frontiers | Frontiers in Ecology and Evolution

TYPE Original Research
PUBLISHED 04 February 2026
DOI 10.3389/fevo.2026.1711240

OPEN ACCESS

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RECEIVED 23 September 2025
REVISED 17 December 2025
ACCEPTED 12 January 2026
PUBLISHED 04 February 2026

CITATION
Gao X, Dong Y, Li N, Wen F, Shang S, Wu Q and Zhang H (2026) Study on the composition characteristics of the intestinal microbiota in three species of cranes under captive conditions. *Front. Ecol. Evol.* 14:1711240. doi: 10.3389/fevo.2026.1711240

Study on the composition characteristics of the intestinal microbiota in three species of cranes under captive conditions

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Cranes are globally threatened yet understudied in terms of their gut microbiome, which is crucial for their health in captivity. This study examines the gut microbiota composition of three captive crane species using 16S rRNA gene sequencing of fecal samples. The research addresses the critical role of gut microbiota in nutrient metabolism, immune regulation, and environmental adaptation, particularly under captive conditions where dietary shifts may disrupt microbial balance. Fecal samples from 45 juvenile cranes were collected, and genomic DNA was extracted for 16S rRNA gene sequencing. Bioinformatic tools were used to analyze microbial diversity, composition, and functional pathways. Significant interspecific differences were observed. The common crane's microbiota was dominated by Bacillota (93.05%), enhancing cellulose degradation, while the Demoiselle Crane exhibited higher diversity with Pseudomonadota (21.95%) and Fusobacteriota (2.83%), reflecting omnivorous adaptations. The Red-crowned Crane showed intermediate Bacillota (78.32%) and notable *Plesiomonas* (12.48%), linked to aquatic diets. Alpha diversity was lowest in Demoiselle Cranes, and beta diversity revealed pronounced structural divergence. Functional analysis identified species-specific adaptations, such as alcohol detoxification in wetland cranes and pyruvate fermentation in arid-adapted Demoiselle Cranes. Captivity influences crane microbiota composition, with interspecific differences driven by evolutionary dietary specialization. The findings underscore the need for tailored dietary management in conservation programs to maintain microbial health and host adaptability. This study provides a scientific basis for optimizing captive crane care and supporting biodiversity conservation efforts.

KEYWORDS

captive diet, crane conservation, gut microbiota, interspecific variation, microbial adaptation

1 Introduction

The gut microbiota, often called the host's "invisible organ" plays a pivotal role in essential physiological processes, including nutrient metabolism, vitamin synthesis, pathogen suppression, and immune regulation (Liu Y. et al., 2021; Mokhtari et al., 2021; Sun et al., 2025). The structural stability of the gut microbiota is closely linked to the host's ability to adapt to environmental changes (Zhang K. et al., 2024). Regarding nutrient absorption, the gut microbiota assists animals in breaking down indigestible dietary components, such as cellulose and hemicellulose, transforming them into bioavailable nutrients, thereby increasing overall nutrient uptake efficiency (Li L. et al., 2021; Sun et al., 2022; Muramatsu and Winter, 2024). For example, certain gut microbes can produce specific enzymes capable of degrading cellulose present in plant cell walls, thereby releasing absorbable sugars. During energy metabolism, the gut microbiota regulates the host's energy balance, influencing energy production, storage, and utilization (Riedl et al., 2021; Lin et al., 2022). These microorganisms can convert complex organic compounds into energy-rich substances such as short-chain fatty acids through fermentation and other metabolic activities, offering supplementary energy sources for wild animals (Scarpellini et al., 2024). Furthermore, the gut microbiota plays a vital role in immune modulation (Lesniak et al., 2022; Dapa and Xavier, 2024). It promotes the development and maturation of the immune system, enhances the functionality of immune cells, and aids in defending against pathogenic invasion, thereby preventing disease. Additionally, adaptive alterations in the gut microbiota enable animals to better cope with environmental fluctuations (Zhang K. et al., 2024). For instance, in response to changes in dietary availability or environmental stressors, the gut microbiota can adjust its composition and functional profile to maintain the host's physiological equilibrium (Dapa and Xavier, 2024; Zhang K. et al., 2024). In wild animals, the diversity and composition characteristics of the intestinal microbiota have been confirmed to be significantly related to factors such as diet, habitat type, and seasonal changes (Mi et al., 2023). However, artificial intervention in captive environments may disrupt this natural balance. For instance, high-energy feed provided artificially may reduce microbiota diversity, a single food source may lead to a decrease in the abundance of specific functional bacteria (such as cellulose-decomposing bacteria), and long-term captivity may reduce microbiota complexity by limiting exposure to environmental microorganisms (Liu C. et al., 2021; Rasmussen and Chua, 2023; Bajagai et al., 2024). During wildlife rescue operations, animals' living environments and dietary conditions often experience significant alterations, which inevitably influence their intestinal microbiota. Understanding these microbiota changes under rescue conditions holds considerable importance. The Gruidae family, a globally threatened group of birds, comprises 15 extant species, the majority of which are included in the appendices of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) or listed as key protected wild animals in China. Cranes play a crucial ecological role; their population dynamics and health status not only indicate the stability of wetland ecosystems but also contribute to broader biodiversity conservation efforts (Qiu et al., 2024). However, habitat loss, environmental pollution, and human disturbances pose serious threats to their survival. In this context, studying the composition and variation patterns of crane intestinal microbiota can enhance our understanding of their environmental adaptability and health conditions, thereby providing a scientific foundation for the development of effective conservation strategies. Studies have demonstrated that the gut microbiota of migratory birds, including various crane species such as the common crane, demoiselle crane, black-necked crane, and white-naped crane, undergoes substantial changes in response to the annual life cycle and prevailing environmental conditions (Yanco et al., 2024). This dynamic variation illustrates the host's capacity to adapt to diverse habitats through the regulation of its microbiota (Yanco et al., 2024). Captive conservation, as a core means of *ex-situ* conservation, has effectively alleviated the risk of extinction of some crane species in the wild through measures such as artificial breeding and population reinforcement (Hu et al., 2025).

Currently, captive populations of species such as the red-crowned crane (*Grus japonensis*), the demoiselle crane (*Anthropoides virgo*), and the common crane (*Grus grus*) have become important supports for species continuation. However, the differences between captive environments and natural habitats may lead to an imbalance in the intestinal microbiota of cranes, thereby affecting their digestion and absorption, immune function, and even reproductive success, becoming a key issue restricting the health management of captive populations. Current research on the microbiota of cranes mainly focuses on single species or comparisons between wild and captive ones (Mi et al., 2023; Yanco et al., 2024). There is a lack of microbiota comparisons among different crane species under the same captive conditions (Mi et al., 2023; Yanco et al., 2024). Despite consistent feed formulations under standardized captive conditions, differences in gut microbiota structure may still exist among crane species, likely due to their distinct evolutionary histories and natural dietary variations, such as dietary specialization. Given the increasing challenges in wildlife conservation, cranes represent important conservation targets, and research into their intestinal microbiota holds significant value for conservation initiatives. This study aims to characterize and compare the gut microbiota of three captive crane species through 16S rRNA gene sequencing. The primary objectives are to (1) identify dominant microbial taxa and predict functional profiles, (2) evaluate species-specific compositional differences, and (3) investigate potential implications for the health and management of captive cranes.

2 Materials and methods

2.1 Sample collection

Source of animals. The juvenile cranes involved in this study were seized from wildlife poachers by local law enforcement authorities and subsequently transferred to the Wildlife Rescue

Center of the Institute of Inland Lakes for rehabilitation. The center is located in the cold and arid region of Northern Hulun Buir, Inner Mongolia.

Animal information and sample collection. In July 2023, we collected 45 fresh fecal samples from three crane species during their rehabilitation period at the center. The samples included 15 samples from juvenile common cranes (H group), 15 samples from juvenile demoiselle cranes (S group), and 15 from samples from juvenile red-crowned cranes (D group). At the time of sampling, the birds had been in captivity for approximately 4–6 weeks, and their general health status was stable with normal feeding behavior. To minimize the influence of dietary factors on intestinal microbiota at the time of sample collection, all fecal specimens were collected during the early morning hours (08:00–09:00) on the same day prior to feeding. According to the center's medical records, no antibiotics had been administered to any of these individuals for at least one month prior to sampling. The birds were fed a standardized diet consisting of chopped loach, corn grains, vegetables (e.g., carrots), and vitamin supplements. Fresh fecal samples were collected aseptically using sterile cotton swabs, immediately transferred into 1.5 ml sterile centrifuge tubes, properly labeled, and kept on ice in a portable cooler to suppress microbial activity during transport. Although all birds were provided with the same standardized diet, preliminary behavioral observations revealed interspecific differences in feeding preferences. Common cranes consumed relatively more corn and vegetables, accounting for approximately 60% of their dietary intake, whereas red-crowned cranes exhibited a marked preference for loach, which constituted about 70% of their intake. Demoiselle cranes demonstrated a more balanced utilization of all dietary components. These behavioral patterns indicate species-specific feeding preferences even under uniform dietary conditions, potentially contributing to divergences in gut microbiota composition. All samples were transported to the laboratory and transferred to a -80 °C freezer for long-term storage within a maximum of 4 hours post-collection. Local law enforcement authorities had seized these juvenile cranes from wildlife poachers and were temporarily housed at the rescue center for rehabilitation. This research was supported and approved by the Academic Ethics Committee of Qufu Normal University.

2.2 Sample sequencing and quality control

Genomic DNA was extracted from 45 fecal samples using the CTAB (cetyltrimethylammonium bromide method) method (Li H. et al., 2021; Yang et al., 2024)(Technical support is provided by Uniteomics Biotech Company Limited), and its purity and concentration were assessed via 1% agarose gel electrophoresis (Wang et al., 2023). The extracted DNA was diluted to 1 ng/μL using sterile water. The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified using the universal primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Xiang et al., 2019; Yin et al., 2024). The Phusion® High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs) and a high-efficiency, high-fidelity enzyme were employed to ensure both amplification accuracy and efficiency. The optimal minimum cycle number was determined through preliminary experiments to ensure sufficient product yield across most samples. Equimolar quantities of the resulting PCR products were pooled, and target fragments were excised from a 2% agarose gel and purified using the Qiagen Gel Extraction Kit with the following program: initial denaturation at 98 °C for 1 min; followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s; with a final extension at 72 °C for 5 min. Library construction was then carried out using the TruSeq® DNA PCR-Free Sample Preparation Kit. The final library quantification was performed using Qubit assays and quantitative PCR (qPCR) following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines on an Applied Biosystems QuantStudio 5 Real-Time PCR System, with standard curves generated for absolute quantification. Libraries that met the predefined quality criteria were subsequently sequenced on the Illumina NovaSeq 6000 platform (Li et al., 2023).

2.3 Sequencing analysis

For the paired-end reads obtained from sequencing, we initially separated the data for each sample based on the Barcode sequence and the PCR amplification primer sequence (Bushnell et al., 2017). Then, we employed FLASH software (v1.2.7) to assemble the reads from each sample, resulting in raw tag sequences (Raw Tags) (Bushnell et al., 2017). Following the quality control procedures for Tags as implemented in QIIME2 (version QIIME2-202202), we applied stringent filtering criteria to the Raw Tags, including truncation and length-based filtering, ultimately generating high-quality Tags data (Clean Tags) (Lasa et al., 2023). Subsequently, chimeric sequences were identified and removed to produce the final effective dataset (Effective Tags >50bps). The Effective Tags were then subjected to denoising using the DADA2 module within QIIME2 to generate Amplicon Sequence Variants (ASVs) and corresponding feature tables (Demircan et al., 2019).

Species annotation was carried out using the QIIME2 software platform. For both 16S and 18S analyses, the Silva 138.1 database was used as the reference dataset. Additionally, based on the ASV annotation information and absolute abundance, we statistically analyzed the proportion of sequences at different taxonomic levels in each sample to the total sequence number to evaluate the species annotation resolution of the samples. Finally, we analyzed the similarity among different samples through species abundance clustering heatmaps and constructed the clustering tree of the samples. We selected the top 10 genera with the highest relative abundance for specific species and performed species classification tree analyses for each sample and across the three groups. Additionally, using the ternaryplot function from the vcd package in R, we identified the top 10 species with the highest average abundance at each taxonomic level within the three groups and generated ternary plots to visually illustrate the differences in

dominant species among the groups at various classification levels. To investigate the phylogenetic relationships at the genus level, representative sequences of the top 100 genera were obtained through multiple sequence alignment, and a phylogenetic tree was constructed to reflect the evolutionary relationships among species. These approaches integrated various bioinformatics tools and statistical methods to provide a comprehensive and in-depth understanding of the species composition and diversity across samples.

2.4 Data analysis

In the present study, the Alpha diversity metrics were employed to evaluate the richness and evenness of microbial communities within each sample group. Diversity indices, including Shannon, Simpson, Chao1, and Pielou, were calculated using QIIME2 software (Liu et al., 2022). Additionally, R software (Version 2.15.3) was utilized to generate dilution, rarefaction curve, rank abundance, and species accumulation curves to assess sequencing depth and the uniformity of species distribution across samples. Box plots were constructed to represent each group's alpha diversity characteristics visually. Statistical significance between groups was evaluated using the Wilcox test for pairwise comparisons. Beta diversity analysis assesses the differences among samples and the evolutionary relationships among microbial communities. Based on Qiime1 software (Version 1.9.1), we calculated the Unweighted Unifrac and Weighted Unifrac distances, and constructed distance matrices and UPGMA sample clustering trees. To assess the significant differences in microbial community structure among three groups. A permutational multivariate analysis of variance (ADONIS/PERMANOVA) was performed to test the null hypothesis of no differences in the centroids of the groups, using Bray-Curtis's dissimilarity matrices and 9999 permutations. Analysis of similarities (ANOSIM) was applied to calculate the global R statistic, which indicates the degree of separation between groups, with an R-value close to 1 suggesting strong separation. Multi-response permutation procedures (MRPP) were used to confirm the within-group homogeneity, providing a chance-corrected within-group agreement statistic (A). A value of $A > 0$ indicates that within-group similarity is greater than expected by chance. Finally, an analysis of molecular variance (AMOVA) was conducted to partition the total genetic variance into components within and between the pre-defined hierarchical groups. Subsequently, we used R software to generate PCoA analysis plots (Chen et al., 2019). We employed the LEfSe software to detect potential biomarkers with LDA scores ≥ 4 to identify significant differences in species abundance across groups (Segata et al., 2011). Functional abundance prediction was performed using PICRUSt2 v2.5.0 with KEGG 2021 release (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States). Initially, PICRUSt2 aligned the ASV sequences against its internal reference database, placed the ASVs into the reference phylogenetic tree, inferred gene family copy numbers for each ASV, predicted the gene content of individual ASVs, and subsequently estimated the abundance of each gene family across samples. Subsequently, gene family profiles were mapped to functional databases (including KEGG and COG) to derive both functional annotations and quantitative abundance data for each sample. For all analyses that involve multiple hypothesis testing (including but not restricted to LEfSe and pairwise Wilcoxon rank - sum tests), p-values were adjusted for multiple comparisons by using the Benjamini - Hochberg method to control the false discovery rate (FDR). Significance was determined at an FDR-adjusted q-value < 0.05. PERMANOVA (Adonis) was employed to test for overall differences in community structure among groups.

3 Results

3.1 Sequence data processing overview

We performed 16S rRNA gene sequencing on 45 fecal samples collected from three crane species. Following the removal of low-quality sequences, a total of 2, 988, 098 raw reads were obtained for initial quality control. After filtering out chimeric sequences, 2, 531, 426 high-quality sequences (Effective Tags) were retained for downstream analysis. These Effective Tags encompassed 1, 070, 934, 706 base pairs, with an average read length of 423 bp and an average GC content of 51.62%. The proportion of Effective Tags relative to the raw reads was 84.72%. The species richness observed in the sample suggests that the sequencing data volume is approaching an adequate and representative level as the curve stabilizes (Figure 1).

3.2 Analysis of the composition and abundance of gut microbiota in three crane species

At the phylum level (Figure 2a), the gut microbiota of the three crane species was dominated by Bacillota, with significant interspecies differences in relative abundance: common crane (H, 93.1%) > Red-crowned Crane (D, 78.3%) > Demoiselle Crane (S, 68.8%). Pseudomonadota was the second most abundant phylum, with an opposite abundance ranking compared to Bacillota (Demoiselle Crane S, 22.0% > Red-crowned Crane D, 20.3% > common crane H, 6.0%). The Demoiselle Crane (S) exhibited higher microbiota diversity, with significantly higher abundances of Fusobacteriota (2.8%), Actinobacteriota (3.3%), and Bacteroidota (1.3%) compared to the common crane and Red-crowned Crane (all $\leq 1.1\%$ for the latter two groups). Additionally, the total abundance of Campylobacterota (0.7%) and other rare phyla (such as Patescibacteria, Chloroflexota, etc.) in the Demoiselle Crane group (about 3.3%) was much higher than that in the common crane group (0.3%) and the Red-crowned Crane group (0.1%). The microbiota composition of the common crane group was highly monotonous, with Bacillota accounting for over 93%, and the total abundance of all other phyla was less than 7%.

At the genus level (Figure 2b), the intestinal microbiota of the three crane species exhibited significant interspecific variation. Only

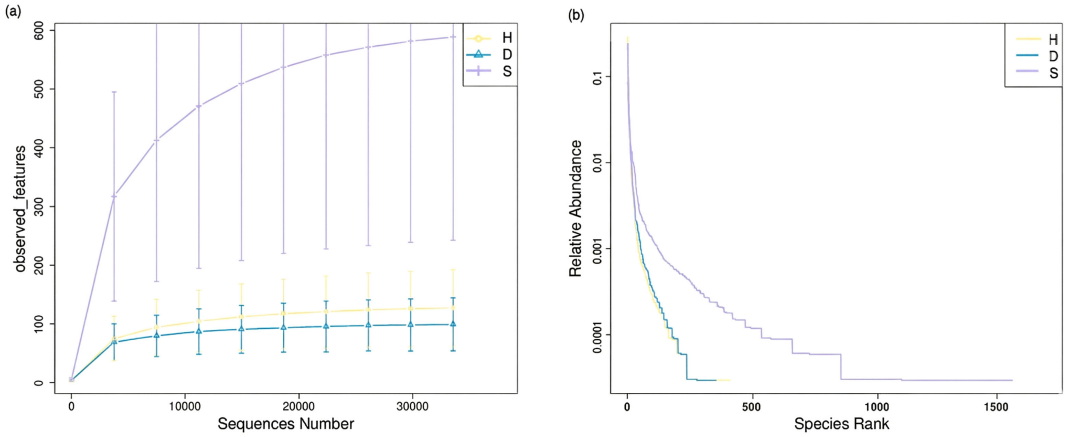

FIGURE 1
Rank abundance analysis among three Crane Species. Note: **(a)** Rarefaction curve, **(b)** rank abundance. H (common crane), S (Demoiselle Crane), and D (Red-crowned Crane). In the dilution curve, the x-axis represents the number of sequencings reads randomly sampled from a certain sample, and the y-axis represents the number of ASVs that can be constructed based on this number of sequencing reads, which is used to reflect the sequencing depth. Different samples are represented by curves of different colors. In the Rank Abundance curve, the x-axis represents the rank number of ASVs sorted by their abundance, and the y-axis represents the relative abundance of the corresponding ASVs. Different samples are represented by lines of different colors.

Ligilactobacillus and *Catellicoccus* were identified as common dominant genera, although their relative abundances varied markedly across species. In the common crane (H), *Ligilactobacillus* (42.4%) and *Catellicoccus* (28.5%) were the predominant genera, collectively accounting for 70.9% of the total microbiota. In the Red-crowned Crane (D), these two genera were present at slightly lower relative abundances—32.9% for *Ligilactobacillus* and 28.1% for *Catellicoccus*. In contrast, the *Demoiselle* Crane (S) exhibited a substantial decrease in the abundance of both genera, with *Ligilactobacillus* accounting for only 8.19% and *Catellicoccus* representing merely 0.01%. Characteristic genera in the Red-crowned Crane (D) included *Plesiomonas* (12.5%) and *Escherichia-Shigella* (6.0%), the latter of which is a potentially pathogenic group. The *Demoiselle* Crane (S) displayed a distinct dominant microbial composition, primarily consisting of *Romboutsia* (15.8%), *Enterococcus* (13.4%), and *Streptococcus* (5.3%), and exhibited the highest microbial diversity, with other genera collectively accounting for 42.9%. The gut microbiota of the common crane (H) continued to exhibit a high degree of compositional uniqueness, with the dominant two genera collectively accounting for over 70% of the microbial community. This was followed by *Clostridium_sensu_stricto*_1 (5.9%) and Lactococcus (4.4%). Notably, the relative abundance of *Escherichia-Shigella* (5.9%) in the Demoiselle Crane (S) was comparable to that observed in the Red-crowned Crane (D). However, the abundance of functionally relevant bacterial genera, such as *Paraclostridium* (0.03%) and Clostridium_sensu_stricto_1 (7.0%), was markedly lower in the Demoiselle Crane compared to the other two species.

3.3 Analysis of alpha differences among different groups

In the alpha diversity analysis (Figure 3), the richness and diversity of the intestinal microbiota in the demoiselle crane (S) were significantly lower compared to those in the common crane (H) and the red-crowned crane (D). The Chao1 index (Figure 3d) indicated that the S group was 19.53 units lower than the H group ($p < 0.001$) and 23.27 units lower than the D group ($p < 0.001$). Similarly, the Shannon index (Figure 3b) confirmed that the S group exhibited the lowest diversity, with differences of -21.53 compared to the H group and -19.47 compared to the D group ($p < 0.001$). The evenness of the microbial community, as measured by Pielou's index (Figure 3c), further supported this trend, showing that the S group was significantly lower than both the H group (-20.0, $p < 0.001$) and the D group (-15.8, $p < 0.001$). Importantly, no significant differences were observed in any of the alpha diversity indices between the common crane and the red-crowned crane (e.g., the difference in the Shannon index between the D and H groups was 2.07, $p = 0.53$; the difference in the Chao1 index was -3.73, $p = 0.23$), suggesting a comparable stability in their microbiota structures. The mean alpha diversity indices for each group are provided in Supplementary S1.

3.4 Significance test of intergroup community structure differences

In the present study, we aimed to determine whether the gut microbiota structure differs significantly among the three crane species (Common Crane, Demoiselle Crane, and Red-crowned Crane), and to identify specific microbial taxa that drive these differences. ANOSIM analysis demonstrated that the gut microbiota structures of the Demoiselle Crane (S) and the common crane (H) exhibited highly significant differentiation. Based on the PCoA plots using both unweighted and weighted UniFrac metrics (Figures 4a, b), the gut microbiota compositions clearly separate among the three crane groups: H (Common Crane),

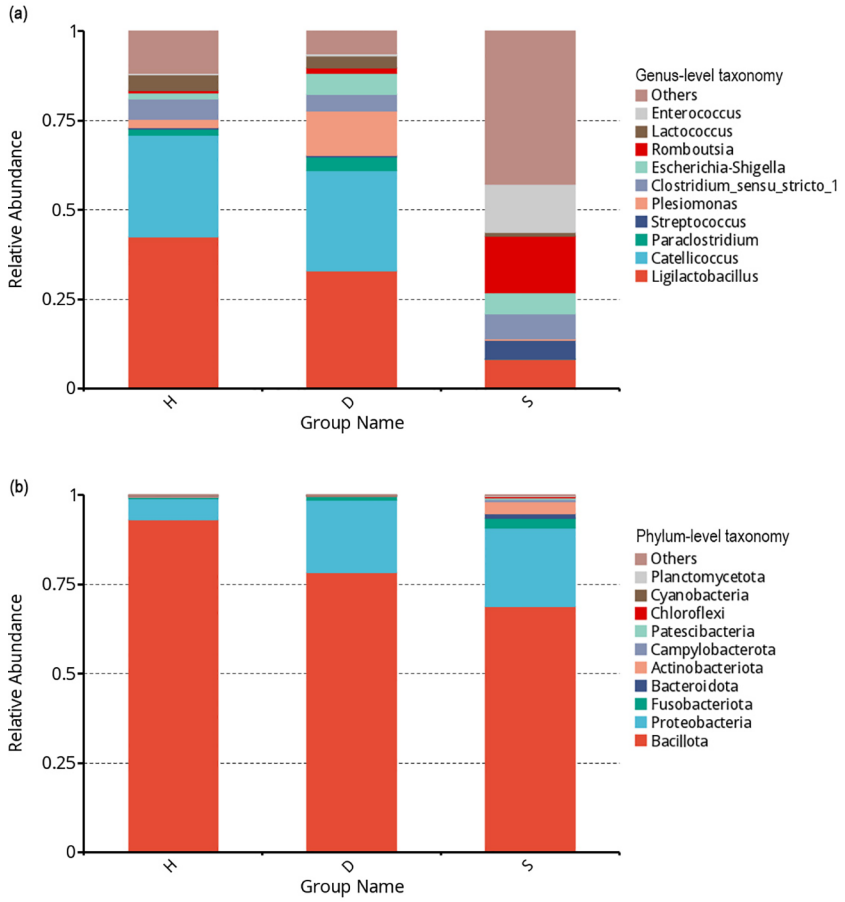

FIGURE 2
Analysis of the Composition and Abundance of Gut Microbiota in three Crane Species. Note: The cladograms reveal distinct microbial community compositions among the three crane groups: H (common crane), S (Demoiselle Crane), and D (Red-crowned Crane). **(a)** means Phylum-level compositional different in gut microbiota. **(b)** means Genus–level compositional different in gut microbiota.

S (Demoiselle Crane), and D (Red-crowned Crane). In the unweighted UniFrac analysis (exploring presence/absence of taxa), the first two principal coordinates (PC1 and PC2) explain 22.66% and 14.86% of the variation respectively, showing distinct clustering among the groups, indicating significant differences in microbial community membership. Conversely, the weighted UniFrac analysis (considering relative abundance) reveals a much stronger separation along PC1, which accounts for 86.01% of the variation, highlighting substantial differences in the relative abundance of microbial lineages between the crane species. Overall, these results demonstrate that the gut microbiota structure is significantly distinct and host-specific across the three crane groups. Based on the Wilcoxon test results displayed, significant differences in microbial taxa (potentially abundance or diversity indices) are observed among the three crane groups (Figure 4). This pattern indicates that a substantial number of microbial taxa are present at significantly different levels between the crane species. The results statistically support the host-specific gut microbiota composition previously suggested by the PCoA analysis, confirming that the gut microbial community structure is significantly influenced by crane species.

3.5 LEfSe analysis among three crane groups

The cladograms reveal distinct microbial community compositions among the three crane groups: H (common crane), S (Demoiselle Crane), and D (Red-crowned Crane) (Figure 5). In the H vs D comparison, Mycoplasmatae (phylum) and Actinobacteria (class) show higher relative abundance in H (Figure 5a), while D exhibits enrichment in other taxonomic groups (e.g., Bacillota). The H vs S comparison highlights unique clustering patterns, suggesting niche-specific adaptations in S's gut microbiome (Figure 5b). Notably, all groups share conserved microbial lineages, indicating phylogenetic constraints, but divergence in branch lengths and ring-layer colors underscores functional differences. These findings align with ecological

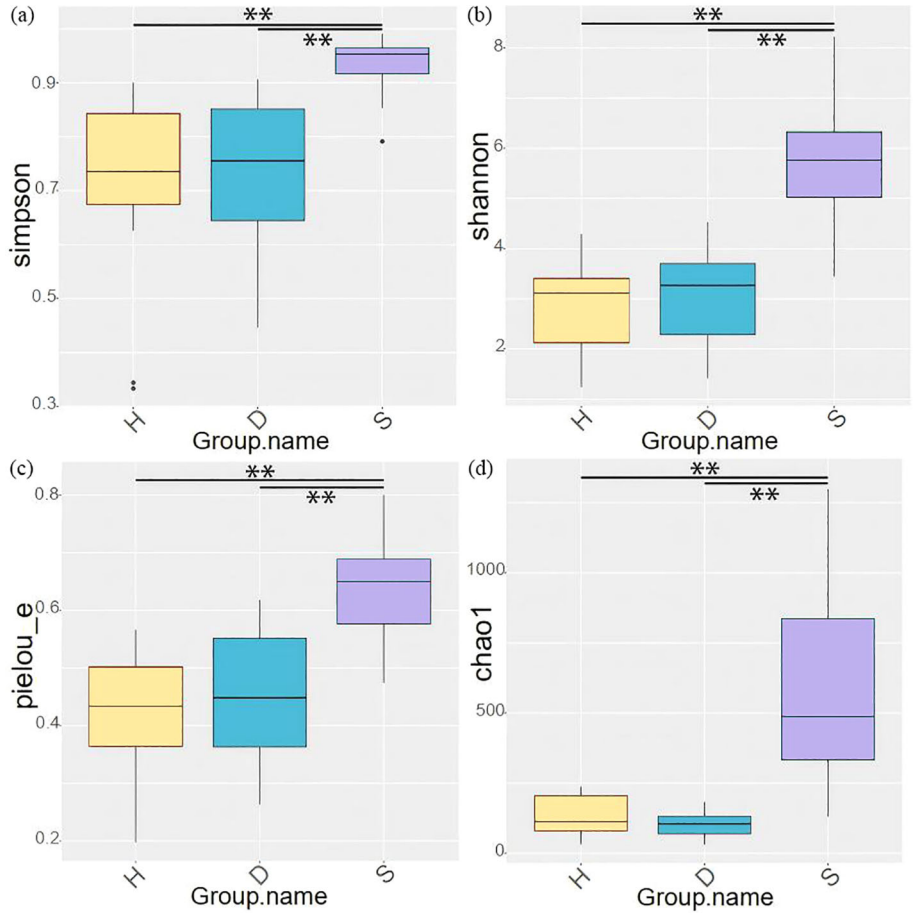

FIGURE 3
Analysis of differences among three Crane Species. Note: The cladograms reveal distinct microbial community compositions among the three crane groups: H (common crane), S (Demoiselle Crane), and D (Red-crowned Crane). **(a)**: Simpson analysis; **(b)**: Shannon analysis; **(c)**: Pielou_e analysis; **(d)**: Chao1 analysis. ** means significance level p<0.01.

specialization, as H and D inhabit wetland ecosystems, whereas S favors arid regions, shaping distinct microbial signatures.

3.7 Function analysis among three crane groups

The microbiomes' three groups exhibit distinct functional adaptations, reflecting their ecological niches and physiological demands (Figure 6). Our results showed that the COG1028 is dominated in H and D groups, suggesting active alcohol detoxification, possibly from fermented dietary sources in wetland habitats (Figure 6a). The PWY-5100 (pyruvate fermentation) dominates in the S group, supporting anaerobic energy production in arid environments. The PWY-612/7229 (adenosine de novo synthesis) and PWY-7220/7222 (deoxyribonucleotide synthesis) are enriched in the D group, likely supporting rapid cell turnover during migration (Figure 6b). The COG1136 (lipoprotein export) and COG0531 (amino acid transport) are prominent in the H group, facilitating nutrient uptake in omnivorous diets. The COG1846 (MarR) and COG1132 (multidrug transport) are upregulated in the D group, indicating enhanced xenobiotic resistance. The S group shows enrichment in PWY-7663 (anaerobic gondoate synthesis) and COG0583 (LysR), highlighting stress adaptation in harsh climates. Moreover, the H and D groups share conserved pathways (FASYN-ELONG-PWY, fatty acid elongation), suggesting phylogenetic constraints. These findings underscore how microbiome functionality aligns with species-specific ecological pressures, offering insights into conservation strategies.

4 Discussion

In this study, we examined the composition and characteristics of the intestinal microbiota across three crane species maintained under captive conditions. Our results showed that the intestinal microbiota of the common crane is predominantly composed of the phylum Bacillota (93.05%). Members of the phylum Bacillota, particularly those within Clostridia and Ruminococcaceae, are key cellulose degraders (Ryu and Davenport, 2022; Perez-Lamarque et al., 2023). The Bacillota phylum is known to play a key role in the breakdown of complex plant polysaccharides in mammals and birds, and its high

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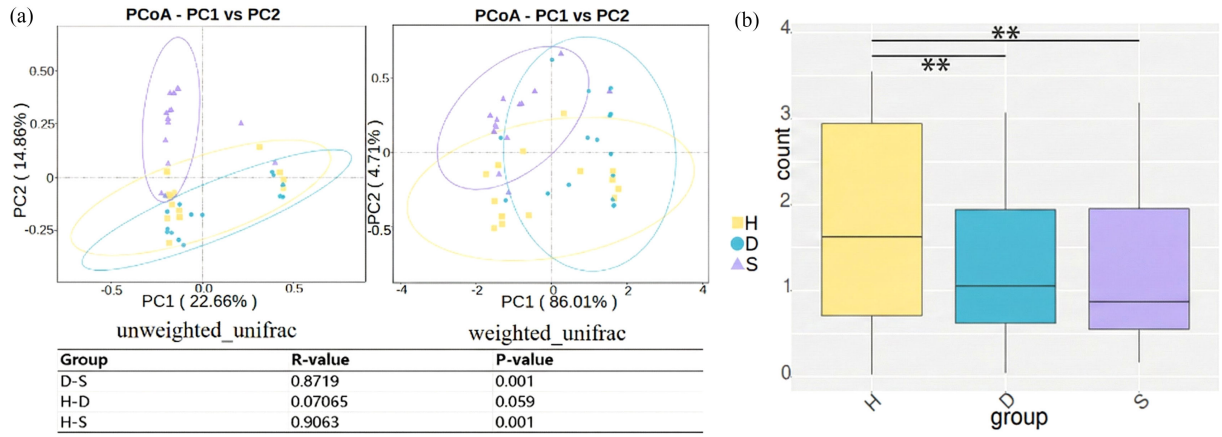

Group	R-value	P-value
D-S	0.8719	0.001
H-D	0.07065	0.059
H-S	0.9063	0.001

FIGURE 4
Analysis of differences among three Crane Species. The cladograms reveal distinct microbial community compositions among the three crane groups: H (common crane), S (Demoiselle Crane), and D (Red-crowned Crane). **(a)**: PCA analysis; **(b)**: Analysis of differences between groups. ** means significance level p<0.01.

abundance has been directly linked to the cellulose digestion requirements of herbivorous animals (Mandelli et al., 2024). The low diversity of the common crane's gut flora (with the combined abundance of other phyla accounting for less than 7%) may be attributed to its specialized herbivorous diet. However, such limited microbial diversity could compromise the species' resilience to environmental disturbances. In contrast, the demoiselle crane exhibits the highest microbial diversity among crane species, with significantly higher abundances of Pseudomonadota (21.95%), Fusobacteriota (2.83%), and Actinobacteriota (3.30%) compared to

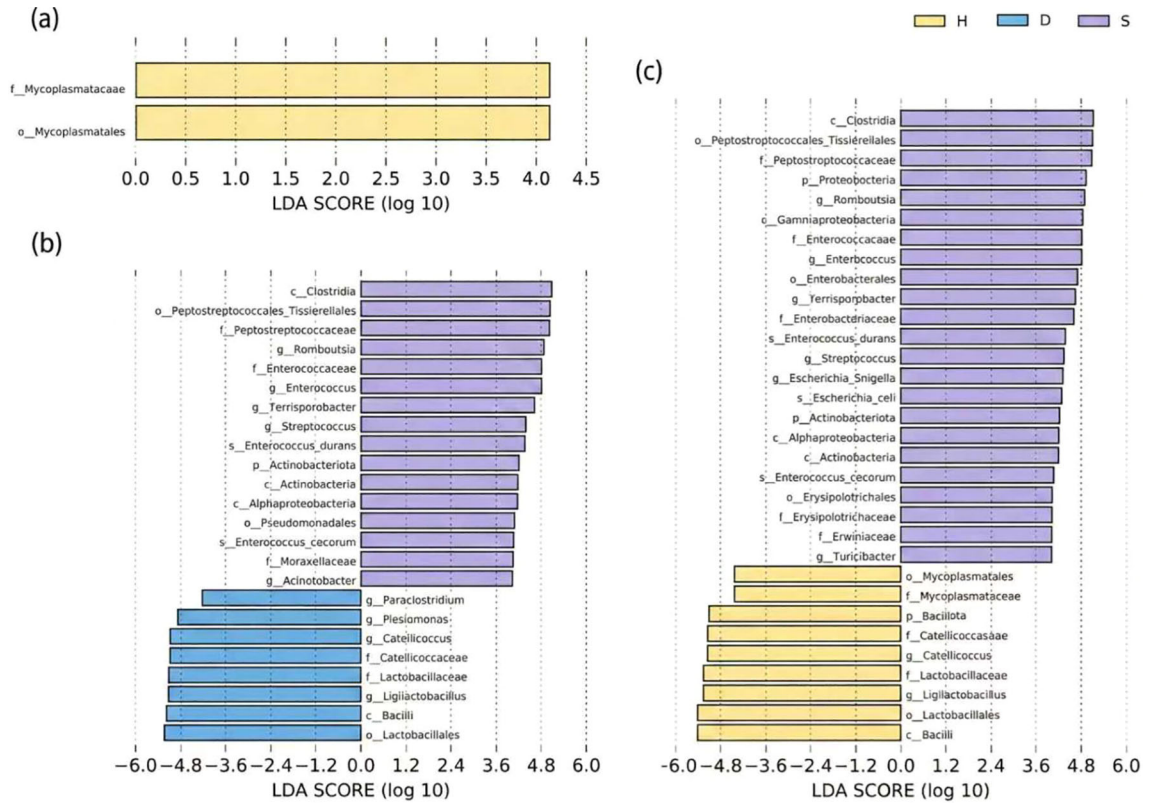

FIGURE 5
LEfSe Analysis among three Crane Species. Note: The cladograms reveal distinct microbial community compositions among the three crane groups: H (common crane), S (Demoiselle Crane), and D (Red-crowned Crane). (LDA) Effect Size (LEfSe) identified specific bacterial taxa that were significantly enriched in each group (LDA score > 3.5). The cladograms reveal distinct microbial community compositions among the three crane groups: H (common crane), S (Demoiselle Crane), and D (Red-crowned Crane). **(a)**: H vs D, **(b)**: D vs S, **(c)**: H vs D.

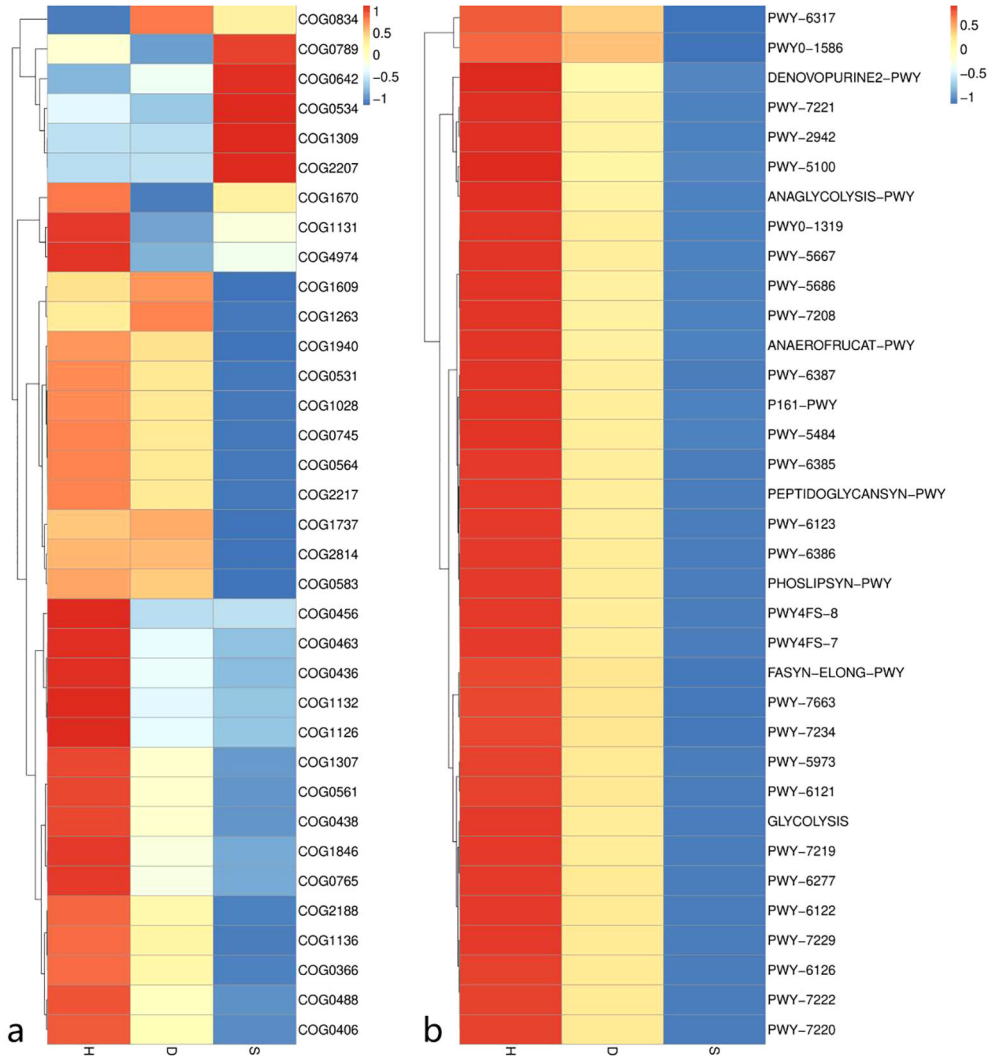

FIGURE 6
Function prediction among three Crane Species based on the COG **(a)** and pathways analysis **(b)**. Note: The graph displays sample information along the horizontal axis and functional prediction annotation details along the vertical axis. The clustering tree on the left represents hierarchical clustering results for the samples. The heatmap values correspond to Z-scores derived from standardizing the relative abundance of each species across rows. Specifically, for a given taxonomic group, the Z-score of a predicted functional pathway is calculated as the difference between its relative abundance in that group and the mean relative abundance of all predicted pathways within the same group, normalized by the standard deviation of those pathway abundances: H (common crane), S (Demoiselle Crane), and D (Red-crowned Crane). All numbers and their corresponding features were submitted in Supplementary S5 and S6.

other crane species (Liu et al., 2024; Shen H. et al., 2024). This greater diversity may reflect its adaptation to an omnivorous or variable diet. As reported in the literature, the gut microbiota of wild birds is influenced by multiple factors, including dietary habits and habitat environment (Bodawatta et al., 2022; Rasmussen and Chua, 2023). Nevertheless, the high prevalence of Pseudomonadota warrants attention due to its potential association with pathogenic bacteria (Chen K. et al., 2025). In the red-crowned crane, the abundance of Pseudomonadota (20.27%) falls between the common crane and the white-naped crane, while Bacillota still dominate (78.32%). However, this microbial composition's ecological role requires further investigation concerning the species' dietary characteristics (Zhang et al., 2025). Studies suggest that differences in gut microbiota among species within the same ecological niche may arise from dietary resource partitioning to minimize interspecific competition.

The gut microbiota composition across crane species appears to be shaped by distinct dietary, environmental, and physiological factors. From a dietary perspective, the common crane, an herbivore, exhibited a marked predominance of Lactic acid bacteria (42.41%), a genus with efficient carbohydrate-fermenting capabilities that facilitate the digestion of plant-based nutrients (Zhu et al., 2021; Chen Z. et al., 2025). Conversely, the demoiselle crane, an omnivore, harbored a high-abundance of Romboutsia (15.85%), a butyrate-producing genus that regulates immune homeostasis, and Streptococcus (5.30%), which contributes to energy metabolism. This consortium may collectively support metabolic flexibility for utilizing diverse food resources (Zhu et al., 2024).

Regarding environmental influences, the red-crowned crane's enrichment of Plesiomonas (12.48%) is closely associated with its reliance on the aquatic food web, as this bacterium is commonly found in freshwater environments and can be ingested with fish and aquatic invertebrates (Liu et al., 2024). Furthermore, the demoiselle crane's high overall microbial diversity (42.87% composed of 'other genera') aligns with the "high diversity-high adaptability" hypothesis, potentially providing the functional redundancy needed to cope with the environmental heterogeneity encountered during migration (Bodawatta et al., 2022; Liu et al., 2024). Ultimately, these factors culminated in contrasting community structures: a simplified, specialized profile in the common crane (70.88% in two genera) versus a highly diverse, flexible one in the demoiselle crane. This pattern reflects a high degree of ecological specialization in a stable niche. Overall, our findings support the hypothesis that sympatric crane species achieve ecological differentiation partly through divergence in their gut microbiota.

The diet type of wild vertebrates is the most influential factor associated with the alpha diversity of their intestinal microbiota. The relatively low alpha diversity observed in the demoiselle crane may reflect a microbial signature of its specialized ecological niche and dietary habits, indicating potential adaptive trade-offs the host makes within its specific habitat. The microbiota similarity between the common crane and the red-crowned crane can be attributed to the combined effects of ecological overlap and phylogenetic conservation. This observation supports the hypothesis proposed in previous studies that "microbes contribute to niche differentiation, " highlighting the role of intestinal microbiota as a key mediator in host adaptation to environmental conditions (Zhang et al., 2025). Beta diversity analysis, which assesses the compositional differences among microbial communities—often through metrics such as weighted UniFrac distance—reveals structural heterogeneity across different groups. Specifically, inter-group comparisons based on weighted UniFrac distance demonstrate statistically significant differences. These findings align with studies on other avian species, such as swans and chickens, and further indicate distinct microbial community structures among different bird species or groups. These differences are often linked to variations in the abundance of the phylum Bacillota and fluctuations in the genus Lactobacillus. Such variations may be influenced by host species identity, age, or environmental factors. According to the literature, Bacillota and Lactobacillus are considered key microbial taxa. Their relative abundance contributes to beta diversity, which measures compositional differences between microbial communities. Furthermore, their abundance may reflect functional aspects of the community, such as nutritional metabolism and host health status (Yang et al., 2022; Chesworth et al., 2024).

The gut microbiota of different bird species exhibits significant variation influenced by host species, ecological niches, and environmental factors. Microbiota differentiation among crane species: The gut microbial composition of the common crane and the red-crowned crane reveals that the common crane harbors a higher abundance of members from the phylum Tenericutes and the class Actinobacteria. This variation may be associated with differences in host immune regulation or nutrient metabolism pathways, although further functional analysis is required (Bodawatta et al., 2022; Mi et al., 2023). The distinct clustering pattern observed in the demoiselle crane suggests that its microbiota may be specifically adapted to arid environments. Differences in food resources, such as seeds and insects found in arid regions, may drive the colonization of specific microbial groups, including those capable of degrading drought-resistant plant fibers. In the wild, the Common Crane and Red-crowned Crane are primarily found in wetland ecosystems, while the Demoiselle Crane is mainly distributed in arid and semi-arid regions. The aquatic plants and microbial communities present in wetland environments may enter the food chain and contribute to the enrichment of specific microbiota, such as bacteria capable of degrading aquatic plant fibers (Mi et al., 2023; Rasmussen and Chua, 2023; Zhang et al., 2025). Environmental factors, including diet and water sources, significantly influence the gut microbiota structure of wild birds, resulting in interspecific microbiota differentiation within the same habitat. Similar microbiota patterns are observed under captive conditions, suggesting that evolutionary constraints during host development or genetic processes may play a role in maintaining intestinal homeostasis.

This study investigated the intestinal microbiota of three crane species maintained under captive conditions: the common crane, the Red-crowned Crane, and the Demoiselle Crane. Functional predictions suggested the potential for alcohol detoxification capabilities (COG1028) in the common crane and Red-crowned Crane, which is consistent with their natural diets in wetland habitats, potentially including fermented plant materials. This could indicate a predicted metabolic adaptation of the microbiota to specific environmental substrates (Garrido-Romero et al., 2024). In contrast, the Demoiselle Crane showed a predicted predominance of pyruvate fermentation (PWY-5100), a pathway associated with anaerobic energy production, aligning with the metabolic demands of surviving in resource-limited arid environments. Existing literature suggests that intestinal microbiota can modulate metabolic pathways to meet host energy requirements (Gregor et al., 2022; Rasmussen and Chua, 2023; Kadyan et al., 2025), particularly in wild birds, where microbiota contributes to energy homeostasis during resource fluctuations (Bodawatta et al., 2022; Huang et al., 2022). Additionally, the inferred enrichment of nucleotide synthesis pathways (PWY-612/7229, PWY-7220/7222) in the Red-crowned Crane group may support elevated cellular turnover rates, aligning with the high energy demands and immune system adaptations associated with migratory activity. Research has shown that microbiota-derived metabolites, such as short-chain fatty acids, regulate host cell proliferation and facilitate tissue repair (Shen Y. et al., 2024; Kim et al., 2025).

Predicted prominent functions of lipoprotein export (COG1136) and amino acid transport (COG0531) in the common crane group suggest an enhanced microbiota nutrient acquisition capacity under an omnivorous diet. Existing literature indicates that the host's dietary composition directly influences the

functional specialization of the gut microbiota, with omnivorous hosts exhibiting a broader range of nutrient metabolism modules (Huang et al., 2022; Zhang et al., 2023).The predicted increased abundance of multidrug transporters (COG1132) and regulatory factors (COG1846) in the Red-crowned Crane group implies a potential adaptation to environmental pollutants, such as pesticides. Numerous studies have demonstrated that the microbiota can mitigate the toxicity of exogenous substances through enzymatic metabolism or the formation of physical barriers (Arun et al., 2021; Theys et al., 2025). Inferred enrichment of glycerate synthesis (PWY-7663) and transcription factors (COG0583) in the Demoiselle Crane group aligns with the physiological demands for oxidative stress and osmotic pressure regulation in arid environments. Under environmental stress conditions, the microbiota may produce protective metabolites or activate host stress signaling pathways to preserve physiological homeostasis (Arun et al., 2021; Li et al., 2022; Zhang X. et al., 2024). We acknowledge that these functional predictions are inferred from 16S rRNA gene amplicon sequencing data using PICRUSt2, rather than being derived from direct metagenomic or biochemical measurements. As such, they represent hypothesized metabolic capabilities of the microbiota.

It is important to note that this study used fecal samples as a non-invasive proxy for gut microbiota. Although fecal samples offer valuable insights into microbial composition, they may not fully reflect the microbial communities present in other regions of the gastrointestinal tract, such as the small intestine or cecum. This limitation is commonly encountered in wildlife microbiota research, and future studies incorporating direct sampling from multiple gut segments could provide more comprehensive insights into spatial variations in microbial distribution.

5 Future perspectives

While this study provides initial insights into the gut microbiota of three crane species, it also highlights several avenues for future research to gain a more comprehensive understanding. First, future studies should expand the scope of sampling to include both adult and wild populations of these crane species. Comparing the gut microbiota of captive juveniles with their wild counterparts and adults would be crucial for discerning the influences of age, diet, and environment (captive vs. natural), and for assessing the true adaptability and health of captive-bred individuals intended for reintroduction. Second, moving beyond 16S rRNA gene sequencing to shotgun metagenomics would enable a far more precise taxonomic identification at the species level and, more importantly, allow for a direct and comprehensive profiling of microbial functional potential. Integrating metabolomic analyses of fecal samples would further reveal the actual metabolic outputs of the gut microbiome, directly linking microbial community structure to host physiology and health. By addressing these perspectives, future research can translate these foundational findings into actionable conservation strategies, ensuring that gut microbiome science becomes an integral part of efforts to safeguard these magnificent birds. It should be noted that all sampled individuals were juvenile cranes rescued from wildlife traffickers and had been exposed to significant stressors, including capture and transportation. These experiences may have already influenced their gut microbiota prior to sampling. Meanwhile, due to the inability to quantify the specific food intake of each individual crane, dietary control as a variable in this study remained incomplete. The observed interspecies differences in feeding behavior may have influenced the variations in gut microbiota composition. Future studies should employ controlled feeding experiments or stable isotope analysis to accurately measure individual dietary intake, thereby enabling a clearer distinction between the independent effects of feeding behavior and host species identity. Although the birds underwent a 4–6-week rehabilitation period under stable conditions to mitigate acute stress effects, the potential for lasting impacts on microbial composition remains. Therefore, results should be interpreted with caution, and future studies involving wild or captive-bred individuals without such traumatic histories are necessary to confirm these findings.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The animal study was approved by the Academic Ethics Committee of Qufu Normal University (2023114). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

XG: Conceptualization, Formal analysis, Investigation, Writing – original draft. YD: Conceptualization, Writing – review & editing. NL: Methodology, Writing – original draft. FW: Resources, Software, Writing – original draft. SS: Validation, Writing – review & editing. QW: Funding acquisition, Validation, Writing – original draft. HZ: Writing – review & editing.

Funding

The author(s) declared that financial support was received for this work and/or its publication. This research was funded by The Postdoctoral Innovation Project of Shandong Province (SDCX-ZG-202400113), the Postdoctoral Fellowship Program of CPSF under

Grant Number (GZC20240887), the National Natural Science Foundation of China (32271557), Beijing Zoo Circus Wildlife Technology Beijing Municipal Key Laboratory Open Research Project (ZDK202501), National Key Research and Development Program of China(2023YFF1305000).

Acknowledgments

The authors have reviewed and edited the output and take full responsibility for the content of this publication.

Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declared that generative AI was not used in the creation of this manuscript.

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SUPPLEMENTARY S1
Chinese ethics approval.

SUPPLEMENTARY S2
English ethics approval.

SUPPLEMENTARY FIGURE 1
ASV analysis of three groups.

SUPPLEMENTARY TABLE 1
The mean alpha diversity indices for each group.

SUPPLEMENTARY TABLE 2
COG relative of Picrust2 group.

SUPPLEMENTARY TABLE 3
Pathways relative of Picrust2 group.

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