Significant Differences in Bacterial and Potentially Pathogenic Communities Between Sympatric Hooded Crane and Greater White-Fronted Goose

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The gut microbiota of vertebrates play a crucial role in shaping the health of their hosts. However, knowledge of the avian intestinal microbiota has arguably lagged behind that of many other vertebrates. Here, we examine the intestinal bacterial communities of the hooded crane and the greater white-fronted goose at the Shengjin Lake of China, using high-throughput sequencing (Illumina Mi-Seq), and infer the potential pathogens associated with each species. Intestinal bacterial alpha-diversity in the greater white-fronted goose was significantly higher than that in hooded crane. The intestinal bacterial community compositions were significantly different between the two hosts, suggesting that host interactions with specific communities might have profound implications. In addition, potential pathogens were detected in both guts of the two hosts, suggesting that these wild birds might be at risk of disease and probably spread infectious disease to other sympatric vertebrates. The gut of hooded crane carried more potential pathogens than that of the greater white-fronted goose. The potentially pathogenic community compositions were also significantly different between the two hosts, suggesting the divergence of potentially pathogenic communities between hooded crane, and greater white-fronted goose. Finally, bacterial and potentially pathogenic structures showed strong evidence of phylogenetic clustering in both hosts, further demonstrating that each host was associated with preferential and defined bacterial and potentially pathogenic communities. Our results argue that more attention should be paid to investigate avian intestinal pathogens which might increase disease risks for conspecifics and other mixed species, and even poultry and human beings.

Keywords: migratory bird, sequencing, wetland, intestinal bacteria, pathogen

INTRODUCTION

The gut microbiota of vertebrates is one of the most densely populated microbial assemblages (Whitman et al., 1998), and plays an essential role in the health of their hosts (Heijtza et al., 2011). The intestinal microbes contribute to many necessary functions for their hosts, including aiding in digestion (Turnbaugh et al., 2006; Stanley et al., 2012), vitamin synthesis and metabolism (Eberl and Boneca, 2010), protection against pathogens (Guarner and Malagelada, 2003; Koch and Schmid-Hempel, 2011), training of the immune system (Atarashi et al., 2011; Chung et al., 2012), organ
development (Stappenbeck et al., 2002; Rahimi et al., 2009), and regulation of host physiology (Backhed et al., 2004; Meinl et al., 2009). The microbiota may even affect mate choice and induce hybrid inviability (Sharon et al., 2010; Brucker and Bordenstein, 2013). In vertebrates, the intestinal microbial assemblage patterns are shaped by a series of complex and dynamic interactions throughout life, including diet (De Filippio et al., 2010), lifestyle (Ley et al., 2008; Nicholson et al., 2012), and seasonal fluctuations (Hird et al., 2014). Intestinal microbial communities are largely shaped by their host species, and microbial communities tend to be more similar between more closely related hosts (Eckburg et al., 2005).

Birds represent an interesting study system for intestinal microbes because they have unique life history traits and developmental strategies that are different from other vertebrates (Kohl, 2012). However, research of the avian intestinal microbiota has arguably lagged behind that of many other vertebrates. Recent studies of avian intestinal microbiota are mainly focused on ornamental and economically important birds (e.g., kakapo, hoatzins, poultry, etc), most of which showed that gastrointestinal microbial communities bring benefits to their hosts (Jin et al., 1998; Angelakis and Raoult, 2010; Torok et al., 2011; Zhang et al., 2011; Cao et al., 2012; Stanley et al., 2012). However, there are also pathways through which the colonization of intestinal microbes might be of detriment, triggering a series of avian diseases (Ford and Coates, 1971; Potti et al., 2002; Cao et al., 2012; Singh et al., 2013). Migratory birds travel long distances and utilize diverse habitats, which potentially exposes them to a broad range of pathogens and could spread infectious disease to conspecifics and/or other bird species, or even poultry and human beings (Altizer et al., 2011). However, the assumption that migrating birds facilitate pathogenic propagation has not been definitely verified.

The hooded crane (Grus monacha) and greater white-fronted goose (Anser albifrons) are two large long-distance migratory colonial wading wild birds. The hooded crane is defined as a vulnerable species in the IUCN (International Union for Conservation of Nature and Natural Resources) Red List of Threatened Species and is a first-class national protected species of Threatened Species and is a first-class national protected species in China, India, and Japan. The hooded crane and greater white-fronted goose breeds mainly on the Siberian arctic coast, eastern Siberia and Russia, and wintering in China, Japan, and South Korea (Zheng et al., 2015). The East Asia species of greater white-fronted goose breeds mainly on the Siberian arctic coast, and hibernates in China, India, and Japan. The greater white-fronted goose is one of the most abundant wintering bird in the Yangtze River floodplain. However, in recent decades, the wintering population of greater white-fronted geese has decreased markedly (Cao et al., 2008), with the population falling from around 140,000 in 1987 to about 18,000 in 2010 due to habitat loss and hydrological changes (Zhao et al., 2012). The wintering period of these two migratory birds is from October to April in the Yangtze River floodplain. Anthropogenic activities trigger rapid degradation of lake wetlands, leading to the significant reduction in food availability, which forces wintering birds to change their dietary structure and flock together for foraging (Barzen et al., 2009; Zhou et al., 2010; Yang et al., 2015).

Shengjin Lake, an internationally important wetland, is a river-connected shallow lake in the middle of the Yangtze River floodplain (Fang et al., 2006). Shengjin Lake is the most important wintering ground for hooded cranes and greater white-fronted goose, providing them with suitable feeding habitats during the wintering period (Chen et al., 2011). Previous studies have demonstrated that hooded cranes and greater white-fronted goose forage together in this area (Chen et al., 2011; Yang et al., 2015; Zheng et al., 2015), which offered the opportunity to compare intestinal bacterial and potentially pathogenic communities between these two hosts. A better understanding of intestinal microbes as well as pathogens of wild birds is important for clarifying avian ecology and disease propagation. In this study, high-throughput sequencing method (Illumina Mi-Seq) was used to analyze the intestinal bacterial communities of wintering hooded crane and greater white-fronted goose at the Shengjin Lake. In particular, we want to examine the bacterial communities and infer the potential pathogens in the guts of these two hosts.

**MATERIALS AND METHODS**

**Ethics Statement**

Fecal samples of hooded crane and greater white-fronted goose were collected after foraging to avoid human disturbance. Non-invasive sample collection did not involve the hunting of experimental animals. Permission was obtained from the Shengjin Lake National Nature Reserve of Anhui Province.

**Site Selection and Sample Collection**

The Shengjin Lake (30.15–30.30°N, 116.55–117.15°E) is a river-connected shallow lake, which flows into the Yangtze River (Supplementary Figure S1). The lake is an internationally important wetland, which serves as indispensable wintering and stopover habitat for migratory birds on the East Asia-Australasian flyway (Chen et al., 2011; Fox et al., 2011). The average annual temperature and precipitation are 16.14°C and 1600 mm, respectively (Fang et al., 2006).

Fecal samples from hooded crane and greater white-fronted goose were collected on the 10th of March, 2018 at the Shegan, Shengjin Lake (Supplementary Figure S1). Hooded crane mainly eats Vallisneria natans and Potamogeton malaianus (Zheng et al., 2015) while greater white-fronted goose feeds primarily on Carex spp. (Zhang and Lu, 1999; Cheng et al., 2009) in the early wintering period (i.e., from October to next January) at Shengjin Lake. However, the wading birds alter their dietary structure to exploit paddy fields as foraging habitat due to food shortage in the later wintering period (i.e., February to April; Zhou et al., 2010; Yang et al., 2015). There are lots of paddy fields around the Shegan region, so hooded cranes and greater white-fronted goose forage together here.

Before sampling, a telescope was used to search the flocks of hooded crane and greater white-fronted goose. The fresh fecal samples were collected immediately after foraging of wild birds. The interval distance for samples was more than 5 meters to avoid individual repetition. The fecal samples were kept in a cooler and transported refrigerated to the lab as quickly as possible.
The outside of each sample was cut and discarded to avoid contamination; the rest was homogenized within each plastic valve bag and stored at −20°C for DNA extraction.

**Fecal DNA Extraction**

DNA extractions were carried out on 200 mg of fecal samples using the Qiagen QIAamp® DNA Stool Mini Kit following the DNA isolation protocol. The extracted DNA was dissolved in 60 μl of elution buffer, quantified by NanoDrop ND-1000 (Thermo Scientific, United States), and stored at −20°C.

**Bird Species Determination**

Primer sets BIRDF1–BIRDR1 were used to amplify COL gene to confirm bird species (Hebert et al., 2004). PCR reaction was carried out in 50 μl reaction mixtures containing 100 ng of fecal DNA, each deoxynucleoside triphosphate at a concentration of 200 μM, forward or reverse primers at a concentration of 0.4 μM and 2 U of Taq DNA polymerase (TaKaRa, Japan). The cycling parameters were as follows: 95°C for 5 min, followed by 35 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 and then blasted (>97% sequence identity) in National Center for Biotechnology Information (NCBI). Only the fecal sample with sequence belonged to hooded crane or greater white-fronted goose was kept for high-throughput sequencing. A total of 30 fecal samples, 15 from hooded crane and 15 from greater white-fronted goose, were determined in this study.

**PCR and Amplicon Library Preparation**

An aliquot (50 μg) of purified DNA from each sample was used as template for amplification. Primer sets F515/R907 equipped with sequencing adapters and unique identifier tags were used to amplify the V4-V5 hypervariable regions of the bacterial 16S rRNA genes fragments (Biddle et al., 2008) for the Illumina Mi-Seq platform (PE 300) at Majorbio (Shanghai, China). PCR reaction was carried out in 50 μl reaction mixtures containing each deoxynucleoside triphosphate at a concentration of 200 μM, forward or reverse primers at a concentration of 0.4 μM and 2 U of Taq DNA polymerase (TaKaRa, Japan). The following cycling parameters were used: 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 45 s; with a final extension at 72°C for 10 min. To check for contamination, PCR negative controls were performed without added DNA template. Negative PCR controls did not contain detectable PCR product and were not processed for sequencing. Triplicate reaction mixtures per sample were pooled together and purified using an agarose gel DNA purification kit (TaKaRa). The PCR products were pooled in equimolar amounts (10 pg for each sample) before sequencing.

**Processing of Sequence Data**

Bacterial raw data were processed by the Quantitative Insights Into Microbial Ecology (QIIME v.1.9; Caporaso et al., 2010). The poor-quality sequences (below an average quality score of 30 and the length <250 bp) were removed. High quality sequences were clustered into Operational Taxonomic Units (OTUs; 97% similarity; de novo approach) using UCLUST (Edgar, 2010). Chimera and singleton OTUs were deleted. The most abundant sequence within each OTU was selected as the representative sequence identified by the ribosomal database project Classifier (Wang et al., 2007), and aligned by PyNAST (Caporaso et al., 2010). To equally rarefy samples, randomly selected subsets of 5,600 sequences (lowest sequence read depth; repetition with 20 times) per sample were used to compare bacterial community compositions and diversity for all samples.

**Potentially Pathogenic Species Determination**

All identified bacterial species were manually searched as key words in Web of Science. These bacterial species which have been demonstrated by references as pathogens in human and/or animals were set aside for further analysis. A total of 11 potentially pathogenic species have been detected in this study (Supplementary Table S1). The *Clostridium perfringens* might cause disease in humans, birds, pigs, dogs, goats, etc. (Craven et al., 2000; Songer, 2010; Mafruza et al., 2012; Kiu and Hall, 2018; Liu et al., 2018). The *Prevotella copri* and *Staphylococcus aureus* probably invade humans and mice (Scher et al., 2013; Tong et al., 2015). The *Helicobacter pylori*, *Elizabethkingia meningoseptica*, *Bacillus cereus* and *Prevotella nigrescens* are mainly human pathogens (Kotiranta et al., 2000; Kusters et al., 2006; Stingu et al., 2013; Jean et al., 2014). Fish are the primary hosts for *Flavobacterium columnare* and *Piscirickettsia salmonis* (Durborrow et al., 1998; Smith et al., 1999). The *Plesiomonas shigelloides* might be a pathogen in humans and fish (Claesson et al., 1984; Hu et al., 2014; Behara et al., 2018). The *Mucispirillum schaedleri* might cause disease in mice (Loy et al., 2017).

**Statistical Analysis**

Identification of intestinal bacterial taxa that differed significantly between host species was performed by linear discriminant analysis (LDA) effect size (LEfSe), which uses the non-parametric Kruskal-Wallis rank sum test with the default setting (an alpha value of 0.05 and an effect size threshold of 2) to identify biomarkers (Segata et al., 2011). The differences in bacterial and pathogenic community compositions between host species were analyzed by non-metric multidimensional scaling (NMDS) and analysis of similarity (ANOSIM; permutations = 999) using the vegan package (Version 2.0-2; Oksanen et al., 2011) in R v.2.8.1 (R Development Core Team, 2006). The nearest taxon index (NTI) was calculated to test phylogenetic structure using the picante package (Purcell et al., 2007) in R v.2.8.1 (R Development Core Team, 2006). More positive NTI values indicate phylogenetic clustering, while more negative NTI values indicate phylogenetic overdispersion (Webb, 2000). One-way ANOVA was used to analyze alpha-diversity and NTI values which followed normal distribution across samples (Kolmogorov-Smirnov test; Supplementary Table S2). The Mann-Whitney-Wilcoxon test was used to analyze the relative abundance of pathogenic species which followed non-normal distribution (Kolmogorov-Smirnov test; Supplementary Table S2).
RESULTS

Intestinal Bacterial Alpha-Diversity

We obtained a total of 443,460 quality-filtered bacterial sequences across all samples for the primer pair F515/R907, ranging from 5622 to 25132 sequences per sample (Supplementary Table S3). A total of 5,325 bacterial OTUs was found, ranging from 265 to 885 across all samples (97% similarity), 32.3% of which (1722) were found in both species. The unique bacterial OTUs were 1545 (29.0%) and 2058 (38.6%) for the hooded crane and greater white-fronted goose, respectively. One-way ANOVA showed that bacterial alpha-diversity in the gut of the greater white-fronted goose was significantly higher than that of the hooded crane (Figure 1).

Intestinal Bacterial Community Structure

The dominant intestinal bacterial phyla were Firmicutes (79.60%), Proteobacteria (11.83%), Bacteroidetes (4.71%), and Actinobacteria (1.21%). The dominant intestinal bacterial classes were Clostridia (77.33%), Alphaproteobacteria (8.60%), Bacteroidia (4.47%), Bacilli (2.20%), and Gammaproteobacteria (1.72%). LEfSe analysis identified specific intestinal bacterial taxa that were differentially abundant across the two hosts. The results showed that bacteria in eight phyla (i.e., Fibrobacteres, Fusobacteria, Gemmatimonadetes, OP11, OP3, Spirochaetes, Thermi and Verrucomicrobia), and 16 classes (i.e., Holophagae, Acidimicrobia, Thermoleophilia, Chloroflexi, Ellin6529, Fibrobacteria, Fusobacteria, Gemm_1, ZB2, OP11_3, OP11_4, Koll11, Spirochaetia, SC3, Deinococci and Verruca_5) were significantly more abundant in the gut of the hooded crane (Figure 2 and Supplementary Figure S2). Soil bacteria from four phyla (i.e., Armimonadetes, Bacteroidetes, Proteobacteria, and Synergistetes) and nine classes (i.e., Soilbacteres, Coriobacteria, SJA_176, Bacteroidia, Cytophagia, Ignivibacteria, Erysipelotrichi, Synergistia, and Deltaproteobacteria) were significantly more

Data Availability

The raw data were submitted to the Sequence Read Archive (SRA) of NCBI under the accession number SRP159542.
abundant in the gut of the greater white-fronted goose (Figure 2 and Supplementary Figure S2).

The bacterial community compositions were significantly different between the guts of the hooded crane and the greater white-fronted goose (ANOSIM: $P = 0.001$; Figure 3A). The bacterial Bray-Curtis dissimilarity within host species was bigger in the gut of the hooded crane than in the greater white-fronted goose (Supplementary Figure S3). The NTI was calculated to test the bacterial phylogenetic structure in the two species. The NTI values were positive for all samples tested, which showed that bacterial communities were phylogenetically clustered in both hooded crane and greater white-fronted goose (Figure 3B). In addition, NTI was lower in the gut of the hooded crane than in the greater white-fronted goose, which indicated that phylogenetic clustering was weaker in the gut of the hooded crane than in the greater white-fronted goose (Figure 3B).

Intestinal Potential Pathogen

A total of 6168 (1.39% relative to all bacterial reads) potentially pathogenic sequences were found across all samples, ranging from 7 to 910 sequences per sample (Supplementary Table S3). Potentially pathogenic sequences were significantly higher in the gut of the hooded crane than in the greater white-fronted goose.

These sequences grouped into 81 potentially pathogenic OTUs, 39.51% of which (32) were found in both host species. The gut of the hooded crane (37) had more unique pathogenic OTUs than the greater white-fronted goose (12, Figure 4A). One-way ANOVA showed that potentially pathogenic OTU richness was significantly higher in the gut of the hooded crane than in the greater white-fronted goose (Figure 4B).

A total of 11 potentially pathogenic species was detected in the guts of the hooded crane and the greater white-fronted goose. The primary dominant pathogenic species was C. perfringens which might be detrimental for birds (Supplementary Table S1). The other potential pathogens might cause diseases in human and/or specific animal species (Supplementary Table S1). In addition, the hooded crane gut carried more relative abundance of C. perfringens and M. schaedleri than in the greater white-fronted goose. The relative abundance of P. copri and P. shigelloides were significantly higher in the gut of the greater white-fronted goose than in the hooded crane (Supplementary Table S4). The potentially pathogenic community compositions were significantly different between hooded crane and greater white-fronted goose (ANOSIM: $P = 0.001$; Figure 4C). The potentially pathogenic NTI values were positive for all the samples, indicating that potentially pathogenic communities were also phylogenetically clustered in both the guts of hooded crane and greater white-fronted goose (Figure 4D). In addition, potentially pathogenic phylogenetic clustering was stronger in the gut of the hooded crane than in the greater white-fronted goose (Figure 4D).

DISCUSSION

In this study, we found significant differences in the intestinal bacterial community composition and diversity between hooded crane and greater white-fronted goose, demonstrating that bacterial taxa showed strong host-preference, suggesting that hosts were the crucial factor in shaping the intestinal bacterial structure (Eckburg et al., 2005). Previous study has shown that heritable taxa might be a reason to cause the shift in bacterial communities between different hosts (Goodrich et al., 2014). In this study, we found strong evidence for phylogenetic clustering of bacterial communities in both guts of the hooded crane and the greater white-fronted goose (Figure 3B), suggesting that different hosts were associated with specific and defined intestinal bacterial communities, which might be included by hosts mediated metabolic pathways and/or dietary selection (Nicholson et al., 2012; Goodrich et al., 2014).

The primary dominant intestinal bacterial phylum was Firmicutes (79.60%) in both the hooded crane and the greater white-fronted goose, which was consistent with prior studies in other birds, such as the chicken (Lan et al., 2002), seabirds penguin (Dewar et al., 2013, 2014) and turkey (Wilkinson et al., 2017). Intestinal Firmicutes contributes to the decomposition of complex carbohydrates, polysaccharides and fatty acids (Flint et al., 2008), which improves hosts' ability to extract nutrients from food (Tap et al., 2009). A high proportion of Proteobacteria (11.83%), which played an important role in
energy accumulation, was found in both guts of hooded crane and white-fronted goose (Chevalier et al., 2015), indicating that these wintering birds might consume lots of energy to deal with frost during wintering periods.

In this study, only 39.51% of total potentially pathogenic OTUs was found in both guts of the hooded crane and the greater white-fronted goose (Figure 4A). In addition, these two hosts carried significantly different potentially pathogenic compositions and diversity (Figure 4), suggesting the divergence of potentially pathogenic communities between hooded crane and greater white-fronted goose. We also found an interesting result where the hooded crane carried less bacterial diversity and more potentially pathogenic diversity relative to the greater white-fronted goose. Previous studies demonstrated that healthy mice contained more bacterial diversity while disease reduced a host’s intestinal bacterial diversity (Manichanh et al., 2006; de Vos and de Vos, 2012; Jeffery et al., 2012; Guan et al., 2016), indicating that there might be a negative relationship between intestinal bacterial and pathogenic diversity. Healthy hosts contain various bacterial groups while disease might break the balance of these groups to decrease bacterial diversity (Mangin et al., 2004).

The primary dominant potential pathogen was *C. perfringens* which might be detrimental for birds (Craven et al., 2000; Ryan and Ray, 2004; Songer, 2010; Mafruza et al., 2012; Liu et al., 2018), suggesting that these wild birds might be at risk of disease. Hooded crane harbored much more abundance of *C. perfringens* relative to greater white-fronted goose (Supplementary Table S4), indicating that wintering hooded crane might be suffering more severe pathogenic invasion. Hooded cranes are a vulnerable species in the IUCN Red List of Threatened Species, so much more attention should be paid to protect hooded cranes. The *C. perfringens* triggers tissue necrosis, bacteremia, emphysematous cholecystitis and gas gangrene, not only to infect avian species, but also human beings (Ryan and Ray, 2004; Songer, 2010). Particularly, three potential pathogens in the feces of the two hosts might cause severe diseases in fish (Supplementary Table S1). The Shengjin Lake is an important fish farming base and these pathogens in avian feces could easily enter into the lake. There were also several potential pathogens which might cause diseases in human and/or specific animal species (Supplementary Table S1). Because of the migration of birds, they might widely propagate their intestinal pathogens and increase the risk of disease in other animals, even

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**Figure 4** The intestinal pathogenic bacterial characteristics in guts of two hosts: pathogenic OTU overlapping (A), operational taxonomic unit (OTU) richness (B), community composition (C), and nearest taxon index (NTI, D). HC, hooded crane; GG, greater white-fronted goose; ANOSIM, analysis of similarity.
human beings (Caron et al., 2010; Mora et al., 2013; Huang et al., 2014; Ekong et al., 2018).

In conclusion, intestinal bacterial as well as potentially pathogenic communities were significantly different between the hooded crane and the greater white-fronted goose. This work helps to build a more complete picture of intestinal microbial communities, as well as potentially pathogenic communities in migratory birds. However, there were certain limitations in this research. Only two bird species with 15 replicates were chosen for analysis. In addition, the intestinal bacterial communities of wild birds were studied within one wintering region rather than across multiple regions. Lastly, we did not show pathogenic interaction among wild birds along the wintering timescale to distinguish the degree of cross infection. These limitations should be clarified in future studies.

AUTHOR CONTRIBUTIONS

XX and LZ designed the experiments. XX, FZ, and RF completed the field sampling. FZ and XX performed the data analysis and prepared the figures. XX wrote the manuscript. SY and LZ contributed to the revision of manuscript.

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SUPPLEMENTARY MATERIAL

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