Variation in the intestinal microbiota of tadpole and adult Hynobius maoershanensis

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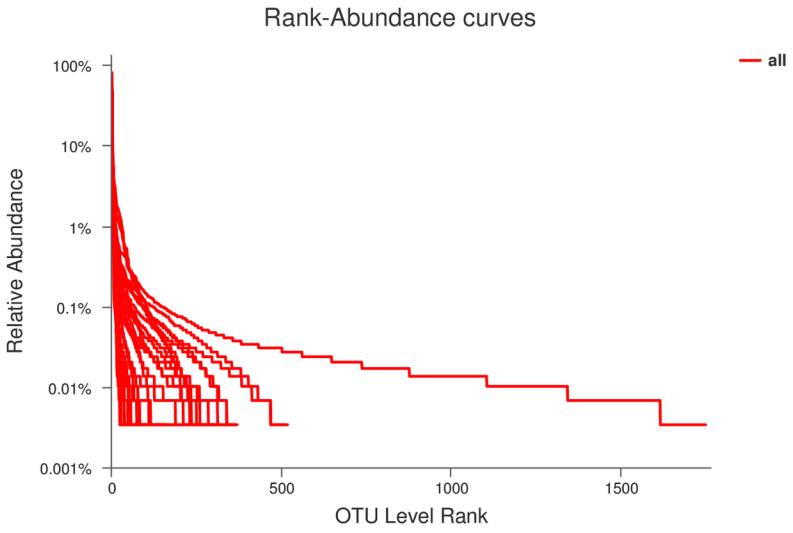
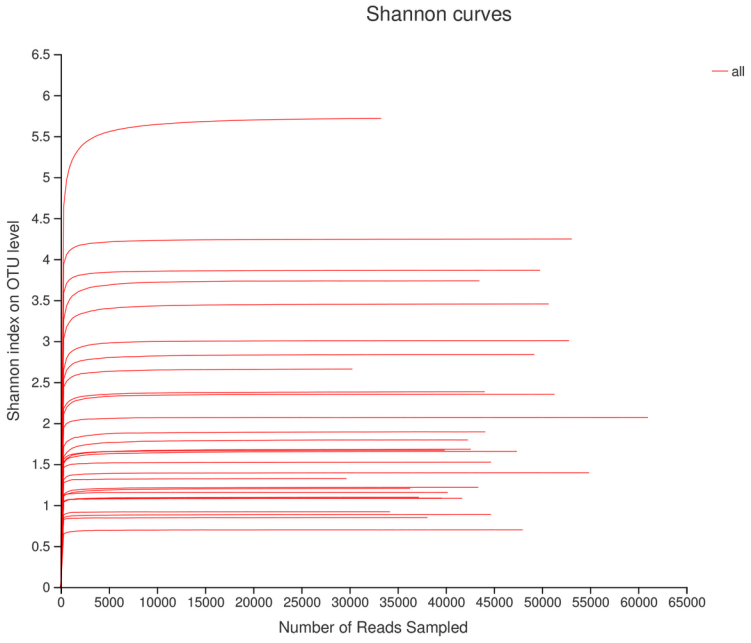
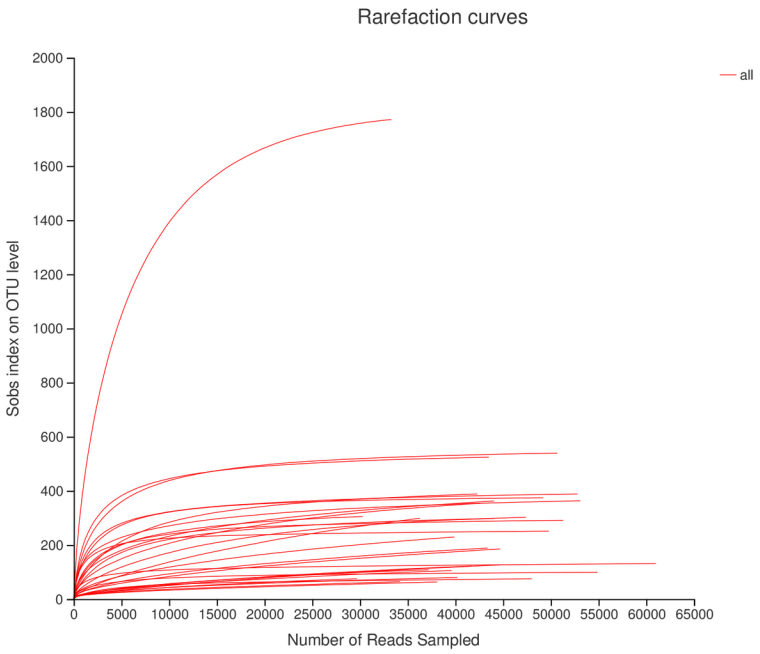
Abstract

The intestinal microbiota play an important role in the life of amphibians. The composition of the intestinal microbial community may vary by developmental stage. In this study, 16S rRNA high-throughput sequencing was used to study the intestinal microbiota of Hynobius maoershanensis tadpoles and adults that exclusively inhabit the Maoer Mountain swamp at an altitude of approximately 2000 m. The results indicated that there was no significant difference in intestinal microbiota between tadpoles and adults. Nevertheless, the abundance of intestinal microbiota in adults was much higher than that in tadpoles. Specifically, at the phylum level, Bacteroidetes was more abundant in adults than in tadpoles. At the genus level, Proteobacteria, Actinobacteria, Cyanobacteria, and Planctomycetes were more abundant in tadpoles, whereas Burkholderiaceae, Caedibacter, Bacteroides, and Serratia were more abundant in adults. A functional prediction analysis revealed that there was no significant difference between tadpoles and adults; however, the function of the intestinal microbiota in H. maoershanensis includes amino acid transport and metabolism, general function prediction only, transcription, energy production and conversion, liquid transport, and metabolism. The aquatic and terrestrial living environment of tadpoles and adults may be the main reason for the difference in intestinal microbiota between tadpoles and adults. Our study provides evidence of variations in the intestinal microbiota of tadpoles and adult amphibians, highlighting the influence of historical developments on the intestinal microbiota and the need for increased understanding of the importance of physiological characteristics in shaping intestinal microbiota of amphibians, which consequently help us to understand the adaptative mechanism of amphibians from an aquatic to a terrestrial environment.







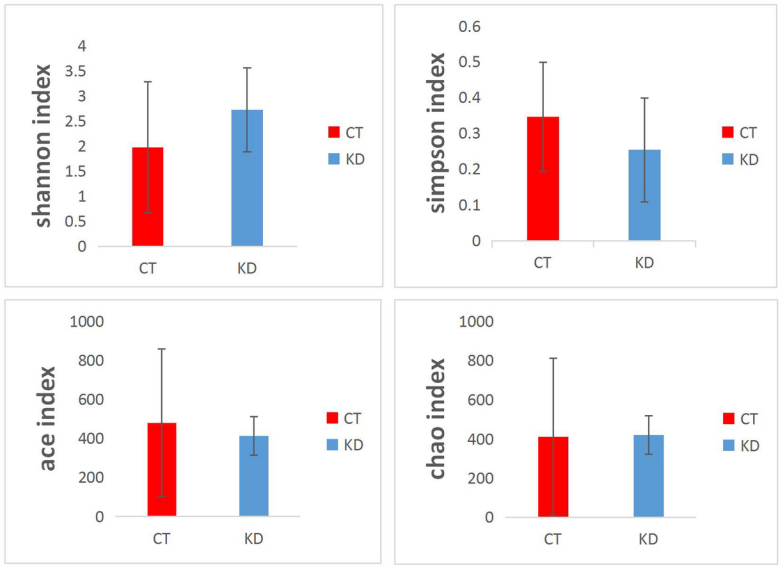


Figure 2. The alpha diversity of intestinal microbiota in tadpoles and adults of H. maoershanensis was not significantly different between the two groups

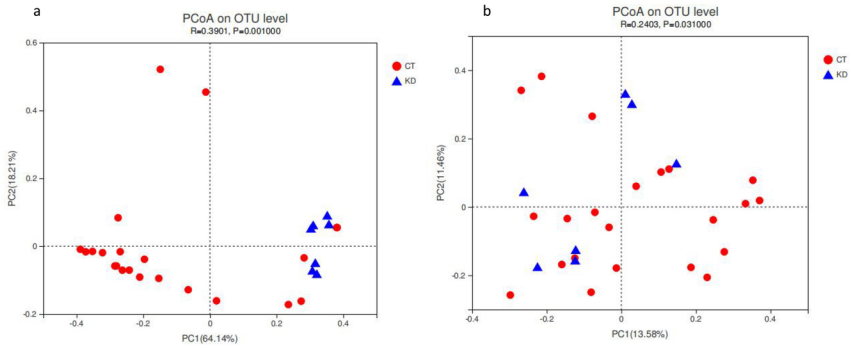
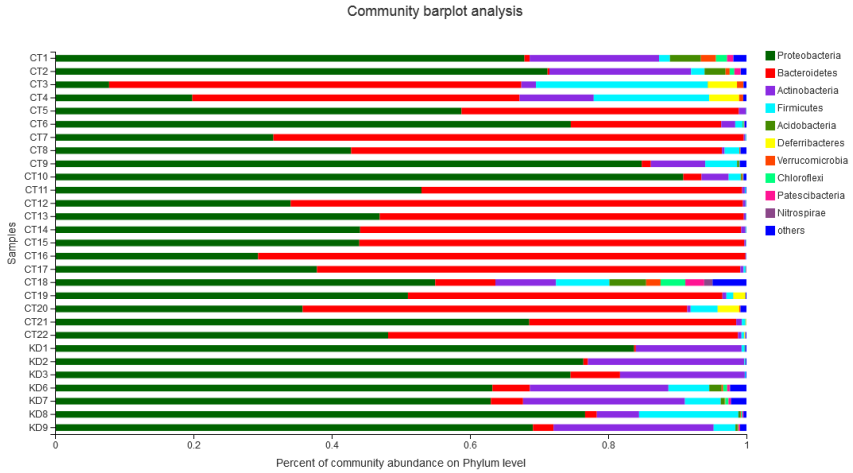
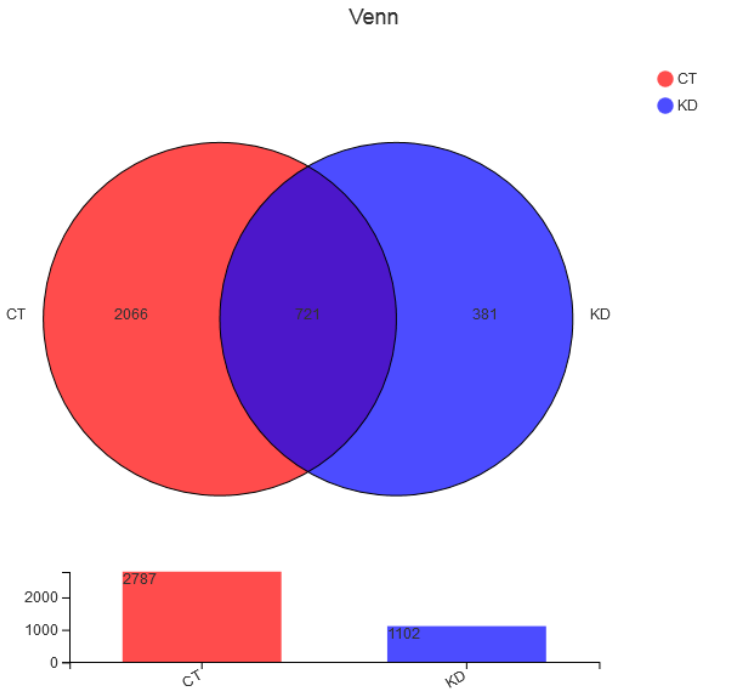
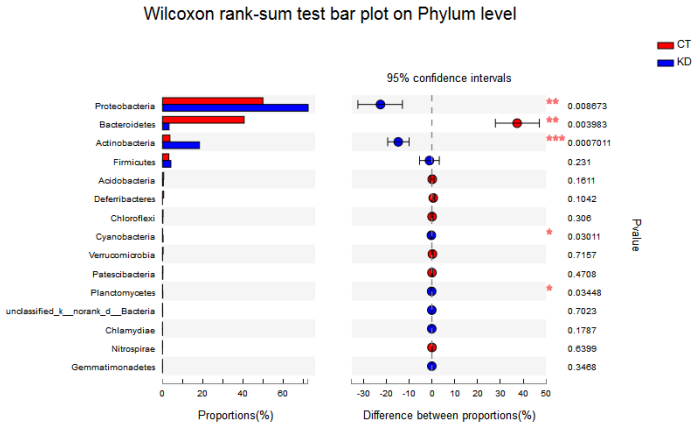
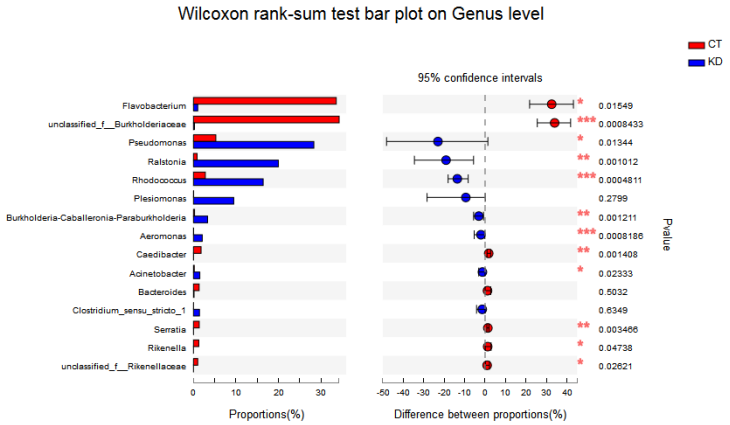


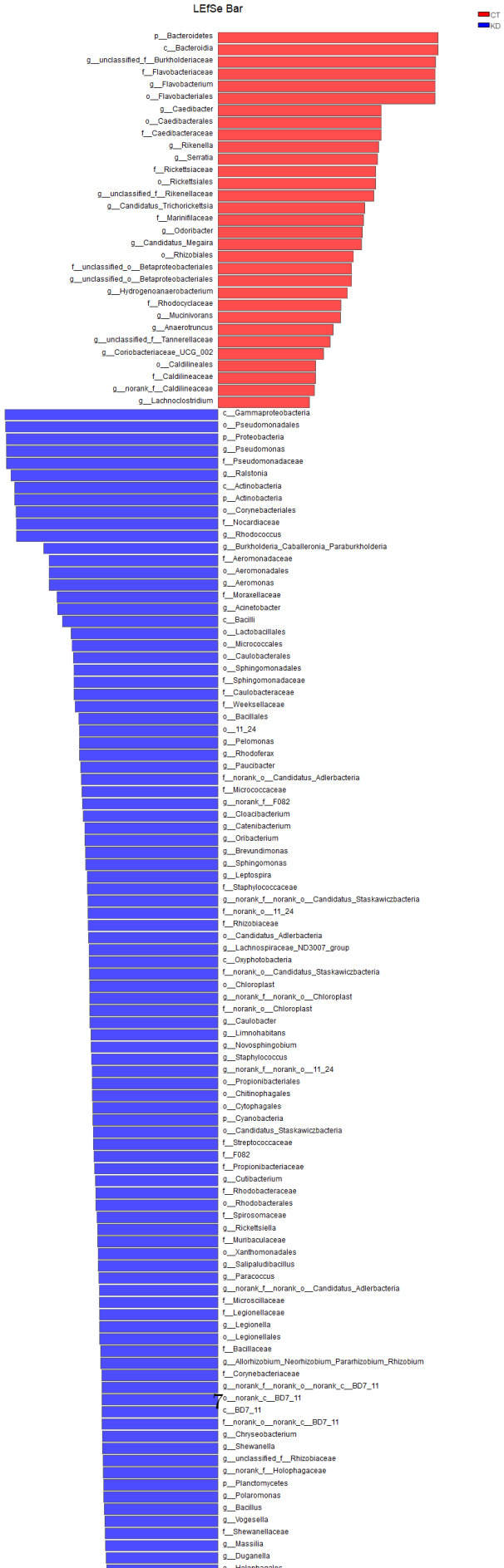
Figure 3. PCoA analysis of microbial community structure differentiation and individual similarity in tadpoles and adults of H. maoershanensis. (a) Based on weighted_unifrac. (b) Based on unweighted_unifrac.











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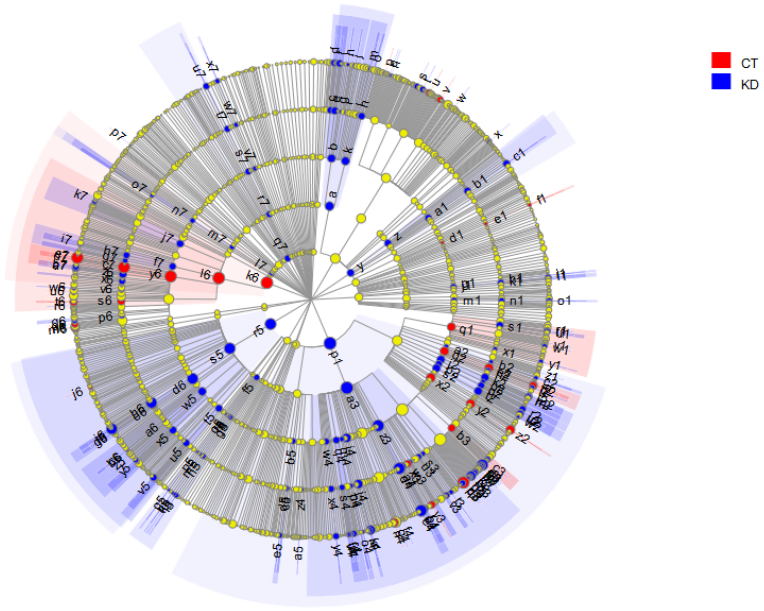
Cladogram



a : c__Bacilli
b : o__Bacillales
c : f__Bacillaceae
d : g__Bacillus
e : f__Staphylococcaceae
f : g__Staphylococcus
g : f__Sporolactobacillaceae
h : g__Sallpaludibacillus
i : f__Listeriaceae
j : g__Brochothrix
k : o__Lactobacillales
l : f__Carnobacteriaceae
m : g__Granulicatella
n : f__Streptococcaceae
o : g__Lactococcus
p : g__Lachnoclostridium
q : g__Lachnospiraceae_ND3007_group
r : g__Oribacterium
s : g__Ruminococcaceae_UCG_005
t : g__Ruminococcaceae_UCG_013
u : g__Hydrogenoanaerobacterium
v : g__Anaerotruncus
w : g__Tisslerella
x : g__Catenibacterium
y : p__Cyanobacteria
z : c__Oxyphotobacteria
a1 : o__Chloroplast
b1 : f__norank_o__Chloroplast
c1 : g__norank_f__norank_o__Chloroplast
d1 : o__Caldilineales
e1 : f__Caldilineaceae
f1 : g__norank_f__Caldilineaceae
g1 : o__11_24
h1 : f__norank_o__11_24
i1 : g__norank_f__norank_o__11_24
j1 : o__Holophagales
k1 : f__Holophagaceae
l1 : g__norank_f__Holophagaceae
m1 : o__Subgroup_2
n1 : f__norank_o__Subgroup_2
o1 : g__norank_f__norank_o__Subgroup_2
p1 : p__Proteobacteria
q1 : o__Rhizobiales
r1 : g__Rhodopseudomonas
s1 : f__Rhizobiaceae
t1 : g__unclassified_f__Rhizobiaceae
u1 : g__Allorhizobium_Neorhizobium_Pararhizobium_Rhizobium
v1 : g__Bosea
w1 : g__norank_f__Beijerinckiaceae
x1 : f__Devosiaceae
y1 : g__Devosia
z1 : g__Acidocella
a2 : o__Tistrellales
b2 : f__Geminicoccaceae
c2 : g__Candidatus_Alysiosphaera
d2 : o__Rickettsiales
e2 : f__Rickettsiaceae
f2 : g__Candidatus_Trichorickettsia
g2 : g__Candidatus_Megaira
h2 : f__SM2D12
i2 : g__norank_f__SM2D12
j2 : o__Caulobacterales
k2 : f__Caulobacteraceae
l2 : g__Asticcacaulis
m2 : g__Brevundimonas
n2 : g__Caulobacter
o2 : f__Hyphomonadaceae
p2 : o__Rhodobacterales
q2 : f__Rhodobacteraceae
r2 : g__Paracoccus
s2 : o__Sphingomonadales
t2 : f__Sphingomonadaceae
u2 : g__Novosphingobium
v2 : g__Sphingomonas
w2 : g__Sphingopyxis
z3 : o__Pseudomonadales
a4 : f__Moraxellaceae
b4 : g__Enhydrobacter
c4 : g__Acinetobacter
d4 : f__Pseudomonadaceae
e4 : g__Pseudomonas
f4 : g__Rickettsiella
g4 : g__Enterobacter
h4 : g__Serratia
i4 : o__Aeromonadales
j4 : f__Aeromonadaceae
k4 : g__Aeromonas
l4 : g__Tolumonas
m4 : o__Alteromonadales
n4 : f__Shewanellaceae
o4 : g__Shewanella
p4 : f__Alteromonadaceae
q4 : o__Xanthomonadales
r4 : g__Stenotrophomonas
s4 : f__Rhodanobacteraceae
t4 : g__Dokdonella
u4 : g__Dyella
v4 : g__unclassified_f__Rhodanobacteraceae
w4 : o__Legionellales
x4 : f__Legionellaceae
y4 : g__Legionella
z4 : f__mle1_27
a5 : g__norank_f__mle1_27
b5 : o__Bdellovibrionales
c5 : f__Bacteriovoracaceae
d5 : f__Bdellovibrionaceae
e5 : g__Bdellovibrio
f5 : c__Parcubacteria
g5 : o__Candidatus_Kaiserbacteria
h5 : f__norank_o__Candidatus_Kaiserbacteria
i5 : g__norank_f__norank_o__Candidatus_Kaiserbacteria
j5 : o__Candidatus_Jorgensenbacteria
k5 : g__norank_f__norank_o__Candidatus_Jorgensenbacteria
l5 : o__Candidatus_Staskawiczbacteria
m5 : f__norank_o__Candidatus_Staskawiczbacteria
n5 : g__norank_f__norank_o__Candidatus_Staskawiczbacteria
o5 : o__Candidatus_Adlerbacteria
p5 : f__norank_o__Candidatus_Adlerbacteria
q5 : g__norank_f__norank_o__Candidatus_Adlerbacteria
r5 : p__Actinobacteria
s5 : c__Actinobacteria
t5 : o__Propionibacteriales
u5 : f__Propionibacteriaceae
v5 : g__Cutibacterium
w5 : o__Micrococcales
x5 : f__Micrococcaceae
y5 : g__Neomicrococcus
z5 : g__Leucobacter
a6 : f__Promicromonosporaceae
b6 : g__Ieoptericola
c6 : g__Promicromonospora
d6 : o__Corynebacteriales
e6 : f__Corynebacteriaceae
f6 : g__Lawsonella
g6 : g__Corynebacterium_1
h6 : f__Nocardiaceae
i6 : g__Rhodococcus
j6 : g__Coriobacteriaceae_UCG_002
k6 : p__Bacteroidetes
l6 : c__Bacteroidia
m6 : g__Rikenella
n6 : g__unclassified_f__Rikenellaceae
o6 : g__Mucinivorans
p6 : f__Prolixibacteraceae
q6 : g__BSV13
r6 : g__norank_f__Prevotellaceae
s6 : f__Marinifilaceae
t6 : g__Odoribacter
u6 : g__unclassified_f__Tannerellaceae
v6 : f__F082

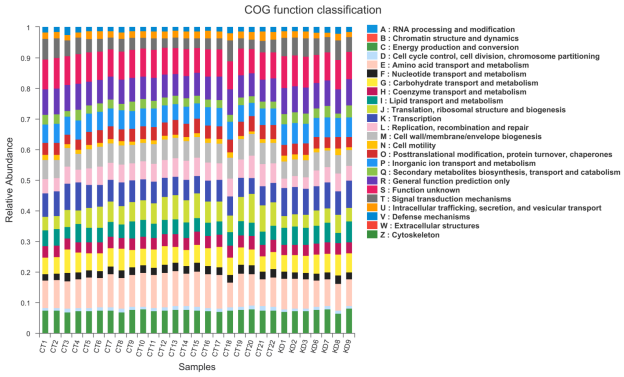


Table 1. Alpha diversity index of *H. Maoershanensis* gut microbiota

Index	CT	KD	p value
Shannon	1.97 ± 1.31	2.72 ± 0.84	0.203
Simpson	0.35 ± 0.15	0.25 ± 0.15	0.316
ACE	480.37 ± 377.26	412.74 ± 97.85	0.543
Chao	410.60 ± 400.85	420.32 ± 98.561	0.933

Variation in the intestinal microbiota of tadpole and adult Hynobius maoershanensis

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Abstract: The intestinal microbiota play an important role in the life of amphibians. The composition of the intestinal microbial community may vary by developmental stage. In this study, 16S rRNA high-throughput sequencing was used to study the intestinal microbiota of *Hynobius maoershanensis* tadpoles and adults that exclusively inhabit the Maoer Mountain swamp at an altitude of approximately 2000 m. The results indicated that there was no significant difference in intestinal microbiota between tadpoles and adults. Nevertheless, the abundance of intestinal microbiota in adults was much higher than that in tadpoles. Specifically, at the phylum level, Bacteroidetes was more abundant in adults than in tadpoles. At the genus level, Proteobacteria, Actinobacteria, Cyanobacteria, and Planctomycetes were more abundant in tadpoles, whereas *Burkholderiaceae*, *Caedibacter*, *Bacteroides*, and *Serratia* were more abundant in adults. A functional prediction analysis revealed that there was no significant difference between tadpoles and adults; however, the function of the intestinal microbiota in *H. maoershanensis* includes amino acid transport and metabolism, general function prediction only, transcription, energy production and conversion, liquid transport, and metabolism. The aquatic and terrestrial living environment of tadpoles and adults may be the main reason for the difference in intestinal microbiota between tadpoles and adults. Our study provides evidence of variations in the intestinal microbiota of tadpoles and adult amphibians, highlighting the influence of historical developments on the intestinal microbiota and the need for increased understanding of the importance of physiological characteristics in shaping intestinal microbiota of amphibians, which consequently help us to understand the adaptative mechanism of amphibians from an aquatic to a terrestrial environment.

Key Words: *Hynobius maoershanensis*, tadpole, adult, intestinal microbiota

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1. Introduction

A detailed understanding of how the intestinal microbial community of an organism is formed and utilized throughout its life cycle is essential to understanding how human and natural disturbances affect endangered amphibian species (Zhang et al., 2020). Phylogeny and evolution (Gutierrez et al., 2021; Xiao et al., 2021; Yatsunenko et al., 2012; Gaulke et al., 2018), dietary preferences and choices (Li et al., 2014), ambient temperature (Hagi et al., 2004; LeaMaster et al., 2005; Khol et al., 2016), transition from larval stage to adult stage (Buchholz et al., 2006; Atkinson et al 1998), and hibernation (Weng et al. 2016. Tong et al., 2019) can affect the composition of the intestinal microbiome. The metamorphosis of amphibians causes drastic physiological and morphological changes, such as gastrointestinal remodeling, dietary changes, and gastrointestinal physiological index changes (Gilbert et al., 1996; Zhang et al. 2020). These complex changes associated with age or during metamorphosis directly and critically affect changes in intestinal microbiota (Zhang et al. 2018; Yatsunenko et al., 2012).

The negative correlation between intestinal length and the proportion of animal prey in the diet is one of the most widely accepted ecomorphological relationships in vertebrates (Davis et al., 2013). The digestive tract is an important interface between the host and the external environment (Adler et al., 2005; Davis et al., 2013). Changes in the intestinal structure are closely related to the changes in the dietary structure, such as phytophagous tadpoles with longer digestive tracts to insectivorous adults with shorter digestive tracts (Wagner et al., 2009; Kohl et al., 2013), which commonly leads to a reorganization of intestinal microbiota.

Previous studies have shown that there are significant differences in the intestinal microbiota during the metamorphosis of frogs (Anura) (Khol et al., 2013; Chai et al., 2018). For example, there were significant differences in the intestinal microbial communities between tadpoles and frogs (mature), that Proteobacteria decrease with age, whereas Firmicutes and Bacteroidetes increase(Kohl et al., 2013; Warne et al., 2019). During the development stage from aquatic to terrestrial larvae (frogs), Proteobacteria and Actinomycetes decrease with age, but the Bacteroidetes and Clostridium increase with age (Chai et al., 2018). This shows that the intestinal microbiota of organisms change during growth and development. Generally, the microbiota of tadpoles was close to that of bony fish (Proteobacteria), such as Dicentrarchus labrax (Carda et al., 2014), and that of frogs were closer to amniotic animals (Bacteroidetes), such as wild mice (Weldon et al., 2015). Identifying the changes in the microbial community composition and abundance during amphibian development can reveal interactions between hosts and microorganisms in wild animals.

In a study of the oxygen-related phenotype of intestinal microbiota during the metamorphosis of ornamented pygmy frogs (*Microhyla fifissipes*), Zhang et al. (2020) found that from tadpole to adult, the

proportion of anaerobic bacteria gradually decreased as the species evolved from an aquatic to a terrestrial environment. This may have resulted from the specific fasting experienced by amphibians during the climax of metamorphosis(Kohl et al. 2014; Koh et al. 2016). Tadpoles do not eat or eat less and mostly rely on tissue degradation to obtain energy for metamorphosis and development. They also obtain energy through the oxidation of accumulated fat and carbohydrates, resulting in the reduction of facultative anaerobes (Warne et al., 2017; Warner et al., 2019; Zhang et al., 2021). Generally, the abundance of anaerobic bacteria in the intestinal microbiota of aquatic organisms is relatively higher compared with that of terrestrial animals. For example, the content of facultative anaerobic Proteobacteria, Legionella, and Mycoplasma in the intestinal microbiota of Larval aquatic organism *juvenile lamprey* is the highest (Tetlock et al., 2012), and the content of Proteobacteria of discus fish (*Symphysodon haraldi*) and the Chinese mitten crab (*Ercheioir sinensis*) is generally high (Zhang et al., 2021; Li et al., 2007). The intestinal microbiota of the terrestrial giant panda and pig consist primarily of anaerobes, Firmicutes, followed by Proteobacteria (Zhu et al., 2011; Isaacson et al., 2012). It can be seen that the composition of intestinal microbiota is significantly different in aquatic organisms and terrestrial organisms, which may be closely related to the living environment of these organisms (Isaacson et al., 2012).

Besides the research on tailless amphibians, there are a small number of studies on intestinal microbiota in the metamorphosis process of tailed amphibians (Zhang et al., 2018). The research on the Chinese giant salamander indicated that Bacteroides (47.76%) account for the intestinal microbiome during the first year, Proteobacteria from the second (32.88%) to third (30.78%) years, and Firmicutes in the fourth year (34.70%) (Zhang et al., 2018). The changes are roughly the same as those of tailless amphibians (kohl et al., 2013; Chai et al., 2018; Zhang et al., 2020). These studies show that the intestinal microbiota of amphibians are constantly changing during metamorphosis.

In this study, we examined the microbiota of tadpoles (forelimb bud stage) and adults of wild *H. Maoershanensis*. *H. maoershanensis* is a tailed amphibian with abnormal development in that tadpoles live in water and adults are amphibious. We hypothesized that the progression of intestinal flora from tadpoles to adults would show a reduction of Proteobacteria and an increase of Firmicutes, similar to other amphibians, and the intestinal flora would be consistent with the adaptive characteristics of aquatic to terrestrial organisms.

2. Materials and Methods

2.1 Sample collection and preservation

H. Maoershanensis belongs to the *Hynobius Hynobius* genus and only lives in an area around the high mountain marshes of Xingan County, Guangxi Zhuang Autonomous Region (25°52′N, 110°24′E) at an altitude of 1950–2000 m (Fang et al., 2006). *H. Maoershanensis* primarily lives in the surrounding area of the alpine swamp, and the vegetation nearby consists mainly of hemlock forest and mountaintop dwarf forest (Chen et al., 2021).

We collected 22 adults during the breeding period, and seven tadpoles hatched in the same period for sampling in December 2019. Anal swabs were collected by nondestructive sampling along with tadpoles, the cloaca was wiped with an alcohol cotton pad, a sterile cotton swab was inserted, rotated for three to five circles, and placed into a sterile preservation tube (Colston et al., 2015). The samples were frozen immediately after collection, transported to the laboratory, and stored in a refrigerator at −80℃(Song et al., 2018). After sampling, all *H. Maoershanensis* were returned to their original place.

2.2 DNA extraction, amplification, and sequencing

Total DNA of the bacterial genome from all fecal samples was extracted using the E.Z.N.A .® Soil DNA kit (Omega Bio-tek). The V3–V4 hypervariable region of the 16S rRNA gene was amplified by the polymerase chain reaction (GeneAmp 9700; ABI) using the general bacterial primers (338F, 5′-ACTCCTACGGGAGGCAGCAG-3′; 806R, 5′-GGACTACHVGGGTWTCTAAT-3′) (Mori et al., 2014). Initial PCR was performed using Transgen ap221–02 TransStart® FastPfu Fly DNA Polymerase. A 20 μl reaction mixture contained 10 ng template DNA, 4 μl 5×fastPfu buffer, 2 μl 2.5 mM dNTPs, 0.8 μl each primer (5 μM), and 0.2 μl bovine serum albumin. The PCR product was eluted from a 2% agarose gel and purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences). The purified PCR fragment was collected and adjusted to an equal molar concentration (Majorbio BioPharm Technology Co., Ltd, Shanghai provide sequencing services), and the paired ends were sequenced (2 × 300) using an Illumina Miseq platform (Illumina).

2.3 Data analysis

On the basis of the overlap between PE reads, the paired reads were merged into a single sequence and the quality of the reads and the effect of the merge were filtered by quality control. According to the barcodes and primer sequences at the beginning and end of the sequence, the effective sequences were obtained and the sequence direction was corrected. The data were flattened according to the minimum number of sample sequences. The base was filtered if the tail mass value was less than 20 and set to a window of 50 bp. The back-end base was cut from the window if the average quality value in the window was lower than 20. The reads below 50 bp were filtered after quality control the reads containing an “N”

base were removed. The minimum overlap length was 10 bp, the maximum mismatch ratio of the overlap region was 0.2, the allowable mismatch number of the barcode was 0, and the maximum mismatch number of the primers was 2 (Trimmomatic software, Illumina) (Vickery et al. 2017).

The original fastq file uses flash for pair-end double-ended sequence splicing. Uparse (version number: 7.0.1090; http://www.drive5.com/uparse/) operational taxonomic unit (OTU) clustering, and Qiime (version number: 1.9.1; http://qiime.org/install/index.html) were used. The water abundance table for each taxonomic species was generated and the beta diversity distance was calculated. An RDP classifier (version 2.11; https://sourceforge.net/projects/rdp-classifier/) was used for sequence classification annotation. Using the Greengenes database (version 135; http://greengenes.secondgenome.com/), each 16S rRNA gene sequence was classified using rRNA database alignment.

The alpha diversity indices (Shannon index, Simpson index, abundance-based coverage estimator (ACE), and Chao estimator) were calculated using the mothur program (version v.1.30.1; http://www. mothur.org/ wiki/ Schloss_SOP# Alpha_ diversity). The diversity distance matrix of beta was calculated by Qiime (http://qiime.org/install/index.html), and a tree was drawn using the R language. Principal coordinate analysis (PCoA) was used to calculate and visualize weighted and unweighted UniFrac distance matrices. Permutational multivariate analysis of variance (PERMANOVA) was used to further determine the difference of intestinal flora between the two populations and a histogram of bacterial composition was generated according to the results of the classification analysis. PERMANOVA was performed using the vegan software package. The Wilcoxon rank-sum test and FDR adjusted p values were used to test the difference of genes, KEGG pathways, and COG function analysis (http://picrust.github.io/picrust/) between the two groups to predict their function. All data were analyzed using the Majorbio I-Sanger Cloud Platform (http://www.i-sanger.com).

3. Results

3.1 Sequence quality evaluation

A total of 22 adult and 7 tadpole samples were collected. A total of 1390147*2 sequences were obtained of which the effective sequence number was 1390147 and the average length of the sequence was 424.11. OTUs with a 97% similarity level are often used for bioinformatics analysis. The analysis of alpha diversity and dilution curves of these OTUs revealed that the sequencing quantity was sufficient. (Figure 1).

3.2 Analysis of alpha and beta diversity

The alpha diversity analysis of 3168 OTUs showed that there were no significant differences in the Shannon ($p = 0.203$), Simpson ($p = 0.316$), ACE ($p = 0.543$), and Chao ($p = 0.933$) indices between tadpoles

and adults (Table 1). PCoA based on unweighted and weighted UniFrac distances showed that the intestinal microflora were highly aggregated by population as shown by the beta diversity (Figures 2 and 3).

3.3 Intestinal microbial community structure

OTUs (n = 3168) were obtained from the samples that comprised 47 phyla and 1688 species. Proteobacteria (772, 24.3%), Firmicutes (544, 17.1%), and Bacteroidetes (498, 15.7%) were the main components, followed by Actinobacteria (238, 7.5%) and Acidobacteria (144, 4.5%) (Figure 4a). The dominant genera were *Burkholderiaceae* (15, 0.4%), *Flavobacterium* (16, 0.5%), and *Pseudomonas* (6, 0.1%) (Figure 4b). Tables 2 and 3 show other phyla and genera. Tadpoles have a community dominated by Proteobacteria, Actinobacteria, and Cyanobacteria, whereas the adults have a community dominated by Proteobacteria and Bacteroidetes (Figure 4).

3.4 Composition differences of intestinal microbiota

According to the Wilcoxon rank-sum test and the results of taxonomic analysis, 2787 species were detected in adults, 1102 species were detected in tadpoles, 721 species were common to both groups, 2066 species were endemic to adults, and 381 species were endemic to tadpoles (Figure 5) according to the minimum sample sequence (CT5:29539). The species of intestinal microbes in adults were significantly more abundant compared with those in the tadpoles. At the phylum level, the Bacteroidetes of the adults were more abundant than the tadpoles, whereas Proteobacteria, Actinobacteria, Cyanobacteria, and Planctomycetes were more abundant in the tadpoles (Figure 6a, Table 2a). At the genus level, the *Flavobacterium*, *unclassified_ F_ Burkholderiaceae*, *Caedibacter*, *Serratia*, *Rikenella*, and *Unclassified_ F_ Rickenellaceae* in the adults were more abundant than in the tadpoles, whereas *Pseudomonas*, *Ralstonia*, *Rhodococcus*, *Burkholderia-Caballeronia-Paraburkholderia*, *Aeromonas*, and *Acinetobacter* were more abundant in the tadpoles (Figure 6b, Table 2b). Lefse was used to determine the difference in the relative abundance of bacterial taxa at the phylum, class, order, family, and genus level between the two groups. The results were similar to that of the Wilcoxon rank-sum test in which adults exhibited abundant *Bacteroidetes*, *bacteroidia*, *unclassified_ F_ Burkholderiaceae*, *Flavobacterium CEAE*, *Flavobacterium*, *Flavobacteria*, and *caedibacter*, whereas tadpoles exhibited more abundant *Proteobacteria*, *Pseudomonas*, *Pseudomonas* daceae, *Ralstonia*, and *actinobacteria* (Figure 7).

3.5 Differences in the functional distribution of intestinal flora

The Wilcoxon rank-sum test was used to identify differences in KEGG pathways to determine a function of the intestinal flora in tadpoles and adults. There was no significant difference between KEGG pathway levels 1 and 2 (Table 3a, 3b). It can be seen from the box diagram that the functional composition

of the sample COG of the *H. maoershanensis* tadpole and adult is relatively similar. The main functions included amino acid transport and metabolism, general function prediction only, transcription, energy production and conversion, liquid transport, and metabolism (Figure 8).

4. DISCUSSION

In this study, we found that there was no significant difference in the diversity of intestinal flora between tadpoles and adults, but there were significant differences in the intestinal microbial communities. Several studies have shown that to adapt to the habit of eating grass, amphibian tadpoles require longer intestines, so that food can remain longer for digestion and fermentation (Castaneda et al. In 2006). The intestinal microbiota of tadpoles have a higher richness and diversity compared with adults who adapt to meat and have shorter intestines (Kohl et al. 2013; Ventes et al. 2016), but this diversity change in *H. maoershanensis* was not reflected in this study. Some studies have shown that the adult digestive tract of *H. maoershanensis* is short (Chen et al., 2021), which is consistent with the short intestinal tract of carnivores (Altig and Kelly 1974; Alford 1999). It has been hypothesized that the reason for the lack of difference in diversity is the sampling time, which was during the underwater breeding period for *H. maoershanensis*. This is consistent with the living environment of tadpoles as a large proportion of intestinal microbiota accumulates from the surrounding environment (Bo et al., 2021; Hagi et al., 2005; LeaMaster et al., 2016). Consequently, the adult intestinal microbiota during the nonreproductive period should be analyzed.

The intestinal microbiota of mammals, aquatic animals, and amphibians are dominated by anaerobic bacteria, which account for more than 99% of the bacteria and include Proteobacteria, Bacteroides, Firmicutes, and Actinomycetes (Chen et al., 2021; Backhed et al., 2005; Langille et al., 2014; Frese et al., 2015; Zhang et al., 2020; Sun et al., 2020). The intestinal microbiotas of *H. maoershanensis* are mostly anaerobic bacteria, in which the community of tadpoles dominated by mainly Proteobacteria and Actinobacteria, whereas the adults contain Proteobacteria and Bacteroidetes.

The relative abundance of Proteobacteria, Actinobacteria, Cyanobacteria, and Planctomycetes decreased significantly through the development from tadpole to adult. Tadpoles maintain a community dominated by Proteobacteria and Actinomycetes, which is consistent with tailed amphibians: larvae of salamander and the Chinese giant salamander (Andrias davidianus)(Bletz et al. 2016; Zhang et al., 2018), whereas the adults maintain a community dominated by Bacteroidetes.

Proteobacteria decreases during metamorphosis, which may be an adaptation to nutrient absorption and food intake (Zhang et al. 2018). On the basis of the results of this study, it can be inferred that during the development of tadpoles into adults, food tends to contain a higher amount of carbohydrates, fat, and protein.

Proteobacteria is the main microbiota in water and is found in many aquatic animals (Friedman et al. 2009; Küchler et al., 2009), and Proteobacteria are associated with RNA processing and degradation as well as outer membrane and lipopolysaccharide synthesis, for example, tadpoles need to synthesize a large number of cell membranes, lipids and proteins for body development, after developing into adults, they gradually tend to be stable and then decline with the increase of age(Fang et al., 2005). The reduction of Proteobacteria after metamorphosis confirms our hypothesis that the intestinal microbiota of tadpoles more closely matches that of aquatic animals, and the reduction of bacteria found in an aquatic environment is related to adapting to the terrestrial environment.

Actinomycetes play an important role in the degradation of cellulose, which decrease with age, suggesting that the plant feeding habits of the salamander(Tang et al., 2015) decrease during metamorphosis. Actinobacteria mediate the degradation of complex polymers such as chitin (polysaccharide with high nitrogen content), lignin, and cellulose. It is widely distributed in fish and mollusks and with the biological control of other bacteria and fungi by producing antimicrobial agents (Tang et al., 2015). Studies have pointed out that tadpoles feed on insects, amphibians, earthworms and shrimp(Ning et al., 2020) , and the shells of insects, tentacles of mollusks, crustaceans, scales of fish, amphibians, and fungi, which are rich in chitin (Tang et al., 2015 ; Muzzarelli et al., 2012).

The levels of Cyanobacteria decrease with age in *H. maoershanensis,* Cyanobacteria can fix nitrogen and are widely distributed, they are found in harsh environments, primarily at low temperatures in the water. Cyanobacteria decrease with age in *H. maoershanensis* suggests that the living environment of the adult has transferred from aquatic environment to land. Planctomycetes use nitrite (NO^{2-}) to oxidize ammonium ion (NH^{4+}) to generate nitrogen for energy under anoxic conditions. The energy metabolism of larvae is higher than that of adolescents and tends to be stable after adulthood (Warne et al., 2017; Warner et al., 2019; Chai et al., 2018). In this study, the observed reduction of Planctomycetes from tadpole to adult indicates that the oxygen in the adult living environment is more abundant and energy metabolism is reduced, which may be the result of adult adaptation to a land environment.

Bacteroidetes are involved in the fermentation of carbohydrates, the utilization of nitrogen-containing substances, and the biotransformation of steroids. Most intestinal bacteria are glycolytic, which means that they obtain carbon and energy through the hydrolysis of carbohydrates and can turn to host polysaccharides when dietary polysaccharides are scarce (Backhed et al., 2005), similar to amniotic animals. But there was no significant difference in intestinal microbial diversity between tadpoles and adults of these species

(Isaacson et al., 2012; Scupham et al., 2008). The abundance of Bacteroidetes increases with development. Bacteroides are the main organism in intestine. Previous studies have demonstrated that bacteroidea play a role in protein degradation in vertebrates, which is related to the intake of high amounts of fat and high (Jang et al., 2021; McDermott et al., 2014; Hooper et al., 2004; Sears et al., 2005). The increase of Bacteroidetes confirmed the transformation of feeding habits from high fiber to a high-fat and high-protein diet. Bacteroides are adapted to high-altitude environments (Zhang et al., 2018) and *H. maoershanensis* is distributed in high-altitude geographical locations (Zhang et al., 2018). It may also be affected by the phylogeny of the host (Xiao et al., 2021; Gaulke et al., 2018). However, to identify the specific reasons, more studies at the different developmental stages are needed.

Through the study of the digestive tract tissue structure of *H. maoershanensis*, we determined that the adult food of *H. maoershanensis* is mainly earthworms, frog tadpoles, and mosquito larvae (Chen et al., 2021). However, there is a lack of feeding data for tadpoles; thus, the basis for the change of intestinal microbiota is unclear.

We also found that during the transformation from tadpole to adult, potential pathogenic bacteria, such as *Acinetobacter*, *Aermonas*, and *Flavobacterium* in the intestine changed. Recent studies have shown that ectopic colonization of intestinal microbiota occurs in the active stage of the disease (Shock et al., 2021; Salome et al., 2021). For example, *Flavobacterium* is a strictly aerobic bacterium that causes various diseases (Gibbs. 1973). It is dominant in the intestinal microbiota of many terrestrial vertebrates (Li et al., 2020; Lei et al., 2020), and this change may represent adaptation from an aquatic to a terrestrial environment. However, it also suggests that the pathogenic risk of *H. maoershanensis* during development is high. A more detailed analysis should be done on the intestinal flora at various age intervals.

Although there was no significant difference in intestinal microbial diversity between tadpoles and adults of *H. maoershanensis*, the richness and bacterial community structure of the two populations are quite different, especially the decrease of Proteobacteria abundance and the increase of Bacteroidetes abundance. These findings provide evidence for the changes of intestinal microbiota in tadpoles and adult amphibians. Concerning the impact of the aquatic to terrestrial living environments, the host phylogeny and diet may be the primary reasons for the differences in intestinal microbiota between tadpoles and adults. This deepens our understanding of the importance and necessity of physiological characteristics in shaping amphibian intestinal microbiota and provides insight into the adaptation mechanism of amphibians from aquatic to terrestrial environments.

ETHICAL STATEMENT

According to the ticket code (4521033) of the Maoershan National Nature Reserve in Guangxi, we were allowed to enter the research site and collect samples. The study did not involve any animal tissues. All animals were returned to the original collection site after collection.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTIONS

Bo Yang: Formal analysis-Equal, Writing-original draft-Equal; Meihong Ning: Investigation-Equal; Yu Chen: Investigation-Equal; Zhenzhen Cui: Investigation-Equal; Zhengjun Wu: Writing-review & editing-Equal Huayuan Huang: Conceptualization-Equal, Writing-review & editing-Equal; Writing-review & editing-Equal.

DATA AVAILABILITY STATEMENT

The raw sequencing data from the current study are avail┐able in the Dryad repository at https://doi.org/10.5061/dryad.zs7h44j9t

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Table 1. Alpha diversity index of *H. Maoershanensis* gut microbiota

Index	CT	KD	p value
Shannon	1.97 ± 1.31	2.72 ± 0.84	0.203
Simpson	0.35 ± 0.15	0.25 ± 0.15	0.316
ACE	480.37 ± 377.26	412.74 ± 97.85	0.543
Chao	410.60 ± 400.85	420.32 ± 98.561	0.933