Vol. 53: 13–22, 2024
https://doi.org/10.3354/esr01284

ENDANGERED SPECIES RESEARCH
Endang Species Res

Published February 1

Temporal changes in the gut microbiota of overwintering great bustard *Otis tarda dybowskii*

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ABSTRACT: The gut microbiota of wild birds is sensitive to external environmental factors. Under the stress of cold temperatures in winter, seed-eating birds residing in stable habitats for a prolonged period experience gradual deterioration of dietary resources as time progresses. Investigating the dynamics of the gut microbiota of endangered birds throughout the wintering phase can offer an essential foundation for designing specific conservation measures. In this study, fresh faecal samples were collected from populations of great bustard *Otis tarda dybowskii* in stable wintering habitats during early, mid, and late winter. The gut microbiota of the great bustard at different time points was comparatively analyzed using high-throughput sequencing of the 16S rRNA gene. The results showed that the gut microbiota of overwintering great bustard comprised primarily 4 phyla: *Firmicutes* (80.13 %), *Bacteroidetes* (10.54 %), *Actinobacteria* (6.86 %), and *Proteobacteria* (1.23 %), accounting for 98.75 % of the total microbial abundance across all samples. While there were no significant differences in the gut microbiota during the early and mid-winter periods, significant changes occurred during the late wintering period. There was a significant decline in cofactor and vitamin metabolism, along with lower bacterial richness indices. In the late wintering period, the great bustard population in the wild may face increased survival pressures. Providing food supplementation before spring migration could potentially play a crucial role in saving this endangered species.

KEY WORDS: Great bustard · Overwintering period · Gut microbiota · High-throughput sequencing

1. INTRODUCTION

The gut microbiota of avian species plays a crucial role in maintaining host health by facilitating nutrient absorption, immune regulation, detoxification, and pathogen defense (Grond et al. 2018, Bodawatta et al. 2022). The composition of gut microbiota varies across avian species due to several factors, including genetics, behavior, habitat, and diet (Teyssier et al. 2018, Davidson et al. 2020, Lu et al. 2022, Schmiedová et al. 2022). Current studies concerning the gut microbiota in birds have primarily concentrated on domesticated poultry and captive animals (Sun et al. 2022). This focus is attributed to strict conservation regulations and the challenges associated with sample acquisition. Therefore, studies on the gut microbiota of wild birds are scarce, with Passeriformes and Charadriiformes being the most commonly studied species (Song et al. 2020, Matheen et al. 2022). Investigating the gut microbiota of birds with different ecotypes can provide a comprehensive understanding of the relationship between birds and their symbionts.

Birds possess a remarkable dispersal ability and exhibit a wide distribution range, and their complex life history and diverse habitat environments endow them with unique gut microbiota (Song et al. 2020, Mallott & Amato 2021). Due to adaptations to flight capabilities, the gut structure of birds changes, and food re-

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sides in the gut for shorter periods, leading to a greater susceptibility of the gut microbiota to diet and habitat conditions (Bodawatta et al. 2021b). A study of great tits *Parus major* found that the initial gut microbiota changed dramatically with dietary control, providing an extra level of plasticity in wild birds to adjust their dietary niche (Bodawatta et al. 2021a). The effect of different urban and rural diets on the gut microbiota of sparrows showed that the high-fat and low-fiber urban diets led to a decrease in microbial diversity, whereas the low-fat and high-fiber rural diets contributed to an increase in microbial diversity. The significant increase in members of *Enterococcaceae* and *Staphylococcaceae* in the rural diet group was generally considered positively associated with plant polysaccharide metabolism (Teyssier et al. 2020). Therefore, dietary changes can have a significant and meaningful effect on the gut microbiota of wild birds (Lewis et al. 2017, Matheen et al. 2022).

The great bustard *Otis tarda* is the largest surviving ground-dwelling bird with the ability to fly and is listed as Vulnerable on the IUCN Red List of Threatened Species (Dunning 2007, Martín et al. 2007). Alonso & Palacín (2022) have reported a 34.6% decline in the global population of great bustards since 2005, with populations facing greater threats (Alonso & Palacín 2022). The great bustard includes the nominal subspecies *O. tarda tarda* and the Asian subspecies *O. tarda dybowskii*, the latter accounting for less than 10% of the global population (Alonso & Palacín 2010). The Asian subspecies mainly breeds in China, Mongolia, and Russia, and almost all of them migrate to China for the winter, showing high habitat loyalty (Collar et al. 2017, Liu et al. 2022, Wang et al. 2023). During winter, the great bustard relies on farmland as a habitat, mainly feeding on winter wheat seedlings and grain seeds scattered on the ground, and is vulnerable to human disturbances such as chemical agents, poaching, and land use changes (Liu et al. 2018, Lu et al. 2021).

Previous studies have demonstrated significant variations in the gut microbiota of great bustards across different wintering regions, emphasizing the influential role of diet as a contributory factor in driving the differentiation of the gut microbiota (Liu et al. 2020, Li et al. 2021). In middle and high latitudes, plants and invertebrates enter a dormancy period under low winter temperatures, leading to a decline in both food quality and abundance for omnivorous birds (Robinson & Sutherland 1999). Consequently, changes in gut microbial community structure may occur during different overwintering periods. In this study, 16S rRNA gene high-throughput sequencing technology was used to analyze the gut microbiota of the overwintering great bustard, focusing on the core microbial community structure as well as variations in gut microbial composition and abundance across multiple overwintering periods. Our findings may offer valuable baseline information for monitoring the intestinal health of overwintering great bustards.

2. MATERIALS AND METHODS

2.1. Sample collection

A total of 31 fresh faecal samples were collected in early, mid and late winter from great bustard populations in stable wintering habitats, which were divided into 3 groups based on the time of collection. The POT group, consisting of 9 samples (POT 1–POT 9), was collected in November 2019, during the early wintering period. The EOT group, consisting of 13 samples (EOT 1–EOT 13), was collected in January 2020, during the mid-wintering period. Finally, the LOT group, consisting of 9 samples (LOT 1–LOT 9), was collected in March 2020, during the late wintering period. The Cangzhou area is located in the northeast of the North China Plain, where a double-cropping system consisting of winter wheat and summer maize is usually adopted, and the wide farmlands provide a suitable overwintering habitat for great bustards (Wang et al. 2008, Mi et al. 2017).

Wintering great bustard populations typically moved between various stable habitats, and the population size recorded ranges from 13 to 64 individuals. High-power monoculars were used to track field populations of great bustards, selecting a population with more than 20 individuals, and no other species were mixed in the population. After the population had completed foraging and flown away, fresh feces with a moist appearance were collected from the ground using disposable sterile tweezers. The middle non-environmental portion of the faeces was taken, placed in sterile centrifuge tubes and transported back to the laboratory through a portable vehicle-mounted refrigerator. Subsequently, the samples were stored at −80°C for preservation.

2.2. Sample DNA extraction, amplification and sequencing

The total DNA in the samples was extracted using the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek)

according to the instructions. The concentration and purity of DNA were measured using an ultramicro spectrophotometer (NanoDrop2000), and the quality of DNA extraction was further verified by 1% agarose gel electrophoresis. The highly variable V3–V4 region of the bacterial 16S rRNA gene was amplified using the primers 338 F (5′-ACT CCT ACG GGA GGC AGC AG-3′) and 806 R (5′-GGA CTA CHV GGG TWT CTA AT-3′) (Mori et al. 2014). The PCR reaction mixture comprised 4 µl of 5× FastPfu buffer, 2 µl of 2.5 mM deoxynucleoside triphosphates (dNTPs), 0.8 µl of forward primer (5 µM), 0.8 µl reverse primer (5 µM), 0.4 µl FastPfu DNA polymerase, 10 µl DNA template (1 ng μl^{-1}), and sufficient double-distilled water (ddH_2O) to make a total volume of 20 µl. The thermocycling conditions consisted of an initial denaturation at 95°C for 3 min, followed by 27 cycles (each comprising denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s) and a final extension step at 72°C for 10 min. The resulting PCR products were assessed via 2% agarose gel electrophoresis before purification using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences) and quantification employing the QuantiFluor™-ST (Promega). The PE 2300 library was prepared, and paired-end sequencing was performed on an Illumina MiseqPE 300 (Illumina) platform according to the standard protocols by Majorbio Bio-Pharm Technology Co.

2.3. Data processing and analysis

Raw sequences were processed according to the standard procedures of QIIME 2 (Bolyen et al. 2019). Following demultiplexing, Fastp v 0.20.0 software was utilized to quality filter the raw sequences, while FLASH v1.2.7 software was used for sequence merging (Magoč & Salzberg 2011). The resulting data were processed using DADA 2 to obtain amplicon sequence variants (ASVs) (Callahan et al. 2016). These ASVs were then classified and annotated against the Silva 16S rRNA database (SSU138) using a plain Bayesian classifier with a confidence threshold of 70% (Glöckner et al. 2017). Additionally, to eliminate potential contamination, chloroplastic and mitochondrial amplicon sequence variants were removed from the data set.

Alpha diversity indices based on ASVs were calculated using the Mothur v1.30.2 tool (Schloss et al. 2009), which included community richness (Sobs), evenness (Shannoneven), diversity (Shannon), and coverage (Coverage). Rarefaction curves were generated against the Sobs index for each sample to evaluate the adequacy of sequencing data. Venn diagrams were employed for identifying shared and unique ASVs among groups. To visualize community composition at various taxonomic levels, community histograms and heatmap plots were created. Principal coordinates analysis (PCoA) was performed to assess the variability in gut microbiota among sample groups based on the Bray-Curtis distance. The statistical significance of this variation was evaluated using the analysis of similarity (ANOSIM) method. Biomarkers that were statistically different in abundance across groups were identified using linear discriminant analysis effect size (LEfSe), which was implemented in the Galaxy platform (Afgan et al. 2018). The PICRUST 2 (phylogenetic investigation of communities by reconstruction of unobserved states) software was used to predict the functional and abundance information of ASVs in each sample based on marker gene sequences against the KEGG database (Douglas et al. 2020). To determine the statistical significance of differences among multiple groups, the Kruskal-Wallis test in the R (V. 3.3.1) package 'stats' was employed ($p = 0.05$), and the Scheffe post hoc test was used to analysis the difference between any 2 groups. P values were corrected for multiple testing using the false discovery rate (FDR).

3. RESULTS

3.1. Sequencing analysis

DNA extraction, 16S rRNA gene amplification, and sequencing were conducted on faecal samples collected from the great bustard during the winter of 2019–2020. After the raw sequences underwent splicing and quality control measures, a total of 1 545 706 optimized sequences were obtained, with sequence numbers ranging from 31 110 to 108 899 (Table S1 in the Supplement at www.int-res.com/articles/suppl/n053p013_supp.pdf). These optimized sequences were subsequently subjected to noise reduction before undergoing secondary sampling. The sequences were randomly re-sampled, ensuring that the minimum number of sequences was used to prevent statistical differences resulting from divergent sequencing depths. After annotation, a total of 1486 ASVs were identified, distributed among 14 phyla, 27 classes, 78 orders, 122 families, and 242 genera. The rarefaction curves revealed that the amount of sequencing was sufficient to reflect most of the information on the microbial composition of all samples (Fig. 1A).

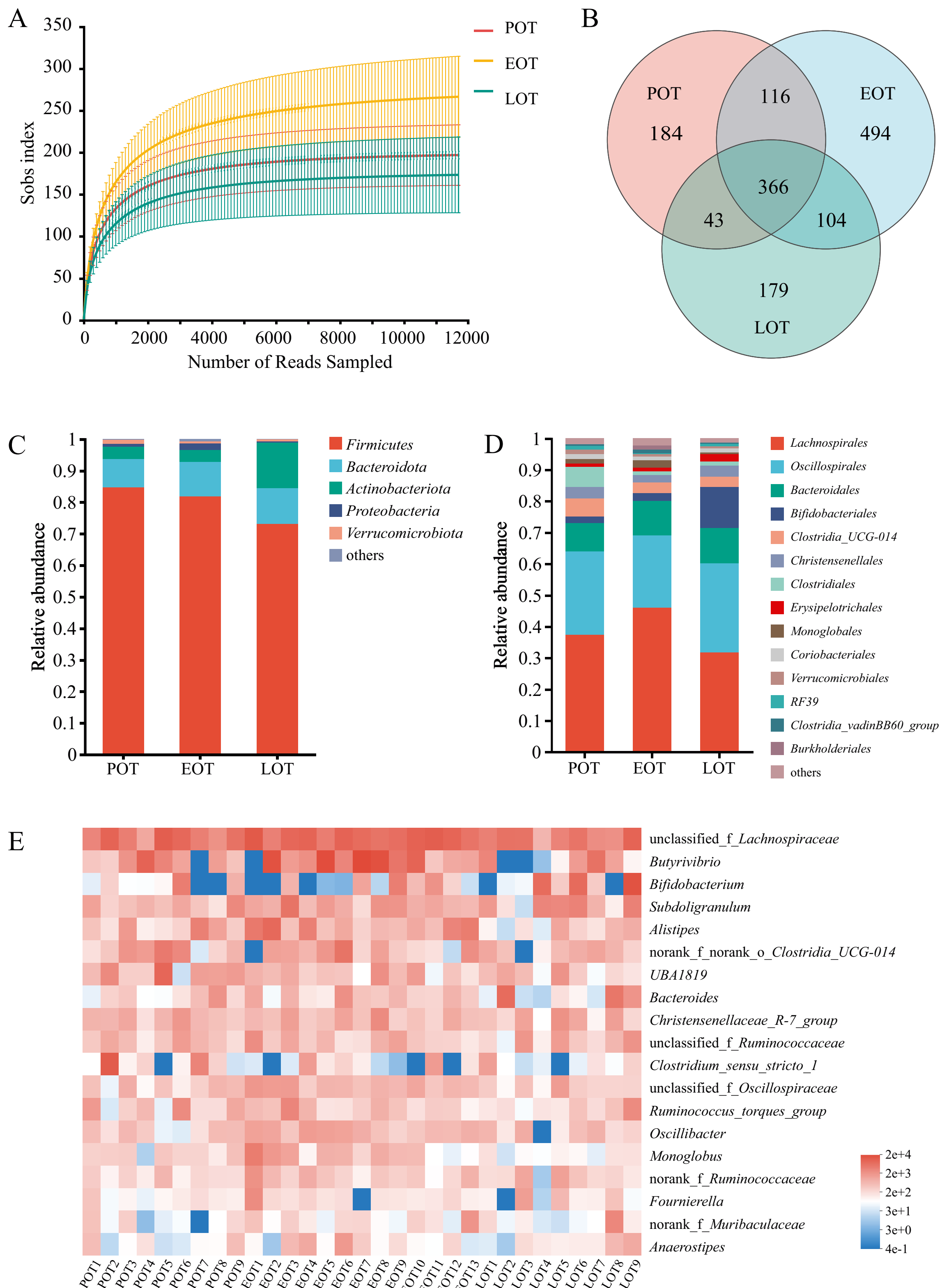

Fig. 1. (A) Rarefaction curve depicts the alterations observed in the Sobs index for the 3 sample groups (POT, EOT, LOT) as the sequencing depth is elevated. (B) Venn diagram of amplicon sequence variants (ASVs) among the 3 sample groups. (C) Relative abundance of the microbial communities at the phylum level. (D) Relative abundance of the microbial communities at the order level. (E) Heatmap showing the microbial community structure of core genera in all samples

3.2. Microbial composition and relative abundance

The gut microbial community structure of the overwintering great bustard was analyzed to determine the core species composition, defined as those with an average relative abundance of more than 1% in all samples. The study found that *Firmicutes* (80.13%), *Bacteroidetes* (10.54%), *Actinobacteria* (6.86%) and *Proteobacteria* (1.23%) were the predominant phyla, accounting for 98.75% of the total microbial abundance across all samples (Fig. 1C). At the order level, *Lachnospirales* (39.35%), *Oscillospirales* (25.62%), *Bacteroidales* (10.53%), *Bifidobacteriales* (5.46%), *Clostridia* UCG-014 (4%), *Christensenellales* (3.11%), *Clostridiales* (2.75%), *Monoglobales* (1.57%), *Erysipelotrichales* (1.44%) and *Coriobacteriales* (1.34%) constituted the average core species composition, which accounted for 95.16% of the total microbial abundance across all samples (Fig. 1D). Moreover, 19 core genera were identified in the total samples, accounting for 81.6% of the microbial composition of all samples (Fig. 1E). The Venn diagram illustrated the number of unique and shared ASVs among different sample groups. Notably, the EOT group exhibited the highest count of both unique and shared ASVs, whereas the LOT group displayed the lowest tally of such ASVs (Fig. 1B).

3.3. Comparative analysis of the gut microbiota

The investigation of alpha diversity indexes in the gut microbiota of the overwintering great bustard exhibited that bacterial microbial abundance (Sobs) spanned from 91 to 393, with the LOT 4 sample showing the lowest abundance and the EOT 3 sample showing the highest abundance. Community diversity (Shannon) indices ranged from 2.24 to 4.93, with the LOT 4 sample displaying the lowest diversity and the EOT 3 sample exhibiting the highest diversity. The community evenness (Shannoneven) indexes ranged from 0.5 to 0.82, with the LOT 4 sample showing the lowest evenness and EOT 3 showing the highest evenness. The Coverage index indicated that all samples exhibited a community coverage greater than 0.99, thus indicating that the bacterial microbial composition in the samples was accurately reflected (Table S2).

The comparative analysis of disparities in alpha diversity indices among sample groups revealed that the community richness was notably lower during the early wintering period than in the mid-wintering period ($p = 0.0069$). Conversely, it was significantly higher during the mid-wintering period than in the late wintering period ($p = 0.0004$, Fig. 2A). Community diversity ($p = 0.1122$, Fig. 2B), as well as evenness ($p = 0.3087$, Fig. 2C) in all 3 periods, did not display significant differences. The PCoA analysis demonstrated noteworthy differences in the bacterial microbial community composition among the sample groups during different periods ($p = 0.003$, Fig. 2D). Specifically, there were no significant differences in microbial community composition between early and mid-wintering periods ($p = 0.059$). However, significant variations were observed between early and late wintering periods ($p = 0.03$), along with highly significant dissimilarities between mid-wintering and late wintering periods ($p = 0.004$, Table 1).

According to the LEfSe analysis, it was determined that 3 biomarkers (*Akkermansia*, unclassified_o_ *Bacteroidales*, and *Enterococcus*) were identified in the early wintering sample group, while 14 biomarkers (*Monoglobus*, *Tyzzerella*, *Colidextribacter*, *Odoribacter*, etc.) were discovered in the mid-wintering sample group. However, no biomarkers were found in the corresponding late wintering sample group (Fig. 3A). The results of the PICRUSt analysis at KEGG levels 2, which were included in the metabolic pathways, showed that there was a significant decline in the metabolism of cofactors and vitamins in the late wintering sample group compared to the early ($p = 0.048$) and mid-wintering groups ($p = 0.0132$). The nucleotide metabolic pathway was significantly higher in the late overwintering sample group than in the mid-wintering group ($p = 0.0125$). However, no significant differences in other metabolic functions were observed among the 3 groups (Fig. 3B).

4. DISCUSSION

Satellite telemetry monitoring of the eastern migration route of great bustards indicates that they normally reach their wintering grounds by late October each year and depart in March of the following year, while exhibiting conservative preferences for wintering habitats (Wang et al. 2023). Field tracking of great bustard populations showed that during winters, they typically exhibit low mobility and tend to occupy several fixed habitats within a limited range of several dozen kilometers. This finding is consistent with the outcomes of population monitoring conducted in Shaanxi Province, China, which indicate that the great bustard tends to exhibit fidelity to regional wintering habitats (Kessler et al. 2013). To reduce the impact of environmental heterogeneity on the host gut micro-

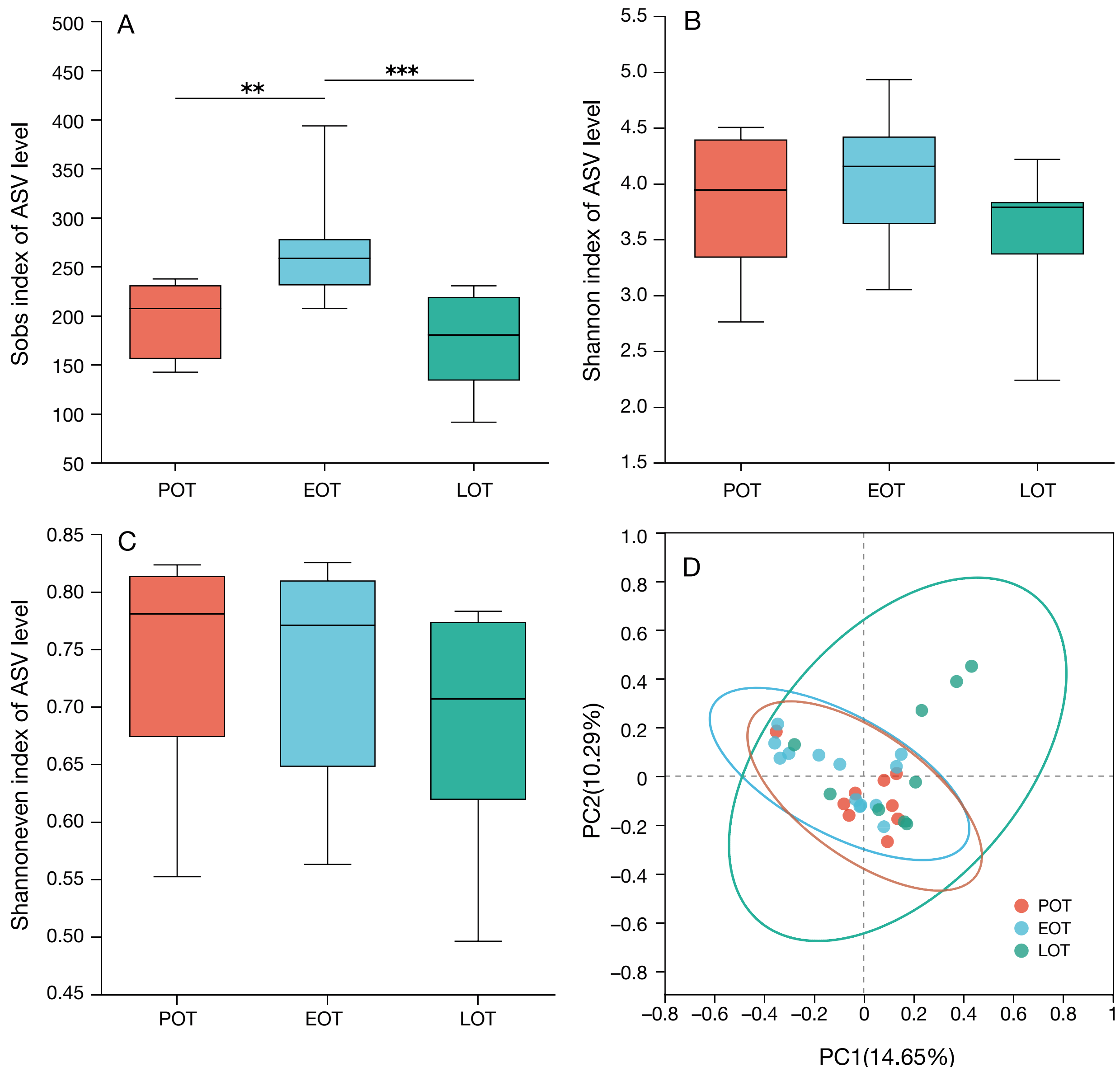

Fig. 2. Boxplot shows the difference in the alpha diversity indices of gut microbiota in the great bustard during different overwintering periods (**p < 0.01, ***p < 0.001). (A) Community richness (Sobs), (B) Shannon, and (C) Shannoneven indices. ASV: amplicon sequence variants. The horizontal line above each box represents the maximum value; the horizontal line inside each box represents the median; and the horizontal line below each box represents the minimum value. (D) Principal co-ordinates analysis (PCoA) analysis of gut microbial communities across the different overwintering periods

Table 1. Community dissimilarity test of gut microbiota during different overwintering periods based on analysis of similarity (ANOSIM)

Groups	R statistic	Significance level (p = 0.05)
POT vs. EOT vs. LOT	0.2022	0.003
POT vs. EOT	0.1281	0.059
POT vs. LOT	0.1337	0.03
EOT vs. LOT	0.3048	0.004

biota, we selected these samples collected from stationary winter wheat farms within the same region.

The gut microbiota of overwintering great bustards predominantly comprised *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* phyla, collectively accounting for 98.75% of the bacterial community across all samples. Among these, *Firmicutes* emerged as the most dominant species. At higher taxonomic levels, the gut microbiota of great bustards exhibited considerable similarity to that of

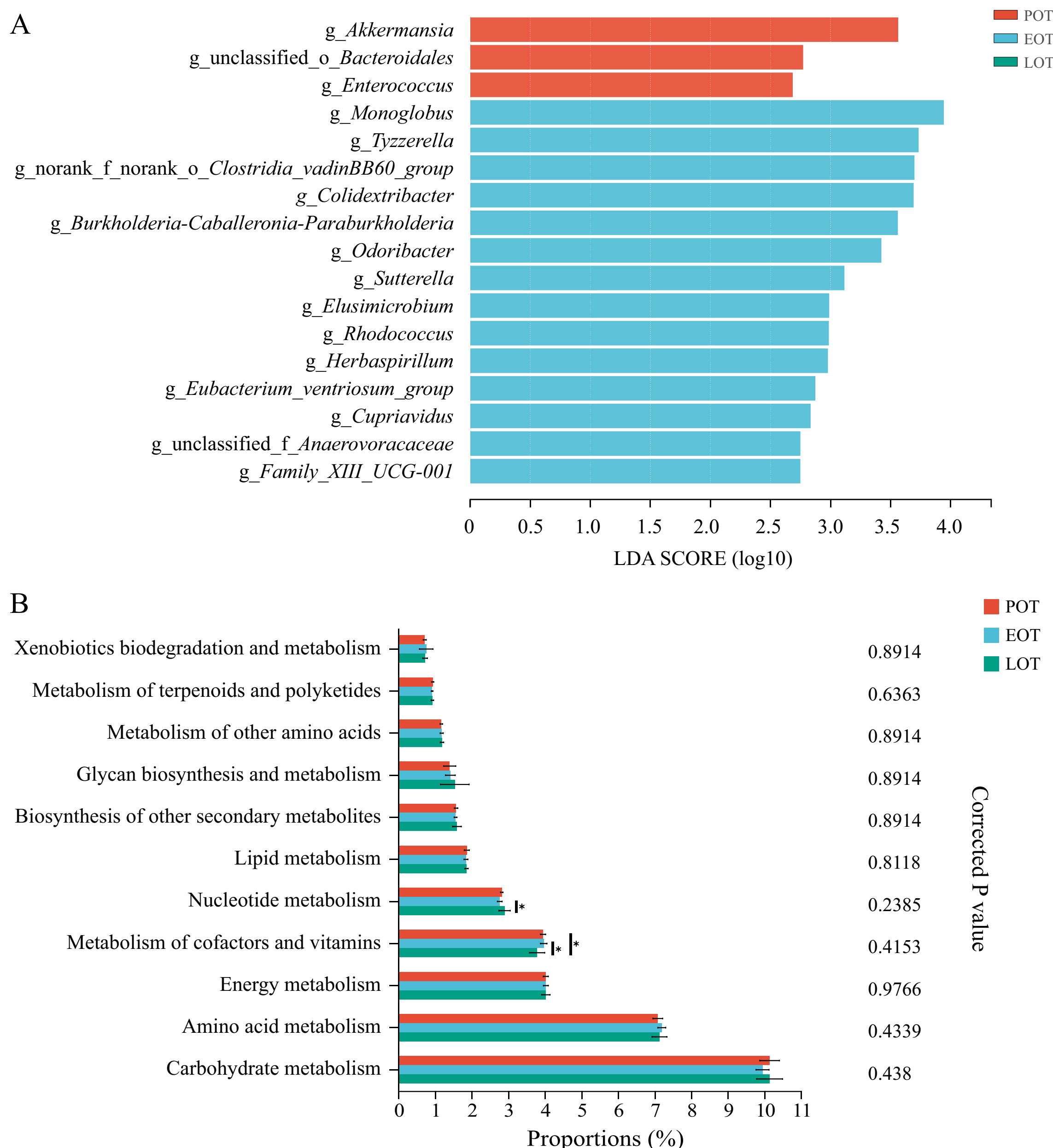

Fig. 3. (A) Linear discriminant analysis (LDA) effect size (LEfSe) analysis was conducted to identify gut microbial biomarkers in different overwintering periods (LDA > 2, p < 0.05). (B) Comparative analysis of the relative abundance of different metabolic pathways attributed to the gut microbiota during the different overwintering periods (*p < 0.05). Error bars are SD

other avian species (Best et al. 2017, Cho & Lee 2020, Fu et al. 2020). No studies have been found on the functioning of *Firmicutes* in wild birds, but in poultry several studies have shown a positive correlation between *Firmicutes* abundance and host weight gain (Li et al. 2019, Elokil et al. 2022). At the genus level, unclassified_f_*Lachnospiraceae* represented the most abundant taxon within the gut microbiota of the great bustard in relative terms. *Lachnospiraceae* have been associated with gut health through the production of short-chain fatty acids (SCFAs) and the degradation of plant fiber (Biddle et al. 2013). This family plays a crucial role in host nutrient absorption and has been identified as a source of candidate probiotic isolates with the potential to enhance poultry production performance (Stanley et al. 2016). Notably, the *Lachnospiraceae* family dominates the gut microbiota across a wide range of species within the Galliformes (Wienemann et al. 2011, Lundberg et al. 2021).

The great bustard, being a long-distance migrant, may harbor unique microbiota in its gut during the initial phase of arrival at its overwintering habitat. Three biomarkers, Akkermansia, unclassified_o_*Bacteroidales*, and *Enterococcus*, were identified through LEfSe analysis within the early wintering sample group. *Akkermansia* members are commonly found inhabiting the mucus layer of the digestive tract, where they facilitate the degradation of mucins and play a critical role in maintaining the intestinal epithelial barrier (Xing et al. 2019, Luo et al. 2022). The increased relative abundance of unclassified_o_*Bacteroidales* provides a potential benefit to chickens, enhancing their egg production performance (Guo et al. 2022). Notably, we observed elevated abundances of *Enterococcus* spp. in the samples collected during the early overwintering period. *Enterococcus* spp. belong to a class of opportunistic pathogens capable of forming biofilms during infection and rely on many virulence determinants for this process (Stępień-Pyśniak et al. 2019). Moreover, *Enterococcus* spp. are commonly present within the gastrointestinal tract of wild birds and possess multiple antibiotic-resistance traits that may accelerate the spread of antibiotic-resistant genes throughout their habitat (Santos et al. 2013). The presence of these genetic elements may pose challenges in effectively combating and managing antibiotic resistance.

The mid-winter sample group exhibited a considerable rise in the abundance of ASVs compared to the early winter period. Alterations in habitat environment and dietary resources towards the end of the autumn migration could potentially affect the host's gut microbiota. The modified dietary composition may lead to the inclusion of novel members or promote the expansion of some of the original members (Lewis et al. 2017). Multiple biomarkers were found in the mid-winter sample group. Within the chicken gut microbiota, *Monoglobus* members are identified as having pectin-degrading capabilities (Lysko et al. 2021). The *Clostridia_vadinBB60_group* members are shown to be beneficial in the accumulation of fat in chicken meat (Wen et al. 2023). Members of *Odoribacter*, *Anaerotruncus*, and *Sutterella* are extensively involved in SCFA metabolism, which assists in regulating host energy metabolism and maintaining intestinal health (Allaire et al. 2018, Medvecky et al. 2018). In addition, it is essential to draw attention to *Rhodococcus*, which is widespread amongst birds and may potentially cause infections in humans and various mammals (Walsh et al. 1993, Santos et al. 2012). Furthermore, a significant decrease in the abundance of ASVs was observed during the late wintering period. There were no biomarkers found with significantly higher relative abundance in the late winter sample group.

During the whole overwintering period, the great bustard primarily feeds on cereal seeds and seedlings of winter wheat that remain on the field from the previous autumn (Liu et al. 2018). Naturally, as winter progresses, the abundance and quality of cereal seeds decrease, leading to an adverse impact on the survival of cereal-eating birds (Robinson & Sutherland 1999). The PCoA analysis indicated that the gut microbiota of the great bustard remained stable during the early and mid-wintering periods, suggesting that the bird's food resources might have been relatively consistent during those periods. However, by late winter, the abundance and quality of the great bustard's food resources could potentially deteriorate due to competition from wildlife such as ring-necked pheasants, magpies, hares, and voles that also reside in the same area, as well as due to mould that can develop in some cereal seeds. These may be important factors that lead to significant differences in gut microbiota during the late wintering period. Additionally, a comparison of gut microbiota function during different overwintering periods revealed a noteworthy decrease in cofactor and vitamin metabolism in the later wintering sample group when compared to both the early and mid-wintering periods. The potential implications of partially diminished functionality in the gut microbiota of the great bustard may have adverse effects on the overall health of the host.

5. CONCLUSION

During the late wintering period, the gut microbiota of great bustard living in conservative overwintering habitats underwent significant changes, and some metabolic functions were suppressed. These phenomena may have adverse effects on the health of the host. In future conservation efforts, preserving the survival of great bustard populations during the late wintering period should be a primary concern, and selecting appropriate food supplementation before spring migration may play a crucial role in saving this endangered species.

Data availability. The data presented in the study are deposited in the NCBI database (accession numbers: PRJNA 719683, PRJNA 982535): accessed via https://www.ncbi.nlm.nih.gov/bioproject/PRJNA719683/ and https://www.ncbi.nlm.nih.gov/bioproject/PRJNA982535/.

Acknowledgements. We extend our gratitude to all the students from our laboratory who generously contributed to the successful completion of this research project. Additionally, we express our appreciation to the reviewers for their critical reviews and insightful comments, which have significantly enhanced the quality and rigor of this paper. This work was Funded by Science Research Project of the Hebei Education Department (grant number: QN2023106).

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Editorial responsibility: Richard Reina, Clayton, Victoria, Australia
Reviewed by: 2 anonymous referees

Submitted: July 1, 2023
Accepted: October 11, 2023
Proofs received from author(s): December 20, 2023