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

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16   **Abstract:**

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18   animal gut harbors abundance and diverse bacteria. Numerous internal and external factors

19   influence intestinal bacterial communities, including diet, seasonal fluctuations and habitat sites.

20   However, the factors that influence the gut bacterial communities of wild bird are poorly

21   characterized.

22   **Methods:** By high-throughput sequencing and statistical analysis, we investigated the variations

23   in gut bacterial communities of the Hooded cranes at three wintering stages in Caizi (CZL) and

24   Shengjin Lake (SJL), which are two shallow lakes in the middle and lower Yangtze River

25   floodplain.

26   **Results:** Our results revealed significant differences in gut bacterial community structure and

27   diversity among different sampling sites and wintering stages. Seasonal changes have a

28   significant impact on the gut microbe composition of Hooded cranes in the two lakes. ANOSIM

29   analysis demonstrated that the samples in CZL had greater differences in the gut bacterial

30   composition than that in SJL. Our data showed strong evidence that host's gut filtering might be

31   an important factor in shaping bacterial community according to mean nearest taxon distance

32   (MNTD). The PICRUSt analysis showed that the predicted metagenomes associated with the gut

33   microbiome were carbohydrate metabolism, amino acid metabolism and energy metabolism over

34   the entire wintering period at the two lakes.

35

36   **Conclusions:** The results demonstrated that both seasonal changes and habitat sites have

37   significant impact on the gut bacterial communities of Hooded cranes. In addition, predictive

38   function of gut microbes in Hooded cranes varied over time. These results provide new insights

39   into the gut microbial community of the cranes, which serves as a foundation for future studies.

40

41   **Running title:** Gut bacterial communities

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 environment 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 microbial structure and function (Kohl 2012). Like other vertebrates, the avian gut is colonized with abundant microbes. Previous studies of the intestinal microbiota of birds have mainly focused on ornamental and economical birds, such as parrot (Waite et al. 2012), penguin (Dewar et al. 2013) and turkey (Wilkinson et al. 2017). However, little information has been reported in wild birds. The rapid development in molecular methodologies has provided new insight for the gut microbiota of birds. Recent research demonstrated that the dominant phyla of the avian gut microbiota were Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes (Waite and Taylor, 2014). Many studies have focused on captive birds which showed that the avian gut microbiota are affected by diet (Yang et al. 2016) and gut microbiota exhibited temporal stability (Kreisinger et al. 2017). Captivity also has been considered to be an important factor affecting the structure of avian gut microbes. Wild birds remained less studied despite for the association with pathogen transmission. Using the 16s rRNA high through sequencing, we tried to understand the diversity and potential functions of the gut microbiota in Hooded cranes and the effects of environmental factors on bird gut microbial communities.

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. Hooded cranes 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 change over spatial-temporal scales. The cranes had to modify their foraging patterns when food recourses change during the

73   wintering periods (Wan et al. 2016; Zheng et al. 2015). A reduction in the food availability

74   forces animals to modify their foraging patterns (Zheng et al. 2015). In the early and mid-winter

75   periods, the most abundant, accessible and frequented food resources were found in paddy fields,

76   while in the late period the more abundant food were available in meadows. Hence, Hooded

77   cranes have changed their dietary structure and habitat. In China, Hooded cranes are omnivorous

78   birds, but feed mainly on plants (Huang & Guo 2015; Zhao et al. 2002).

79   Shengjin and Caizi Lakes, the two-shallow river-connected lakes in the middle and lower

80   Yangtze River floodplain, respectively, are the most important wintering ground for cranes.

81   These lakes provide the birds with suitable feeding habitats during winter seasons (Chen et al.

82   2011). As a result of the lake degradation in the last decade, the cranes lost many suitable

83   foraging habitats. In this study, we used high-throughput sequencing methods to analyze the gut

84   bacterial community of Hooded cranes at three wintering stages in the two lakes. Based on

85   spatial-temporal scales, we tested two hypotheses: (1) Gut bacterial diversity and composition, as

86   measured by alpha- and beta-diversity, differs significantly in different wintering sites; (2) Gut

87   bacterial composition exhibit the same pattern in different wintering location.

88

89   **Materials & Methods**

90   **Ethics statement**

91   Fecal samples were collected after foraging to ensure that the Hooded cranes were devoid of

92   human disturbance. It does not involve direct hunting or capture. Permission for the collection of

93   fecal samples was obtained from the local government for wildlife management.

94

95   **Study areas**

96   The study was carried out in Shengjin (30.25–30.50°N, 116.92–117.25°E) and Caizi (30.75–

97   30.97°N, 117.00–117.15°E) Lakes, which were located in the middle and lower Yangtze River

98   floodplain (Figure 1). Both lakes are globally important wintering and stopover habitats for

99   migratory wading birds on the East Asian-Australasian Flyway (Cao & Fox 2009; Fox et al.

100   2011b). Shengjin and Caizi Lakes are river-connected shallow lakes with a northern subtropical

101   monsoon climate. The average annual temperature is 14–18 °C and the average annual rainfall is

102   approximately 1000–1400 mm.

103   The seasonally inundated wetlands provide plenty of food (e.g., *Oryza sativa*, *Polygonum*
104   *criopolitanu*, and *Potentilla supina*) for the wading birds (Zhao et al. 2013; Zheng et al. 2015).
105   There are approximately 600 individuals of Hooded cranes wintering in the two lakes each year.
106   The habitat utilization rate of the cranes has shifting over spatial and time scales (Zhao et al.
107   2013; Zheng et al. 2015). During the wintering period, shifts in food density and resources
108   triggers cranes to adjust their foraging habitats (Wan et al. 2016; Zhao et al. 2013; Zheng et al.
109   2015). The wintering period can be divided into three stages according to the migratory rhythm
110   and hydrological processes of the cranes: the early stage from November to December; the
111   middle stage from January to February; and the late stage from March to April (Zhao et al. 2013;
112   Zheng et al. 2015). The foraging habitats of Hooded cranes are relatively stable at certain
113   wintering stage in Shengjin and Caizi Lakes (Cao et al. 2010).

114

115   **Sample collection and preservation**
116   Fecal samples were collected during the three-wintering periods. The foraging sites of the crane
117   flocks were observed with a telescope before sampling to ensure that there were no other species.
118   To avoid human disturbance, the fecal samples were collected immediately upon the completion
119   of foraging. Fecal samples were collected at a minimum distance of approximately 1–2 m to
120   avoid individual sampling repetition (Zhang & Zhou 2012). All samples were obtained from
121   inside the feces to avoid soil contaminants. The samples were kept in a cooler, transported under
122   refrigeration to the lab within several hours and stored at -80 °C. A total of 87 fecal samples
123   were used in this study. 43 samples were collected from Shengjin Lake (SJL) and 44 samples
124   from Caizi Lake (CZL). In Shengjin Lake, the samples were 13 in the early wintering period
125   (SJL-E), 14 in the middle wintering period (SJL-M) and 15 in the late wintering period (SJL-L).
126   In Caizi Lake, the samples were 14 in the early wintering period (CZL-E), 15 in the middle
127   wintering period (CZL-M), and 15 in the late wintering period (CZL-L).

128

129

130

131   **DNA extraction and species determination**
132   DNA was extracted from the fecal samples using the Qiagen QIAamp® DNA Stool Mini Kit
133   following the DNA isolation protocol for pathogens. DNA was eluted in 150 μL of sterilized

134   deionized water and then stored at -80 °C. The COI gene was amplified using the BIRDF1–

135   BIRDR1 primer pair to determine host species. PCR amplification was performed in a 50 μL

136   reaction volume containing 100 ng of fecal DNA, 25 μL of 2× EasyTaq mix (TransGen), 10 μM

137   BIRDF1   (TTCTCCAACCACAAAGACATTGGCAC)   and   10   μM   BIRDR1

138   (ACGTGGGAGATAATTCCAAATCCTG). The cycling conditions were as follows: 95 °C for 5

139   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

140   extension period at 72 °C for 10 min. The PCR products were sequenced by Sangon Biotech Co.

141   Ltd. (Shanghai). The sequences were aligned by NCBI. All of the samples were confirmed to

142   contain Hooded cranes DNA based on the sequence analysis.

143

144   **16S rRNA sequencing**

145   Primer sets 338F/806R equipped with sequencing adapters and unique identifier tags were used

146   to amplify the V3-V4 hypervariable regions of the bacterial 16S rRNA genes fragments for the

147   Illumina Hiseq platform (PE250). PCR was carried out in 50 μL volume containing 60 ng of

148   fecal DNA, 25 μL of 2 × Premix Taq and 1 μL each of the forward and reverse primers (10 μM).

149   The cycling conditions were as follows: 94 °C for 3 min, followed by 30 cycles of 95 °C for 30 s,

150   55 °C for 45 s and 72 °C for 45 s, with a final extension at 72 °C for 10 min. Triplicate reaction

151   mixtures per sample were pooled together and purified using the EZNA Gel Extraction Kit

152   (Omega, USA). Quantification was performed with a NanoDrop. Sequences were processed

153   using Quantitative Insights Into Microbial Ecology (QIIME). After denoising, poor quality

154   (below an average quality score of 30) and short sequences (shorter than 200bp) were removed

155   and those sequences which exactly matched with their barcode and primer were kept. The

156   barcode and primer sequences were then removed. Sequences were clustered into OTUs using a

157   97% identity threshold by UPARSE. Singleton OTUs were deleted and chimers were filtered to

158   remove erroneous OTUs due to sequence errors using the USEARCH in QIIME. The most

159   abundant sequence within each cluster was selected as the representative sequence for that OTU.

160   The taxonomic identify of each OTU was checked against the ribosomal database project (RDP)

161   Classifier. The raw data has been submitted to SRA of NCBI with accession number

162   SUB2178492.

163

164   **Data analysis**

165   Non-metric multidimensional scaling (NMDS) based on the Bray-Curtis dissimilarity (calculated
166   from the relative abundance matrix) and Analyses of Similarities (ANOSIM; permutations = 999)
167   were performed to compare the community composition in different treatments in R 3.4.1 (vegan
168   2.4-3) (Dixon 2010). In addition, permutational analysis of multivariate dispersions (PERMDISP;
169   permutations = 999) was used to test the gut bacterial community structure heterogeneity varied
170   over spatial-temporal scale. This test was using a Bray-Custis similarity matrix. The significance
171   of PERMDISP was determined via the ANOVA F-statistic to compare among-group differences
172   in the distance from observations to their corresponding group centroids. One-way and two-way
173   ANOVA was used to test observed species, PD whole tree, Shannon and Chao 1 indices among
174   different treatments. The nearest taxon index (NTI) and betaNTI are used to test the assembly
175   processes of the gut bacterial community. NTI was calculated to assess the spatial and temporal
176   changes in bacterial phylogenetic structure using Picante software. The NTI can be used to test
177   for phylogenetic clustering or overdispersion. Positive NTI values and low quantiles ($P < 0.05$)
178   indicate that co-occurring species are more closely related than expected by chance (clustering),
179   whereas positive values and high quantiles ($P > 0.05$) indicate that the co-occurring species are
180   less closely related than expected by chance (overdispersion). Positive betaNTI values indicate
181   greater than expected phylogenetic turnover, and betaNTI was calculated in phylocom. LEfSe
182   (linear discriminant analysis effect size) was used to identifies genomic features characterizing
183   the differences between two or more biological conditions (Segata et al. 2011). To detect KEGG
184   pathways with significantly different abundances between the two lakes, LEfSe analysis was
185   used according to the online protocol (https:// huttenhower. sph. harvard. edu/ galaxy/). Nearest
186   sequenced taxon index (NSTI) was the sum of phylogenetic distances for each organism in the
187   OTU table and the closest genetic relationship of the sequencing reference genome, as measured
188   by the substitutions per site in the 16S rRNA gene and weighted by the frequency of that
189   organism in the OTU table (Langille et al. 2013). Functional predictions were made based on the
190   16S rRNA OTU membership using PICRUSt (Phylogenetic Investigation of Communities by
191   Reconstruction   of   Unobserved   States)   according   to   the   online   protocol
192   (http://picrust.github.io/picrust/). One-way ANOVA was used to detect the influence of the
193   sampling site and season on bacterial taxonomy (phylum) variation. All univariate statistical
194   analyses were conducted using SPSS 20.0.
195

196   **Results**

197   **Bacterial alpha-diversity**

198   Gut bacterial alpha-diversity was calculated at a depth of 10,000 randomly selected sequences

199   per sample. Alpha-diversity was estimated by the observed species index, phylogenetic diversity,

200   Shannon and Chao 1 index. Two-way ANOVA showed that all alpha diversity was significantly

201   different with time, and only phylogenetic diversity and Chao 1 index were significantly

202   different in spatial variation. Alpha diversity was significantly different under the interaction of

203   wintering period and spatial distance (Table S1). At the early wintering periods, the alpha-

204   diversity of the SJL samples were significantly higher than that of the CZL samples ($P < 0.001$)

205   (Figure 2), but there was no significant difference in middle and late wintering periods between

206   the two lakes ($P > 0.05$ in both causes). Alpha-diversity was the highest in the middle period

207   across the temporal changes in CZL, and the Chao 1 index of the SJL samples showed

208   significant different across the temporal change (Figure S1).

209

210   **Bacterial community composition**

211   In total, we obtained 4,717,488 reads of high-quality sequences with an average of 46,193 reads

212   was found. Potentially chimeric sequences and singleton OTU (698,694) were then discarded.

213   The dominant gut bacterial phyla were Firmicutes (59.82% ± 32.35%), Proteobacteria (26.82% ±

214   24.87%), Actinobacteria (5.43% ± 8.19%), Fusobacteria (3.78% ± 12.22%), and Bacteroidetes

215   (2.24% ± 6.21%) (Figure 3A, C). Within Firmicutes, the dominant bacterial classes were

216   *Clostridia* (41.79 ± 31.94%) and *Bacilli* (17.96 ± 21.17%). The classes of *Epsilonproteobacteria*

217   (12.61 ± 21.80%), *Gamaproteobacteri*a (8.69 ± 13.87%) and *Alphaproteobacteria* (4.28 ± 8.04%)

218   were dominant in Proteobacteria (Figure 3B, D). However, the distribution of each taxon among

219   the four groups was uneven, as indicated by Figure 3. At the family level, the top 30 families

220   were shown for all samples (Figure S2). At the lower level, only 84.7% of sequences could be

221   assigned to 995 genera. The dominate genera were *Clostridium* (15.5%), *SMB53* (13.62%),

222   *Helicobacter* (11.86%), *Lactobacillus* (7.22%), *Epulopiscium* (4.82%), *Enterococcus* (4.76%),

223   *Fusobacterium* (3.67%), *Pseudomonas* (2.54%), *Turicibacter* (2.08%), *Serratia* (2.03%),

224   *Agrobacterium* (1.77%) and *Lysinibacillus* (1.59%).

225   One-way ANOVA showed that relative abundance of Firmicutes was significantly higher in

226   SJL samples than in CZL samples, whereas the relative abundance of Proteobacteria from the

227   SJL samples was significantly lower than the CZL samples (Figure 4, S3). At Shengjin Lake, the

228   relative abundance of the Firmicutes increased significantly along the winter period. In order to

229   explore the differences in spatial and temporal scale, we conducted LEfSe tests to detect the

230   difference in relative abundance of microbial taxa. At Shengjin Lake, four indicator bacterial

231   taxa (i.e., *Pseudomonadaceae, Pseudomonas, Sphingobacteriales, Sphingobacterii*) were found

232   in the gut of the Hooded crane in SJL-E samples and five indicator bacterial taxa (i.e.,

233   *Clostridiales, Clostridia, Lachnospiraceae, Epulopiscium, Peptostreptococcaceae*) were found

234   in the gut of Hooded crane in SJL-L samples (Figure 5A, B). At Caizi Lake, three indicator

235   bacterial taxa (*Fusobacteriaceae, Fusobacterium, Peptostreptococcaceae*) were found in the

236   CZL-E samples, four indicator bacterial taxa (i.e., *Sphingobacteriaceae, Sphingobacterii,*

237   *Sphingobacteriales, Microccaceae*) were found in the CZL-M samples and 11 indicator bacterial

238   taxa (*Gammaproteobacteria, Enterobacteriaceae, Enterobacteriales, Bacilli, Lactobacillales,*

239   *Enterococcaceae, Enterococcus, Lactobacillales, Enterococcus, Lactobacillus, Lactobacillaceae,*

240   *Enterobacter*) were found in the CZL-L samples (Figure 5C, D).

241   The bacterial community compositions were significantly different between the guts of

242   Hooded crane from SJL samples and CZL samples. NMDS revealed that SJL samples tended to

243   be less different compared to the CZL samples (Figure 6E). ANOSIM analysis confirmed that

244   seasonal changes had a significant impact on the microbial composition of the Hooded cranes in

245   two lakes (Table 1). The bacterial community composition of SJL samples and CZL samples

246   were significantly different ($P = 0.001$) in the three wintering periods (Table 1), the bacterial

247   community compositions in different wintering period were significantly different in both

248   Shengjin and Caizi Lake (R = 0.238, $P = 0.001$) (Table 1).

249   According to PERMDISP, average distance to the corresponding group centroid have no

250   significant differences among different wintering periods at Shengjin Lake ($P = 0.167$). However,

251   mean distance to the corresponding group centroid have significant differences among different

252   wintering periods at Caizi Lake ($P < 0.001$). Furthermore, no significant differences could be

253   detected among average distance to the corresponding group centroid of gut bacterial

254   communities in different sample collected sites.

255

256   **Assemblage processes of the gut bacterial community**

257 NTI was used to evaluate the gut bacterial phylogenetic structure. All of the NTI values were
258 positive, which showed that the bacterial communities were phylogenetically clustered (Figure 7,
259 S4). At the early wintering stage, the CZL-E samples NTI values were less positive compared to
260 the SJL-E samples, which indicated that phylogenetic clustering was weakest in the CZL-E
261 samples (Figure 7). In addition, the NTI values of SJL-L samples were less positive compared to
262 the CZL-L samples at late wintering stage, which indicated that phylogenetic clustering was
263 weakest in the SJL-L samples. Phylogenetic clustering was similar in SJL samples during three
264 wintering periods, whereas CZL-L samples phylogenetic clustering were more similar in CZL
265 samples.

266

267 **Variation in predicted metagenomes between the two lakes**
268 In addition, NSTI also influenced PICRUSt accuracy. The NSTI for our samples was 0.18 ± 0.07
269 similar to the previously reported analyses in soils (mean NSTI = 0.17 ± 0.02). In this study, a
270 total of 41 functional genes were predicted in Hooded crane population. The majority of
271 functions were membrane transport (12.63%), carbohydrate metabolism (9.36%), amino acid
272 metabolism (9.19%), replication and repair (8.08%), energy metabolism (7.10%) and translation
273 (5.18%) during the wintering period. ANOSIM analysis revealed that the potential functions of
274 the gut bacterial communities of the two lakes were significantly different during early and late
275 wintering periods (P = 0.001) (Table 2). However, during the middle wintering period, there was
276 little difference in the potential functions of the gut bacterial in the two lakes (P = 0.116). In
277 early wintering period, Hooded crane from Caizi Lake were predicted to have a microbiota with
278 enhanced capability for metabolic disease, carbohydrate metabolism, amino acid metabolism,
279 circulatory transport, cellular processes and singling and a lower rate of microbial genes
280 associated with genetic information processing (Table S2). In middle wintering period, Hooded
281 crane from Caizi Lake were predicted to have a microbiota with enhanced capability for amino
282 acid metabolism, sensory system, transport and catabolism, membrane transport and genetic
283 information processing.   When comparing predicted metagenomes in the late period, the
284 microbiota of Hooded crane from Caizi Lake was predicted to have a capability for metabolic
285 disease, carbohydrate metabolism, amino acid metabolism, replication and repair and glycan
286 biosynthesis and metabolism.

Discussion

287

288 Hooded cranes have to modify their foraging behavior to adapt to variations in food availability

289 over a spatial-temporal scale as a result of wetland loss and degradation (Wan et al. 2016; Zheng

290 et al. 2015). Having a diverse gut microbiome may be another adaptive mechanism. In this study,

291 the gut bacterial composition and structure of the wintering Hooded Crane from the Shengjin and

292 Caizi Lakes were explored. Differences in microbial community structures and interactions were

293 also identified.

294   The gut microbial community of the Hooded crane was dominated by Proteobacteria,

295 Firmicutes and Actinobacteria, which was similar to that of mammals (Waite & Taylor 2014).

296 The phylum of Firmicutes was dominated in Hooded crane gut at the two lakes and was

297 consistent with the many avian species (Dewar et al. 2014; Waite & Taylor 2014; Wilkinson et al.

298 2017). Firmicutes are associated with the breakdown of carbohydrates, polysaccharides, sugars

299 and fatty acids, which are utilized by the host as energy sources (Flint et al. 2008). The

300 *clostridium* genus belonging to Firmicutes can digest simple carbohydrates (Aristilde 2017;

301 Bäckhed et al. 2004) as well as complex polysaccharides (Aristilde 2017; Ramos et al. 2015),

302 which may lead to high proportion of carbohydrates metabolism. All above results indicated that

303 the Hooded cranes, like other waterbirds, feed on high-energy foods such as *Vallisneria* tubers

304 (Fox et al. 2011a; Li-Lin et al. 2008). In order to survive in the dry and cold weather, the Hooded

305 cranes need considerable amounts of energy (Cai et al. 2014; Fox et al. 2011a). However,

306 Proteobacteria plays an important role in energy accumulation (Bryant & Small 1956; Chevalier

307 et al. 2015), and the high proportion of Proteobacteria may be due to the cranes need a lot of

308 energy to cope with the cold winter. The classes of *Epsilonproteobacteria*, *Gamaproteobacteri*a

309 and *Alphaproteobacteria* were dominant in Proteobacteria, similar to other avian, such as

310 kittiwake, bean goose, kakapo (Dongen et al. 2013; Yang et al. 2016). The dominant families

311 were *Xanthomonadaceae* and *Comamonadaceae*. These two families are strong competitors with

312 flexible metabolism and are considered essential for host's digestion in poor nutritional diet. The

313 phylum Fusobacteria associated with butyrate production, with butyrate playing an important

314 role in ion absorption and immune regulation. It is also an important anti-inflammatory agent

315 (Canani et al. 2011). The phylum of Actinobacteria also found in the Hooded cranes and

316 associated with pathogens, such as the genera of *Nocardia* and *Rhodococcus* (Santos et al. 2012).

317 The habitats of domestic waterbirds overlapping with Hooded cranes at the two lakes, and large

318   assemblance of migratory birds may represent a source of pathogenic microbes that can be

319   transmitted by each other through feces (Zhao *et al*., 2017).

320   However, the detailed composition of these phyla was notably altered with space-time

321   according to our study. The samples from Shengjin Lake were dominated by Firmicutes, while

322   those from Caizi Lake were dominated by Firmicutes and Proteobacteria. Compared to Shengjin

323   Lake, the samples from the Caizi Lake appear to be enriched in Proteobacteria and the relative

324   abundance of Firmicutes decreased (Figure S1). Increased the relative abundance of

325   Proteobacteria in the gut microbiota increase the risk of inflammatory bowel disease (Zhou et al.

326   2016). According to LEfse analysis, genus of *Pseudomonas* was enriched in the gut of Hooded

327   crane from SJL-E samples. Pervious study shows that the *Pseudomonas* genus codes for

328   levansucrases (Visnapuu et al. 2015). We also found the class of sphingobacteriales were highest

329   in the gut of Hooded crane from SJL-E samples and CZL-M samples (Figure 5) because of the

330   increase in polysaccharide (Carney et al. 2014). In addition, there was no significant difference

331   in the alpha diversity of the SJL samples in different wintering periods, while the alpha diversity

332   of the CZL was significantly difference in samples in different wintering periods (Figure S1).

333   Furthermore, significant differences in gut microbial communities were identified, as reflected

334   by NMDS clustering and microbial interactions (Figure 4, Table 1). These results showed that

335   the seasonal and temporal change may be important factors in shaping host bacterial structure. In

336   recent years, grazing animal waste and poultry litter effects on the environment (Sauer et al.

337   1999). As most members of genera (i.e. *Agrobacterium*) are present in soil, these bacteria may

338   have originated from the environment, implying horizontal transmission could also influence the

339   structure of animal gut microbiota (Yang et al. 2016). Thus, grazing animal waste and poultry

340   litter also affects the structure of the gut microbiota. Seasonal fluctuations also had a significant

341   effect on the composition of gut microbes at the two lakes.

342   In addition, all the bacterial community assemblages showed significant phylogenic

343   clustering, indicating that bacterial communities were strongly structured by gut environment

344   filtering (Yan et al. 2016). Although the bacterial community compositions showed significantly

345   different between the two sites in the early wintering period, however, the spatial changes did not

346   affect bacterial phylogenetic clustering, while gut environmental filtering influenced bacterial

347   communities in both lakes in the middle and late periods. The effect of temporal changes on

348   bacterial phylogenetic structure was not consistent in Shengjin and Caizi lakes. These results

349 suggest that the environment factors can influence the composition of bacterial community and
350 might be an important factor for bacterial phylogenetic structure.
351   The bacterial community in the guts of Hooded cranes may have many important functions.
352 In this study, PICRUSt were used to deduce potential gene profiles from 16S rRNA sequencing.
353 The result showed that the most abundant functional classes were related to membrane transport,
354 carbohydrate, amino acid and energy metabolism. The higher proportion of energy metabolism
355 pathway-related predicted genes may meet the Hooded crane's energy needs for flight. The
356 energy metabolism pathways were much higher in the late wintering than in the other two
357 periods, which may be induced by the fact that the food density gradually decreases over winter
358 period, leading to an increase in the foraging efforts of Hooded cranes (Wan et al. 2016).
359 Moreover, the PICRUSt results revealed that the glycan biosynthesis and metabolism-related
360 genes were present in both lakes but with higher proportion at Caizi Lake. Therefore, we propose
361 that this pathway in the gut microbiota may significantly contribute to increase digestive
362 efficiency and assimilation, which may play an important role in providing energy and nutrients
363 to cope with the cold weather (Wan et al. 2016).
364

365 **Conclusions**
366 In this study, we showed that gut bacterial community composition and diversity of Hooded
367 cranes changed over spatial-temporal scales. We found that spatial-temporal changes might be
368 important factors to influence gut bacterial community composition. In addition, dietary seasonal
369 fluctuation also affected the gut bacterial community composition. This study provides a
370 foundation understanding of gut bacterial community composition and potentially bacterial
371 functions in Hooded cranes. Future work should focus on how these actual functions relate to the
372 gut microbial community composition.
373

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378

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492   **Figure legends**

493   **Figure1.** The sampling areas of in this study.

494   **Figure2.** Variations in alpha-diversity ((A) Phylogenetic diversity, (B) OTU richness, (C) Chao

495   1 and (D) Shannon) of different wintering periods. Different letters represent significant

496   differences by Tukey's HSD comparisons ($P < 0.05$). Error bars indicate standard deviation.

497   **Figure3.** Relative abundance of gut bacteria at the phylum, class and genus levels at two lakes.

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

499   dominant classes in all samples; Rings represent corresponding phylum (Firmicutes in blue and

500   Proteobacteria in red) for each of the most frequently represented class. (C) Relative abundance

501   at the phylum and (D) relative abundance at the genera at different periods in the two lakes.

502   **Figure4.** Relative abundance of the dominant phyla ((A) Firmicutes, (B) Ptoteobacteria, (C)

503   Actinobacteria, (D) Fusobacteria) in different wintering periods. Samples are grouped according

504   to sampling location and wintering periods. Different letters represent significant differences by

505   Tukey's HSD comparisons ($P < 0.05$). Error bars indicate standard deviation.

506   **Figure5.** LEFSe analysis of the Hooded Crane guts bacteria in different wintering periods (LDA >

507   2, $P < 0.05$). (A) LDA score representing the taxonomic with significant difference in different

508   wintering periods at the Shengjin Lake. (B) Cladogram representing the taxonomic hierarchical

509   structure of the phylotype biomakera identified among three wintering periods from the Shengjin

510   Lake; Red, phylotypes statistically overrepresented in early wintering period; Green, phylotypes

511   statistically overrepresented in late wintering period. (C) LDA score representing the taxonomic

512   with significant difference in different wintering periods at the Caizi Lake. (D) Cladogram

513   represting the taxonomic hierarchical structure of the phylotype biomakera identified among

514   three wintering periods from the Caizi Lake; Red, phylotypes statistically overrepresented in

515   early wintering period; Green, phylotypes statistically overrepresented in late wintering period;

516   Blue, phylotypes statistically overrepresented in middle wintering period.

517   **Figure6.** Differences in gut bacterial community composition. (A) Non-metric multidimensional

518   scaling plot bacterial community composition from Shengjin and Caizi Lakes at three wintering

519   periods. (B) Non-metric multidimensional scaling plot showing bacterial community

520   composition from Shengjin and Caizi Lakes at the early wintering period. (C) Non-metric

521   multidimensional scaling plot for the hooded crane from Shengjin and Caizi Lakes at the middle

522   wintering period. (D) Non-metric multidimensional scaling plot for the hooded crane from

523 Shengjin and Caizi Lakes at the late wintering period. (E) Non-metric multidimensional scaling

524 plot for the hooded crane from Shengjin Lake at three wintering periods. (F) Non-metric

525 multidimensional scaling plot for the hooded crane from Caizi Lake at three wintering periods.

526 **Figure7.** Bacterial phylogenetic structure evaluated by the NTI in different wintering periods. (A)

527 At the early wintering period. (B) At the middle wintering period. (C) At the late wintering

528 period.

529

Figure 1

Sampling areas

The sampling areas of in this study.

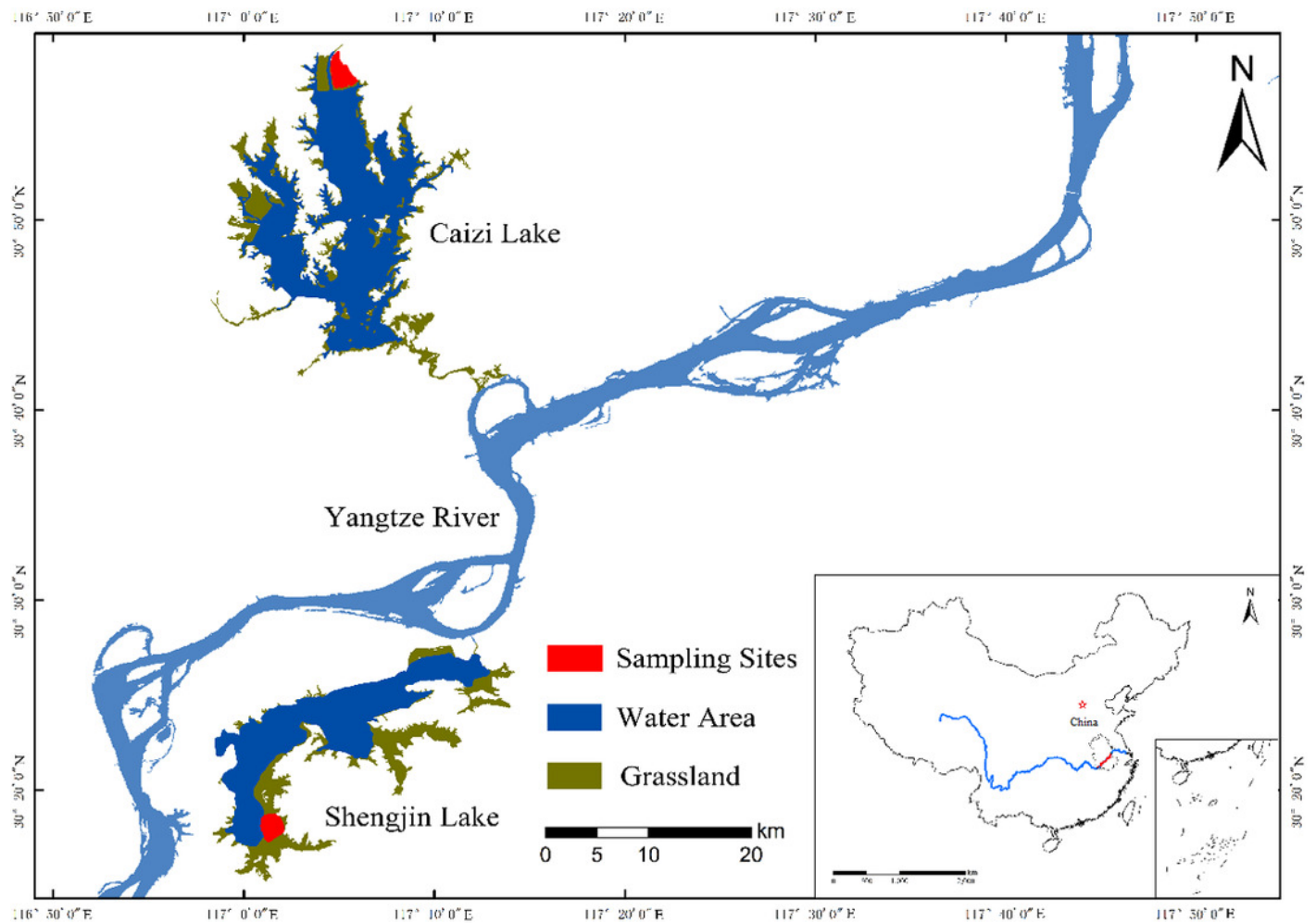



Figure 2

Alpha-diversity of different wintering periods.

Variations in alpha-diversity ((A) Phylogenetic diversity, (B) OTU richness, (C) Chao 1 and (D) Shannon) of different wintering periods. Different letters represent significant differences by Tukey's HSD comparisons ($P < 0.05$). Error bars indicate standard deviation.

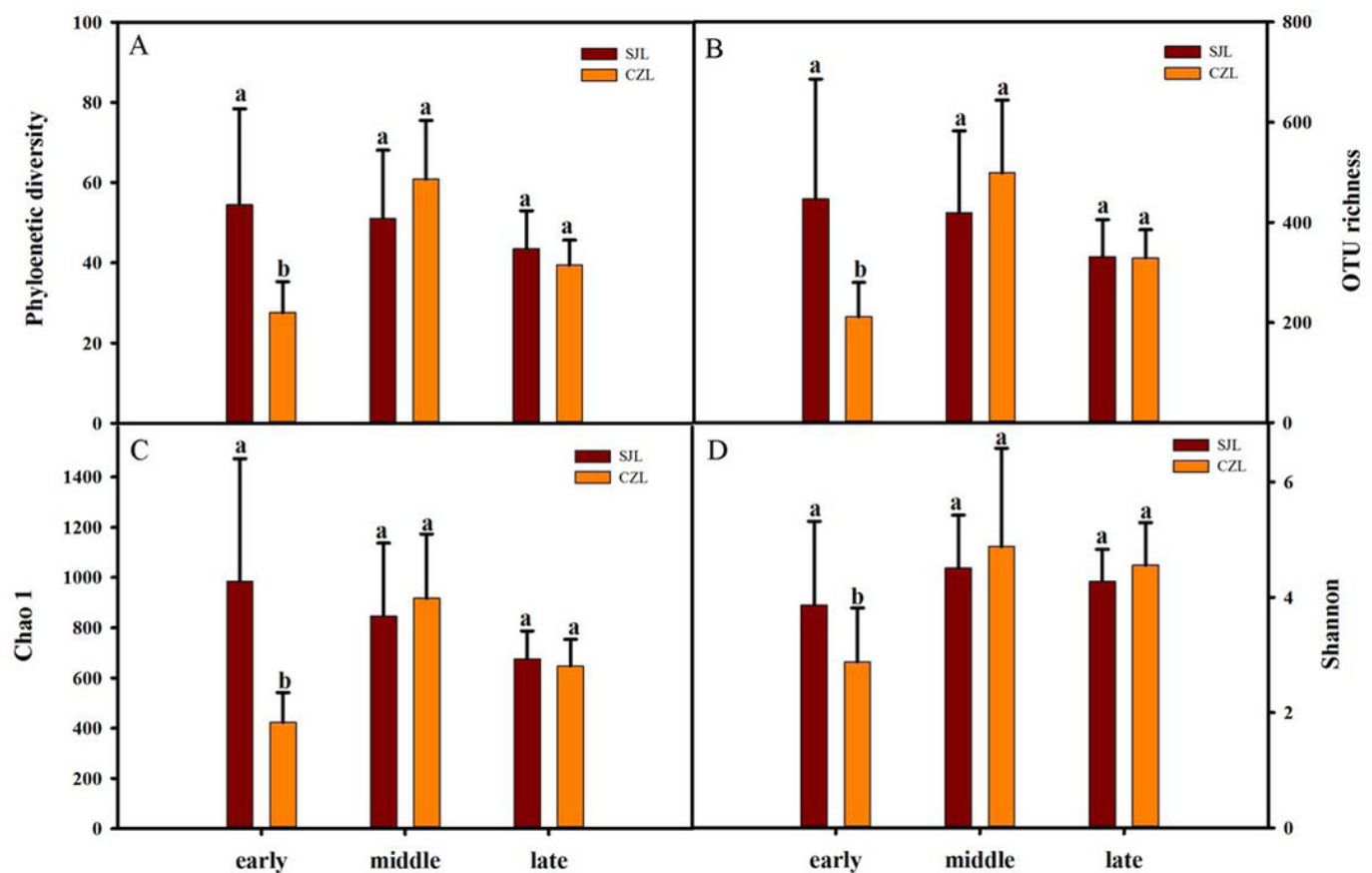



Figure 3

Relative abundance of gut bacteria.

Relative abundance of gut bacteria at the phylum, class and genus levels at two lakes. (A) Relative abundance of the dominant phyla in all samples. (B) Relative abundance of the dominant classes in all samples; Rings represent corresponding phylum (Firmicutes in blue and Proteobacteria in red) for each of the most frequently represented class. (C) Relative abundance at the phylum and (D) relative abundance at the genera at different periods in the two lakes.

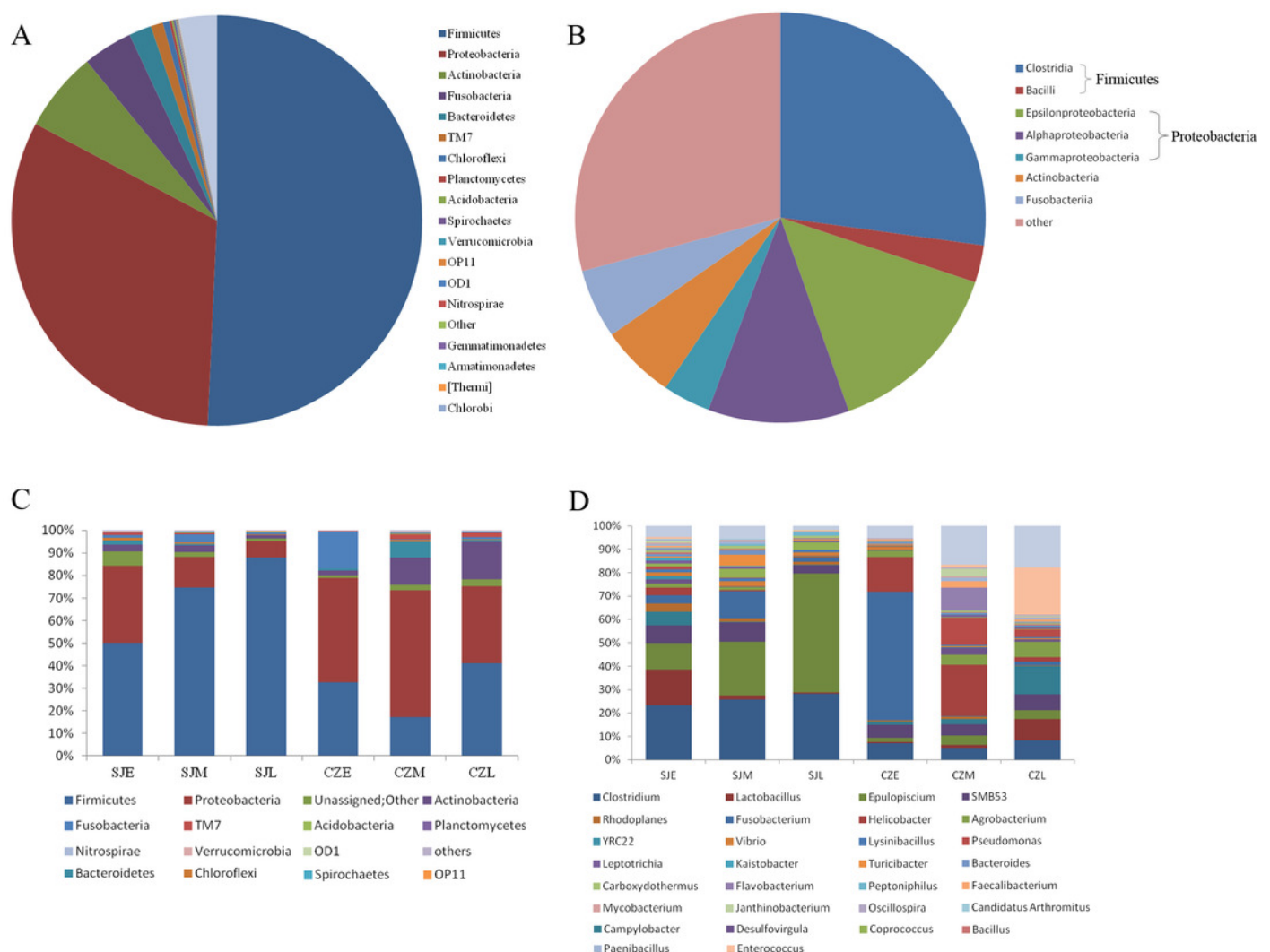



Figure 4

Relative abundance of the dominant phyla.

Relative abundance of the dominant phyla ((A) Firmicutes, (B) Ptoteobacteria, (C) Actinobacteria, (D) Fusobacteria) 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.

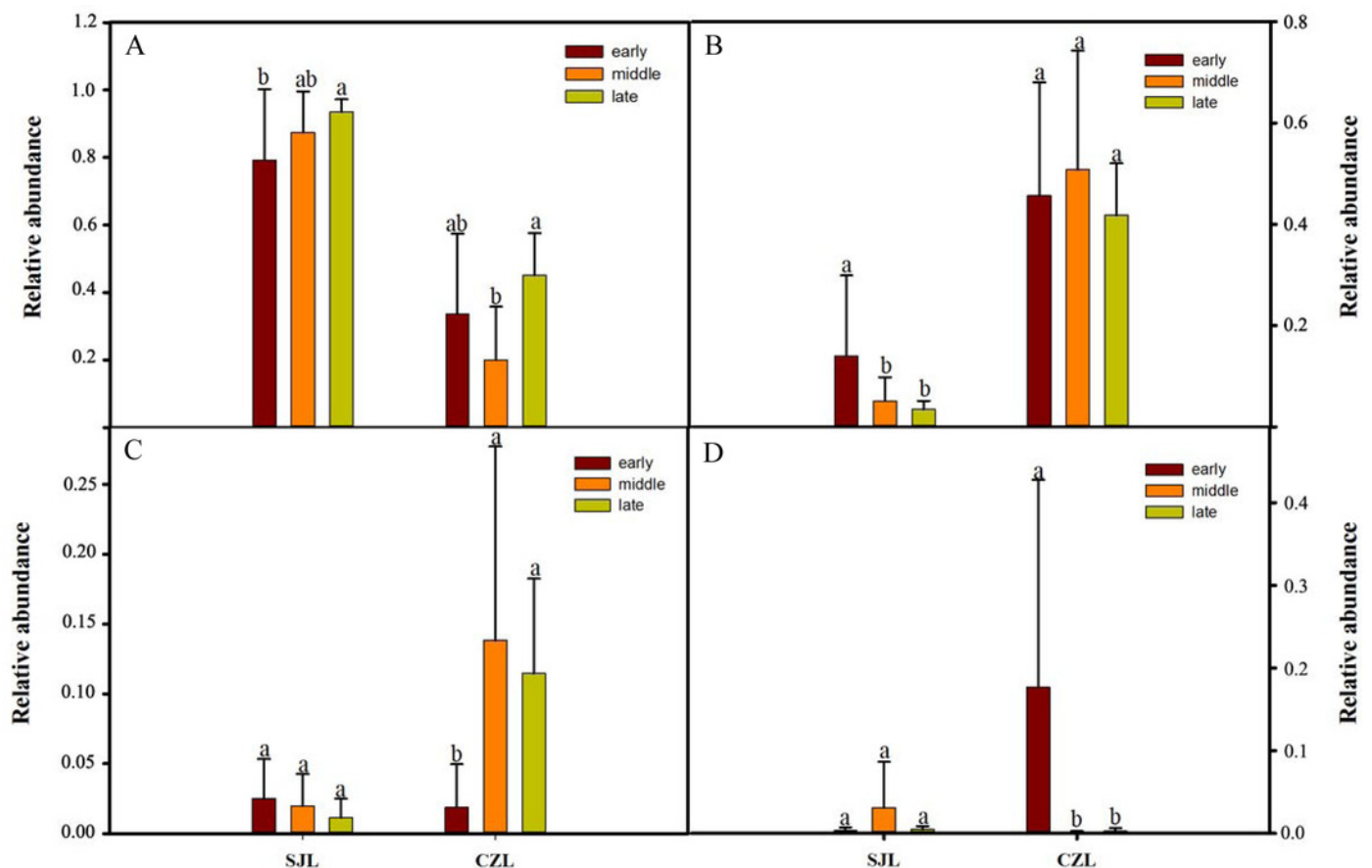



Figure 5

LEFSe analysis of the Hooded Crane guts bacteria.

LEFSe analysis of the Hooded Crane guts bacteria in different wintering periods (LDA > 2, $P <$ 0.05). (A) LDA score representing the taxonomic with significant difference in different wintering periods at the Shengjin Lake. (B) Cladogram representing the taxonomic hierarchical structure of the phylotype biomakera identified among three wintering periods from the Shengjin Lake; Red, phylotypes statistically overrepresented in early wintering period; Green, phylotypes statistically overrepresented in late wintering period. (C) LDA score representing the taxonomic with significant difference in different wintering periods at the Caizi Lake. (D) Cladogram represting the taxonomic hierarchical structure of the phylotype biomakera identified among three wintering periods from the Caizi Lake; Red, phylotypes statistically overrepresented in early wintering period; Green, phylotypes statistically overrepresented in late wintering period; Blue, phylotypes statistically overrepresented in middle wintering period.

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

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

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

Differences in gut bacterial community composition.

Differences in gut bacterial community composition. (A) Non-metric multidimensional scaling plot bacterial community composition from Shengjin and Caizi Lakes at three wintering periods. (B) Non-metric multidimensional scaling plot showing bacterial community composition 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.

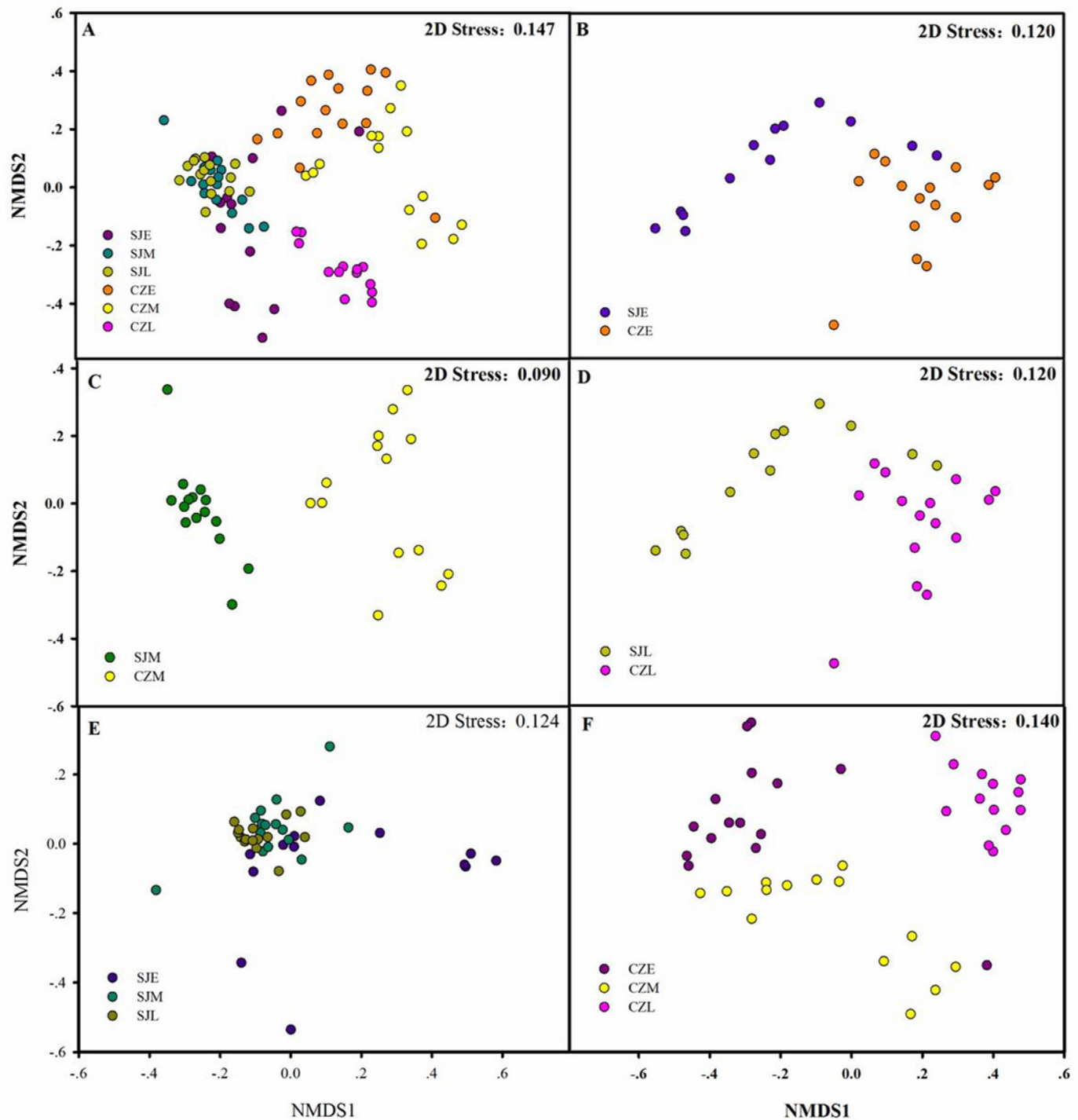
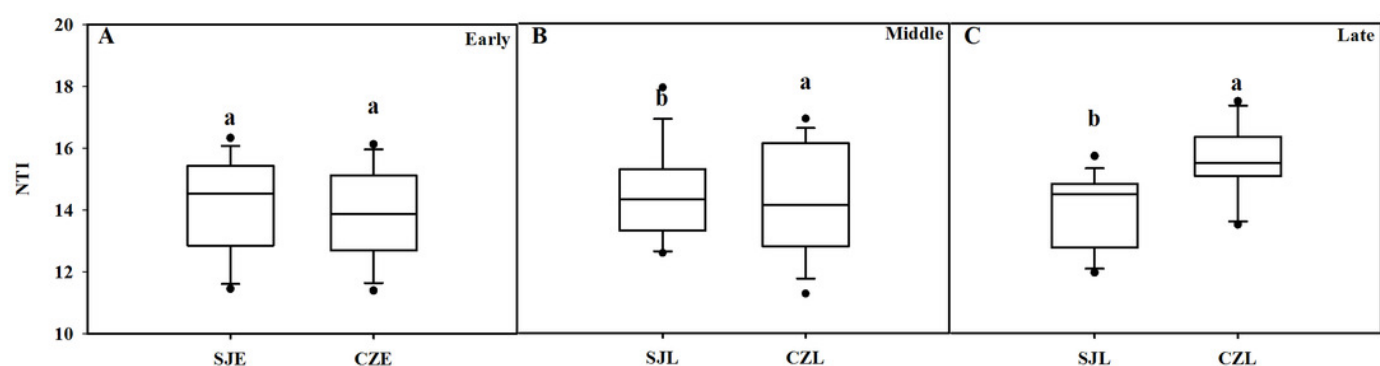



Figure 7

Bacterial phylogenetic structure evaluated by the NTI

Bacterial phylogenetic structure evaluated by the NTI in different wintering periods. (A) At the early wintering period, (B) At the middle wintering period, (C)At the late wintering period.

ANOSIM analysis

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

1 Table1. Differences in the microbial community composition based on the similarity test of
2 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			

ANOSIM analysis

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

1   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