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Variation in gut microbial communities of Hooded crane (*Grus monacha*) over spatial-temporal scales

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Background: Microbes have been recognized as important symbionts to regulate host life. Animal gut harbors abundance and diverse bacteria. Numerous internal and external factors could influence intestinal bacterial communities, including diet, seasonal fluctuations and habitat sites. However, the factors that influence the gut microbiota are poorly characterized in wild bird. **Methods:** By high-throughput sequencing and statistical analysis, we investigated the variations in gut bacterial community composition and diversity of the Hooded cranes at three wintering stages in Caizi (CZL) and Shengjin Lake(SJL), which are two shallow lakes in the Yangtze floodplain. **Results:** Our results revealed significant differences in the fecal bacterial community structure and diversity among different sampling sites and seasons. ANOSIM analysis explored that the samples in CZL had greater difference in the gut microbial composition than the in SJL. Our data also indicated that the host's gut environmental filtering might be an important factor to shape the gut bacterial community according to mean nearest taxon distance (MNTD). In addition, PICRUSt analysis revealed that the predicted metagenomes associated with carbohydrate metabolism, amino acid metabolism and energy metabolism over the entire wintering period at the two lakes. Seasonal changes have a significant impact on the gut microbes of hooded cranes in the two lakes. **Conclusions:** The results demonstrated that both seasonal changes and habitat sites have significant impact on the gut microbes of hooded cranes. In addition, predictive function of gut microbes in Hooded cranes varied over time. These results provide new insights on the gut microbial composition of the cranes, and our results serve as a foundation for future studies.

Title: Variation in gut microbial communities of Hooded crane (*Grus monacha*) over spatial-temporal scales

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Abstract:

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Methods: By high-throughput sequencing and statistical analysis, we investigated the variations in gut bacterial community composition and diversity of the Hooded cranes at three wintering stages in Caizi (CZL) and Shengjin Lake(SJL), which are two shallow lakes in the Yangtze floodplain.

Results: Our results revealed significant differences in the fecal bacterial community structure and diversity among different sampling sites and seasons. ANOSIM analysis explored that the samples in CZL had greater difference in the gut microbial composition than ~~the~~ in SJL. Our data also indicated that the host's gut environmental filtering might be an important factor to shape the gut bacterial community according to mean nearest taxon distance (MNTD). ~~In addition,~~ PICRUSt analysis revealed that the predicted metagenomes associated with carbohydrate metabolism, amino acid metabolism and energy metabolism over the entire wintering period at the two lakes. Seasonal changes have a significant impact on the gut microbes of hooded cranes in the two lakes.

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Keywords: Gut bacteria, Habitat, Migratory bird, Hooded crane, sequencing

Running title: Gut bacterial communities in Hooded cranes

Introduction:

The gut microbiota confers mutualistic functions involved in substance synthesis and metabolism (Eberl & Boneca 2010), resistance to the intrusion of the pathogen (Guarner & Malagelada 2003; Koch & Schmid-Hempel 2011) and modulation of immune development (Eberl & Boneca 2010). In vertebrates, the gut harbors diverse and abundant microbes that interact with host and environmental factors to form a complex ecosystem (Qin et al. 2010). Previous studies showed that gut microbiota are shaped by diet, life style (Nicholson et al. 2012) and several environmental factors (e.g., seasonal fluctuations and location) (Hird *et al*., 2014).

Birds have unique life history traits that are different from other vertebrates, such as migratory behavior, complex dietary habits, physiological traits and complex network of habitats, all of which may impact gut microbiota structure and function (Kohl 2012). Like other vertebrates, ~~birds~~ gut is colonized with abundant microbes. Previous studies of the intestinal microbiome of birds have focused mainly on ornamental and economical birds, such as parrot (Waite et al. 2012), penguin (Dewar et al. 2013) and turkey (Wilkinson et al. 2017), however, with less information on wild birds. ~~Past decade with~~ rapid development in molecular methodologies, ~~which~~ has ~~been~~ provided new insight for the gut ~~microbial~~ of birds. Recent research reported that the dominant phyla of the avian gut microbiota were Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes (Waite and Taylor, 2014). The avian gut microbiota affected by diet (Yang et al. 2016) and gut microbiota exhibited temporal stability (Kreisinger et al. 2017). Even so, wild birds remained under ~~study~~ despite ~~they cause~~ pathogen transmission. ~~With the~~ 16s rRNA high throughput sequencing, we tried to understand the diversity and potential functions of the gut microbiota in Hooded ~~cranes. In addition,~~ the effects of environmental factors on bird gut microbial communities ~~are~~ largely unknown.

Hooded cranes (*Grus monacha*) are large long-distance migratory colonial wading birds. They are defined as a vulnerable species in the IUCN Red List of Threatened Species (Birdlife 2012) and Category I key National Protected Wild Animal Species in China, ~~which~~ breed in south-central and south-eastern Siberia and Russia, and winter in Japan, China and South Korea (Hammerson & Ryan 2004). In China, the cranes still inhabit natural lakes and the nearby paddy fields (Zheng et al., 2015). The food resources

changed over spatial-temporal scales. The cranes had to modify their foraging patterns when food recourses change during the wintering periods (Wan et al. 2016; Zheng et al. 2015). A reduction in the availability of food forces animals to move to other habitats that contain more food such as paddy fields (Zheng et al. 2015). Hence, Hooded cranes have changed their dietary structure (Zhou et al. 2016a). Hooded cranes are omnivorous birds, but it feeds mainly on plants in China (Huang & Guo 2015; Zhao et al. 2002). However, it is not yet completely understood how seasonal fluctuations and different habitats affect the microbial communities in avian guts.

Shengjin and Caizi Lakes, the two shallow river-connected lakes in the middle and lower Yangtze River floodplain, respectively, are the most important wintering ground for cranes (Barter et al. 2005; Fang et al. 2006). These lakes provide the birds with suitable feeding habitats during winter seasons (Chen et al. 2011). As a result of the lake degradation in the last decade, the cranes lost many suitable foraging habitats. In this study, we used high-throughput sequencing methods to analyze the gut bacterial community of Hooded cranes at three wintering stages in the two lakes. Based on spatial-temporal scales, we tested three hypotheses: (1) Gut bacterial diversity and composition, as measured by alpha and beta diversity, differs significantly in different wintering sites; (2) Gut bacterial are influenced by seasonal changes and exhibit the same pattern in different wintering location; (3) Dietary changes in different seasonal affect the gut bacteria of Hooded cranes.

Materials & Methods

Ethics statement

Fecal samples were collected at the end of the foraging period to ensure that the Hooded cranes were devoid of human disturbance. It did not face direct hunting or capture. Permission for the collection of fecal samples was obtained from the local government for wildlife management.

Study areas

The study was carried out in Shengjin (30.25–30.50°N, 116.92–117.25°E) and Caizi (30.75–30.97°N, 117.00–117.15°E) Lakes, which were located in the middle and lower

Yangtze River floodplain (Figure 1). Both lakes are globally important wintering areas for migratory wading birds on the East Asian-Australasian Flyway (Cao & Fox 2009; Fox et al. 2011). Shengjin and Caizi Lakes are two river-connected shallow lakes with a northern subtropical monsoon climate and an average annual temperature 14–18 °C. The average annual rainfall is approximately 1000–1400 mm, with most falling from April to September.

The seasonally inundated wetlands provide plenty of food (e.g., *Oryza sativa*, *Polygonum criopolitanu*, and *Potentilla supina*) for the wading birds (Zhao et al. 2013; Zheng et al. 2015). There are approximately 600 individuals of Hooded cranes wintering in the two lakes each year. The habitat utilization rate of the cranes has been ~~shifted~~ over spatial and time scales (Zhao et al. 2013; Zheng et al. 2015). During the wintering period, food density and resources change, causing cranes to adjust their foraging habitats (Wan et al. 2016; Zhao et al. 2013; Zheng et al. 2015). The wintering period can be divided into three stages according to the migratory rhythm and hydrological processes of the cranes: the early stage from November to December; the middle stage from January to February; and the late stage from March to April (Zhao et al. 2013; Zheng et al. 2015). The foraging habitats of Hooded cranes are relatively stable at certain wintering stage in Shengjin and Caizi Lakes (Cao et al. 2010).

Sample collection and preservation

Fecal samples were collected during the three-wintering periods. The foraging sites of the crane flocks were observed with a telescope before sampling to ensure that there were no other species. To avoid human disturbance, the fecal samples were collected immediately upon the completion of foraging. Fecal samples were collected at a minimum distance of approximately 1–2 m to avoid individual sampling repetition (Zhang & Zhou 2012).. All samples were obtained from inside the feces to avoid soil contaminants. The samples were kept in a cooler, transported under refrigeration to the lab as quickly as possible and stored at -80 °C. A total of 87 fecal samples were used in this study. 43 samples were collected from Shengjin Lake (SJL) and 44 samples from Caizi Lake (CZL). In SJL, the ~~sampleswere~~ 13 in the early wintering period (SJL-E), 14 in the middle wintering period (SJL-M)and 15 in the late wintering period (SJL-L). In CZL, the samples were 14 in the

early wintering period (CZL-E), 15 in the middle wintering period (CZL-M), 15 in the late wintering period (CZL-L).

DNA extraction and species determination

DNA was extracted from the fecal samples using the Qiagen QIAamp® DNA Stool Mini Kit following the DNA isolation protocol for pathogens. DNA was eluted in 150 μL of sterilized deionized water and then stored at -80 °C. For the host species determination test using fecal DNA, an approximately 680 bp region of the COI gene was amplified using the BIRDF1–BIRDR1 primer pair. PCR amplification was performed in a 50 μL reaction volume containing 100 ng of fecal DNA, 25 μL of 2× EasyTaq mix (TransGen), 10 μM BIRDF1 (TTCTCCAACCACAAAGACATTGGCAC) and 10 μM BIRDR1 (ACGTGGGAGATAATTCCAAATCCTG). The cycling conditions were as follows: 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 45 s and 72 °C for 90 s, with a final extension period at 72 °C for 10 min. The PCR products were sequenced by Sangon Biotech Co. Ltd. (Shanghai). The sequences were aligned by NCBI. All of the samples were confirmed to contain Hooded cranes DNA based on the sequence analysis.

16S rRNA sequencing

Primer sets 338F/806R equipped with sequencing adapters and unique identifier tags were used to amplify the V3-V4 hypervariable regions of the bacterial 16S rRNA genes fragments for the Illumina Hiseq 2500 platform (PE250). PCR was carried out in 50 μL volume containing 60 ng of fecal DNA, 25 μL of 2 × Premix Taq and 1 μL each of the forward and reverse primers (10 μM). The cycling conditions were as follows: 94 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 45 s and 72 °C for 45 s, with a final extension at 72 °C for 10 min. Triplicate reaction mixtures per sample were pooled together and purified using the EZNA Gel Extraction Kit (Omega, USA). Quantification was performed with a NanoDrop. Raw sequences were analyzed and processed following the pipeline coupling Mothur and Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al. 2010; Schloss et al. 2009). Trimmomatic software was used to filter out poor-quality sequences, filter the N reads, quality scores less than 30 and sequences less than 200 bp. The sequences were clustered into OTUs with a default confidence threshold

of 97% using UPARSE and a select representative sequence of the OTU. Singleton OTU was removed using usearch (http://www.drive5.com/usearch/manual/chimera_formation.html), and the chimeric sequence was removed with UCHIME (http://www.drive5.com/usearch/manual/uchime_algo.html). The most abundant sequence within each cluster was selected as the representative sequence for that OTU. The NCBI SRA database accession number is SRP095247.

Data analysis

Non-metric multidimensional scaling (NMDS) based on the Bray-Curtis dissimilarity (calculated from the relative abundance matrix) and Analyses of Similarities (ANOSIM; permutations = 999) were performed to compare the community compositions in different treatments in R 3.4.1 (vegan 2.4-3) (Dixon 2010). Observed species, PD whole tree, Shannon and Simpson indices were also calculated. The nearest taxon index (NTI) and betaNTI are used to test the assembly processes of the gut bacterial community. NTI measures the mean nearest taxon distance (MNTD). NTI was calculated to assess the spatial and temporal changes in bacterial phylogenetic structure using Picante software. The NTI can be used to test for phylogenetic clustering or overdispersion. Positive NTI values and low quantiles ($P < 0.05$) indicate that co-occurring species are more closely related than expected by chance (clustering), whereas positive values and high quantiles ($P > 0.05$) indicated that the co-occurring species are less closely related than expected by chance (overdispersion). Positive betaNTI values indicated greater than expected phylogenetic turnover, and betaNTI was calculated in phylocom. LDA Effect Size (LEfSe) was used to ~~identifies~~ genomic features characterizing the differences between two or more biological conditions (Segata et al. 2011). To detect KEGG pathways with significantly different abundance between the two lakes, LEfSe analysis was used according to the online protocol (https:// huttenhower. sph. harvard. edu/ galaxy/). Nearest sequenced taxon index (NST1) was the sum of phylogenetic distances for each organism in the OTU table and the closest genetic relationship of the sequencing reference genome, as measured by the substitutions per site in the 16S rRNA gene and weighted by the frequency of that organism in the OTU table (Langille et al. 2013). Functional predictions were made based on the 16S rRNA OTU membership using

PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) according to the online protocol (http://picrust.github.io/picrust/). One-way ANOVA was used to detect the influence of the sampling site and season on bacterial taxonomy (phylum) variation. All univariate statistical analyses were conducted using SPSS 20.0.

Results

Bacterial alpha-diversity

Gut microbial alpha-diversity was estimated by the observed species index, phylogenetic diversity, Shannon and Chao 1 index. At the early wintering periods, the alpha-diversity of the SJL samples were significantly higher than that of the CZL samples ($P < 0.001$) (Figure 2), but there was not significant difference ~~of the alpha-diversity~~ in middle and late wintering periods between two lakes ($P > 0.05$ in both causes). ~~The~~ alpha-diversity ~~were~~ the highest in the middle period across the temporal changes in CZL, and the Chao 1 index of the SJL samples showed significant difference across the temporal change (Figure S1).

Bacterial community composition

A total of ~~4018,794~~ filtered reads with an average of 46,193 reads was found, ranging from 7392 to 77,085 (median 10,000) in this study. The dominant bacterial phyla belonged to Firmicutes (59.82% ± 32.35%), Proteobacteria (26.82% ± 24.87%), Actinobacteria (5.43% ± 8.19%), Fusobacteria (3.78% ± 12.22%), and Bacteroidetes (2.24% ± 6.21%) in the gut microbiota of Hooded cranes (Figure 3). Within Firmicutes, the dominant classes were *Clostridia* (41.79 ± 31.94%) and Bacilli (17.96 ± 21.17%). The classes of *Epsilonproteobacteria* (12.61 ± 21.80%), *Gamaproteobacteria* (8.69 ± 13.87%) and *Alphaproteobacteria* (4.28 ± 8.04%) were dominant in Proteobacteria. However, the distribution of each taxon among the four groups was uneven, as indicated by Figure 3. At the lower level, only 84.7% of sequences could be assigned to 995 genera. The dominate genera were *Clostridium* (15.5%), *SMB53* (13.62%), *Helicobacter* (11.86%), *Lactobacillus* (7.22%), *Epulopiscium* (4.82%), *Enterococcus* (4.76%),

Fusobacterium (3.67%), *Pseudomonas* (2.54%), *Turicibacter* (2.08%), *Serratia* (2.03%), *Agrobacterium* (1.77%) and *Lysinibacillus* (1.59%).

The relative abundance of Firmicutes from the SJL samples was significantly higher than the CZL samples, whereas the relative abundance of Proteobacteria from the SJL samples was significantly lower than the CZL samples (Figure 4, S2). At Shengjin Lake, the relative abundance of the Firmicutes gradually increased across the temporal change. In order to explore the differences in spatial and temporal scale, we conducted LEfSe tests to detect the difference in relative abundance of microbial taxa. At Shengjin Lake, four indicator bacterial taxa were found in the SJL-E samples and five indicator bacterial taxa were found in the SJL-L. At Caizi Lake, three indicator bacterial taxa were found in the CZL-E samples, 11 indicator bacterial taxa were found in the CZL-M samples and four indicator bacterial taxa were found in the CZL-L samples (Figure 5).

Both NMDS and ANOSIM revealed that CZL samples were dissimilar to the SJL samples, and during late wintering period the two lakes showed greater dispersion. NMDS revealed that SJL samples tended to have less difference compared to the CZL samples, and the SJL samples in the middle and late wintering periods clustered more closely (Figure 6E). ANOSIM analysis confirmed that Seasonal changes had a significant impact on the microbial composition of the Hooded cranes in two lakes(Table 1). The bacterial community composition of SJL samples and CZL samples were significantly different (P = 0.001) in the three wintering periods (Table 1). At Shengjin Lake, the samples in different wintering period were significantly different (R = 0.238, P = 0.001), as were the samples from Caizi Lake (Table 1).

Assemblage processes of the gut bacterial community

NTI was used to evaluate the gut bacterial phylogenetic structure. All of the NTI values were positive, which showed that the bacterial communities were phylogenetically clustered (Figure 7, S3). At the early wintering stage, the CZL-E samples NTI values were less positive compared to the SJL-E samples, which indicated that phylogenetic clustering was weakest in the CZL-E samples (Figure 7). In addition, the NTI values of SJL-L samples were less positive compared to the CZL-L samples at late wintering stage, which indicated that phylogenetic clustering was weakest in the SJL-L samples.

Phylogenetic clustering were similar in SJL samples during three wintering periods, whereas CZL-L samples phylogenetic clustering were more similar in CZL samples.

Variation in predicted metagenomes between the two lakes

In addition, NST1 also influenced PICRUSt accuracy. The NST1 for our samples was 0.18 ± 0.07. In this study, a total of 41 functional genes were predicted in Hooded crane population. The majority of functions were membrane transport (12.63%), carbohydrate metabolism (9.36%), amino acid metabolism (9.19%), replication and repair (8.08%), energy metabolism (7.10%) and translation (5.18%) during the wintering period. ANOSIM analysis revealed that the potential functions of the gut microbial communities of the two lakes were significantly different during early and late wintering periods (P = 0.001). However, during the middle wintering period, there was almost no difference in the potential functions of the gut bacterial in the two lakes (P = 0.116). Seasonal changes had a significant impact on the gut microbes of hooded cranes in both Shengjin and Caizi lakes (Table 2).

Discussion

Gut bacterial have now been shown to have a great influence on host health through various functional roles in terms of nutrient intake (Heijtz et al. 2011; Kohl 2012). Microbes can be influenced by many factors, such as diet (C et al. 2010; Kau et al. 2011) and environment (Godoy-Vitorino et al. 2012; Maul et al. 2005), while the importance of these factors is largely unknown in the Hooded cranes.

The gut microbial community of the Hooded cranes was dominated by Firmicutes , Proteobacteria and Actinobacteria, which was similar to that of mammals (Waite & Taylor 2014). The phylum Firmicutes was dominant in Hooded crane gut in the two lakes, as is consistent with the many avian species. However, the detailed composition of these phyla was notably altered with space-time according to our study. At Shengjin Lake, the gut bacterial community was dominated by Firmicutes. The relative abundance of the Fimicutes increased and the relative abundance of the Proteobacteria decreased gradually with the wintering period (Figure 3). Increased the relative abundance of Proteobacteria in the gut microbiota increase the risk of inflammatory bowel disease (Zhou et al. 2016b). Significant differences in gut microbial communities were identified, as reflected by NMDS clustering and microbial interactions (Figure 4, Table 1). The gut bacterial structures in the SJL samples were significantly different from those in the CZL samples. In recent years, grazing animal waste and poultry litter effects on the environment (Sa[illegible] et al. 1999). Seasonal fluctuations also had a significant effect on the composition of gut microbes at the two lakes. Besides, alpha diversity of Hooded cranes between Shengjin and Caizi lakes also has a little change.

Firmicutes are associated with the breakdown of carbohydrates, polysaccharides, sugars and fatty acids, which are utilized by the host as energy sources (Flint et al. 2008). The *clostridium* genus belonging to Firmicutes can digest simple carbohydrates (Aristilde 2017; Bäckhed et al. 2004) as well as complex polysaccharides (Aristilde 2017; Ramos et al. 2015), which may lead to high proportion of carbohydrates metabolism. All above results showed that the cranes, like other waterbirds, fed on high-energy foods such as plant roots (Fox et al. 2011; Li-Lin et al. 2008). In order to survive in the dry and cold weather, the cranes needed to take in considerable amounts of energy (Cai et al. 2014; Fox et al. 2011). However, Proteobacteria play a role in energy accumulation (Bryant &

Small 1956; Chevalier et al. 2015), and the high proportion of Proteobacteria may be due to the cranes need a lot of energy to cope with the cold winter. The phylum Fusobacteria associated with butyrate production, with butyrate playing an important role in ion absorption and immune regulation. It is also an important anti-inflammatory agent (Canani et al. 2011).

The phylum of Actinobacteria also found in the cranes and associated with pathogens, such as the genera of *Nocardia* and *Rhodococcus* (Santos et al. 2012). The two lakes are of importance for staging and habitat for the wintering wading birds (Barter et al., 2005; Fang et al., 2006). ~~Pathogen may be harmful to poultry or other vertebrates (Altizer et al. 2011; Z & J 1999).~~ The habitats of domestic waterbirds overlap those ~~for~~ Hooded cranes at the two lakes, and large assemblance of migratory birds may represent a source of pathogenic microbes that can be transmitted through feces (Zhao *et al*., 2017). Whether these bacteria perform certain functions for the host remains unclear. PICRUSt analysis confirmed that SJL-L samples ~~in~~ ~~high~~ proportion of ~~disease.~~ Compared to Shengjin Lake, the CZL samples appeared to be enriched in Proteobacteria. All the bacterial community assemblages showed significant phylogenic clustering, indicating that bacterial communities were strongly structured by gut environment filtering (Yan et al. 2016). Although the bacterial community compositions showed significantly different between the two sites in the early wintering period, ~~however,~~ the spatial changes did not affect bacterial phylogenetic clustering, while gut environmental filtering influenced bacterial communities in both lakes in the middle and late periods. The effect of temporal changes on bacterial phylogenetic structure was not consistent in Shengjin and Caizi lakes. These results suggest that the environment factors can influence the composition of bacterial community and might be an important factor for bacterial phylogenetic structure.

The bacterial community in the guts of Hooded cranes may have many important functions. In this study, PICRUSt were used to deduce potential gene profiles from 16S rRNA sequencing. The result showed that the most abundant functional classes were related to membrane transport, carbohydrate, amino acid and energy metabolism. The higher proportion of energy metabolism pathway-related predicted genes may meet Hooded ~~crane s'~~ energy needs for flight. The energy metabolism pathways were much higher in the late wintering than in the other two periods, which may be induced by the

fact that the food density gradually decreases over wintering, leading to an increase in the foraging efforts of Hooded cranes (Wan et al. 2016). Moreover, the PICRUST results revealed that the glycan biosynthesis and metabolism-related genes were present in both lakes and in high proportion at Caizi Lake. Therefore, we propose that this pathway in the gut microbiota may significantly contribute to increased digestive efficiency and assimilation, which may play an important role in providing energy and nutrients to cope with the cold weather (Wan et al. 2016).

Conclusions

In summary, in this study we show that the bacterial community composition in Hooded cranes' gut changed across the two lakes in terms of alpha-diversity and beta-diversity. We found environment factors might be the importance influence factors about the gut bacterial composition. In addition, dietary seasonal fluctuation also affected the gut bacterial composition. This study provides a foundation understanding of gut bacterial composition and the gut bacterial functions in Hooded cranes. Future work should focus on how these actual functions relate to the gut bacterial community composition. Finally, we hope that this study will provide the basic information for the protection of the Hooded Cranes.

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Additional information and declarations

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Author Contributions

YD and LZ designed the experiment. YD and GZ complete the field sampling. YD and XX performed data analysis and prepared figures. YD wrote the manuscript. XX and LZ contributed to the revision of manuscript.

Conflict of Interest Statement

The authors declare that the study was conducted in the absence of any commercial of financial relationships that could be constructed as potential conflicts of interest.

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Figure 1(on next page)

The study areas of the hooded crane fecal sample collection.

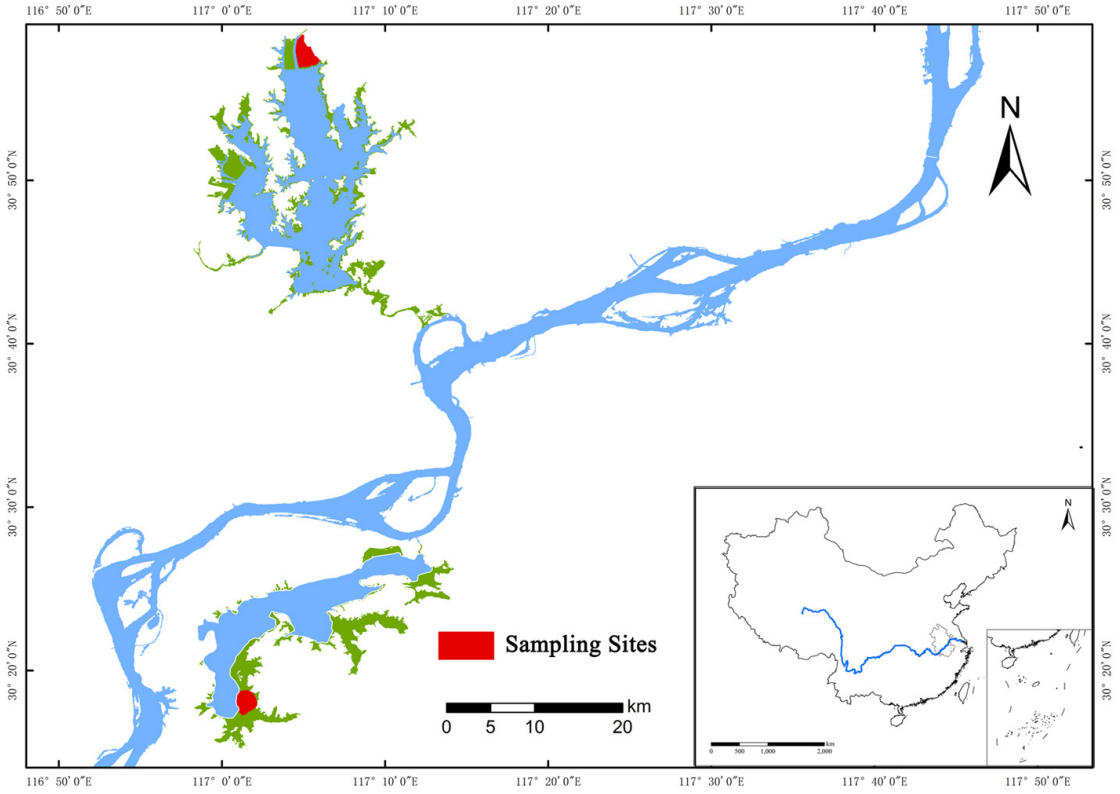
116° 50' 0"E
117° 0' 0"E
117° 10' 0"E
117° 20' 0"E
117° 30' 0"E
117° 40' 0"E
117° 50' 0"E
30° 50' 0"N
30° 40' 0"N
30° 30' 0"N
30° 20' 0"N
N
Sampling Sites
km
0 5 10 20

Figure 2(on next page)

Variations in diversity (Pylogenetic diversity , OTU richness, Chao 1 and Shannon) in different wintering periods.

Different letters represent significant differences by Tukey's HSD comparisons ($P < 0.05$).

Error bars indicate standard deviation.

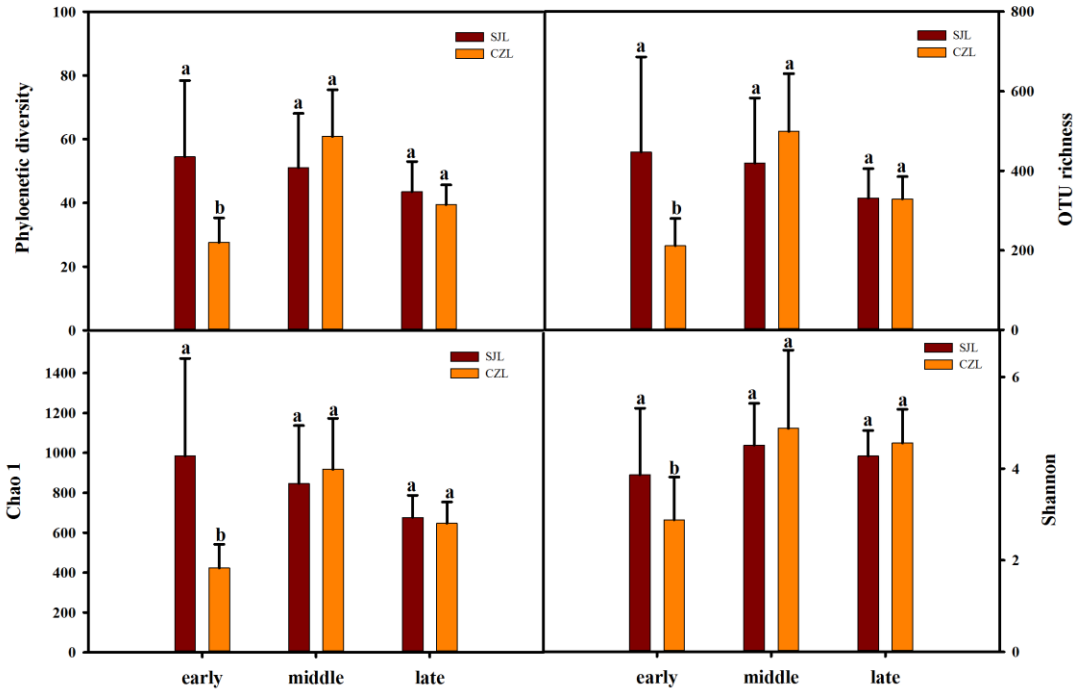
Phyloenetic diversity
OTU richness
Chao 1
Shannon
SJL
CZL
a
b
early
middle
late

Figure 3(on next page)

Gut microbiota composition of the Hooded Crane.

(A) Relative abundance of the dominant phyla in all samples. (B) Relative abundance of the dominant classes in all samples.

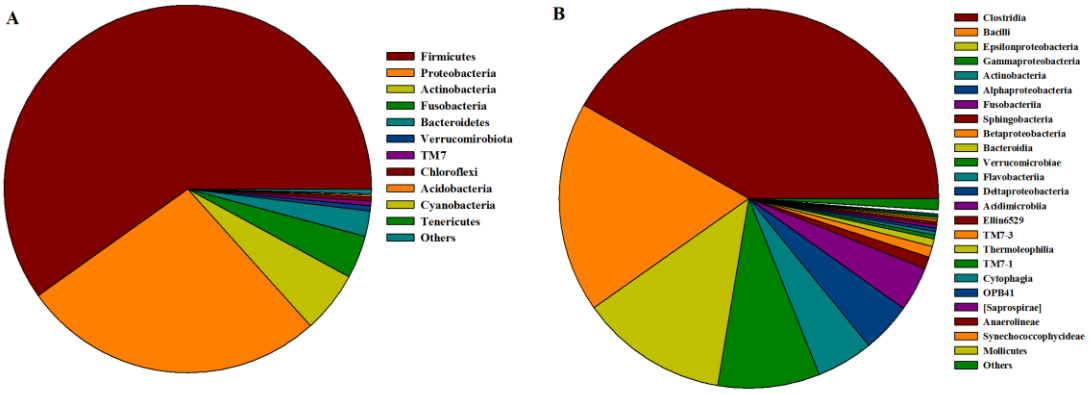
A
Firmicutes
Proteobacteria
Actinobacteria
Fusobacteria
Bacteroidetes
Verrucomirobiota
TM7
Chloroflexi
Acidobacteria
Cyanobacteria
Tenericutes
Others
B
Clostridia
Bacilli
Epsilonproteobacteria
Gammaproteobacteria
Actinobacteria
Alphaproteobacteria
Fusobacteriia
Sphingobacteria
Betaproteobacteria
Bacteroidia
Verrucomicrobiae
Flavobacteriia
Deltaproteobacteria
Acidimicrobiia
Ellin6529
TM7-3
Thermoleophilia
TM7-1
Cytophagia
OPB41
[Saprospirae]
Anaerolineae
Synechococcophycideae
Mollicutes
Others

Figure 4(on next page)

Relative abundance of the dominant phyla in different wintering periods.

Samples are grouped according to sampling location and wintering periods. Different letters represent significant differences by Tukey's HSD comparisons ($P < 0.05$). Error bars indicate standard deviation.

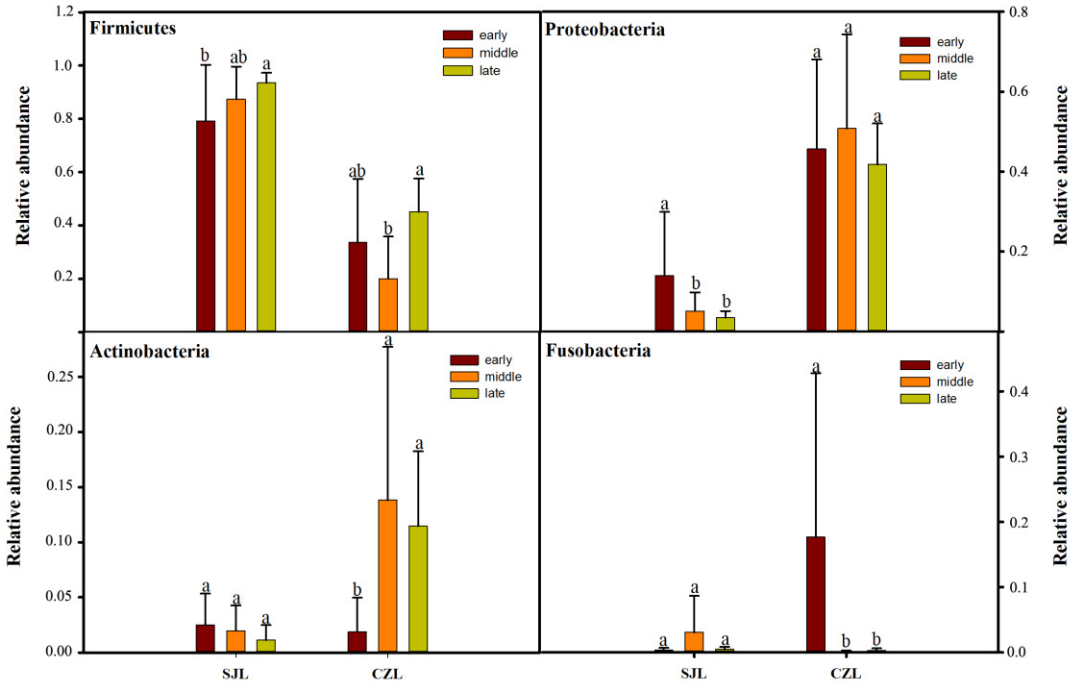
Firmicutes
Proteobacteria
Actinobacteria
Fusobacteria
early
middle
late
Relative abundance
SJL
CZL

Figure 5(on next page)

LEFSe analysis of the Hooded Crane gut bacterial in different wintering periods (LDA > 2, P < 0.05).

(A) the samples Shengjin Lake. (B) the samples from Caizi Lake.

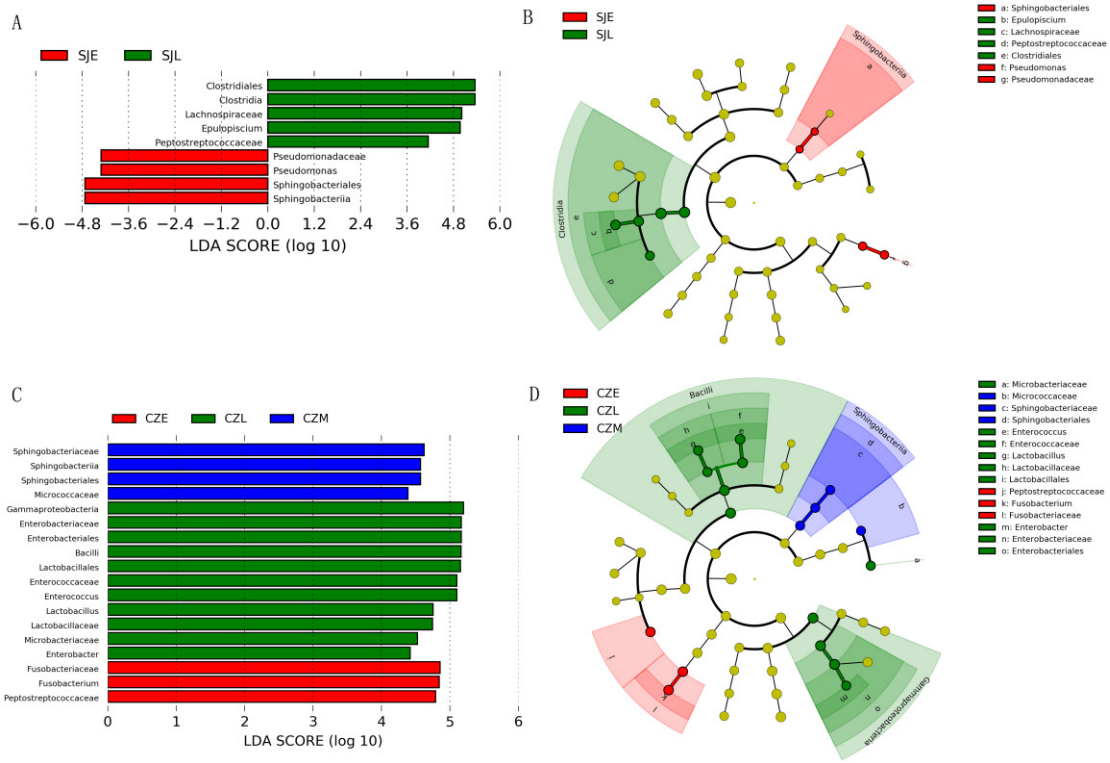
A
SJE
SJL
Clostridiales
Clostridia
Lachnospiraceae
Epulopiscium
Peptostreptococcaceae
Pseudomonadaceae
Pseudomonas
Sphingobacteriales
Sphingobacteriia
−6.0 −4.8 −3.6 −2.4 −1.2 0.0 1.2 2.4 3.6 4.8 6.0
LDA SCORE (log 10)
B
SJE
SJL
Sphingobacteria
Clostridia
a: Sphingobacteriales
b: Epulopiscium
c: Lachnospiraceae
d: Peptostreptococcaceae
e: Clostridiales
f: Pseudomonas
g: Pseudomonadaceae
C
CZE
CZL
CZM
Sphingobacteriaceae
Sphingobacteriia
Sphingobacteriales
Micrococcaceae
Gammaproteobacteria
Enterobacteriaceae
Enterobacteriales
Bacilli
Lactobacillales
Enterococcaceae
Enterococcus
Lactobacillus
Lactobacillaceae
Microbacteriaceae
Enterobacter
Fusobacteriaceae
Fusobacterium
Peptostreptococcaceae
0 1 2 3 4 5 6
LDA SCORE (log 10)
D
CZE
CZL
CZM
Bacilli
Sphingobacteria
Gammaproteobacteria
a: Microbacteriaceae
b: Micrococcaceae
c: Sphingobacteriaceae
d: Sphingobacteriales
e: Enterococcus
f: Enterococcaceae
g: Lactobacillus
h: Lactobacillaceae
i: Lactobacillales
j: Peptostreptococcaceae
k: Fusobacterium
l: Fusobacteriaceae
m: Enterobacter
n: Enterobacteriaceae
o: Enterobacteriales

Figure 6(on next page)

Differences in fecal microbial communities.

(A) Non-metric multidimensional scaling plot for the hooded crane from Shengjin and Caizi Lakes at three wintering periods. (B) Non-metric multidimensional scaling plot for the hooded crane from Shengjin and Caizi Lakes at the early wintering period. (C) Non-metric multidimensional scaling plot for the hooded crane from Shengjin and Caizi Lakes at the middle wintering period. (D) Non-metric multidimensional scaling plot for the hooded crane from Shengjin and Caizi Lakes at the late wintering period. (E) Non-metric multidimensional scaling plot for the hooded crane from Shengjin Lake at three wintering periods. (F) Non-metric multidimensional scaling plot for the hooded crane from Caizi Lake at three wintering periods.

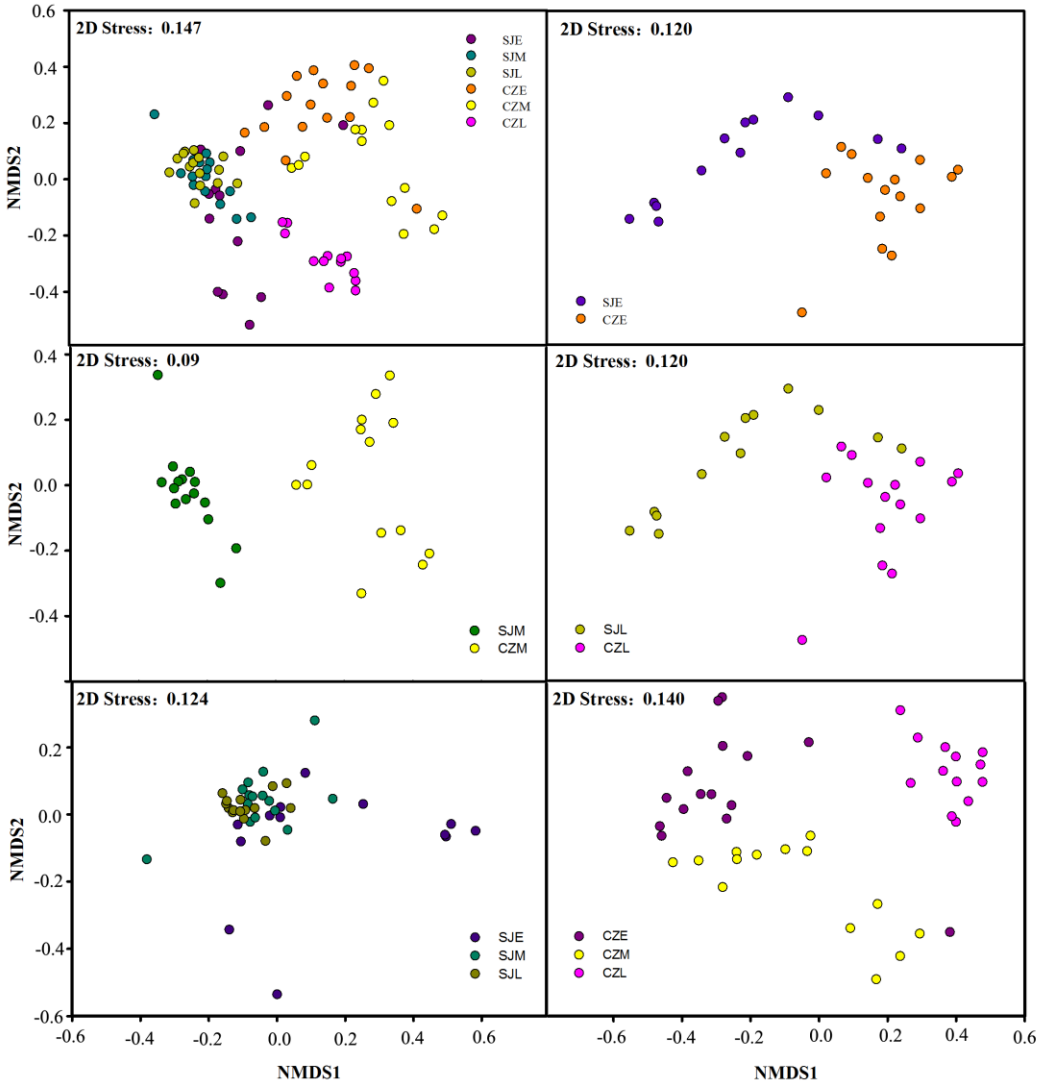
2D Stress: 0.147
SJE
SJM
SJL
CZE
CZM
CZL
2D Stress: 0.120
SJE
CZE
2D Stress: 0.09
SJM
CZM
2D Stress: 0.120
SJL
CZL
2D Stress: 0.124
SJE
SJM
SJL
2D Stress: 0.140
CZE
CZM
CZL
NMDS1
NMDS2

Figure 7 (on next page)

Bacterial community phylogenetic structure evaluated by the NTI in different wintering periods.

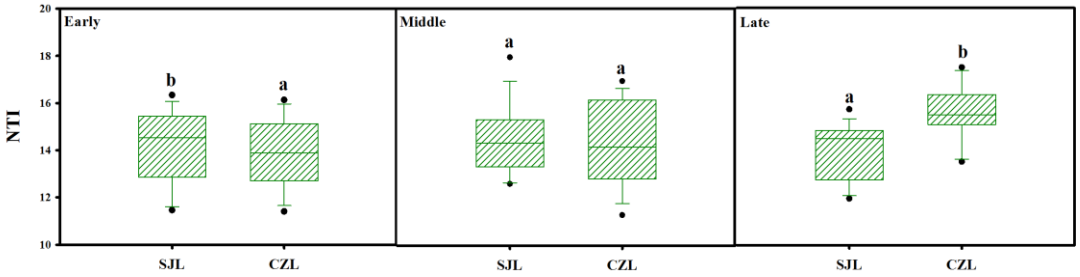
Early
Middle
Late
NTI
20
18
16
14
12
10
b
a
a
a
a
b
SJL
CZL
SJL
CZL
SJL
CZL

Table 1(on next page)

similarity test of ANOSIM.

Table 1. Differences in the microbial community composition based on the similarity test of ANOSIM.

Table 1. Differences in the microbial community composition based on the similarity test of ANOSIM.

	temporal variation			spatial variation	
	R	*P*		R	*P*
SJE vs SJM	0.264	0.001	SJE vs CZE	0.591	0.001
SJM vs SJL	0.124	0.009	SJM vs CZM	0.85	0.001
CZE vs CZM	0.4	0.001	SJL vs CZL	0.967	0.001
CZM vs CZL	0.845	0.001			
SJE vs SJM vs SJL	0.238	0.001			
CZE vs CZM vs CZL	0.739	0.001			

Table 2(on next page)

similarity test of ANOSIM.

Table 2. Differences in the microbial functions based on the similarity test of ANOSIM.

Table 2. Differences in the microbial functions based on the similarity test of ANOSIM.

	temporal variation			spatial variation	
	R	P		R	P
SJE vs SJM vs SJL	0.181	0.005	SJE vs CZE	0.444	0.001
CZE vs CZM vs CZL	0.218	0.001	SJM vs CZM	0.058	0.116
			SJL vs CZL	0.221	0.001