Gut Microbiota Structure of Dead Migratory Birds in Dali Nouer Lake, Chifeng City, China, based on Bacterial 16S rRNA Amplicon Sequencing

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Gut microbiota structure of dead migratory birds in Dali Nouer Lake, Chifeng City, China, based on bacterial 16S rRNA amplicon sequencing

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Abstract

In August 2018, hundreds of migratory birds died in the area of Dali Nouer Lake, Chifeng City, China. We collected the remains of dead birds along with water and aquatic plants from the birds' environment. The bacterial communities of all samples were profiled by high-throughput sequencing of the V3–V4 hypervariable region of the 16S rRNA amplicon. At the genus level, *Bacteroides, Clostridium, Plesiomonas, Vibrio, Fusobacterium,* and *Aeromonas* were the dominant genera in dead birds, the lake water, and aquatic plants in 2018. However, the relative abundances of these bacterial genera were significantly reduced compared with the levels obtained from healthy migratory bird feces, lake water, and aquatic plants from the same place and time period in 2019. Combined with environmental factors such as the changes in salt content and pH, the invasion and reproduction of those pathogens may have promoted the decline and death of the birds.

Keywords: migratory birds, 16S rRNA amplicon, gut microbiota

Introduction

As migratory birds can carry and spread pathogenic microorganisms over long distances, this has raised international awareness of their health problems (Dijk et al., 2018; Allarda et al., 2019, Alvarez et al., 2010). In August 2018, hundreds of migratory birds died at Dali Nouer Lake of Chifeng City, Inner Mongolia autonomous region, China. Among the species of birds were *Larus ridibundus*, *Pluvialis squatarola*, *Tadorna ferrginea*, *Anas poecilorhyncha*, *Aix galericulata,* and *Tadorna ferrginea*. The dead birds were found to have congestion or bleeding in the intestinal mucosa or an enlarged liver and pancreas, but no obvious abnormalities were observed in other visceral organs. Upon first observation, some birds had no obvious clinical lesions, only displaying signs of malnutrition and wasting. To prevent a pandemic of zoonotic bacteria, attention should be paid to the bacterial flora carried by migratory birds, especially during outbreaks. It is known that interactions between animals and their gut microbiota play an important role in regulating host physiological processes (Round & Mazmanian, 2009; Sommer & Bäckhed, 2013). The host's gut microbiota will be altered when external pressures change (Zaneveld, McMinds, & Vega, 2017). We collected the remains of the dead birds along with samples of water and aquatic plants from the lake to examine the structure of the microbiota and the presence of pathogens. Because there were no relevant data before the present pandemic, in the following year we collected and analyzed the stools of healthy migratory birds (no outbreak) and the same environmental samples mentioned above from the same locations as the references.

Materials and Methods

Sample collection and preparation

In 2018, 19 freshly dead migratory birds (not corrupted) were collected at Dali Nouer Lake of Chifeng City, Inner Mongolia autonomous region, China. Their visceral organs and intestinal tracts were sampled. Meanwhile, lake water and aquatic plant samples were also collected from three different points; two were from Dali Nuoer Lake (Nanhekou and Beihekou) where dead birds were found, and the third was from the contiguous Ganggeng Nuover Lake without dead birds. Based on morphological observations, we assigned lesion samples: intestinal tract (A3C, A5C, A8C, A11C, A12C, A13C, A14C, A18C, A19C) and visceral organs (A2G, A2F, A3F, A5G, A5F, A8G, A8F, A11G, A11F, A13G, A13F, A13Y, A14G) (Table 1).

In 2019, we also collected stool samples from healthy migratory birds, lake water, and aquatic plants from the same points (Figure 1). The host species of feces were determined by molecular methods (Verma et al., 2003), and 10 feces samples from identical hosts were mixed into one for sequencing. The detailed information of all samples is listed in Table 1.

The pH of lake water was determined using a pH meter, and salinity was determined using a conductivity meter. Meanwhile, 100 μL water samples were diluted into three concentrations using physiological saline, then spread on Brain-Heart-Agar-Medium, and incubated at 37°C overnight. Finally, single colonies (CFU) on the agar plates were counted. After all indicators were tested, the 500 mL water samples were concentrated to 50 mL and stored at −80°C until DNA extraction.

Extraction of genomic cDNA and 16s rRNA sequencing

The genomic DNA was extracted using different DNA kits (Omega Biotek) according to the sample characteristics. DNA was amplificated using paired 341F/806R primers that am plified a 425 bp V3−V4 region of the 16S rRNA bacterial gene, and amplicons were the n sequenced using an Illumina HiSeq PE250 platform (Caporaso et al., 2012; the full prot ocol for these primers is available at www.bioplatforms.com).

Sequence processing

The effective sequences were clustered into operational taxonomic units (OTUs) with Usearch using a 97% identity threshold. Before downstream analysis, all samples were randomly re-sampled at the minimum depth of all samples to minimize the bias of unequal sequencing depth. Taxonomic information of each OTU was assigned using the Ribosomal Database Project classifier (Version 2.2) after classification according to the number of sequences in each OTU.

Sequences were clustered followed by chimera filtering. In using OTUs for species classification, each OTU was considered to represent one species. We chose representative sequences for each OTU and used the RDP (http://rdp.cme.msu.edu) classifier to annotate taxonomic information for each representative sequence (James et al., 2014; Qiong et al., 2007). Based on OTU abundances and taxonomic annotation of OTUs, we obtained relative abundance profiles at the phylum, class, order, family, genus, and species levels. This made it easy to understand the overall pattern of each classification level annotated.

Phylogenetic relationships were used to reveal the differences between OTU sequences; combined with the species annotation information represented by each OTU sequence, we constructed a species classification tree. The dominant species were selected according to the results of the species classification.

Species diversity analysis

We calculated the values of sample alpha diversity indices by QIIME software, and the results were used to create the corresponding dilution curve. The dilution curve used the relative proportions of various known OTUs obtained from the 16S rRNA sequences to calculate the expected value of each alpha diversity index when extracting *n* OTUs (*n* is less than the total number of measured reads sequences) and then based on a set of *n* values (generally a set of equivariance series less than the total sequence number) and the expected value of the corresponding alpha diversity index. We prepared an alpha diversity index statistical table. In addition, the 16S rRNA gene amplicon sequence data were also analyzed based on weighted Unifrac distance, alpha diversity (Chao1 index, Shannon index, and Simpson index), and principal coordinates analysis (PCoA).

Isolation and identification of culturable *Vibrio*

The 16S rRNA amplicon sequencing identified the genus *Vibrio.* To confirm the species, 36 samples from 2018 and 2019 were detected by isolation on CHROMagar *Vibrio* agar plates and identification using BD Phoenix-100. Then, the genes *ompW*, *infC*, *ctxA*, *hlyA*, and *chxA* et al. were detected by PCR (the primers and PCR conditions are listed in Table S1). *V. cholerae* isolates were subjected to O1/O139 antigen serotyping using *V. cholerae* O antisera.

Results

Quality of high-throughput sequencing profiles

High-throughput amplicon sequencing from 36 samples yielded a total of 1, 839, 668 good-quality sequences. A total of 3, 883 OTUs were identified from nine migratory bird intestinal tracts, 13 migratory bird visceral organs, 3 migratory bird feces samples, 4 aquatic plants, and 7 water samples from 2018 (2, 518 OTUs) and 2019 (1, 365 OTUs) (annotation results of OTUs are listed in Table S2).

Bacterial community composition

The samples from 2018 to 2019 displayed different structures of gut microbiota. At the phylum level, *Proteobacteria* (45.17%), *Bacteroidetes* (21.73%), *Firmicutes* (20.25%), *Fusobacteria* (9.96%), and *Verrucomicrobia* (2.89%) were the dominant bacterial phyla in 2018 (Figure 2). In 2019, *Bacteroidetes*, *Firmicutes*, *Fusobacteria* and *Verrucomicrobia* decreased to 3.37%, 16.98%, 0.10% and 0.5%, respectively, and *Proteobacteria* increased to 54.90%.

At the genus level, there were 20 genera with relative higher content in samples from 2018. These included *Bacteroides* (17.87%), *Clostridium* (5.87%), *Plesiomonas* (9.52%), *Vibrio* (7.73%), *Fusobacterium* (7.00%), *Aeromonas* (6.26%), *Escherichia* (4.76%), *Wohlfahrtiimonas* (3.31%), *and Cetobacterium* (2.95%) (Figure 2) of the percentages of *Aeromonas* and *Cetobacterium* decreased to 0.40% and 0.10%, respectively, and the percentage of others was less than 0.10% in 2019.

Differences in species diversity

To estimate the bacterial diversity in each sample, alpha diversity indices were calculated based on the OTUs. The Chao1 index varied from 102.200 to 966.074. The Shannon index varied from 0.803 to 7.379, and the Simpson index varied from 0.142 to 0.986 in 2018. There were significant differences in alpha diversity indices between the samples in 2018 ($p < 0.05$, *t* test). The diversity, richness, and uniformity values of lake water were higher than those for aquatic plants and migratory bird remains. The Chao1 index of A3C was below those from the migratory bird remains. All indices of bird A19C were higher than those of other birds. However, there were no significant differences in alpha diversity indices between the three types of samples in 2019 ($p > 0.05$, *t* test) (Table S3).

According to the weighted Unifrac PCoA (Figure 3), the samples from 2018 were able to be clustered together except for four intestinal tract samples (A14C, A8C, A12C, A3C). Compared to 2018, the distributions of stool samples and environmental samples from 2019 were too scattered to be clustered. Only bird samples RS2.4 and RS3.1 could be clustered together.

pH, salt content, total *Vibrio* amount, and total bacterial counts in the migratory bird habitat

Table 2 shows pH values and salt contents during the sampling periods from 2018 to 2019 in Chifeng City, Inner Mongolia autonomous region, China. In 2018, the salt contents and amounts of *Vibrio* of Ganggeng Lake were lower than in Nanhekou and Beihekou. In 2019, the salt contents and amounts of *Vibrio* at these three sampling points had decreased compared to 2018.

Confirmation of *Vibrio* genus

Vibrio species were identified as *V. alginolyticus, V. cholerae*, and *V. metschnikovii* by BD Phoenix-100 testing. *V. cholerae* was detected through the *ompW* gene in 2018, but *Vibrio cholerae* was not detected in 2019. All *V. cholerae* isolates harbored *hlyA* genes but not *ctxA, tcpA,* or *chxA* genes. Additionally, all isolates were NOVC. By screening the *infC* gene, we also verified the presence of *V. metschnikovii* strains in samples from 2018 and 2019 (Table 3).

NOVC and *V. metschnikovii* were detected in six bird intestines; these two bacteria were only detected in bird NO. 5 and bird NO. 11 at the same time. This suggested that they had not spread throughout the bodies of the migratory birds. Interestingly, NOVC was present in the liver of bird No. 5; *V. metschnikovii* was present in the lungs of bird No. 11 and bird No. 13 but not in the intestines (Table 3). *V. metschnikovii* in the lungs could have been inhaled by migratory birds via the nasal cavity.

Nucleotide Sequence Accession Numbers

The sequences of 16S rRNA amplicon were submitted to FigShare under the public site https://figshare.com/s/4ce5c0dd792898e1c32e.

Discussion

Dali Nouer Lake, Chifeng, China was named as one of the "Important Wetlands in Asia". It is an important migration channel for migratory birds in northern China, harboring 15 orders, 32 families, and 152 species birds. It also has had high yields of mandarin fish and crucian carp. Therefore, environmental changes should be monitored, especially the structure of the microbiota.

At the phylum level, the relative abundance of *Fusobacteria* in dead migratory birds was higher than in healthy migratory birds from 2018 to 2019. *Fusobacteria* disturbed the intestinal microbiota balance via an increase in opportunistic pathogens and a decrease in probiotics (Yu Y.N., 2015). *Fusobacteria* was not the main phylum present in Dali Nouer Lake, and this indicated that these bacteria were carried by migratory birds. At the genus level, the relative abundances of *Vibrio, Aeromonas, Plesiomonas*, and *Bacteroides* were also higher in all samples in 2018. The relative abundances of these bacteria in healthy migratory birds had dropped in 2018. The top 20 genera of the relative contents (except *Vibrio* and *Aeromonas*) in samples were mainly distributed in the migratory bird organs and intestines, and little was present in the environment. Therefore, it is possible these bacteria were carried by migratory birds.

It is worth noting that NOVC could simultaneously be isolated in water and dead migratory birds by PCR. O1/O139 *V. cholerae* that carries the cholera toxin has resulted in seven pandemics. The seventh *V. cholerae* pandemic continues to present day and has exhibited evolved characteristics compared to previous pandemics, rendering it difficult to treat cholera disease outbreaks (Hu D., 2016; Laviad-Shitrit S., 2018). Although NOVC isolates did not produce toxins that cause cholera, the possibility that NOVC can have other pathogenic characteristics cannot be overlooked. NOVC most commonly causes sporadic gastroenteritis, and less commonly is an invasive parenteral infection (Deshayes S., 2015). NOVC bacteremia is still rare, but it has been reported sporadically in some countries (Li X., 2020). The hemolysis and no CTX toxin characteristics of NOVC in the liver of bird No. 5 were consistent with the findings of George N et al. (2013). Thus, it is likely that migratory bird immunity had decreased due to the invasion of *Fusobacteria*, causing NOVC to invade the liver through the blood, not the intestines.

Fortunately, *V. cholerae* was not isolated and identified in the water or the feces of migratory birds in 2019. However, *V. metschnikovii* could be still isolated from the feces of migratory birds and from Nanhekou, Dali Nouer Lake. In contrast to *V. cholerae*, few reports have described the pathogenicity of *V. metschnikovii*. A cytolysin specific for *V. metschnikovii* with hemolytic properties was first described in 1981 (Miyake M, 1988). Although the pathogenicity of *V. metschnikovii* to migratory birds has been less frequently reported, it was also isolated in the feces of healthy migratory birds in 2019. Therefore, the bacteria possibly entered the bodies of migratory birds through feeding on contaminated food, and the bacteria did not cause harm to the migratory birds. The aquatic environment, as the main food source for migratory birds, plays an important role in the composition of the gut microbiota (Risely et al., 2018). The gut microbiota structure of migratory bird samples was similar to that of the environment in 2018, but not in 2019 (Figure 3). This suggests that the migratory birds from 2018 had been in contact with the lake for a period of time sufficient for their gut microbiota structure to have stabilized (Risely et al., 2018).

No avian influenza virus (AIV), Newcastle disease virus (NDV), avian infectious bursal disease virus (IBDV), avian infectious virus (IBV), avian infectious laryngotracheitis virus (ILTV), duck plague virus (DPV), gosling plague virus (GPV), avian paramyxovirus type 4 (apmv-4), or West Nile virus (WNV) were detected in dead migratory birds (data no shown).

Conclusions

By comprehensive analyses of physicochemical characteristics of Dali Nouer Lake and 16SrDNA of samples from 2018 and 2019, the microflora changes of healthy and dead birds caused by the pH changes of Dali Nouer Lake were analyzed. Migratory birds whose immune function had declined due to infection with *Fusobacteria* were infected by opportunistic pathogens. For several reasons, the salinity of Dali Nouer Lake has increased, making the area suitable for the proliferation and growth of bacteria from migratory birds. Therefore, long-term monitoring of the water quality in this migratory bird area is required.

List of abbreviations

PCR: Polymerase chain reaction; OTU: operational taxonomic unit; AIV: avian influenza viruses. NDV: new castle disease virus. IBDV: avian infectious bursal disease virus. IBV: avian infectious virus. ILTV: avian infectious laryngotracheitis virus. DPV: duck plague virus. GPV: gosling plague virus. APMV-4: avian paramyxovirus type 4. WNV: west nile virus. NOVC: non-O1/O139 *V. cholerae.*

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Declarations

Ethics approval and consent to participate

All migratory bird stool samples were collected under the supervision by the Wild Animal Sources and Diseases Inspection Station, National Forestry and Grassland Bureau of China, and did not cause any harm to the animals. All migratory bird epidemic material samples were provided by the local animal disease prevention and control center for bacteriological examination. The experimental protocol was established, according to the ethical guidelines of Helsinki Declaration and was approved by the Changchun Veterinary Research Institute, Chinese Academy of Agricultural Sciences (AMMS - 11 - 2020 - 11).

Consent for publication

Not Applicable.

Availability of data and material

All data generated or analysed during this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

PC, XJG conceived, directed, and carried out the study. LWZ, DC, LHX, JJ, YS and GJL prepared samples for sequence analysis; XJ, JYG, MWL and LZ acquired samples and analyzed the data. All authors have read and approved the final manuscript.

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Figure Legends

Figure 1 Sample collection diagram. The green marks indicate the water sample collection locations in 2018 and 2019.

Figure 2 Species classification and the relationship between different groups in 2018. Different color sectors indicated different samples. The size of the sector indicated the relative abundance ratio of the samples in the classification; the numbers below the classification name indicated the relative abundance percentage.

Figure 3 Weighted Unifrac PCoA. H, B, S, and C represent aquatic plants, organ microbiota, water planktonic flora, gut microbiota, respectively, from 2018. RS1, RS2, and RS3 represent feces planktonic flora, water planktonic flora, and aquatic plants planktonic flora, respectively, from 2019.

Figures

Figure 1

Sample collection diagram. The green marks indicate the water sample collection locations in 2018 and 201

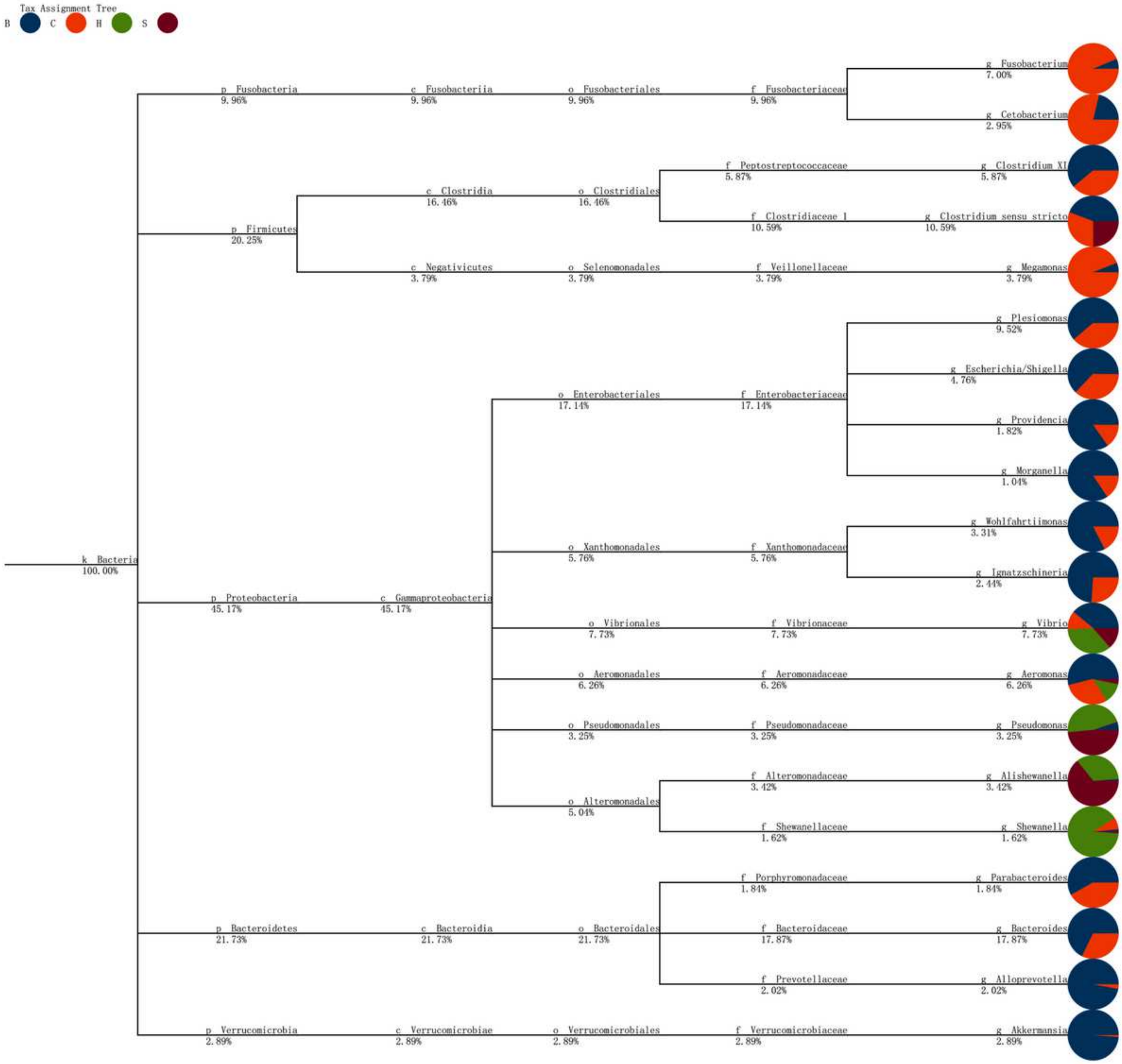

Figure 2

Species classification and the relationship between different groups in 2018. Different color sectors indicated different samples. The size of the sector indicated the relative abundance ratio of the samples in the classification; the numbers below the classification name indicated the relative abundance percentage

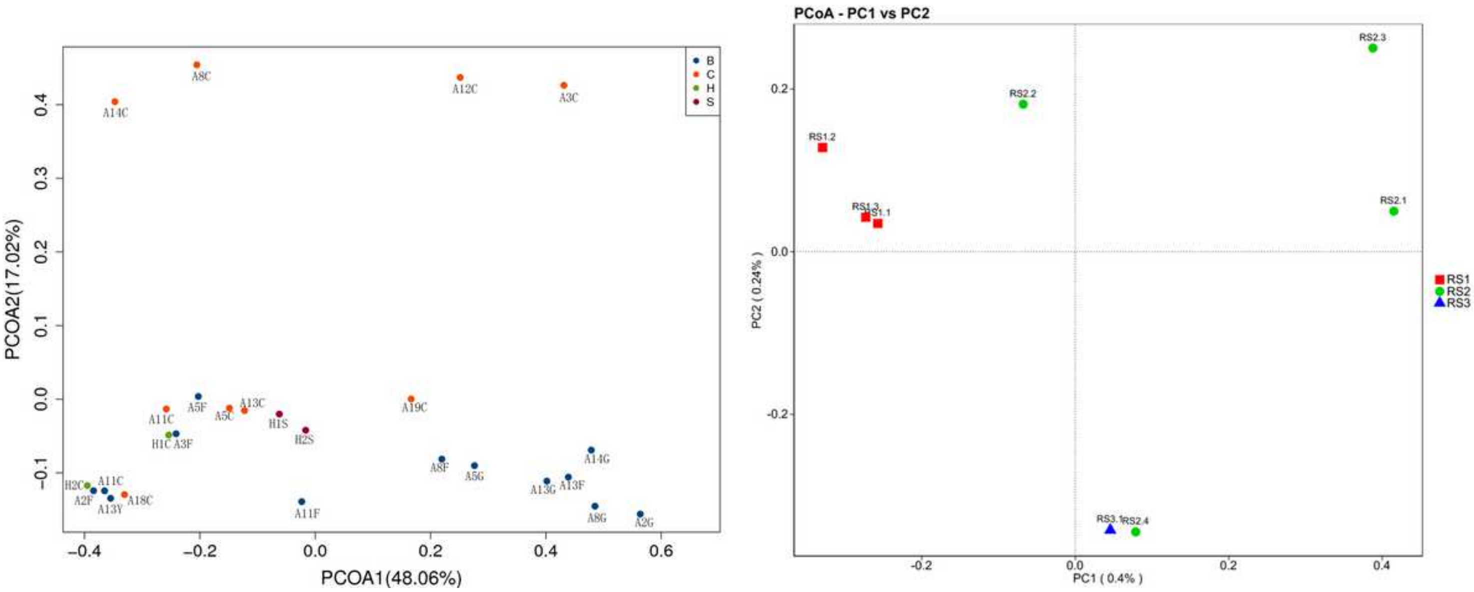

Figure 3

Weighted Unifrac PCoA. H, B, S, and C represent aquatic plants, organ microbiota, water planktonic flora, gut microbiota, respectively, from 2018. RS1, RS2, and RS3 represent feces planktonic flora, water planktonic flora, and aquatic plants planktonic flora, respectively, from 2019

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- SupplementTableS2OTUsspeciesclassificcation.xls
- SupplementTableS3Alphadiversityindex.xls