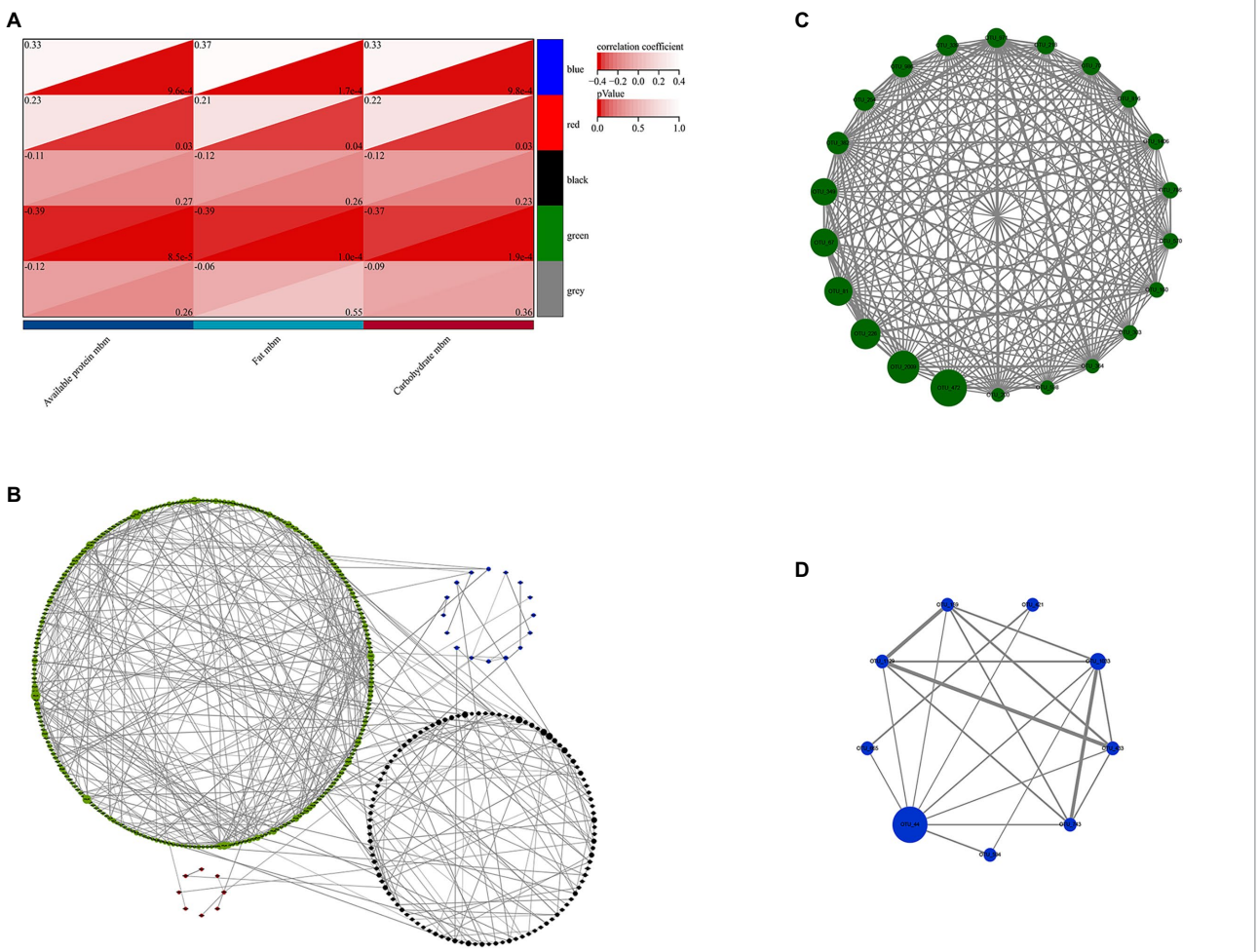

FIGURE 7
Identification of key module and hub OTUs based on WGCNA. **(A)** Correlation between module eigenvalues and traits of golden snub-nosed monkey. Depth of color corresponds to depth of correlation and p value of each module. **(B)** Network graph of the hub OTUs. Each node represented the OTUs whose betweenness centrality value was in the top 10%, and its color represented the corresponding module, the size of each node represented the betweenness centrality value, the size of each line thickness represented the weight value between nodes (OTUs). **(C)** Visualization of full weighted networks of 22 candidate hub OTUs in green module associated with three different nutrients (fat, protein, and carbohydrate). **(D)** Visualization of full weighted networks of 9 candidate hub OTUs in blue module associated with fat.

study reveals that a high proportion of *Firmicutes* means that the host is able to get more energy from the food (Ley et al., 2006). When metabolic disorder produces dysbiosis that disturb the stability of the gut microbes, it is usually accompanied by an increase in *Proteobacteria* (Shin et al., 2015). In our study, golden snub-nosed monkeys have the least nutrient intakes in spring, and the gut microbiota show high abundance in *Firmicutes* and low abundance in *Proteobacteria*. This reflects that gut microbiota of host would be more stable in spring and the host could absorb more energy from low nutrient intakes to sustain life activities. Based on the above findings at the phylum level, we speculate that these gut microbes flourish to compensate for the low nutrient intakes in spring through microbial action to maximize energy utilization.

At the order level, samples in spring show an increase in *Methanobacteriales* and a decrease in *Aeromonadales*. Methanogens can reduce intestinal gas accumulation (Kengen et al., 1994) and maintain an anaerobic environment in the hindgut, facilitate the metabolic of polysaccharides, and improve the utilization efficiency of energy (Samuel and Gordon, 2006; Samuel et al., 2007). And more our study finds that the abundance of *Aeromonadales* is much lower in spring than in other seasons, while the increased abundance of *Aeromonadales* has been verified to be the reason for intestinal inflammation. *Aeromonadales*-related lipopolysaccharides disrupt the intestinal mucosal barrier and cause the increase of intestinal permeability, thereby causing inflammation (Zhang et al., 2021b).

At the genus level, there is an increase in the genus *Methanosphaera*, *Methanobrevibacter*, *Shuttleworthia*, *Ruminococcaceae_UCG-014*, *Treponema_2*, *Ruminiclostridium*, and *Ruminococcaceae_NK4A214_group* in spring when proteins, fats, and carbohydrates are consumed at the lowest amount. The methanogenic bacteria *Methanosphaera*, *Methanobrevibacter*, and *Shuttleworthia* can convert hydrogen and formate into methane. When enriched simultaneously with efficient hydrogen-producing bacteria such as *Christensenellaceae_R-7_group* (Morotomi et al., 2012), they are able to work synergistically to improve the efficiency of gut fermentation of starch and other polysaccharides (Samuel and Gordon, 2006). A large number of methanogenic bacteria can increase the calories obtained from food and enhance the absorption and utilization of nutrients (Mizrahi et al., 2021). They also promote the production of short-chain fatty acids by other fermenting bacteria and

stimulate the production of fats (Zhang et al., 2009; Basseri et al., 2012). *Ruminococcaceae_UCG-014* can produce butyrate, an important energy source for colon cells. In the meantime, they can increase short-chain fatty acids and affect host appetite and satiety through different mechanisms, delaying gastric emptying and thus energy absorption (Canfora et al., 2015; Guo et al., 2022). *Ruminiclostridium* is positively correlated with acetate content in the cecum, providing more energy to the host (Zhang et al., 2021c). As to autumn when diets are high-carbohydrate and high-fat, *Prevotella*, *Phascolarctobacterium*, and *Lactobacillus* were the dominant genus. *Prevotella* is capable of breaking down non-cellulosic polysaccharides and pectins (Flint et al., 2012). Both *Phascolarctobacterium* and *Lactobacillus* are probiotics that can break down fats (Lv et al., 2019; Rodrigues et al., 2021). Although our results show that the change in gut microbiota from spring to autumn is not due to a change in diet structure. However, there are diverse polysaccharides and fats that could be potentially utilized in autumn. Therefore, we suggest that differences in nutrient intakes may be a significant factor that shaped the composition of gut microbial communities during animal growth and development.

4.2. The effects of nutrients on gut microbial function

The analysis for gene function prediction based on KEGG database shows that the gut microbiota in wild golden snub-nosed monkeys mainly take part in metabolism and synthesis of lipid, carbohydrate, protein, amino acids, and other secondary metabolites. We should point out that most of the functional genes in the metagenome in this study appear to be involved in carbohydrate metabolism, but these pathways are not enriched in any seasonal grouping. This result may be due to the fact that we fed the monkeys equal amounts of maize throughout the year, which maintained their energy provided by large amounts of carbohydrates at a relatively constant level. Therefore, the gut microbes also responded stably to the degradation of carbohydrate.

The macronutrients are found significantly different both between spring and summer groups and between summer and winter groups. Also, there is a significant difference in fat intakes between spring and autumn groups. We found that 71, 228, and 210 differentially expressed genes between summer vs. winter group, spring vs. summer group, and spring vs. autumn group and were significantly enriched in 16, 20, and 21 KEGG pathways, respectively. Six of these pathways are important for the response to changes in the nutrients intake, including protein digestion and absorption (ko04974), fatty acid elongation (ko00062), arachidonic acid metabolism (ko00590), linoleic acid metabolism (ko00591), alpha-linolenic acid metabolism (ko00592), and bile secretion (ko04976) (Figure 6). In fact, there are studies reporting that these pathways play an important role in physiological activities of the host. For instance, bile acid is an amphiphilic molecule with strong surface activity (Yang et al., 2020), which can emulsify fat into chylomicrons to increase the contact area between lipase and fat, and facilitates fat digestion and reduces autologous fat catabolism (Velazquez-Villegas et al., 2018; Hu et al., 2019). Alpha-linolenic acid, linoleic acid, and arachidonic acid are essential fatty acids that animals cannot be synthesized by the body and must come from food (Di Pasquale, 2009; Martin et al., 2016). These results indicate that seasonal differences in these pathways may mainly be due to the differences in nutrient intakes.

Noticeably, in season groups with significant differences in nutrient intakes, besides the pathway associated with macronutrient metabolism, we observed enrichment of multiple secondary metabolite biosynthesis pathways such as Phenylpropanoid biosynthesis, Flavone and flavonol biosynthesis, Flavonoid biosynthesis, and Diarylheptanoid and gingerol biosynthesis. As signals to gut microbes, microbial diet-based metabolites or small molecules are key mediators that affect physiological processes in the host (Koh et al., 2016). They can activate or inhibit endogenous signaling pathways, or act as a source of nutrients for host cells (Sonnenburg and Bäckhed, 2016). The biosynthesis of phenylpropane begins with the shikimate pathway, which initially breaks down glucose by the combined action of the glycolysis and pentose phosphate pathway to produce phosphoenolpyruvate and erythrose-4-phosphate of the synthetic initiation metabolite 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP) (Chen et al., 2016). Flavone, flavonol, and flavonoid metabolites all appear as intermediates in phenylpropanoid biosynthesis pathway (Dong and Lin, 2021). In addition, key enzymes for the synthesis of resveratrol (stilbenoid), diarylheptanoid, and gingerol are also the central nodes of the phenylpropane pathway (Yin et al., 2022). Therefore, the enrichment of these pathways may be related to the reutilization of host-ingested carbohydrates by gut microbes. This implies that the intake of macronutrients exceeds the digestibility during seasons when foods were abundant. The macronutrients escape primary digestion and become a substrate for microbial metabolism to produce fermentation by-products and affect host physiological health.

The results of WGCNA analysis indicate that the green module has the highest correlation with fat, protein, and carbohydrate, and it also has the most complex network relationships. Twenty out of 22 candidate members of hub OTUs belong to *Clostridiales* and two belong to *Bacteroidales*. A total of five families have been annotated, namely *Ruminococcaceae*, *Lachnospiraceae*, *Muribaculaceae*, *Peptococcaceae*, and *family_XIII*. Among them, candidate members of *Ruminococcaceae* (OTU_472, OTU_81, OTU_339, OTU_570, and OTU_598) show consistent inter-seasonal trends with the energy provided by carbohydrates in natural foods in terms of their abundance, this indicates that these OTUs also have the lowest abundance in spring when carbohydrate intake was lowest. Previous studies have proved that *Ruminococcaceae* can degrade a variety of polysaccharides and dietary fibers. They are also the producers of short-chain fatty acids (SCFAs) (Scott et al., 2008; Louis and Flint, 2009; Hooda et al., 2012). Our assumptions about the relationship between nutrients and gut microbes are consistent with these findings. OTU_472, OTU_2009, OTU_226, OTU_81, OTU_67, and OTU_349 have largest fluctuation with changes in nutrients intakes. These 6 hub OTUs were annotated to *Ruminococcaceae*, *Lachnospiraceae*, and *Muribaculaceae*. They were at the core of the green module and largely affect the network structure of the co-occurrence bacterial taxa network pf the green module. This could be used as an important indicator to assess the gut nutrient absorption of the golden snub-nosed monkeys.

4.3. The evolution of host adaptation

Based on the present finding that macronutrients are responsible for the changes in golden snub-nosed monkeys' gut microbiota, we consider this is

an important mechanism that helps them survive and increase fitness. This can be inferred from the great number of gut microbes and metabolic pathways annotated in the study. In our study, the gut microbiotas of golden snub-nosed monkeys were annotated to 38 phyla, 140 orders, 352 genera, and 395 metabolic pathways. We refer to previous studies that found golden snub-nosed monkeys have more types of gut microbes and metabolic pathways compared to mammals with relatively homogeneous or food-specific diets such as red pandas (*Ailurus fulgens*) (Kong et al., 2014), koalas (*Phascolarctos cinereus*) (Barker et al., 2013), amur tigers (*Panthera tigris altaica*) (Ning et al., 2020), and musk deers (*Moschus chrysogaster*) (Sun et al., 2019). This is unlikely due to the sequencing depth because the Good's coverage of each bacterial community was >97%. Golden snub-nosed monkeys rely on microbiota functions to obtain sufficient nutrients from foods to cope with the harsh living conditions and variable food types (Liu et al., 2022). We infer that the gut microbiota of golden snub-nosed monkeys has gradually become more diverse and complex during their co-evolution with their hosts to stabilize the host nutrient intakes under seasonal shifts of the diet. The gut microbiota helps the host adapt to broader dietary by enabling the host to digest multiple food types and obtain sufficient nutrients to meet its survival needs.

5. Conclusion

Golden snub-nosed monkeys exist significant difference in food consumed and nutrients intake among seasons that the three macronutrients intake showed a consistent trend that they are higher in summer and autumn and lower in spring and winter. We found seasonal dietary differences caused the macronutrient variation is the main reason for seasonal shifts of gut microbiota. Particularly, phyla *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* are significantly dominant in all samples, but the ratio of Firmicutes to Bacteroidetes was correspondingly highest in spring when nutrient intakes were lowest per metabolic body weight. The dominant genera also showed the same seasonal trends: Methanogens and *Ruminococcus*, which promote nutrient intake efficiency increased in spring when nutrient intakes were lowest. In autumn when high-carbohydrate and high-fat diets were consumed, *Prevotella* that digest complex polysaccharides had a high abundance. These results demonstrated that gut microbes through microbial metabolic functions help the host to compensate for the insufficient macronutrients intake.

Data availability statement

The data presented in the study are deposited in the https://www.ncbi.nlm.nih.gov/bioproject/929077, repository accession number PRJNA929077.

Ethics statement

The animal study was reviewed and approved by the Ethics Committee of Northwest University.

Author contributions

SG conceived the project idea and designed the study. HF and SH collected the samples and performed the experiments. YL, YY, and ZW performed the bioinformatic analysis of the sequencing data. YL and YY wrote the manuscript and analyzed and interpreted the data. YL, YY, SJ, and SG edited the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

Author GD was employed by Guangdong Chimelong Group Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1126189/full#supplementary-material

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The koala gut microbiome is largely unaffected by host translocation but rather influences host diet

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Introduction: Translocation is a valuable and increasingly used strategy for the management of both threatened and overabundant wildlife populations. However, in some instances the translocated animals fail to thrive. Differences in diet between the source and destination areas may contribute to poor translocation outcomes, which could conceivably be exacerbated if the animals' microbiomes are unsuited to the new diet and cannot adapt.

Methods: In this study we tracked how the faecal microbiome of a specialist *Eucalyptus* folivore, the koala (*Phascolarctos cinereus*), changed over the course of a year after translocation. We assessed microbiome composition by 16S rRNA amplicon sequencing of faecal pellets.

Results: We found no significant overall changes in the faecal microbiomes of koalas post-translocation ($n = 17$) in terms of microbial richness, diversity or composition when compared to the faecal microbiomes of koalas from an untranslocated control group ($n = 12$). This was despite the translocated koalas feeding on a greater variety of *Eucalyptus* species after translocation. Furthermore, while differences between koalas accounted for half of the microbiome variation, estimated diets at the time of sampling only accounted for 5% of the variation in the koala microbiomes between sampling periods. By contrast, we observed that the composition of koala faecal microbiomes at the time of translocation accounted for 37% of between koala variation in post-translocation diet. We also observed that translocated koalas lost body condition during the first month post-translocation and that the composition of the koalas' initial microbiomes were associated with the magnitude of that change.

Discussion: These findings suggest that the koala gut microbiome was largely unaffected by dietary change and support previous findings suggesting that the koala gut microbiome influences the tree species chosen for feeding. They further indicate that future research is needed to establish whether the koalas' gut microbiomes are directly influencing their health and condition or whether aspects of the koala gut microbiomes are an indicator of underlying physiological differences or pathologies. Our study provides insights into how animal microbiomes may not always be affected by the extreme upheaval of translocation and highlights that responses may be host species-specific. We also provide recommendations to improve the success of koala translocations in the future.

KEYWORDS

microbial community, body condition, *Phascolarctos cinereus*, marsupial, eucalypt, hindgut fermenter

Introduction

Anthropogenic habitat loss, resource decline, climate change and ecological imbalances such as overpopulation threaten many mammal populations. One practical strategy available to environmental managers to promptly alleviate these threats is to translocate animals (Fischer and Lindenmayer, 2000; Menkhorst et al., 2019). However, in some instances the translocated animals fail to thrive and have higher rates of mortality than those left *in situ* (Fischer and Lindenmayer, 2000; Whisson et al., 2012; Menkhorst, 2017). These higher rates of mortality may in part be due to factors such as stress from capture and transportation, a lack of familiarity with the location of local resources such as denning sites, inexperience with local predators, a loss of territory and social structure and competition with resident conspecifics (Fischer and Lindenmayer, 2000; Massei et al., 2010). There are also a range of other, less studied, reasons for high rates of mortality in translocated animals including differences between the source and destination habitats. For instance, if the diet available to the animals at the destination location is different from that at the source, then translocated animals may have reduced fitness post translocation. This is especially likely to be true for specialist herbivores that rely on their gut microbiome to digest and detoxify otherwise unpalatable material, particularly if the animals' microbiomes are unsuited to the new diet and cannot rapidly adapt (Kohl et al., 2014). Alternatively, in instances where a range of food types are available in the destination habitat and the gut microbiome of the translocated species has been shown to influence diet (Blyton et al., 2019), it is conceivable that the hosts' microbiome may contribute to diet selection and habitat use post-translocation. In this study we track how the fecal microbiome of a specialist arboreal folivore, the koala (*Phascolarctos cinereus*), changes over the course of a year post translocation and investigate the associations between fecal microbiome composition, diet and host post-translocation condition.

Koalas are listed as endangered in the Australian states of Queensland and New South Wales as well as the Australian Capital Territory (Department of Agriculture, W.a.t.E., Australian Federal Government, 2022), where many populations are in decline. By contrast, in several areas of the southern Australian states of Victoria and South Australia, koalas can reach unsustainable population densities, resulting in the over-browsing of preferred food tree species, leading to habitat destruction and starvation (Martin, 1985a, 1985b, 1985c; Menkhorst, 2008; Whisson et al., 2016). In response, translocations of koalas away from high density populations into apparently suitable habitat with low koala densities plays a role in the ongoing management of koalas in these states (Menkhorst, 2008). Many of these translocations have been successful with high survival rates, while others where the destination habitat patch is small or located in agricultural land have had high rates of mortality (Whisson et al., 2012; Menkhorst, 2017). Koalas are also translocated throughout Australia to remove them from danger (unsuitable habitat) or where their habitat has been lost (Phillips, 2017). Further, translocations and breed-to-release programs are being developed to establish and expand threatened koala populations in north-eastern Australia.

Koalas are dietary specialists, feeding almost exclusively on eucalypt leaves, predominately from the genus *Eucalyptus* (Shipley et al., 2009). Koalas in different geographic areas feed on different species in part due to local availability. Additionally, individual koalas within populations can show preferences for different food tree species (Moore and Foley, 2000; Brice et al., 2019; Marsh et al., 2021) and koalas feeding on different species of *Eucalyptus* have functionally and compositionally different microbiomes (Brice et al., 2019). The koala gut microbiome also appears to influence what species of eucalypt the host can consume. This was tested in an experiment where koalas that primarily fed on *Eucalyptus viminalis* in the wild were inoculated with fecal material from donor koalas that fed primarily on *Eucalyptus obliqua*. The microbiomes of treatment koalas changed to resemble those of the donor koalas while those of control koalas did not, and the degree of change was associated with the amount of *E. obliqua* that koalas subsequently ate (Blyton et al., 2019). Thus, in the case of translocations, if the food tree species present at the destination habitat differ from those in the source habitat, then koala gut microbiomes may reduce the likelihood of good health and survival by limiting their hosts' ability to feed and obtain nourishment from the new potential food species, unless the gut microbiomes can change and adapt to the new diet.

Across a range of other species with varied gastrointestinal anatomies and diets, including Tasmanian devils, Pere David's deer, giant pandas and Atlantic salmon, the fecal microbiomes of individuals translocated between geographic areas, or released from captivity, change to resemble those of resident animals at the destination site (Chong et al., 2019; Wang et al., 2019; Yao et al., 2019; Uren Webster et al., 2020). However, koalas are unusual as they inherit their gut microbiomes maternally by feeding on pap (special maternal feces that has a higher microbial density than normal feces and a higher abundance of rare taxa) around the time of pouch emergence (Osawa et al., 1993; Blyton et al., 2022a, 2022b). Wild adult koalas have temporally stable fecal microbiomes over the course of several months when they are left in their established home ranges, despite some variation in the food tree species eaten (Eisenhofer et al., 2022; Blyton et al., 2023). Additionally, in 2013 koalas in Cape Otway, Victoria, that fed on *E. viminalis* starved to death when that species was defoliated due to overbrowsing rather than feeding on the readily available *E. obliqua* (Whisson et al., 2016). We subsequently showed that the fecal microbiomes of koalas that fed on *E. viminalis* in the wild did not change when they were experimentally encouraged to eat some *E. obliqua* in captivity, unless fecal inoculations were provided (Blyton et al., 2019). This suggests that the koala gut microbiome may not readily change after host translocation even when the microbiome is unsuited to the diet available.

In this study we assessed: (1) whether the composition of koala fecal microbiomes shifted after translocation in comparison to those of control koalas, captured but released immediately at the site of

capture at Cape Otway, Victoria; (2) whether the fecal microbiomes of the koalas prior to translocation predicted their post-translocation body condition (a measure of their resilience during translocation); and (3) whether the fecal microbiomes of the koalas prior to translocation predicted their diets in the destination habitat, to further examine the role of the koala gut microbiome in diet selection.

Methods

Study design and sampling

In September 2015, 60 koalas were included in a trial translocation study conducted by the Victorian State Government Department of Environment, Land, Water and Planning (Menkhorst et al., 2019). The aim of the study was to assess the health and survival outcomes for koalas after they were translocated into mixed eucalypt forest from an area exhibiting severe overbrowsing and koala starvation (Whisson et al., 2016). All koalas were captured using a standard noose and flag technique (Madani et al., 2020) from herb-rich woodlands canopied by manna gum (*Eucalyptus viminalis*) and messmate (*Eucalyptus obliqua*) at Cape Otway, Victoria, Australia (38.825°S, 143.525°E). The koalas were assessed by veterinarians and only healthy adult individuals were included in the study (see Menkhorst et al., 2019 for selection criteria and veterinary procedures). All females received a subcutaneous contraceptive implant containing levonorgestrel (a synthetic progesterone) to ameliorate the risk of overabundance at the release site (Middleton et al., 2003; Hynes et al., 2010). The koalas were fitted with collar-mounted VHF radio transmitters. Twenty-four koalas were assigned to the control group and were released at the point of capture. The remaining 36 koalas were translocated a distance of approximately 90 kilometers northeast and released into coastal mixed eucalypt forest near Aireys Inlet, Victoria. All koalas were radio tracked on a regular basis to determine their location and survival status. The koalas were re-captured 1 month and 5 months after their initial capture to assess their health and condition, including weight and head length. All VHF collars were to be removed 1 year post translocation, however, only 9 of the koalas included in this study (control: males = 3, females = 2; Translocated: males = 2, females = 2) were located and caught at that time. This was because the VHF signals from the collars were no longer being transmitted due to premature failure of the collars' batteries.

To assess how the koalas' gut microbiomes and diet changed after translocation, we collected as many fecal pellets (range:1–70) as available from each translocated and control koala located during each sampling period. The sampling periods were: (1) when the koalas were first captured (day 0, number of koalas = 29); (2) within the first 2 days post-translocation (days 1–2, koalas = 17); (3) 1 week post-translocation (days 6–8, koalas = 14); (4) 2 weeks post-translocation (days 14–16, koalas = 11); (5) 1 month post-translocation (days 25–36, koalas = 16); (6) 2 months post-translocation (days 62–70, koalas = 14); (7) 5 months post-translocation (days 129–161, koalas = 26); (8) 9 months post-translocation (days 253–281, koalas = 15; these samples were only used for diet analysis); and (9) 1 year post-translocation, at the conclusion of the study (days 345–356, koalas = 9 with one koala sampled on two occasions during this period). Fecal pellets were either collected opportunistically during koala captures or from plastic mats placed beneath radio-tracked koalas. Pellets were generally frozen and then stored at –20°C within 2 h of excretion and all were frozen within 10 h. It was not always possible to collect samples during each sampling period/time point for an animal as they often did not produce any pellets while researchers were present. Only animals for which an initial sample and at least one post-translocation sample were collected were included in our analyses. Following these criteria we included 17 translocated (female = 8, male = 9) and 12 control koalas (female = 6, male = 6) in our study. On average, samples were available from 4.7 time points per koala.

Microbiome characterization and bioinformatics

We determined the microbiome composition of 137 fecal samples (29 koalas sampled on between 2 and 8 occasions across time points) using a culture-independent DNA based approach. For each fecal sample, total genomic DNA was extracted between May and November 2016 from approximately 50–70 mg of fecal material. The material was taken from the center of a single fecal pellet to avoid any surface contamination. The material was beaten for 5 min at 2,000 rpm using the MoBio PowerLyzer24 in a MoBio bead tube containing 0.1 mm dia. Zirconian/silica beads and 750ul of TLA buffer (Promega). The samples were centrifuged at 10,000 g for 30 s. DNA was then extracted from 150 µl of the supernatant using the Maxwell 16 robotic system and corresponding Tissue DNA kit (Promega) following the manufacturer's instructions. Negative controls were included for each extraction kit.

A 589 bp section of the 16 s rRNA gene (V6 – V8 region) was amplified using 803F (5'-TTAGANACCCNNGTAGTC) and 1392R primers (5'-TTAGANACCCNNGTAGTC, Engelbrektson et al., 2010) from the DNA extracts following the workflow outlined by Illumina (#15044223 Rev.B) except that Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs) was used. PCR products were indexed with unique 8 bp barcodes using the Illumina Nextera XT 384 sample Index Kit A-D (Illumina FC-131-1,002). Indexed amplicons were isolated using Qiagen QIAquick Gel Extraction Kit, as per manufacturer's instructions. Paired-end sequencing was performed at the Australian Centre for Ecogenomics, on the Illumina Miseq using the version 3 reagent kit for 300 cycles within 6 months of DNA extraction. The raw sequencing data from the study can be obtained from the NCBI SRA database, BioProject accession PRJNA901215.

Raw reads were trimmed to remove primer sequences using Cutadapt (Martin, 2011), and quality trimmed to remove poor quality sequence using a sliding window of 4 bases with an average base quality above 15 using the software Trimmomatic (Bolger et al., 2014). All reads were then hard trimmed to 250 bases, and any with less than 250 bases excluded. Trimmed reads were then processed and assigned taxonomic designations by QIIME 2 with default parameters (v.2017.10; Bolyen et al., 2019) using the SILVA 128 database (Quast et al., 2012; Yilmaz et al., 2013). The resulting microbial feature-by-sample table was rarefied to 10,000 reads per sample using the vegan package, version 2.6–2 (Oksanen et al., 2022) in R (version 3.5.0; R Core Team, 2012). All community composition analyses were performed on the rarefied dataset. Microbiome richness was estimated by a count of unique features recovered per sample after rarefaction and by calculating the Chao Index of alpha diversity using the package fossil in R (Vavrek, 2011). Microbiome

diversity for each koala at each timepoint was estimated using the Shannon diversity index as calculated using the vegan package. Weighted and unweighted Unifrac distances (Lozupone and Knight, 2005; Lozupone et al., 2007) between samples were calculated in QIIME 2 from a filtered rarefied sample-by-features table where only features with greater than 10 reads in at least one sample were included.

Diet determination

Diet composition for the translocated koalas was characterized by amplifying and sequencing dietary DNA fragments containing species-specific SNPs from pooled DNA extractions of two fecal pellets (when available) from each of the fecal samples for which microbiome assessment was performed. This was done using the DarTag platform at Diversity Arrays Technologies (Blyton et al., 2023). Further information on the diet analysis method and details of the determination of diet composition for the koalas included in this study is provided in Blyton et al. (2023). To provide an overall assessment of the diet of each koala, the proportions of each of the food tree species were averaged across all samples collected after 1 week post translocation. Samples collected within the first week post translocation were excluded from this assessment as the koalas' diets may have been heavily influenced by their release location. Koalas from the control group were not included in this analysis.

Statistical analysis

Microbiome change in response to host translocation

To assess if the koalas' fecal microbiomes changed in response to translocation, linear mixed effects models were fitted using the lme4 package in R (Bates et al., 2015) with statistical significance calculated using the lmerTest package (Kuznetsova et al., 2013). To assess if microbial richness or diversity changed in response to translocation, the counts of the microbial features for each koala at each sampling time point, the Chao1 Index of alpha diversity and the Shannon diversity index were fitted as response variables. To assess if the composition of the koala fecal microbiomes changed in response to translocation, the unweighted and weighted unifrac distances between the post translocation samples and the day 0 samples for each koala were fitted as response variables and whether the koalas were translocated included as a fixed explanatory variable. Three alternative temporal explanatory variables were considered along with translocation status in separate models. Firstly, the sampling time point was fitted as a factor to determine if there were any particular time points that were distinct. Secondly, the number of days since translocation was fitted as a continuous variable to model a gradual change in microbiome composition. Thirdly, log10 (number of days since translocation) was fitted as a continuous explanatory variable to account for the possibility that the fecal microbiomes had shifted rapidly after translocation and then stabilized. The interaction of Treatment × timepoint or interval since translocation was also included, while koala identity was included as a random effect to account for the repeated measures sampling design. Backward elimination of non-significant terms was performed. The assumption of normality of the model residuals was assessed by the Shapiro–Wilk test and where necessary the response variable rank-transformed to meet this assumption.

To assess whether the microbiomes of translocated koalas were more variable over time than those of control koalas, average unweighted and weighted unifrac distances between sampling time points were calculated for each koala. Linear models were then fitted to the rank-transformed average unifrac distances, with translocation status as the explanatory variable.

To assess whether the relative abundance of particular microbial features increased or decreased in the translocated or control koalas over time we fitted betabionomial mixed effect regression models using the glmmTMB package v.1.1.4 in R Core Team (2012). All features that were detected on at least 2 occasions within three koalas in each of the translocated and control groups of koalas were assessed ($n = 132$). The explanatory variables in the analysis were the number of days since translocation or log10 (number of days since translocation) and whether the koalas were translocated. The interaction of treatment x interval since translocation was also included, while koala identity was included as a random effect. Significance was determined from Bonferroni corrected p-values for multiple comparisons.

Effect of the gut microbiome on host body condition

Koala body condition was calculated from the residuals of a linear regression of koala head length against body weight according to the method of McLean (2003). Linear regression models were then fitted to the change in body condition over the first month post-translocation. The coordinates of the koalas' initial microbiomes on the first 5 dimensions generated from the PCoAs of (1) weighted and (2) unweighted unifrac distances were fitted as explanatory variables along with (3) the Shannon Indices of the initial microbiomes; (4) the Chao Index of the initial microbiomes; (5) the proportions of each food tree species in the koalas' diets averaged over time points; and (6) the first 2 principal coordinates of the koalas' diets. Each set of explanatory variables (1–6) were fitted in separate models with backwards elimination used within each set to select significant predictors where appropriate.

Effect of the gut microbiome on diet

To assess if the composition of the koalas' microbiomes influenced diet selection post-translocation, Redundancy Analysis was performed on the Bray-Curtis distances between the koalas' overall (averaged post-translocation) diets using the vegan package in R. The explanatory variables in the analysis included all principal components generated from the weighted and unweighted unifrac distances between the translocated koalas' microbiomes on day 0 as well as the number of microbial features for each koala on day 0, the Chao1 Index of alpha diversity and the Shannon diversity index of the koalas' microbiomes on day 0. Forward and backward selection was used to select significant explanatory variables based on adjusted p-values.

To assess if the diversity of the koalas' microbiomes influenced the diversity of species from which they fed in the destination habitat, linear regression models were fitted in R. The response variables were the number of tree species present in the diet and the Shannon index of dietary diversity. The explanatory variables were the Chao1 Index of alpha diversity and the Shannon diversity index of the koalas' microbiomes on day 0.

Association between diet and microbiome variation

To assess if variation in the diets of translocated koalas was associated with temporal variation in fecal microbiomes, Redundancy Analysis was performed on the Bray-Curtis distances between koala microbiomes at all time points generated from the square root transformed proportions of the microbial features. The explanatory variables in the analysis included the proportion of each food tree species at each time point as well as the principal coordinates generated from the unscaled food tree species proportions. Koala ID was included as a covariate in this analysis to account for interindividual differences in fecal microbiome composition (Eisenhofer et al., 2022). Forward and backward selection was used to select significant explanatory variables based on adjusted p-values.

Results

Microbiome change in response to host translocation

The richness and diversity of the koalas' fecal microbiomes did not significantly differ between the translocated and untranslocated koala groups (Table 1). Nor did richness or diversity change in a consistent manner over time in either the translocated or control koala groups, whether the sampling time points were considered as discrete factors or as a continuous variable (Figures 1A,B).

Microbial community composition did not change in response to translocation when measured by weighted or unweighted unifrac distances, as there was no significant interaction between translocation status and any of the temporal measures (Table 1). Nor were the post-translocation microbiomes of the translocated koalas more dissimilar to their initial compositions than for control *in situ* koalas. There was no consistent temporal shift in the composition of the fecal microbiomes of either the translocated or control koalas (Figures 1C–E).

The microbiomes of the translocated koalas did not vary more between time points than those of the control koalas based on the unweighted or weighted unifrac distances between time points for each koala (unweighted unifrac: $p = 0.57$; weighted unifrac: $p = 0.17$).

Of the 132 prevalent features assessed (see methods), only 10 changed in relative abundance over the study, with a similar number of changes seen in each treatment group. Four features increased and two decreased in both the translocated and control koalas (rapid increase (log(Days)): 1 from phylum Synergistetes, genus *Cloacibacillus* and 2 unassigned shown in Figure 2; increase (Days): *Butyricicoccus* sp.; rapid decrease (log(Days)): Dialister sp.2; decrease (Days): 1 from phylum Firmicutes, family Lachnospiraceae). Additionally, Ruminococcaceae NK4A214 decreased in both the translocated and control koalas but to a greater extent in those that were translocated. *Akkermansia* sp.1 increased rapidly in the control koalas (log(Days)), while, *Ruminoccus*-1 sp.2 rapidly decreased in the translocated koalas (log(Days); Figure 2). *Ruminiclostridium* 9 sp.3 also decreased in the translocated koalas but not the control koalas. This concurs with the findings above, suggesting that host translocation did not lead to large changes in the koalas' gut microbiomes.

Effect of the gut microbiome on host condition

The translocated koalas in this study had a similar mortality rate to the control group, although, an initial drop in body condition was observed during the first month after translocation, followed by a recovery of condition by 5 months post translocation (Figure 3; Menkhorst et al., 2019). Therefore, we assessed whether the fecal microbiomes of the koalas prior to translocation affected their change in body condition 1 month post translocation.

Dimension 2 of the PCoA generated from the weighted unifrac distances among the translocated koalas' initial microbiomes was a marginally significant predictor of their change in body condition over

TABLE 1 *p*-values from linear mixed effects models of the change in fecal microbiome richness, diversity, and composition in response to host translocation.

Response	Translocation status (TS)[1,2]	Time point (TP)[3]	TS×TP	Day[4]	TS×Day	Log(Day) (LD)[5]	TS×LD
Rank Feature count[6]	0.52	0.72	0.24	>0.99	0.46	0.68	0.11
Rank Chao1 Index[7]	0.58	0.76	0.29	0.91	0.56	0.67	0.14
Rank Shannon Index[8]	0.94	0.61	0.28	0.93	0.69	0.56	0.25
Weighted Unifrac[9,10]	0.15	0.87	0.72	0.61	0.78	0.82	0.23
Unweighted Unifrac[10,11]	0.23	0.44	0.12	0.49	0.44	0.90	0.06

1. Whether each koala was translocated or part of the control group of koalas left in the source habitat
2. Type III p-values given for models including an interaction term with Time point. Backwards elimination was performed for each model to confirm the non-significance of all explanatory variables
3. Samples were grouped into time points that were fitted as a factor
4. Days since translocation were fitted as a continuous explanatory variable
5. Days since translocation were log base 10 transformed and fitted as a continuous explanatory variable
6. Rank Feature count = a measure of microbiome richness and is a count of the number of microbial features/sequence variants present in a sample, which is then rank transformed so that the model confirms to the assumption of normal residuals.
7. Rank Chao1 Index = a measure of microbiome richness that estimates the number of microbial sequence variants present in a sample, correcting for undetected variants, which is then rank transformed so that the model confirms to the assumption of normal residuals.
8. Rank Shannon Index = a measure of microbiome diversity, which is then rank transformed so that the model confirms to the assumption of normal residuals.
9. Weighted Unifrac = a measure of how different two microbiomes are that is calculated based on the phylogenetic branch lengths between the taxa in each sample, weighted by the relative abundance of those taxa. In this analysis distances were calculated between each sampling period and the day 0 sample for each koala
10. two outliers were removed from this analysis to meet the normality of residuals assumption.
11. Unweighted Unifrac = a measure of how different two microbiomes are that is calculated based on the phylogenetic branch lengths between the taxa in each sample. In this analysis distances were calculated between each sampling period and the day 0 sample for each koala

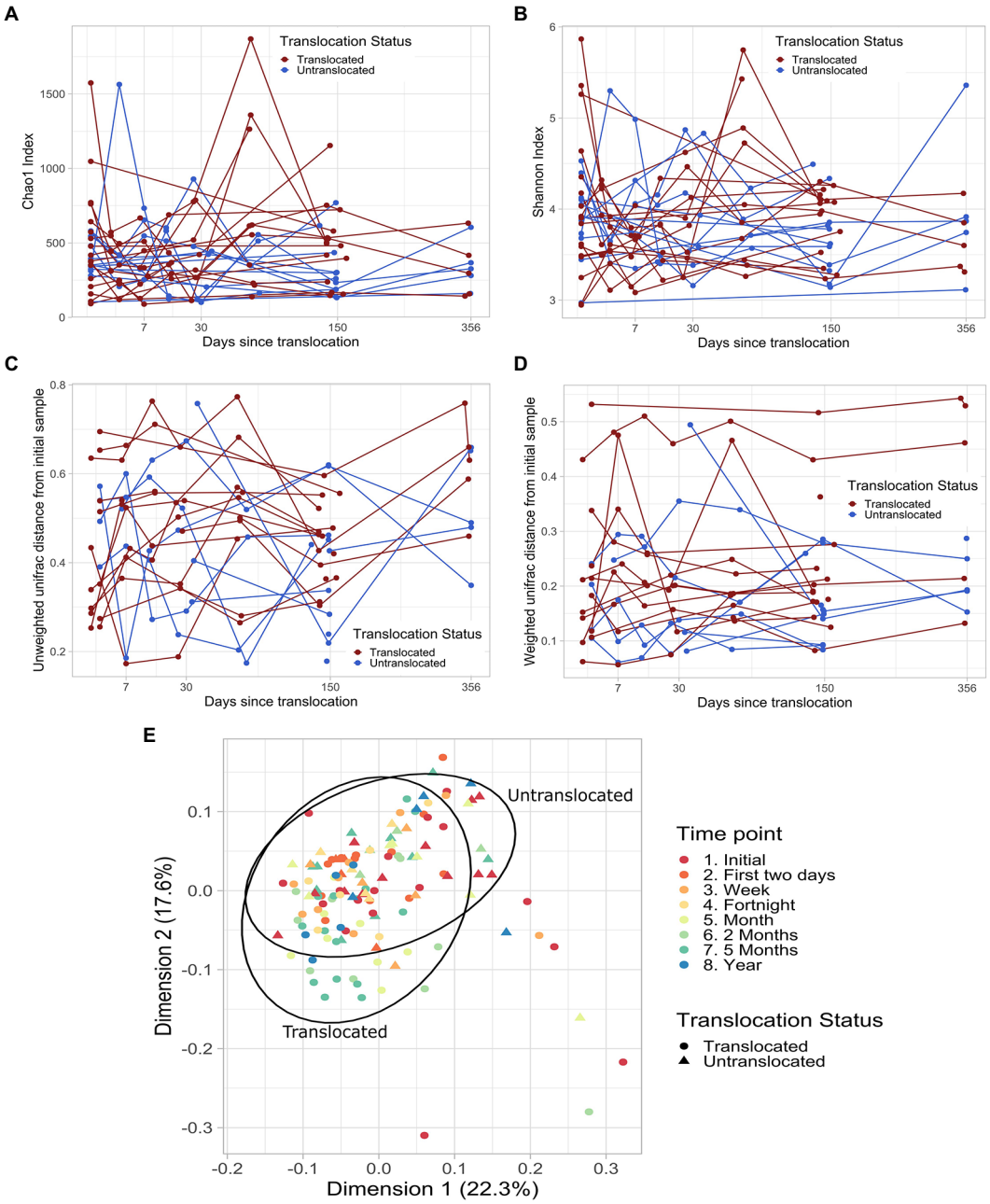

FIGURE 1
Fecal microbiome richness **(A)**, diversity **(B)** and composition **(C** and **D)** over time in individual translocated and untranslated koalas. **(A)** Chao1 diversity with days since translocation. Lines join sampling time points for the same koala. **(B)** Shannon diversity with days since translocation. Lines join sampling time points for the same koala. **(C)** Unweighted unifrac distance between each koala's pre-translocation sample and subsequent samples against the days since translocation. Lines join sampling time points for the same koala. **(D)** Weighted unifrac distance between each koala's pre-translocation sample and subsequent samples against the days since translocation. Lines join sampling time points for the same koala. **(E)** The first two principal components of the weighted unifrac distances for all koalas and time points in this study. 95% ellipses calculated assuming a multivariate t-distribution are shown for samples from translocated and untranslated koalas.

the first month post-translocation ($R^2=0.327$, $t=2.31$, $p=0.041$; Figure 4). As the microbiome dimensions were generated from the weighted unifrac distances they cannot be directly related to any particular microbial taxon. However, the relative abundance of 27 of the 132 abundant microbial features had a greater than 20% positive correlation with Dimension 2 (Supplementary Table S1). These microbial features spanned a range of taxa including the phyla Euryarchaeota, Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Planctomycetes and Proteobacteria with no strong phylogenetic signal observable. None of the other dimensions from the PCoAs of the unweighted and weighted distances among the koalas' initial microbiomes nor initial microbiome richness or diversity were significant predictors of the change in body condition over the first month post-translocation.

No measures of diet composition post translocation were significant predictors of the change in body condition over the first month post translocation.

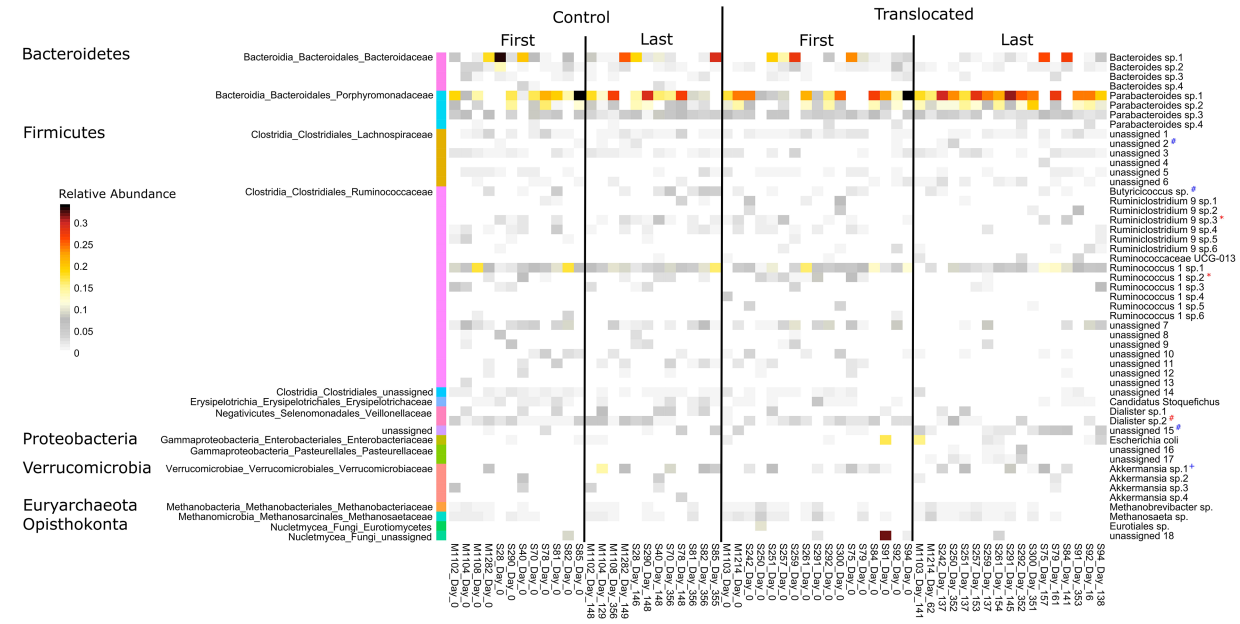

FIGURE 2
Heatmap showing the relative abundance of microbial features found at greater than 3% relative abundance in at least one sample for control (untranslocated) and translocated koalas prior to translocation (day 0) and on the last occasion where they were sampled. Species designations for the features are shown on the right while higher level taxonomy is shown on the left. # indicates features that significantly changed in relative abundance in both translocated and control koalas as identified by betabionomial mixed effects regression analysis. * indicates features that significantly changed in relative abundance only in the translocated koalas, while + indicates those that significantly changed only in control koalas. Blue symbols (#,+,*) indicate those features that significantly increased in relative abundance, while red symbols denote those that significantly decreased as identified by betabionomial mixed effects regression analysis.

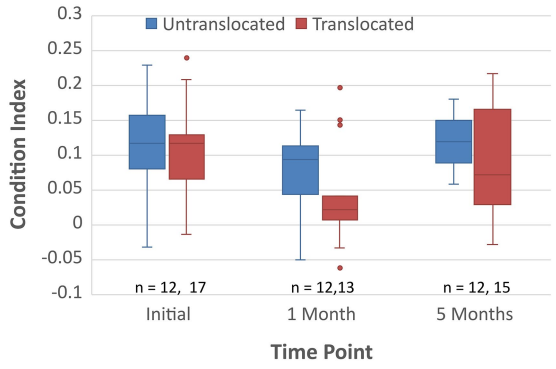

FIGURE 3
Body condition as determined by the method of McLean (2003) for the translocated and untranslocated koalas upon initial capture, 1month post translocation and 5months post translocation.

Effect of the gut microbiome on dietary choice

The diets of the translocated koalas were assessed prior to and after translocation from fecal pellets using a newly developed panel of species-specific SNPs (Blyton et al., 2023). This analysis revealed that the koalas' diets rapidly changed after translocation (Figure 5). Pre-translocation, *E. viminalis* dominated the diets of the majority of koalas, while *E. obliqua* was also eaten by half the koalas. After translocation, the koala diets became more species-rich and variable among individuals with *Eucalyptus globulus*, *Eucalyptus cypellocarpa*, and *Eucalyptus radiata* most often the dominant components.

Redundancy analysis revealed that the microbiomes of koalas prior to translocation (day 0) influenced what species of eucalypt they ate in the new habitat. Both dimensions 3 and 5 of the PCoA generated from the unweighted unifrac distances of the translocated koalas' initial microbiomes, were significant predictors of the post-translocation diets of koalas (Dimension 3: $F=2.72$, $p=0.03$; Dimension 5: $F=2.61$, $p=0.03$). Dimension 3 was associated with diets containing a high proportion of *E. globulus* and Dimension 5 was associated with diets containing a high proportion of *Eucalyptus falciformis/E. radiata* and to a lesser extent *Eucalyptus aromaphloia/E. viminalis* (Figure 6). Together, these two dimensions of microbiome variation accounted for 37.1% of the variation in diet. As the microbiome dimensions were generated from the unweighted unifrac distances they cannot be directly related to specific microbial taxa. However, there was a negative correlation between the number of microbial features belonging to the families Bacteroidaceae (range: 3–10 features; $R^2=0.129$) and Veillonellaceae (range: 0–4 features; $R^2=0.445$) and Dimension 3. The number of microbial features belonging to the families Lachnospiraceae (range: 9–42 features; $R^2=0.210$), Rikenellaceae (range: 1–3 features; $R^2=0.292$) and Ruminococcaceae (range: 6–23 features; $R^2=0.127$) were negatively corelated with Dimension 5.

There was no association between the diversity of the koalas' microbiomes at the time of translocation and the diversity of their diets in the destination habitat; either in terms of the number of tree species eaten (Chao1 index: $p=0.32$; Shannon microbial diversity index: $p=0.31$) or Shannon diet diversity index (Chao1 index: $p=0.48$; Shannon microbial diversity index: $p=0.29$).

Association between diet and microbiome variation

Although there was not a consistent directional change in the fecal microbiomes of translocated koalas in this study, variation in microbiome composition was nonetheless observed for individual koalas between sampling time points. Therefore, in an explorative analysis we investigated whether temporal variation in the microbiomes of translocated koalas could in part be attributed to changes in the diets of these animals. Redundancy analysis revealed that the proportion of *E. obliqua* ($p = 0.03$) and *E. falciformis/E. radiata* ($p = 0.04$) eaten at a particular time point was associated with the relative abundances of the microbial features in the koalas' feces at that time (Figure 7). However, while differences between koalas accounted for 52.9% of the microbiome variation, the proportion of *E. obliqua* and *E. radiata* together only accounted for 5% of the microbiome variation.

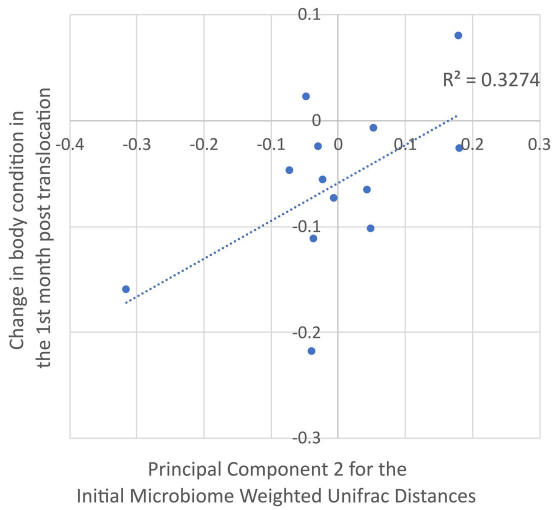

FIGURE 4
Correlation between dimension 2 of the principal components analysis of the translocated koalas' weighted unifrac distances for their initial microbiomes (explaining 29.8% of the variation) and the change in their body condition over the first month post translocation.

Discussion

Across a range of animal species, including humans, the gut microbiome has been found to change and adapt to variation in host diet (Hungate, 1966; David et al., 2014; Xu and Knight, 2015; Barron Pastor and Gordon, 2016). However, in hindgut fermenting herbivores such as the koala where much of the nutritious portion of the diet is absorbed in the small intestine prior to entering the caecum (Cork et al., 1999), the effect of diet on the hindgut and fecal microbiome may be more restricted. In this study the fecal microbiomes of translocated koalas did not change compared to those of koalas that remained in their original habitat. This was despite diversification of the translocated koalas' diets and a change in the species of *Eucalyptus* eaten (Blyton et al., 2023). One explanation for the observed stability of the koala microbiome could be that the change in diet may have represented only a subtle shift in the nutritional composition of the koalas' diets when compared with the large changes examined in other species. However, this explanation is unlikely, as we and others have previously shown that koala food tree species can differ markedly in their nutritional (Petrović, 2014; Brice et al., 2019) and plant secondary metabolite composition (Moore et al., 2005; Marsh et al., 2019). Furthermore, multiple *Eucalyptus* subgenera were eaten by koalas in this study and compositional differences among food tree species are most apparent between species in different subgenera. In this instance, the variation in diet between individuals after translocation and the finding that the koalas' pre-translocation microbiomes were associated with post translocation diet may indicate that the koalas were able to find diets in the destination habitat that suited their existing microbiomes, limiting the extent to which adaptation of their microbiomes were necessary. However, in our previous work, the gut microbiomes of koalas were found to be unaffected by an experimentally induced change in diet to which their microbiomes were not adapted (Blyton et al., 2019). Therefore, the most credible explanation for the lack of change in the microbiomes of the

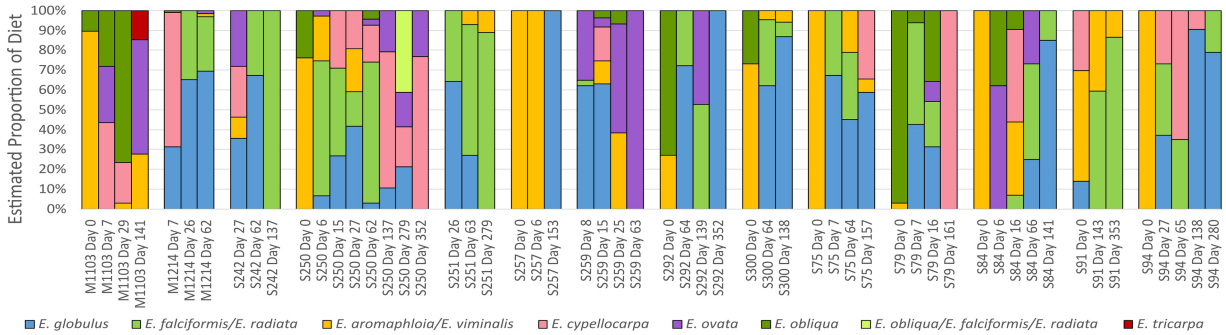

FIGURE 5
Diet composition for the translocated koalas as determined by selectively amplifying and sequencing species-specific SNPs from dietary food tree species using the DarTag platform (Diversity Arrays Technologies). The SNPs were amplified from DNA extracted from faecal pellets in the same collections that were used for microbiome assessment in this study. Day 0 samples were collected from the koalas prior to translocation, while all other samples were collected at the indicated days after translocation. S and M numbers correspond to individual koala IDs. Figure reproduced from (Blyton et al., 2023).

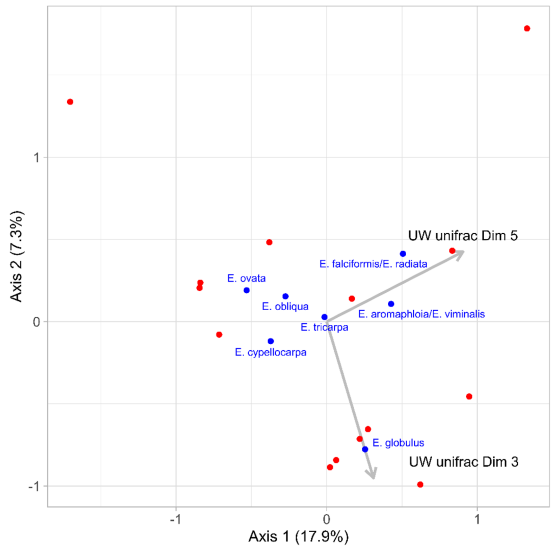

FIGURE 6
Constrained dimensions from the redundancy analysis of the translocated koalas' diets post-translocation (red points). Dimensions 3 and 5 from the principal components analysis of the unweighted unifrac distances of the koalas' initial microbiomes were significant predictors of the koalas' post translocation diets. The loadings for Dimensions 3 and 5 are illustrated by the gray arrows. The loadings of the dietary food tree species are indicated by the blue points and font.

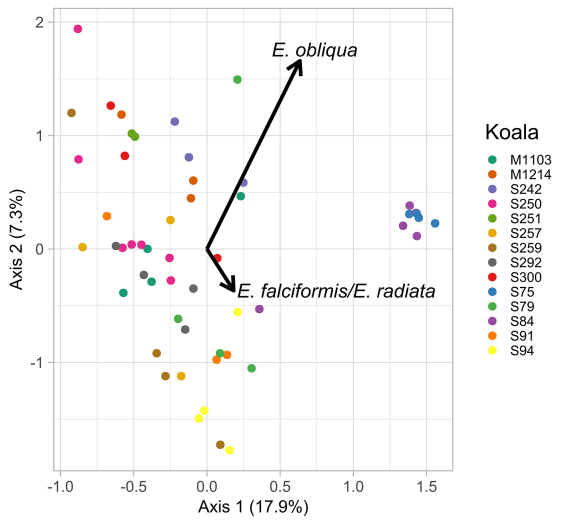

FIGURE 7
Constrained dimensions one and two from the redundancy analysis of the translocated koalas' faecal microbiomes at each sampling time point (colored points). The proportions of *E. obliqua* and *E. radiata* detected in the samples at each time point were significant predictors of microbiome composition and their loadings are illustrated by the black text and arrows.

translocated koalas is that they had a limited ability to respond and adapt to dietary change.

Koalas maternally inherit their gut microbiomes through the ingestion of a special feces (pap) around the time of pouch emergence and prior to the joeys' transition from a milk-based diet onto a diet of *Eucalyptus* leaves (Osawa et al., 1993; Blyton et al., 2022a, 2022b). This appears to lead to varying, but individual koala microbiomes that are temporally stable (Eisenhofer et al., 2022). Eisenhofer et al. (2022) found that captive koalas sourced from different populations but fed on a similar diet maintained distinct microbiomes. In contrast, wild koalas in a single population, feeding on diverse diets, had more similar microbiomes. Indeed, in the present study we found that while temporal variation in diet led to small changes in the translocated koalas' fecal microbiomes, the majority of variation could be attributed to interindividual differences observed prior to translocation that were maintained. Further, diet was only associated with microbial community composition measures that are heavily influenced by the relative abundance of the different microbial species (weighted unifrac distances) suggesting that diet does not alter the presence/absence of microbes. These studies suggest that the gut microbiomes of koalas are primarily determined by their acquisition and development during early life, with subsequent diet having a comparatively weak effect.

While koala gut microbiomes do not readily change with alterations in diet, the fecal microbiomes of koalas with consistently different diets are distinct (Brice et al., 2019). In other species, the gut microbiome has been shown to affect diet selection *via* toxins in the hosts' diet that can be broken down by particular microbial species. For instance, Australian cattle can only eat *Leucaena leucocephala* when bacteria that degrade mimosine are introduced into their rumen (Pratchett et al., 1991; Derakhshani et al., 2016). Additionally, when the foregut pouches of woodrats (*Neotoma* spp.) are inoculated with oxalate degrading bacteria from woodrats eating juniper or oxalate-rich cactus species they are able to maintain condition on those diets, where prior to inoculation they ate little and lost weight (Kohl et al., 2014; Miller et al., 2014). Previously, we experimentally altered the microbiomes of wild caught koalas using fecal transplants and showed that koalas with greater engraftment consumed more of an unfamiliar *Eucalyptus* species that formed the dominant component of the fecal donors' diets. This suggests that the gut microbiome may also influence feeding choices of koalas (Blyton et al., 2019). However, one limitation of our earlier study was that, as a group, the koalas that received fecal transplants did not consume more of the novel *Eucalyptus* species than control koalas, reducing certainty of the causality of the observed diet-microbiome association.

The translocated koalas in this study maintained relatively stable microbiomes over time and post translocation the koalas' diets changed in response to the availability of new food tree species but with individual koalas selecting different mixes of species. Host factors, such as physiological variation between individuals in their ability to detoxify particular plant secondary metabolites (McLean and Duncan, 2006), likely influenced these dietary choices. Variation in the food tree species within the translocated koalas' home ranges also likely influenced their diets, although the translocated koalas initially moved quite large distances and those movements may have been driven in part by diet selection (Menkhorst et al., 2019). Additionally, our finding that the initial fecal microbiomes of the translocated koalas were significant predictors of the koalas' post-translocation diets, accounting for over a third of the variation in diet, provides compelling evidence that the gut microbiome does indeed influence diet selection in koalas. In our fecal transplant experiment, *E. obliqua* intake declined over the first 3 days post-introduction, which we suggested may have been due to the koalas developing an aversion to the new species through post-ingestive feedback (Provenza et al.,

1992; Lawler et al., 1999). Conceivably, the microbiomes of the koalas in this study may have influenced dietary choice in the destination habitat in a similar way. That is the koalas' gut microbiomes may have influenced post-ingestive feedback as a result of their identified effects on digestion, nutrition, and detoxification (Cork and Hume, 1983; Osawa, 1992; Osawa et al., 1995).

Although the composition of the koalas' gut microbiomes may have influenced their diet in the destination habitat, we still lack an adequate understanding of the functional significance of particular microbial taxa, on which these associations with particular eucalypt species are based. In this study, we detected the strongest microbiome associations with diets dominated by either *E. globulus* or *E. falciformis*/*E. radiata*. The genetic method used to identify the species of *Eucalyptus* eaten by the koalas in this study was not able to differentiate *E. falciformis* from *E. radiata*. However, *E. falciformis* was rare in the home ranges of the translocated koalas and it is therefore likely that the koalas were not feeding on that species and only on *E. radiata* (Blyton et al., 2023). *Eucalyptus radiata* is often avoided by koalas (Martin, 1985c) and is less digestible than *E. globulus* because it contains more fiber and higher concentrations of tannins (Foley and Hume, 1987; Petrović, 2014). *E. globulus,* by contrast, is a highly used koala food tree (Hynes et al., 2021) and provides relatively digestible foliage that likely provides more metabolizable energy to koalas, in conjunction with high nitrogen (protein) availability (Petrović, 2014). In this study, the Firmicutes family Veillonellaceae was negatively associated with a diet dominated by *E. globulus* and the families Lachnospiraceae and Ruminococcaceae were negatively associated with *E. radiata*. In previous studies, we have identified a positive association between the relative abundance of the phylum Firmicutes, particularly members of the families Lachnospiraceae and Ruminococcaceae, and the consumption of *E. obliqua*. *Eucalyptus obliqua* is high in fiber and low in available nitrogen compared with the dominant food tree species, *E. viminalis,* consumed by that koala population (Blyton et al., 2019; Brice et al., 2019). While *E. radiata* may appear nutritionally similar to *E. obliqua*, and *E. globulus* nutritionally similar to *E. viminalis*, species-specific differences may account for the differing microbial associations observed in this study. Further, there is substantial functional variation among members of the same microbial family (Biddle et al., 2013). Thus, more detailed functional studies at a finer taxonomic resolution are required to elucidate these microbiome-nutrition associations. Notably though, only the unweighted unifrac distances were associated with diet composition in this study, suggesting that it may be the presence of particular microbial species rather than their relative abundances that is important to diet selection. As such, it is possible that our previous relative abundance findings may reflect underlying differences in the microbial species.

The gut microbiomes were inferred to influence not only the diets of koalas, but also their condition during the first month post-translocation. However, the associated microbiome characteristics were different in each case. Further, there was no evidence that post translocation diets influenced koala condition. This suggests that the observed association between the koalas' body condition and the initial fecal microbiomes were not linked to their diet. Instead, it is conceivable that the koalas' microbiomes were associated with other aspects of the koalas' physiology and/or health. While not directly applicable to this study, koalas suffering from chlamydial infection and those that die from antibiotic treatment appear to have gut microbiomes that differ from those of healthy individuals, with koalas that recover from antibiotic treatment regaining a microbiome community similar to that of healthy koalas (Barker et al., 2013; Alfano et al., 2015; Dahlhausen et al., 2018). Additionally, in our study of gut microbiome development in captive joeys we observed that two captive koalas that later died from non-infectious diseases had dissimilar gut microbiomes to those of other adult koalas in that study and that they passed those distinct microbiomes onto their joeys (Blyton et al., 2022b). Further studies are needed to determine if the gut microbiome could be used as a marker for host health in koalas. Establishing whether the abnormal microbiomes of sick koalas contribute to their condition or are merely a symptom of underlying pathology should also be an important area of further research.

Overall, there were few changes in the relative abundance of particular microbial species in either the translocated or control koalas. However, one species, *Akkermansia* sp.1, is of interest with regard to koala health. Members of the genus *Akkermanisa* have been found to increase in relative abundance in fasted Burmese pythons and Syrian hamsters (Sonoyama et al., 2009; Costello et al., 2010). In humans, *Akkermanisa muciniphila* has been shown to feed on host mucin, allowing it to survive when nutrients become limiting such as during host starvation (Derrien et al., 2004). Captive and wild adult koalas have been reported to carry *Akkermanisa* at an average relative abundance of 1.5%–3.1% and 2.2%, respectively (Eisenhofer et al., 2022; Blyton et al., 2022a, 2022b). In this study, *Akkermansia* increased in relative abundance in the control koalas from an average of 2% to 4.9% in June 2016. Interestingly, koalas feeding on mana gum at Cape Otway (the site of this study) had *Akkermanisa* relative abundances of 1.0% in 2015 (prior to this study), and 3.4% in 2017–2018 (a year after the completion of this study) (Blyton et al., 2019; Brice et al., 2019). Thus, the high relative abundance of *Akkermanisa* observed in the control koalas in this study could indicate that they suffered from some level of starvation. In line with this, Menkhorst et al. (2019) observed that males in the control group of this study had a continued decline in body condition and suggested that this was due to the defoliated habitat at the source site. Notably, however, in 2013 when defoliation and starvation was at its peak at the site, the average relative abundance of *Akkermanisa* was 2.0% in koalas feeding on manna gum (Whisson et al., 2016; Brice et al., 2019). Although, it is not known if the particular koalas sampled at that time were starving.

The findings from our analysis of the microbiomes and diet of the koalas in this successful translocation project provide several insights for improving future koala translocation efforts. As the koala gut microbiome appears to be largely unaffected by dietary changes, we suggest that the *Eucalyptus* species at the destination habitat be matched to those in the source habitat wherever possible. Alternatively, where food tree species cannot be matched between the source and destination habitats, we suggest selecting a destination site that has a range of different food tree species as was done in this study. Mortality at the destination habitat in this study was not greater than at the source habitat over the same period (Menkhorst et al., 2019). The translocated koalas had access to at least 10 species of *Eucalyptus* in the destination habitat and at least six to seven of these were food tree species (Figure 5). Koalas can thrive on a single food tree species where that species has high nutritional quality (Ashman et al., 2020; Whisson and Ashman, 2020). Prior to translocation the koalas' diets were near monophagous for one

of these preferred species; *E. viminalis*. After translocation, the koalas developed individual differences in their diets and there was an association between their post-translocation diets and starting microbiomes. Together these findings suggest that a landscape containing multiple different food tree species from which the koalas could select their diets after translocation may have allowed them to find species to which their microbiomes were suited, contributing to their high survival rates. While careful site selection is essential to the success of any translocation, in some cases it may not be possible to locate destination sites with a range of food tree species. In such cases, the finding from this study and our previous work that the koala microbiome remains stable despite diet changes indicates that fecal inoculations (Blyton et al., 2019) could be useful for adapting their microbiomes to a small number of unfamiliar food tree species in a destination habitat.

Conclusion

This study provides evidence that microbiome-diet associations in the koala are more convincingly explained by the influence of gut microbiome composition on diet selection than the reverse. Our findings also indicate that the koala gut microbiome is largely unperturbed by diet change, which in some instances could be to the detriment of the animal. This suggests that the success of koala translocations would be enhanced by ensuring that food tree availability in destination habitats matches that of the source. If the exact vegetation associations are not available, then ensuring a variety of food tree species are available will increase the likelihood of a microbiome-diet match. Additionally, the association between the koalas' initial microbiome and their post-translocation condition suggests that further research is needed to establish the role of the koala gut microbiome in host health. The stability of the koala's gut microbiome despite major upheaval is in contrast to the plasticity seen in some other species and demonstrates that the response of the gut microbiome to translocation is likely to be host species-specific.

Data availability statement

The data presented in the study are deposited in the NCBI SRA repository, Bioproject accession number PRJNA901215.

Ethics statement

The animal study was reviewed and approved by the Western Sydney University Animal Care and Ethics Committee. Fieldwork was carried out under the approval of the Western Sydney University Animal Care and Ethics Committee (A11253) and with an appropriate permit from the Victorian government (10007714).

Author contributions

MB and BM designed the project. MB, JP, and EH completed the fieldwork including fecal sample collection. RS performed the DNA extractions and sequencing. MB performed the statistical analyses and wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1085090/full#supplementary-material

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