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# Community structure and environmental determinants of the bacterial and fungal gut microflora in Hainan gibbons (*Nomascus hainanus*)

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A B S T R A C T

High-throughput sequencing technology was used to establish the OTU (Operational Taxonomic Units) composition and diversity of bacteria and fungi in the gut of three family groups of Hainan gibbons (*Nomascus hainanus*) in the Bawangling National Nature Reserve on Hainan Island, southern China. Firmicutes (47.23%) and Bacteroidetes (36.54%) predominated at the phylum level, while the predominant genera were Prevotella-7 (15.30%), Lachnospiraceae_NK3A20_group (12.49%), and uncultured_bacterium_f_Erysipelotrichaceae (11.79%). At the phylum level, the dominant fungi were Ascomycota (66.41%), as well as Hanseniaspora (23.33%), Schwanniomyces (13.44%), and Pichia (5.43%) at the genus level. We found significant differences in the bacterial OTU diversity (Shannon index) between family groups living in Tropical Lowland or Tropical Montane Rain Forest, compared to those inhabiting Tropical Montane Evergreen Forest at higher altitudes. Bacterial OTU community composition also differed between family groups, unraveling significant differences among the 30 most dominant bacterial core taxa. Furthermore, we unraveled a significant difference of OTU richness in the fungal microbiome (Chao 1) between family groups living at lower altitudes, i.e., Tropical Lowland or Tropical Montane Rain Forest and those inhabiting the Tropical Montane Evergreen Forest. Our analysis further indicated significant differences in the fungal OTU community composition between the three family groups, especially regarding the three most dominant fungal core taxa. Subsequently, two habitat factors, and nine environmental and anthropogenic variables were used to explore possible causes of disparity in the microbial flora of gibbon groups. A factor reduction procedure resulted into three principal components which were correlated to bacterial and fungal OTU richness and diversity using Spearman's rank-order correlations. Bacterial and fungal OTU diversity was high in areas of high altitude, steep slopes, high tree density, but low tree height, while high fungal OTU richness corresponded to high altitude habitats, i.e., in

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the Tropical Montane Evergreen Forest. Distance to human settlements and to the next water body showed no significant relation with bacterial and fungal richness and diversity.

## 1. Introduction

Researching the gut microflora of endangered wildlife species became increasingly important in recent years owing to close host-microbiome associations and their significance for conservation (Bahrndorff et al., 2016; West et al., 2019; Zhu et al., 2021). The study of gut microbiomes can provide key information on dietary supplementation (Clemente Ursell et al., 2012; Maynard et al., 2012; Le Chatelier et al., 2013; Valdes et al., 2018), host immune functions (Ning et al., 2020) and individual behaviors (Valentina, 2019). Microbiomes affect host fitness, population demography and health as well as the adaptability to environmental changes (Thompson-Chagoyán et al., 2007; Cani and Delzenne, 2009; Sekirov et al., 2010). Accordingly, the study of gut microbiomes plays a pivotal role in the conservation and management of threatened primate populations. Numerous studies have focused on the intestinal microbiome of nonhuman primates, gradually developing into a research hotspot of nonhuman primate research (Goldberg et al., 2007; Villers et al., 2008; Yildirim et al., 2010; Amato et al., 2013; McKenney et al., 2014; Chen et al., 2020). Several factors were identified to shape the microbial community of non-human primates, including the social organisation (Moeller et al., 2016; Degnan et al., 2012; Tung et al., 2015) or the status and age of individuals (Reese et al., 2021), but also environmental factors such as quality and abundance of food (Fish and Lockwood, 2003), season, or altitude (Thoemmes et al., 2018). Recently, i.e., since high-throughput sequencing became widely available, the study of great-ape (*Hominoidea*) gut microbiomes turned out to be increasingly important. This is mainly owed to the evolutionary importance of symbiotic interactions between hosts and their microbiome (Manara et al., 2019), but also because it allows to explore environmental causes for their decline and how to facilitate conservation through science-based management (Moeller et al., 2013; Stumpf et al., 2016; Hicks et al., 2018; Nishida and Ochman, 2019).

Until recently, the Hainan gibbon (*Nomascus hainanus*) was considered the most critically endangered great-ape species in the world (Chan, 2017) and was therefore classified as 'China's National First-class Protected Wildlife Species' (Chan, 2017; Geissmann and Bleisch, 2020). Accordingly, the current IUCN Red List status of Hainan gibbon is 'Critically Endangered' (Geissmann and Bleisch, 2020). The species' range is limited to a patch of primary montane rainforest on Mt. Futouling in the Bawangling National Nature Reserve on Hainan Island in southern China with an extant population of currently 33 individuals, forming five family groups along with a few solitary individuals (Ling and Chen, 2020). Numerous studies were carried out to investigate the phylogeny and conservation status of Hainan gibbons (Fellowes et al., 2008; Mootnick et al., 2012; Bryan et al., 2016; Turvey et al., 2016), but only a few studies have focused on the behavior and ecology of this rare ape species (Liu et al., 1989; Deng et al., 2016).

Core microorganisms are important to evaluate the stability and functional composition of gut microbiomes in the host organism and are used to better understand the interactions between the host and its environment, i.e., the dietary composition and quality, the habitat structure, or the social organization. In this study, we examined the intestinal microbiomes of three family groups inhabiting different habitats and altitudes to describe the microbial community composition of Hainan gibbons and to identify the environmental parameters that may shape this community. In a first step, we used noninvasive sampling, high-throughput sequencing, and bioinformatics to determine the composition of the bacterial and fungal gut microflora in Hainan gibbons. In a second step, we established the alpha and beta diversity for both microbiomes and tested for differences between three habituated family groups. Finally, we tested two habitat factors and nine environmental and anthropogenic covariates for differences between family groups and attempted an interpretation of our findings by conducting a factor reduction procedure on these variables. To explore possible causes of disparity in the microbial flora, we correlated the resulting principal components with the bacterial and fungal richness and diversity of each families' microbiome. Owing to the small sample size, social factors such as group size and composition or home range size and overlap were not analyzed but discussed. However, given the serious conservation situation of Hainan gibbons, our study will—despite extremely low sample sizes—provide useful information to improve and enhance the conservation management of the species in the Bawangling National Nature Reserve.

## 2. Materials and methods

### 2.1. Ethics statement

This study was carried out in accordance with the recommendations on animal care and ethics of Research Institute of Forest Ecology Environment and Protection, Chinese Academy of Forestry.

### 2.2. Study area and species

Hainan Island, located off the coast of southern China, is home to the world's only wild population of Hainan gibbons (*Nomascus hainanus*), persisting in the Bawangling National Nature Reserve (18°57′–19°11′N, 109°03′–109°17′E). The protected area encounters a tropical monsoon climate (i.e., one extended rainy season from May to October) and covers an area of 29,998 ha, with an altitudinal range from 350 m to 1438 m above sea level (Zhou, 2018). Hainan gibbons inhabit the tropical rainforest at an altitude of 650–1200 m (Chan et al., 2005). Depending on altitude, the vegetation comprises mainly of Tropical Lowland Rain Forest (TLRF), Tropical Coniferous Forest (TCF) and Tropical Montane Rain Forest (TMRF), and Tropical Montane Evergreen Forest (TMEF; Jiang et al., 2016).

In this area, the main feeding plants are Lauraceae, Moraceae and Annonaceae, which provide a wide variety of food items mainly comprising of fruits, but also leaves, buds and flowers, depending on plant phenology and availability (Deng and Zhou, 2016).

The social organization of Hainan gibbon is polygynous, forming family groups of one male, two females and their offspring (Bryan et al., 2016). The average group size in the protected area was 5.4 individuals, ranging from four to eight individuals (Bryan et al., 2016). Home range size was close to that observed in related species, with an average of 1.49 $km^2$ (Bryant et al., 2017). At present, Hainan gibbons are concentrated in the Futouling, Nanban and Dongwu areas of the reserve, occupying an overall home range area of about 15 $km^2$ (Fig. 1). The range is shared by five family groups of which three were incorporated in this study. Family group A comprised of six individuals, group B of eight, and group C of 10 individuals. Each group included one adult male and two adult females, with the remaining group members being either sub-adults or juveniles. Based on Jiang et al. (2016), the vegetation type in the home range of family group A comprised 100% TMRF, that of group B 61.3% TMRF and 38.7% TLRF, while that of group C comprised of 10.8% TLRF, 85.0% TMEF and 4.2% TMRF.

### 2.3. Fecal sample collection

A total of 15 fresh fecal samples were arbitrarily collected from three family groups (five samples from each family). Family groups were habituated to the presence of the researchers and single family members could be individually distinguished based on fur coloration (male vs female), body size (parents vs offspring), as well as behavior and other characteristics such as scratches or scars. Habituated groups were followed until a gibbon was observed defecating. One fecal sample of each family member was gleaned from the ground and collected into sterile centrifuge tubes. Tubes were sealed, labelled, and stored in a mobile refrigerator at −20 °C, until further processed in the laboratory. To ensure seasonal uniformity, all samples were collected within one month, i.e., from July to August 2019.

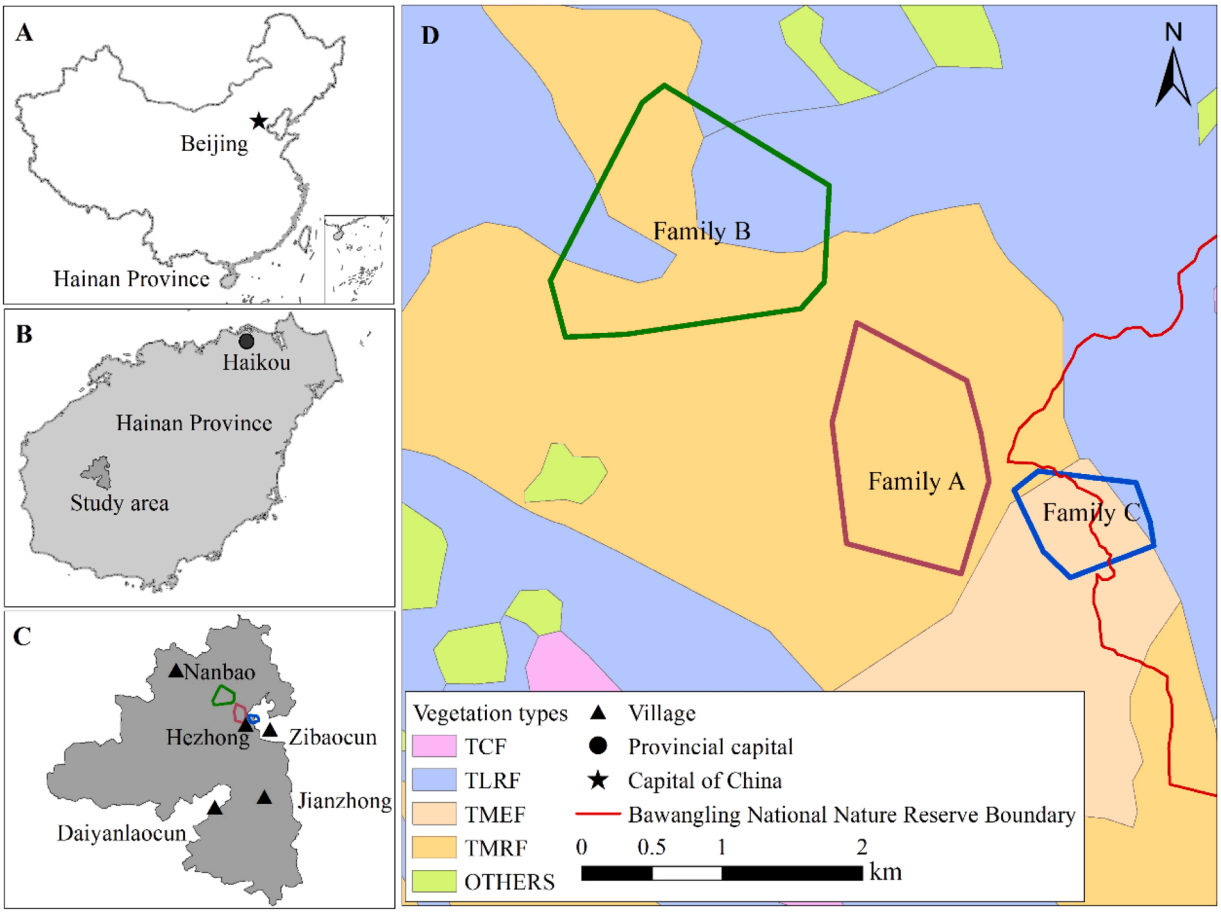


**Fig. 1.** A) The location of Hainan Island in China, B) the location of the Bawangling National Nature Reserve on Hainan Island, C) the location of family group home ranges within the Bawangling National Nature Reserve, and D) the location of family group home ranges (Minimum convex polygones) in relation to the vegetation type defined by Jiang et al. (2016): Tropical Coniferous Forest (TCF), Tropical Lowland Rain Forest (TLRF), Tropical Montane Evergreen Forest (TMEF), Tropical Montane Rain Forest (TMRF), others comprise mainly of human settlements and agriculture.

### 2.4. DNA extraction and sequencing

The total genomic DNA of the fecal flora was purified using a fecal DNA extraction kit (QIAamp DNA Stool Mini Kit, QIAGEN, Hilden, Germany) following the manufacturer's product manual. The integrity of extracted genomic DNA was verified by 1.0% agarose gel electrophoresis, and a single bright electrophoretic band devoid of significant dragging indicated an intact genome without degradation. The concentration and purity of genomic DNA were determined using Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA). The extracted genomic DNA was stored in a freezer at −80 °C for subsequent PCR and sequencing. The V3-V4 region of bacterial 16 S rRNA gene was amplified using primers 338 F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806 R (5′-GGACTACHVGGGTWTCTAAT-3′). The ITS:ITS1 region of fungal 18 S rRNA gene was amplified using primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′). The polymerase chain reaction (PCR) volume was 50 ml containing 10 ml PCR buffer, 0.2 μL High-Fidelity DNA Polymerase, 1 μLdNTP, 10 μL GC Enhancer, 1.5 μL each of 10 μM forward and reverse primers, 60 ng template DNA and the rest volume was DNase-free sterile water. The PCR conditions were as follows: 95 ℃ for 5 min, followed by 25 cycles of 95 ℃ for 30 s, 50 ℃ for 30 s, 72 ℃ for 40 s and 72 ℃ for 7 min. The PCR products was purified with DNA gel extraction kit (Axygen, Shanghai, China). Ultimately, high-throughput sequencing on an Illumina HiSeq 2500 platform (Illumina, Inc., San Diego, CA, USA) was conducted at Biomarker Technologies Corporation (Beijing, China). Raw image data files obtained by high-throughput sequencing were transformed into the original sequence reads using Base Calling analysis. Results were stored in FASTQ (fq) file format, including the sequence information as well as the corresponding sequencing quality information. All raw sequences obtained during this study were submitted to the NCBI Sequence Read Archive (accession number PRJNA688378). To obtain high-quality clean tags, raw sequences were quality filtered using PRINSEQ (Schmieder and Edwards, 2011). All sequences were grouped into operational taxonomic units (OTUs) with a 97% sequence similarity level using the UCLUST program (Edgar, 2010). For the identification of bacteria and fungi taxa, sequences were subsequently matched with sequences deposited at SILVA database (Quast et al., 2013).

### 2.5. Environmental and anthropogenic variables

Two environmental factors, i.e., terrain (slope, ridge, valley), and vegetation type (TMRF, TLRF, TCF and TMEF; see above), as well as nine environmental covariates, i.e., altitude, slope gradient, tree density, tree height, trunk diameter, number of dead wood logs, number of climbers, distance to the next settlements and distance to the next water body, were established at each fecal sampling point to characterize the environmental attributes of each family's home range. The vegetation type was identified using a vegetation map provided by Jiang et al. (2016), while terrain, altitude, slope gradient, distance to the next settlement and distance to the next water body were obtained from Google Earth. Tree density, tree height, trunk diameter (at breast height), number of dead logs, and the number of climbers (mainly lianas) were established within quadrats of 30 × 30 m around each fecal sampling location (900 $m^2$). Tree height (adult trees only) was determined using the 45° triangulation method, while the tree diameter was calculated from the circumference of all adult trees within each quadrat. Values for tree height and trunk diameter at each sampling location were averaged and, together with the other environmental attributes, subjected to factor reduction procedure (see below).

### 2.6. Statistical analysis

#### 2.6.1. Microbial composition and diversity

To assess the alpha diversity of the bacterial and fungal community in the gastro-intestinal tract of Hainan gibbons, the Chao1 index and the Shannon index were calculated from rarefied samples using QIIME (Version 2.0; Caporaso et al., 2010). The Chao1 index was used to estimate the OTU richness, i.e., the total number of OTUs in each sample, while the Shannon index was applied as a proxy of microbial diversity in each sample. Differences in OTU richness and diversity were tested using a One-way ANOVA.

Non-metric Multidimensional Scaling (NMDS), based on the Unweighted UniFrac similarities (bacteria) and the Bray-Curtis dissimilarities (fungi), was performed to determine beta diversity using QIIME (Version 2.0; Caporaso et al., 2010). A one-way analysis of similarity (ANOSIM) was carried out to test for differences in the bacterial and fungal community composition between groups using the 'vegan' package (Clarke and Gorley, 2006) in the software R (version 3.5.2). Finally, Linear Discriminant Analysis (LDA) effect size (LEfSe) analysis was performed to test for significant differences of each index between the three family groups (Segata et al., 2011). A size-effect threshold of 4.0 on the logarithmic LDA scale was used for discriminative functional biomarkers.

#### 2.6.2. Environmental and anthropogenic determinants of microbial composition

To compensate for the small sample size and the inability to run a model on our limited data set, we chose a two-step approach to analyze the impact of environmental and anthropogenic factors on the bacterial and fungal OTU richness and diversity. Initially, two chi-squared tests for goodness of fit were applied to test whether the two environmental factors (i.e., terrain and vegetation type) differed between the three family groups. Besides, we performed a Kruskal-Wallis rank sum tests on each of the nine environmental covariates to test for differences between the three family groups. Prior to the factor reduction procedure, all environmental covariates were z-transformed to standardize the dataset and subsequently subjected to a Principal Component Analysis (PCA; based upon a correlation matrix) to eradicate collinearities among the nine environmental variables. The PCA was conducted using the 'factoextra' package (Kassambara and Mundt, 2020) in R software (version 3.5.2), resulting into three Principal Components (PCs) with an eigenvalue > 1.0 (Table 1), and explaining 74.51% of the total variance. Finally, the three environmental PCs were correlated to the richness (Chao1 index) and diversity (Shannon index) of bacterial and fungal OTUs of each family group using three independent

Spearman's rank-order correlations. All statistical analysis were conducted using R software (version 3.5.2).

## 3. Results

### 3.1. Microbial composition and diversity

#### 3.1.1. Abundance of core bacterial microbiomes

A total of 1122,694 effective bacterial sequences were obtained from 15 fecal samples, corresponding to 74,846 ± 992 bp (mean ± SD; range: 73,988–76,809 bp). The average sequence length was determined as 421.53 ± 1.46 bp. A total of 216 OTUs was identified, whereby the number of OTUs in each sample reached from 146 to 187 OTUs. These OTUs were assigned to 15 phyla, 23 classes, 42 orders, 75 families, 139 genera, and 147 species.

At the phylum level, the bacterial gut community was dominated by Firmicutes (47.23%), Bacteroidetes (36.54%), Proteobacteria (4.30%), Actinobacteria (4.27%), Cyanobacteria (3.99%) and Fibrobacteres (2.16%; Fig. 2a). At the genus level, the bacterial gut community was dominated by Prevotella_7 (15.30%), Lachnospiraceae_NK3A20_group (12.49%), uncultured_bacterium_f_Erysipelotrichaceae (11.79%), hoa5–07d05_gut_group (7.41%), uncultured_bacterium_f_Lachnospiraceae (5.10%), Prevotellaceae_NK3B31_group (4.91%), Prevotellaceae_UCG-001 (4.21%), Nicotiana_otophora (3.96%) and uncultured_bacterium_f_Veillonellaceae (3.07%; Fig. 2b).

#### 3.1.2. Alpha and beta diversity of bacterial communities

No significant differences ($P > 0.05$) in OTU richness of bacterial microbiomes (Chao1 index) were unraveled between family groups (group A: 186.07 ± 6.85, group B: 185.90 ± 8.08, group C: 182.72 ± 6.89; Fig. 3a). By contrast, we found significant differences in OTU diversity (Shannon index) between family group A and C (A: 4.19 ± 0.19, C: 4.87 ± 0.12, $P = 0.01$) as well as between family group B and C (B: 4.35 ± 0.15, C: 4.87 ± 0.12, $P = 0.04$; Fig. 3b). Furthermore, the NMDS indicated dissimilarities in the bacterial community composition between family groups (Fig. 4), revealing a significantly different community composition between family group A and B ($R = 0.22$, $P = 0.01$) as well as between family group A and C ($R = 0.28$, $P = 0.01$). LEfSe analysis revealed significant differences among 30 dominant bacterial core taxa (Fig. 5) and showed bacterial LDA scores to be significantly different between family groups. At phylum level, the biomarker showed significant differences for Cyanobacteria (LDA > 4.0, $P < 0.05$), while at the genus level, significant differences were demonstrated for uncultured_bacterium_f_Lachnospiraceae, Lysinibacillus, Nicotiana_otophora, Lactobacillus, Prevotellaceae_UCG_001, Prevotella_2, Asteroleplasma, Sutterella, and the Prevotellaceae_NK3B31_group (LDA > 4.0, $P < 0.05$).

### 3.2. Fungal composition and diversity

#### 3.2.1. Abundance of core fungal microbiomes

A total of 1,090,071 effective fungal sequences were obtained from 15 fecal samples corresponding to 72,805 ± 3665 bp (mean ± SD; range: 62,380–77,235 bp). The average sequence length was determined as 284.47 ± 55.45 bp. A total of 754 OTUs was identified whereby the number of OTUs in each sample reached from 156 to 363 OTUs. These OTUs were assigned to nine phyla, 25 classes, 61 orders, 117 families, 179 genera, and 155 species. At the phylum level, the bacterial gut community was dominated by Ascomycota (66.41%) and Basidiomycota (1.55%; Fig. 6a). At the genus level, the fungal gut community was dominated by *Hanseniaspora* (23.33%), *Schwanniomyces* (13.44%), *Pichia* (5.43%), *Penicillium* (3.80%), *Clonostachys* (1.75%), *Candida* (1.39%), and *Cladosporium* (1.07%; Fig. 6b).

#### 3.2.2. Alpha and beta diversity of fungal communities

We unraveled a significant difference ($P = 0.011$) in the OTU richness of fungal microbiomes (Chao 1) between family group A and C (A: 251.48 ± 21.43, C: 398.47 ± 43.96) and a significant difference in the Shannon diversity between family group B and C (B: 2.82

**Table 1**
Results of Principal Component Analysis of nine environmental variables obtained from three Hainan gibbon family home ranges. PC loadings > |0.50| are shown in bold font type.

| Variable | PC1 | PC2 | PC3 |
|---|---|---|---|
| Eigenvalue | 2.53 | 2.27 | 1.90 |
| % of variance | 28.10 | 25.30 | 21.11 |
| Altitude | **0.74** | 0.22 | -0.15 |
| Slope gradient | **0.86** | 0.05 | -0.02 |
| Tree density | **0.84** | 0.04 | 0.12 |
| Trunk diameter | -0.25 | 0.16 | **0.89** |
| Tree height | **-0.57** | **0.54** | 0.43 |
| No. dead logs | -0.20 | 0.22 | **-0.87** |
| Distance to settlement | -0.08 | **0.82** | -0.32 |
| Distance to water | -0.30 | **-0.77** | -0.02 |
| No. climbers | 0.09 | **0.76** | 0.12 |

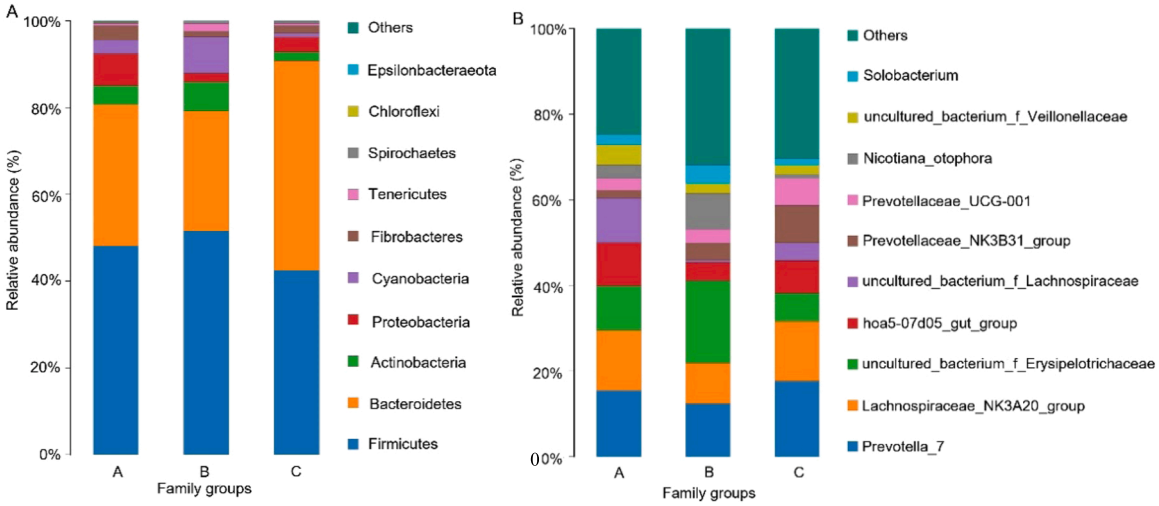


**Fig. 2.** A) Relative abundance of the ten most abundant bacterial phyla, and B) the ten most abundant bacterial genera for each family group. Others, represent bacteria taxa with an abundance of $\leq 1\%$.

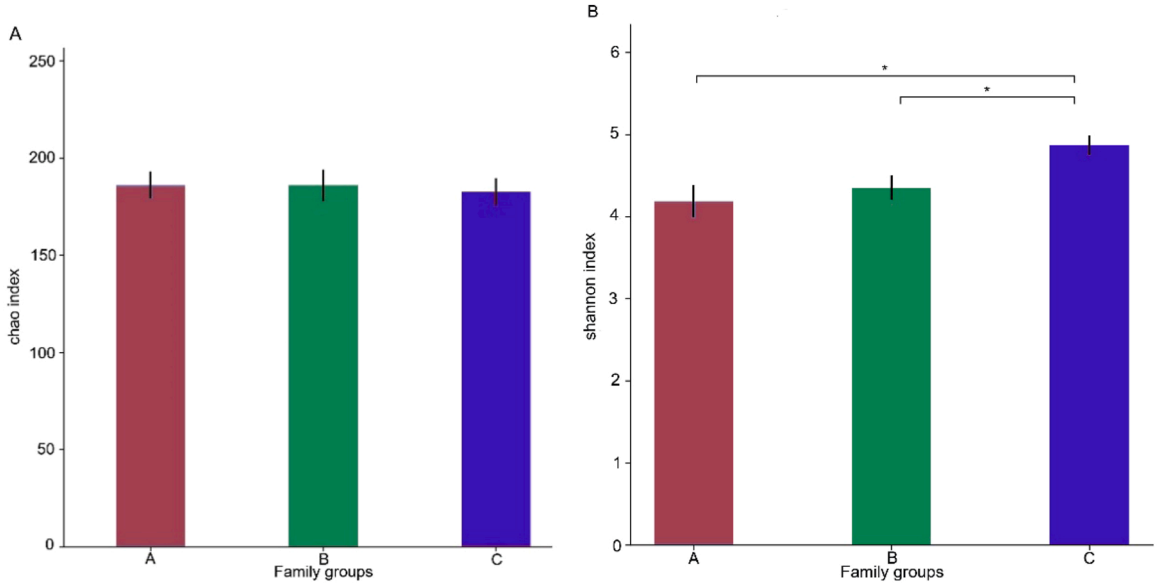


**Fig. 3.** A) OTU richness (Chao 1 index) and B) OTU diversity (Shannon index) of bacterial microbiota in Hainan gibbon. * indicates significant differences: One-Way ANOVA: $P < 0.05$.

$\pm$ 0.22, C: 4.64 $\pm$ 0.59; $P = 0.05$; Fig. 7). Furthermore, NMDS (Bray-Curtis dissimilarities) indicated significant differences in the fungal community composition between all family groups (A vs B: $R = 0.36$, $P = 0.01$; A vs C: $R = 0.47$, $P < 0.001$; B vs C: $R = 0.38$, $P = 0.01$; Fig. 8).

LEfSe analysis revealed significant differences among three dominant fungal core taxa (Fig. 9) and showed LDA scores of fungal OTUs to be significantly different between family groups. At phylum level, the biomarker demonstrated significant differences in *Basidiomycota* (LDA $> 4.0$, $P = 0.02$), while at genus level, no significant differences between family groups were detected (LDA $> 4.0$, $P > 0.05$).

### 3.2.3. *Environmental determinants of gut microflora*

The chi-squared test showed a significant difference in the proportion of vegetation types between the three family groups ($\chi^2 = 18.75$, $df = 4$, $P < 0.001$). A post-hoc multiple comparison procedure (Dunn's test) revealed that group C had a significantly larger proportion of TMEF, and a significantly smaller proportion of TMRF, than the other two family groups. Regarding the terrain type, the chi-squared test revealed only a marginally significant difference between the three family groups ($\chi^2 = 9.25$, $df = 4$, $P = 0.06$), but

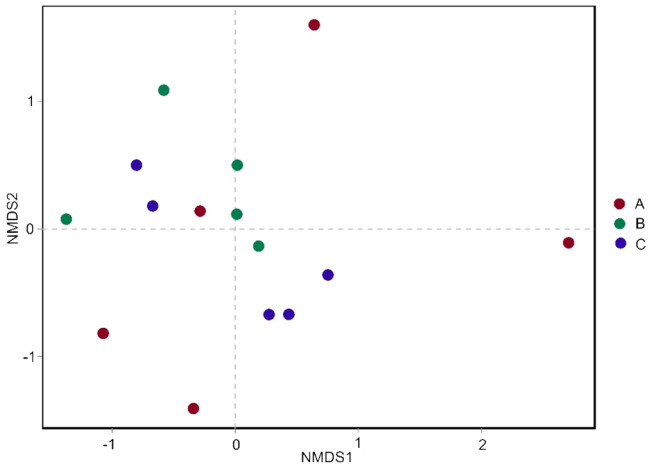


**Fig. 4.** Non-metric multidimensional scaling (NMDS) scatter plot of 15 fecal samples, representing the bacterial OTU community composition in each family group. The distance between points represents the degree of difference based on unweighted Unifrac similarities in each sample. A stress value of 0.1251 indicates that the NMDS analysis was reliable.

disparity was not confirmed after post-hoc multiple comparisons.

The Kruskal-Wallis rank sum test showed a significant difference in tree density between the three family groups ($\chi^2 = 6.08$, $df = 2$, $P = 0.05$). After Bonferroni correction, a post-hoc multiple comparison procedure (Dunn's test) revealed no significant difference between the family groups. Moreover, the three family groups showed a significant difference in the distance to the next settlement ($\chi^2 = 12.52$, $df = 2$, $P = 0.002$) and to the next water body ($\chi^2 = 9.68$, $df = 2$, $P = 0.008$). After Bonferroni correction, the post-hoc test (Dunn's test) revealed group B to be significantly farther from the next human settlement than group C ($Z = 3.54$, $P = 0.001$; Fig. 10a), while group C was significantly farther from the next water body than group A ($Z = 2.88$, $P = 0.01$) and group B ($Z = 2.45$, $P = 0.03$; Fig. 10b). Other environmental variables including altitude, slope gradient, tree height, number of dead logs, trunk diameter, and the number of climbers did not indicate any significant differences between the three family groups.

The PCA condensed nine environmental covariates into three PCs (Table 1). PC1 received high positive factor loadings from altitude, slope, tree density and a negative factor loading from tree height, characterizing elevated areas in steep terrain with dense growth of short trees. PC2 had high positive factor loadings from tree height, distance to settlement, number of climbers and a negative factor loading from distance to water, indicating old and pristine montane rain forest with relatively low human impact. PC3 received a high positive factor loading from trunk diameter and a negative factor loading from the number of the dead logs, indicating old age of the forest and prominent management activities.

Spearman's rank-order correlations revealed that PC1 was significantly correlated to the Shannon index of both, bacteria and fungi (Table 2), suggesting that bacterial and fungal OTU diversity was high in areas of high altitude, steep slopes, and high tree density but low tree height. Such areas are usually found in the TMEF where tree density is high but dominated by short-grown species. Moreover, PC1 was also significantly, positively correlated to the Chao1 index of fungal OTUs (Table 2), indicating a relatively high fungal OTU richness at high altitudes in the TMEF. However, PC2 and PC3 were neither correlated with bacterial and fungal OTU richness nor diversity, suggesting that distance to the nearest water body and distance to the nearest settlement did not influence the richness and diversity of the bacterial and fungal microbiome of Hainan gibbons.

## 4. Discussion

### *4.1. Bacterial and fungal composition of the gut microbiome*

In our study we showed that the bacterial gut microflora of Hainan gibbons comprised of ten core phyla, of which Firmicutes and Bacteroidetes were the two most dominant. This finding is consistent with previous studies on non-human primates such as chimpanzee (*Pan troglodytes*) and gorilla (*Gorilla gorilla*; Moeller et al., 2013), black-and-white colobus (*Colubus guereza*), red-tailed guenon (*Cercopithecus ascanius;* Yildirim et al., 2010), and snub-nosed monkeys (Jin et al., 2019). The combined proportion of bacteria from these two phyla ranged from 70.5% to 98.3% in the intestinal tract of non-human primates studied by Yildirim et al. (2010) and is thus in line with the mean percentage proportion of Firmicutes and Bacteroidetes observed in this study (83.8% of the total bacterial gut microflora). Moreover, Firmicutes and Bacteroidetes are also dominant species in the gut microflora of many other terrestrial mammal species since they play an essential role in the maintenance of the ecological balance and the normal physiological function of the gastro-intestinal tract (e.g., Shanks et al., 2019; Li et al., 2016, 2017, 2019; Yan et al., 2021). Firmicutes are the main fiber-degrading bacteria capable of decomposing cellulose into volatile fatty acids, while Bacteroidetes serve the degradation of carbohydrates and proteins but also promote the immune response of the host organism (Cross, 2002). Other bacterial core phyla found in the gut microbiome of Hainan gibbons accounted for 14.7% of the total bacterial gut microflora, comprising of Proteobacteria, Actinobacteria,

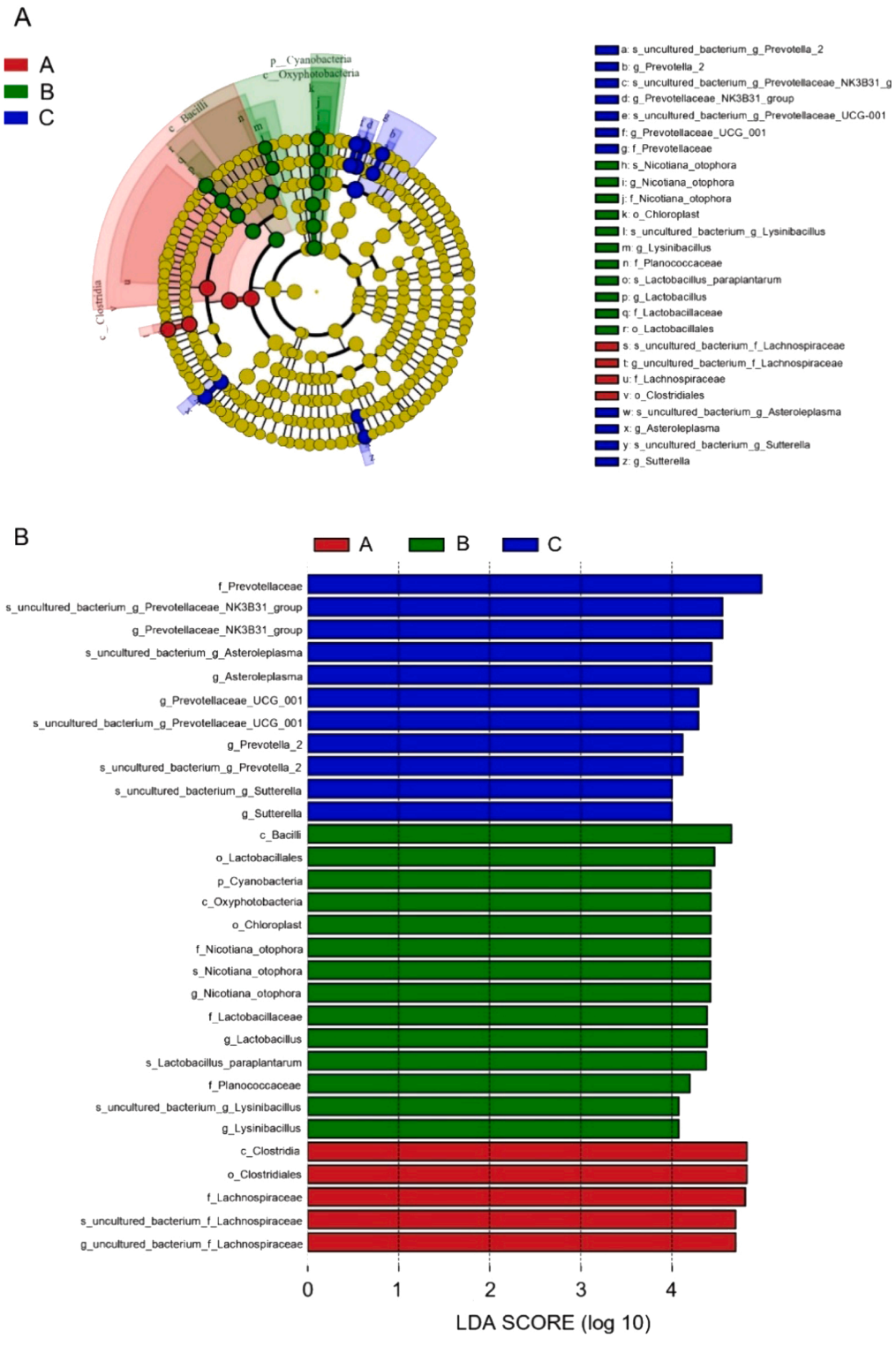


**Fig. 5.** A) Cladogram based on LEfSe analysis, showing bacterial OTUs with significant differences between family groups (red, green and blue). Taxonomic hierarchies were arranged from the inside (lower taxonomic level) to the outside (higher taxonomic level). Yellow nodes represent OTUs with no significant difference. B) Log10-transformed LDA scores for the 30 most abundant bacterial OTUs, i.e., with a threshold value > 4.0. Letters in front of OTUs represent taxonomic level (p = phylum, c = class, o = order, f = family and g = genus). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

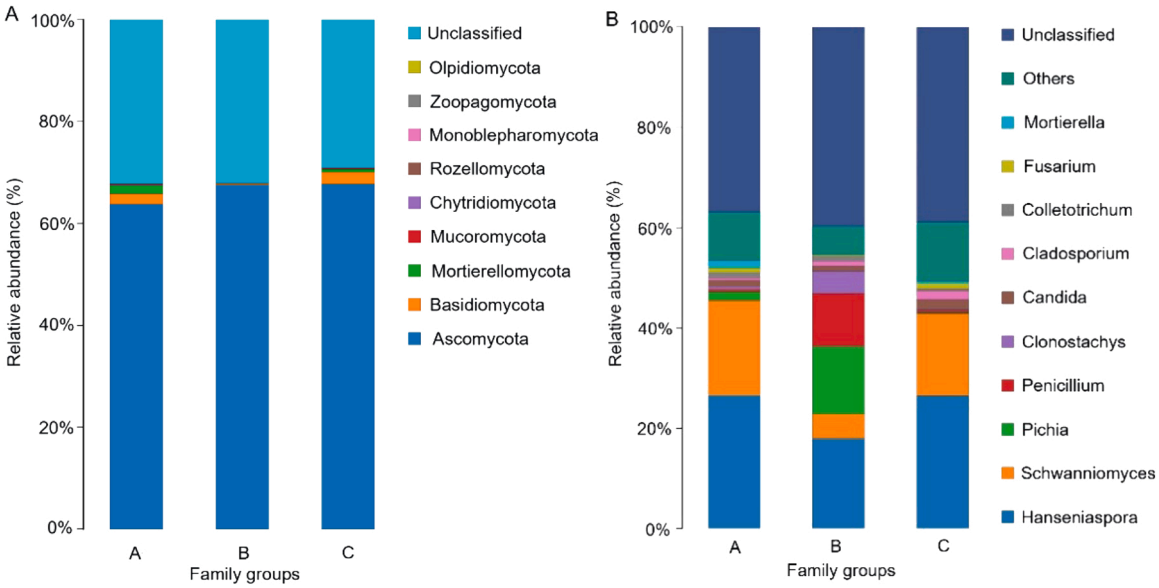


**Fig. 6.** A) Relative abundance of the ten most abundant fungi phyla, and B) the ten most abundant fungi genera for each family group. Others, represent fungi taxa with an abundance of $\leq 1\%$.

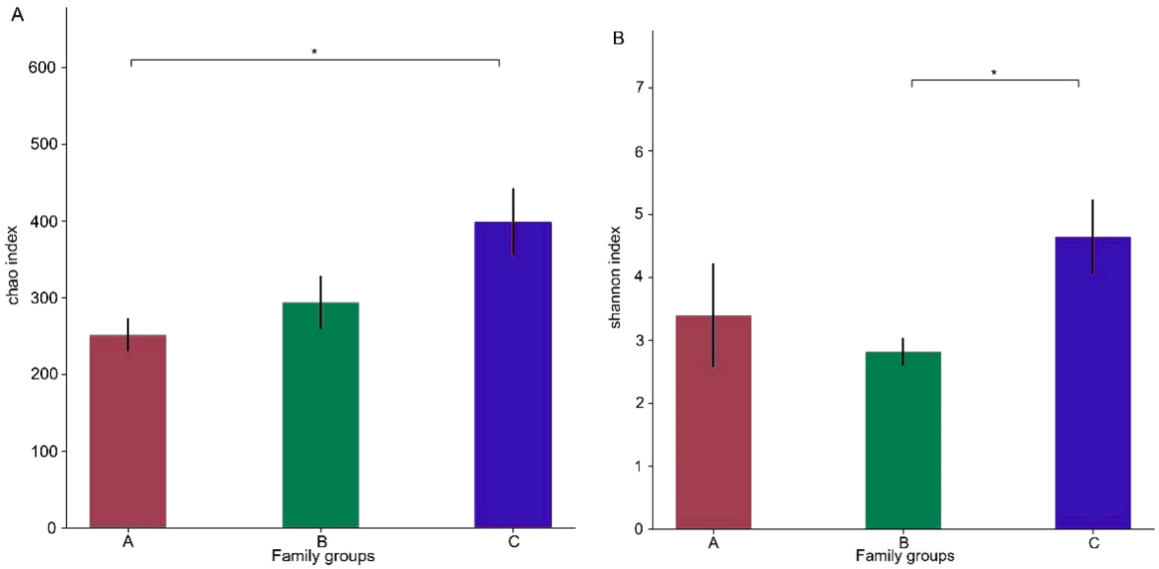


**Fig. 7.** A) OTU richness (Chao1 index) and B) OTU diversity (Shannon index) of fungal microbiota in Hainan gibbon. * indicates significant differences: One-Way ANOVA: $P < 0.05$.

Cyanobacteria, and Fibrobacteres, all of which were also reported from other non-human primates, highlighting the phylogenetic relationship between these host taxa (Yildirim et al., 2010; Manara et al., 2019). Proteobacteria (4.30% of the total bacterial gut microflora) include various pathogens, such as *Escherichia coli*, *Salmonella* spp., *Vibrio cholera*, and *Helicobacter pylori* and may therefore have a sustained impact on the health conditions of the entire population or specific family groups (Katouli, 2010; Nougayrede and Oswald, 2011; Tran et al., 2011; Wang and Huang, 2014). This relatively large proportion of Proteobacteria in the gastro-intestinal tract of Hainan gibbons may have affected the intestinal health of the animals (especially that of family group A; Fig. 2a) and could be therefore detrimental to the continued conservation efforts in the protected area.

The average proportion of Cyanobacteria in the gut of Hainan gibbon was 3.99% although they account for only 0.10% of the mammalian bacterial gut microflora (Amato et al., 2013). This gram-negative group of bacteria performs oxygenic photosynthesis and is closely linked to aquatic environments (Guo, 2018). In his study on rhesus macaques (*Macaca mulatta*), Guo (2018) discovered a substantial proportion of Cyanobacteria (up to 0.91%) in the faecal samples of macaque and attributed this high prevalence to an

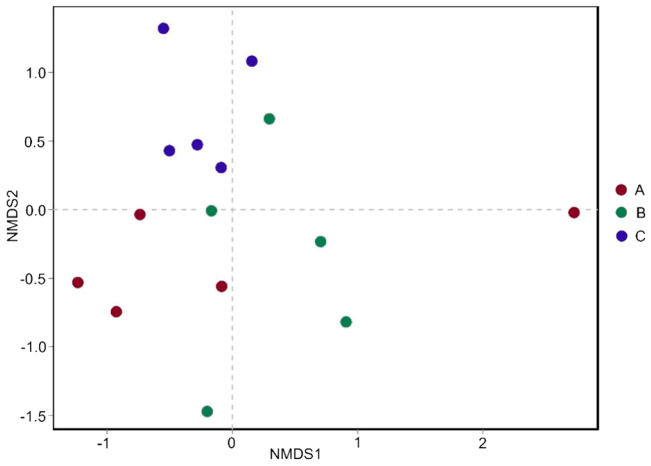


**Fig. 8.** Non-metric multidimensional scaling (NMDS) scatter plot of 15 fecal samples, representing the fungal OTU community composition in each family group. The distance between points represents the degree of difference based on unweighted Unifrac similarities in each sample. A stress value of 0.1413 indicates that the NMDS analysis was reliable.

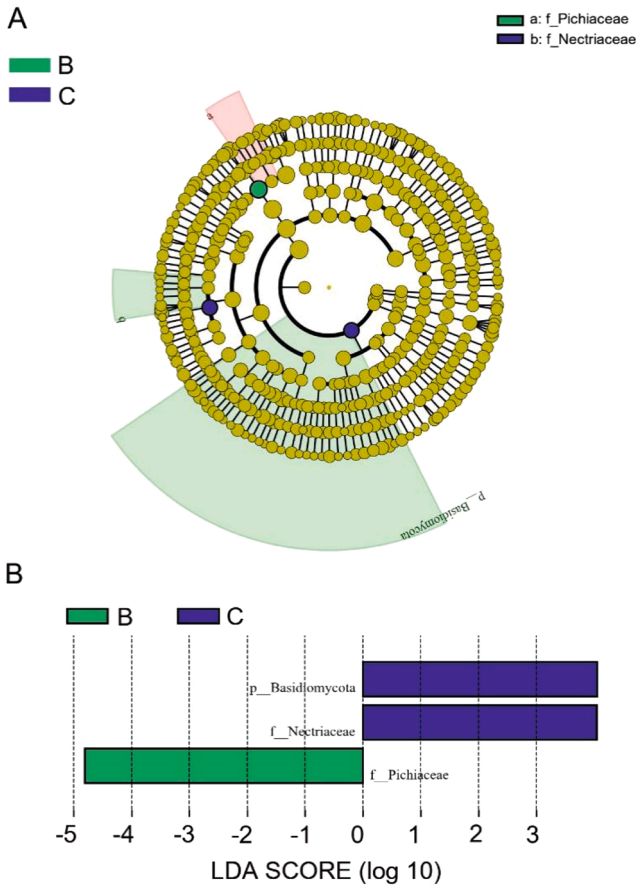


**Fig. 9.** A) Cladogram based on LEfSe analysis, showing fungal OTUs with significant differences between family groups (green and blue). Taxonomic hierarchies were arranged from the inside (lower taxonomic level) to the outside (higher taxonomic level). Yellow nodes represent OTUs with no significant difference. B) Log10-transformed LDA scores for the 30 most abundant fungal OTUs, i.e., with a threshold value > 4.0. Letters in front of OTUs represent taxonomic level (p = phylum, c = class, o = order, f = family and g = genus).

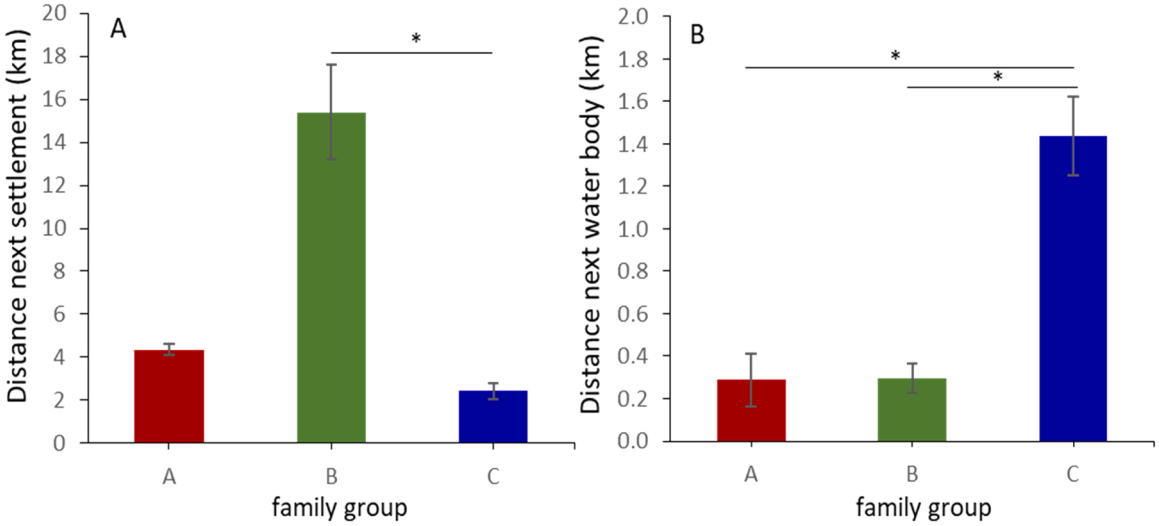


**Fig. 10.** A) Distance to the nearest settlement, and B) distance to the nearest water body of three family groups of Hainan gibbons in the Bawangling National Nature Reserve.

**Table 2**
Spearman's rank correlations between the richness of bacterial and fungal OTUs (Chao 1 index) and the environmental principal components (PC1, PC2 and PC3), as well as between bacterial and fungal OTU diversity (Shannon index) and the environmental principle components.

| | PC1 | | PC2 | PC3 |
|---|---|---|---|---|
| ***Bacteria Chao1*** | | | | |
| ***r*** | -0.439 | | + 0.050 | + 0.032 |
| ***P*** | 0.103 | | 0.826 | 0.913 |
| ***Bacteria Shannon index*** | | | | |
| ***r*** | **+ 0.579** | | -0.307 | -0.316 |
| ***P*** | **0.024** | | 0.265 | 0.251 |
| ***Fungi Chao1*** | | | | |
| ***r*** | **+ 0.643** | | -0.429 | -0.382 |
| ***P*** | **0.012** | | 0.113 | 0.161 |
| ***Fungi Shannon index*** | | | | |
| ***r*** | **+ 0.598** | -0.210 | | -0.048 |
| ***P*** | **0.019** | 0.452 | | 0.864 |

artificial, slow-flowing irrigation system with a high contamination of Cyanobacteria. Given this, it could be argued that the increased proportion of Cyanobacteria in Hainan gibbons (especially in family group B: 8.35%; Fig. 2a). Likewise, our LEfse analysis showed that at the phylum level, the OTU richness of Cyanobacteria of family group B was significantly higher than that of group A and C (Fig. 5), which could be explained by frequent access of group B to anthropogenic water sources. Due to their anatomical and physiological constitution, Hainan gibbons hardly ever stay on the ground, also not for drinking water. It is thus more likely that the high infestation with Cyanobacteria was contracted from rainwater that had accumulated in tree holes. This observation was further supported by the fact that bacterial OTU richness was not affected by the distance to the nearest human settlement or water body (see below and Table 2). Cyanobacteria can produce a variety of biological toxins, including neurotoxins, liver toxins, cytotoxins, and endotoxins, which can pose severe threats to the health of gibbons and that should be considered for habitat management in the reserve. Interesting is also the fact that the gastro-intestinal tract of Hainan gibbons contained Fibrobacteres (2.16%), a bacterial phylum which is mainly prevalent in the rumen of ruminants to help digest cellulose (Jewell et al., 2013). This result is consistent with the strictly herbivorous diet of Hainan gibbons and explains why this species is well adapted to digest large amounts of fruits, leaves, buds, and flowers (Deng and Zhou, 2016).

The fungal gut microflora of Hainan gibbons comprised of two dominant core phyla, i.e., the Ascomycota and the Basidiomycota (67.96%). Apart from those two taxa, other gut fungi could not be clearly classified during this study. Interestingly, these results were similar to those reported for the fungal gut microflora of giant pandas (*Ailuropoda melanoleuca*; Ai et al., 2014), Amur leopards (*Panthera pardus orientalis*; Hua et al., 2020), and domestic herbivores (horses, yaks, goats, and sheep) on the Qinghai-Tibetan Plateau (Yang et al., 2014). These similarities might be due to a general fungal microflora prevalent in the environments of eastern Asia but are outweighed by differences which can be attributed to phylogenetic affinity, the type of food consumed, or the structure of the digestive tract. Generally, studies on the fungal gut microflora of mammals—especially on non-human primates—are rare (Liu et al., 2017,

2019), although fungal infections are known to cause an imbalance of the fungal gut microbiome, eventually leading to morbidity or even mortalities among host organisms (Kapitan et al., 2019). In well balanced microbiomes, gut fungi have an innate immune response mechanism to ensure the normal functioning of the gut and thus the health of the host. For example, dysbiosis of the fungal flora can cause diarrhoea, and abnormalities in the fungal community structure can cause the inflammatory bowel disease (Ai et al., 2014). Hence, it is recommended to expand the study on the diversity and the benefits of fungal gut flora—not only in Hainan gibbons but also in other endangered wildlife species. This will help to better understand the role and function of fungi in food digestion and will thus assist the conservation of those species.

### *4.2. Environmental determinants of alpha and beta diversity*

In our study, we did not unravel any significant difference in the bacterial OTU richness between family groups (Fig. 3a). However, the Shannon diversity of family C was significantly higher compared to that of the other family groups (Fig. 3b). Likewise, the OTU richness of fungal microbiomes was significantly higher in family group C than in family group A (Fig. 7a) and the Shannon diversity was significantly higher in group C than in group B (Fig. 7b). Likewise, the Bray-Curtis dissimilarities and LEfSe analysis revealed significant differences in the fungal community composition between all family groups (Figs. 6, 9). These results suggest family group C to be differently affected by environmental (or social) parameters, raising the question of what environmental variables caused this dissimilarity?

Interestingly, neither environmental PC2 (old and pristine montane rain forest far from water and human settlements) nor environmental PC3 (old forest with prominent human management activities) were significantly correlated to the OTU richness or diversity of bacterial and fungal microbiomes (Table 2), suggesting that—despite that group C resides closest to human settlement and furthest from the next water body (Fig. 10)—human activities or the distance to water play only a minor role in shaping the microbial community composition of Hainan gibbons. Instead, PC1 was significantly correlated with fungal OTU richness (Chao 1 index), and with the bacterial Shannon diversity (Table 2). PC1 is indicative for elevated areas in steep terrain with a dense growth of short trees. These environmental variables are characteristic for high altitude (>1200 m asl) TMEF as described by Jiang et al. (2016). The habitat type is typified by dense stands of laurel trees (Lauraceae) and was described as the main habitat of Hainan gibbons (Deng and Zhou, 2016). Family group C, with the highest bacterial and fungal Shannon diversity is almost entirely restricted to this habitat (TMEF: 85.0%; Fig. 1d), suggesting that the diet obtained from the laurel forest may explain the increased OTU richness and diversity in the gut microbiome of Hainan gibbons. By contrast, family group A and B, occurred at lower altitudes (around 900 m asl) comprising mainly of Tropical Montane Rain Forest (group A: 100% TMRF, group B: 61.3% TMRF; Fig. 1d), possibly instigating the lower bacterial and fungal diversity observed in these two groups. Although this habitat type has the highest plant diversity within the protected area, the soils are characterised by low to moderate fertility (Jiang et al., 2016). Food items obtained from this habitat may therefore have a rather stabilizing effect on the gut microbiome of Hainan gibbons and thus leading to a lower diversity. This finding is strongly supported by a study of Thoemmes et al. (2018), who found that differences in altitude and between seasons explained almost all variation observed in the microbial diversity and community structure of chimpanzees in Tanzania. However, due to the extremely small sample size, these results must be viewed with caution, proposing an in-depth future study to finally unravel the reasons for the deviating microbiomes observed between different family group of Hainan gibbons.

Another possible explanation for the diverting microbiome compositions among gibbon groups could be the sex and age structure or the home range overlap of family groups (Moeller et al., 2016; Thoemmes et al., 2018). Group size ranged from six to ten individuals, whereby each group comprised of one adult male, two adult females, and three to seven sub-adults or juveniles (this study). Home range overlap between groups was marginal (Deng and Zhou, 2016; Deng et al., 2017; Zhou, 2018), at least between group C and the other two families, possibly contributing to the diverting microbiome observed in family group C. Several studies on non-human primates (Moeller et al., 2013, 2016; McKenney et al., 2014; Thoemmes et al., 2018; Reese et al., 2021) indicated that the isolation of groups (i.e., no, or little home range overlap), but also the group size and composition as well as the age and status of its members may have a profound effect on the gut microbiome of apes and other primates. However, due to a lack of sufficient data, this aspect should be interpreted with caution and rendered further attention in forthcoming studies on the Hainan gibbons.

Based on previous studies (reviewed in Fackelmann et al., 2021), we expected the gut microbiome to be affected by the vicinity to human settlements, agriculture, or water sources. However, that seems to have not been the case. Instead, our study insinuated that neither richness nor diversity of the bacterial and fungal microbiomes was affected by human activities or the vicinity to their settlements. This result was rather surprising, given that human activities were shown to disrupt the gastro-intestinal microbiomes of several wildlife species (Amato et al., 2013; Barelli et al., 2015, 2020; Ingala et al., 2019; Juan et al., 2019). Reasons for this include habitat fragmentation and isolation, amplified by additional factors, such as the presence of and contact with humans, their livestock, or other invasive wildlife species (Fackelmann et al., 2021). Despite the weak support and the small sample size, we recommend keeping the contact between Hainan gibbons and humans (as well as their livestock) at a minimum, and to promote the connectivity between preferred gibbon habitats (especially between TMEF and TMRF) so that a social, and thus genetic exchange between family groups can be encouraged.

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## CRediT authorship contribution statement

YL, JK and TW designed the study and performed the experiments. YL, WW, WT, and SP analyzed the data and wrote the manuscript.

## Declaration of Competing Interest

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

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