

A Comparison of the Intestinal Fungal Community in Wild and Captive Himalayan Vultures (*Gyps himalayensis*)

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Abstract

In this study, we systematically analyzed the ecological response mechanism of the intestinal fungal community of the endangered species, the Himalayan vultures (*Gyps himalayensis*), concerning the survival differences between wild and captive environments. Based on its high-throughput sequencing technology, the key features such as species composition analysis, α/β diversity assessment, principal coordinate analysis, and correlation network analysis revealed that the captive environment led to the complexity of the fungal community structure, the turnover of the core flora, and the increase in the complexity of the intergeneric interactions network. It was further shown by FUNGuild functional annotation that the relative abundance of undefined saprotroph functional taxa in the wild population was significantly higher than that in the captive population, corroborating the adaptive advantage of its intestinal fungal community to the scavenging ecological niche. This study reveals for the first time the potential effects of captive stress on Himalayan vultures from the perspective of gut fungi, providing key microbiomic evidence for optimizing the conservation strategy of endangered raptors, and at the same time expanding the functional cognitive system of gut fungi in highland carrion-feeding birds.

Key words: *Gyps himalayensis*, intestinal microbiota, fungal community, captive, high-throughput sequencing

Introduction

As specialized scavengers in nature, vultures play an irreplaceable role in maintaining ecological balance, transmitting cultural values such as Celestial burial, and interrupting the spread of zoonotic diseases by scavenging animal carcasses in both natural environments and areas of human activity (García-Jiménez et al. 2022). However, this keystone species group is facing a severe existential crisis, with the majority of the world's 23 vulture species, divided into New World and Old World vultures, in a precarious conservation status, making them one of the world's most threatened birds (Buechley and Şekercioğlu 2016). Their population decline is due to a combination of threats, including the ingestion of toxic substances such as lead poison-

ing (Plaza and Lambertucci 2019), pesticide poisoning (Plaza et al. 2019), diclofenac poisoning (Moreno-Opo et al. 2021), and trauma from collisions with human infrastructure, gunshot wounds (Ives et al. 2022), other injuries such as climate change (Marneweck et al. 2021), poaching behavior (Henriques et al. 2020), loss of nesting sites, and low reproductive success (Plaza et al. 2022).

The Himalayan vulture (*Gyps himalayensis*) is a species of vulture in the Old World, with a slightly larger population than other species, belonging to the vulture genus of the hawk family of the order Eagleiformes. It is a large raptor endemic to western China and the Tibetan Plateau region, and has been listed as China's national Grade II protected animal, and is also classified as Near Threatened in the International Union

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for the Conservation of Nature (IUCN) Red List, and could potentially reach vulnerable status due to its persistently declining population (Paudel et al. 2016). With an average weight of 7.9 kilograms and a large wingspan, averaging 2.4 meters, the Himalayan vulture is one of the heaviest flying birds in the world (Sherub et al. 2016). As a high-altitude species, the vulture has a wide range of habitats, from 1,200 to 6,000 meters. In summer, Himalayan vultures ascend to the highlands of the Himalayas, Inner Mongolia, and Mongolia. In winter, they descend to the plains of northern India, the Himalayas of Nepal and Bhutan, the Himalayan plateau, and the lower regions of southeastern China (Meng et al. 2017a; Sherub et al. 2017). As a specialized scavenger, the Himalayan vulture feeds on animal carcasses, with domestic yak carcasses accounting for up to 64% of its diet, and wild ungulates and human carcasses accounting for 3%, rarely attacking live prey (Lu et al. 2009; Meng et al. 2017b). Their highly developed senses of sight and smell allow them to pinpoint the location of carrion at high altitudes and quickly cluster to clean up the carcasses, effectively preventing the spread of epidemics, and they are known as natural scavengers (Zou et al. 2021). The breeding cycle of Himalayan vultures has significant plateau adaptability. The breeding period is usually from January to September, with clusters nesting on the sunny side of cliffs. Each nest produces only one egg, which has an incubation period of about 50 days and a brood-rearing period of 3–6 months. The young birds need about half a year to be able to leave the nest and become independent. In recent years, the Xining Wildlife Zoo has successfully bred Himalayan vultures through artificial breeding techniques, providing important data for species conservation. However, captive environments may change the composition of their gut microbes, which in turn affects host health and adaptability.

Gut microbes, also known as the second genome, are closely linked to the host. Gut microbes influence host adaptation and play a crucial role in host growth and development (Hill et al. 2025), metabolism (Tilg et al. 2020), and immune defense (Bodawatta et al. 2022). Available studies suggest that the adaptive characteristics of carrion feeding in Himalayan vultures are related to gut microorganisms (Blumstein et al. 2017). In addition, anthropogenic changes in the living environment are also a way to explore the impact of environmental changes on the study of the gut of birds, a study found that the microbiome alpha diversity of Great Bustard (*Otis tarda dybowskii*) in a captivity en-

vironment decreased significantly, and the structure of the microbial community changed significantly (Lu et al. 2025). Zhang et al. (2022b) investigated the microbial diversity of the gut microbial community in Kestrels (*Falco tinnunculus*) and found differences in gut flora composition and richness before and after captivity. Advances in Metagenomics technology have advanced the depth of microbiome research, and it has been found that viruses and bacteria isolated from captive Himalayan vultures differ greatly from wild Himalayan vultures in richness and diversity (Zhai et al. 2023; Wang et al. 2024).

However, despite the fact that intestinal fungi, as an important part of the gut microbiota, have attracted much attention in recent years because of their key roles in host metabolic regulation (Zhou et al. 2024), immune homeostasis (Hill and Round 2024), and disease development (Li et al. 2023; Liu et al. 2024a), and that the fungal group can alter the bacterial group and influence host physiology (Shekarabi et al. 2024). There are limited reports on the intestinal fungi of Himalayan vultures in the literature, and their functions in Himalayan vultures have not yet been thoroughly analyzed, with most studies focusing on the isolation and functional validation of bacterial communities. This study is the first to systematically analyze the diversity of intestinal fungi and their ecological functions in Himalayan vultures. By comparing the fungal community structure between wild and captive populations, the study reveals the influence of environmental factors on the stability of the microbiome and provides a theoretical basis for optimizing the captive breeding strategy.

Experimental

Materials and Methods

Sample collection. A total of 16 fecal samples of Himalayan vultures were collected in this study, of which 6 (No. W1, W2, W3, W4, W5, W6) were taken from wild individuals in their natural habitats, and 10 (No. Z1, Z2, Z3, Z4, Z5, Z6, Z7, Z8, Z9, Z10) were taken from captive individuals in Xining Wildlife Zoo, Qinghai Province. For the wild Himalayan vulture population, fresh fecal samples were collected after the birds fed and naturally defecated, typically just before flight. To minimize the risk of sampling the same individual repeatedly, droppings that were separated by a certain distance were collected. Due to sexual monomorphism

and inaccessible cliffside nests, only basic information (i.e., all individuals were adults) could be determined in the field, while sex and precise identity remained unknown. For the captive population housed at Xining Wildlife Park, individuals lived in a large, semi-natural communal enclosure covering half a hillside, with food provided every other day and water available *ad libitum*. Sampling was conducted after feeding, similar to the wild population. However, detailed individual records were not maintained at the facility, making it difficult to distinguish specific individuals within the group. The samples were collected by strict aseptic practices: using disposable sterilized gloves and sterile cotton swabs, avoiding the contaminated area of the fecal surface, and accurately collecting about 20 g of sample from the center of the fresh sample. After collection, the samples were immediately sealed in a sterile bag and transported to the laboratory with liquid nitrogen and then deposited in an ultra-low-temperature refrigerator at -80°C to avoid DNA degradation and to ensure the reliability of the data for subsequent macro-genome extraction and analysis.

DNA extraction. Sixteen thawed fecal samples were homogenized, and genomic DNA was extracted using the E.Z.N.A.[®] Soil DNA Kit (Omega Bio-tek, Inc., USA), strictly following the standard procedure of the kit. The product was further purified using a DNA purification kit. The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with a NanoDrop[™] 2000 Spectrophotometer (Thermo Scientific[™], Thermo Fisher Scientific Inc., USA). Qualified samples were stored at -20°C for backup to ensure experimental reproducibility.

ITS amplification and sequencing. Total microbial DNA extracted from Himalayan condor feces was used as a template for PCR amplification of the full-length ITS using primers ITS1F (5'CTTGGTCATTAGAG-GAAGTAA3') and ITS4R (5'TCCTCCGCTTATTGATATATGC3') (Chen et al. 2018). Amplification reactions (20 μl volume) consisted of 5 $\times$ FastPfu buffer 4 μl , 2.5 mM dNTPs 2 μl , forward primer (5 μM) 0.8 μl , reverse primer (5 μM) 0.8 μl , FastPfu DNA Polymerase 0.4 μl , template DNA 10 ng and DNase-free water. The PCR amplification was performed as follows: initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s, and single extension at 72°C for 10 min, and end at 4°C (T100[™] Thermal Cycler, Bio-Rad Laboratories, Inc., USA). After elec-

trophoresis, the PCR products were purified using the AMPure[®] PB Beads (Pacific Biosciences of California, Inc., USA) and quantified with Qubit[™] 4 Fluorometer (Invitrogen[™], Thermo Fisher Scientific Inc., USA). Purified products were pooled in equimolar amounts, and the DNA library was constructed using the SM-RTbell[®] prep kit 3.0 (Pacific Biosciences of California, Inc., USA) according to PacBio's instructions. Purified SMRTbell[®] libraries were sequenced on the Sequel[®] IIe System (Pacific Biosciences of California, Inc., USA) by Majorbio Bio-Pharm Technology Co., Ltd. (China). High-fidelity (HiFi) reads were obtained from the sub-reads, generated using circular consensus sequencing via SMRT[®] Link v11.0 (Pacific Biosciences of California, Inc., USA).

Data statistical analysis. The required raw sequences were obtained for quality control. In this study, we used FLASH v1.2.11 (<https://ccb.jhu.edu/software/FLASH/index.shtml>) to merge paired-end (PE) read lengths from SMRT[®] sequencing based on overlap relationships with a minimum overlap length of 10 bp (Magoč and Salzberg 2011). Successful merged sequences were screened and quality-controlled. Operational taxonomic units (OTUs) were clustered at a 97% similarity level using UPARSE v7.0.1090 (<http://drive5.com/uparse/>) for QC sequences (Edgar 2013; Li et al. 2024), and chimeric sequences were removed during the clustering process. The OTU representative sequences were clustered by comparison with the Unite (Release 8.0; <http://unite.ut.ee/index.php>; Abarenkov et al. 2024) gene database using RDP Classifier v2.13 (<https://sourceforge.net/projects/rdp-classifier>) for taxonomic analysis of OTU representative sequences and species annotation at the level of kingdom, phylum, order, family, genus, and species (Ye et al. 2017; Wang and Cole 2024). The analysis was performed using R v3.3.1 (R Core Team 2014) to generate Venn diagrams and Core plots (Ji et al. 2017). Alpha diversity indices, including the Chao1 index, ACE index, Simpson index, and Shannon index, were calculated using mothur software v1.30.2 (https://www.mothur.org/wiki/Download_mothur) (Schloss et al. 2009; Xuan et al. 2022). The Wilcoxon rank-sum test was used to determine whether there was a significant difference in diversity index between the two groups. Sparse curves were plotted based on the above alpha diversity indices using the R language tool (Ye et al. 2017). For the Beta diversity analysis, the principal coordinate analysis (PCoA) scatter plot was generated based on the Bray–

Curtis distance algorithm (Mohamed et al. 2022), and the average degree of variation (AVD) was calculated as the evaluation index of fungal community stability. Based on the richness data of fungi at different taxonomic levels, the Wilcoxon rank sum test was used to analyze the significant differences in richness between groups at the phylum and genus levels. Community stacked bar charts and rank sum test bar charts were generated using R language. One-way correlation network analysis at the genus level was performed using Networkx v1.11 (Hagberg et al. 2008; Chen et al. 2024), and visual network plots were drawn using Cytoscape v3.3.0 (Shannon et al. 2003). Fungal OTU tables were converted to text format and then uploaded to FUN-Guild (<http://www.funguild.org/>) to predict function (Nguyen et al. 2016; Xie et al. 2021).

Results

Data acquisition and analysis. In this study, we obtained 1,457,783 original sequences from 16 samples totaling 437,334,900 bases. Optimization resulted in 1,277,498 optimized sequences totaling 317,754,440 bases, with a total of 97,539,088 bases in the wild and 220,215,352 bases in the zoo, with all sample sequences ranging from 12,447,591 bases to 24,264,230 bases, and sequence length ranging from 200 bp to 529 bp, with an average sequence length of 248 bp (Table SI). Cluster analysis of the optimized sequences identified a total of 1,381 OTUs, ranging from 16 to 518 OTUs per sample. The Himalayan vulture holobiont fungi consisted of 417 genera from 204 families in 11 phyla, 36 classes, and 91 orders (Table SII). The Vene

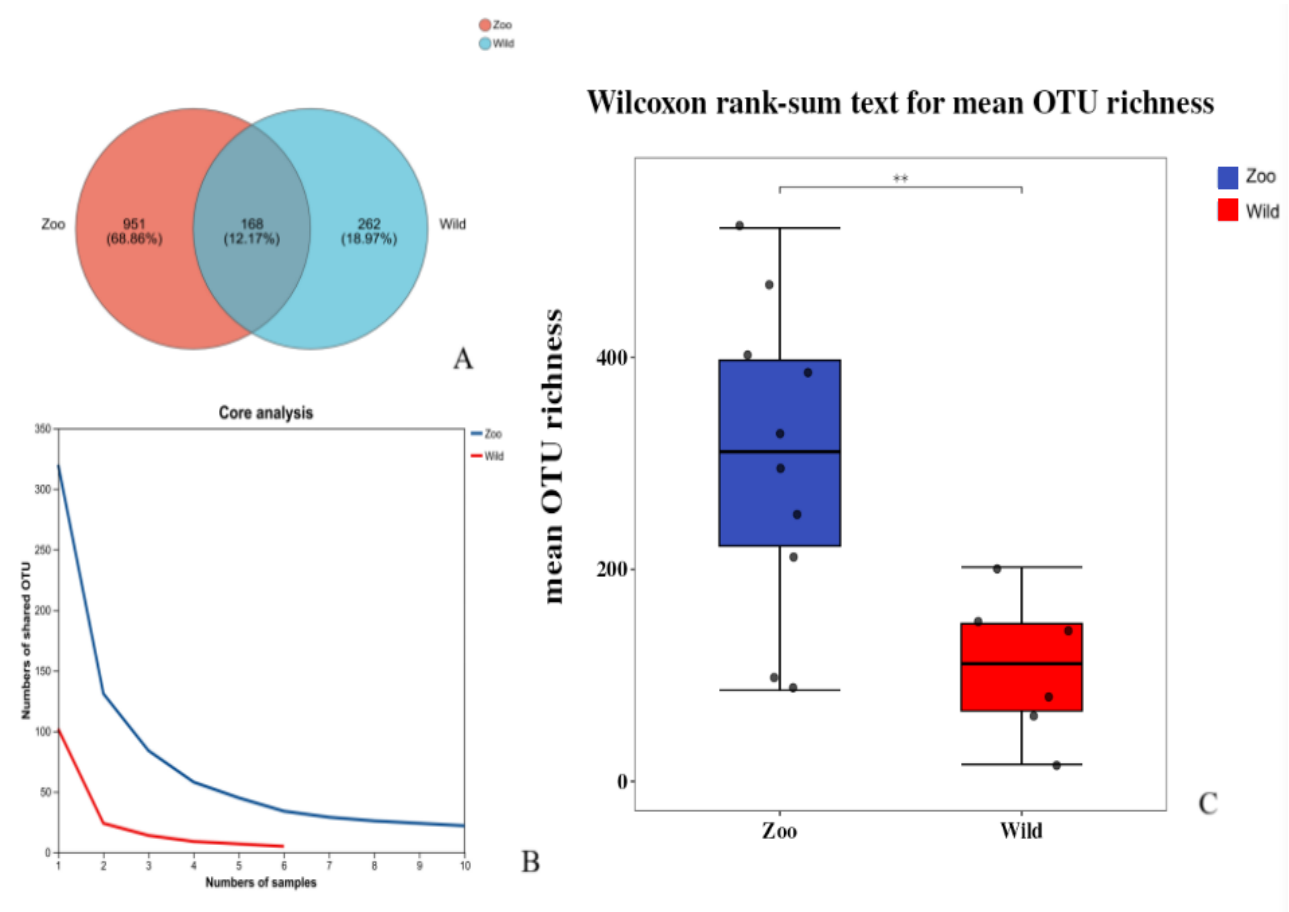


Fig. 1. The analysis of the number of OTU in zoo and wild Himalayan vultures. A) A Venn diagram and histogram of the OTU in different groups; B) core species analysis for both groups; C) Wilcoxon rank-sum text for mean OTU richness.

plot showed a total of 168 OTUs in the wild group and the zoo group, which accounted for 18.97%, with 262 endemic to the wild group and 951 endemic to the zoo group (Fig. 1A).

In addition, the core species analysis was used to assess the adequacy of the measured sample size. Core OTU refers to the core OTUs, the number of OTUs that are common to all samples, which decreases with the increase in the number of samples in terms of the number of shared OTUs. From the core species analysis (Fig. 1B), it is evident that the number of samples in this study is sufficient to reach a plateau, indicating that the sample size is adequate. Based on this, we conducted a differential test on the mean OTU richness. The results revealed a highly significant difference in mean OTU richness between the zoo and wild groups (Fig. 1C, $p < 0.01$), with the zoo group exhibiting higher fungal diversity in their gut microbiota.

Gut fungal composition and alterations at different taxonomic levels. To further study the differences

in the intestinal fungal communities of captive and wild Himalayan vultures, a total of 11 phyla were identified from 16 fecal samples, with 4–8 phyla per sample. At the phylum level, the fungal community of wild Himalayan vultures was predominantly composed of Ascomycota (56.09%), Basidiomycota (39.11%), Mucoromycota (1.20%), and unclassified_k_Fungi (1.90%; which refers to sequences belonging to the kingdom Fungi that could not be annotated to any known phylum; this definition applies throughout the text). In captive Himalayan vultures, the fungal community was dominated by Ascomycota (54.80%), Basidiomycota (14.39%), and Mortierellomycota (9.09%). The unclassified_k_Fungi sequences accounted for 21.52% of the total sequences (Fig. 2A). Furthermore, the Wilcoxon rank-sum test showed that there were significant differences in Basidiomycota and Mucoromycota between the two groups (Fig. 2B, $p < 0.05$).

At the genus level (Fig. 2C), the dominant genera in the wild group samples were *Naganishia*, *Didymel-*

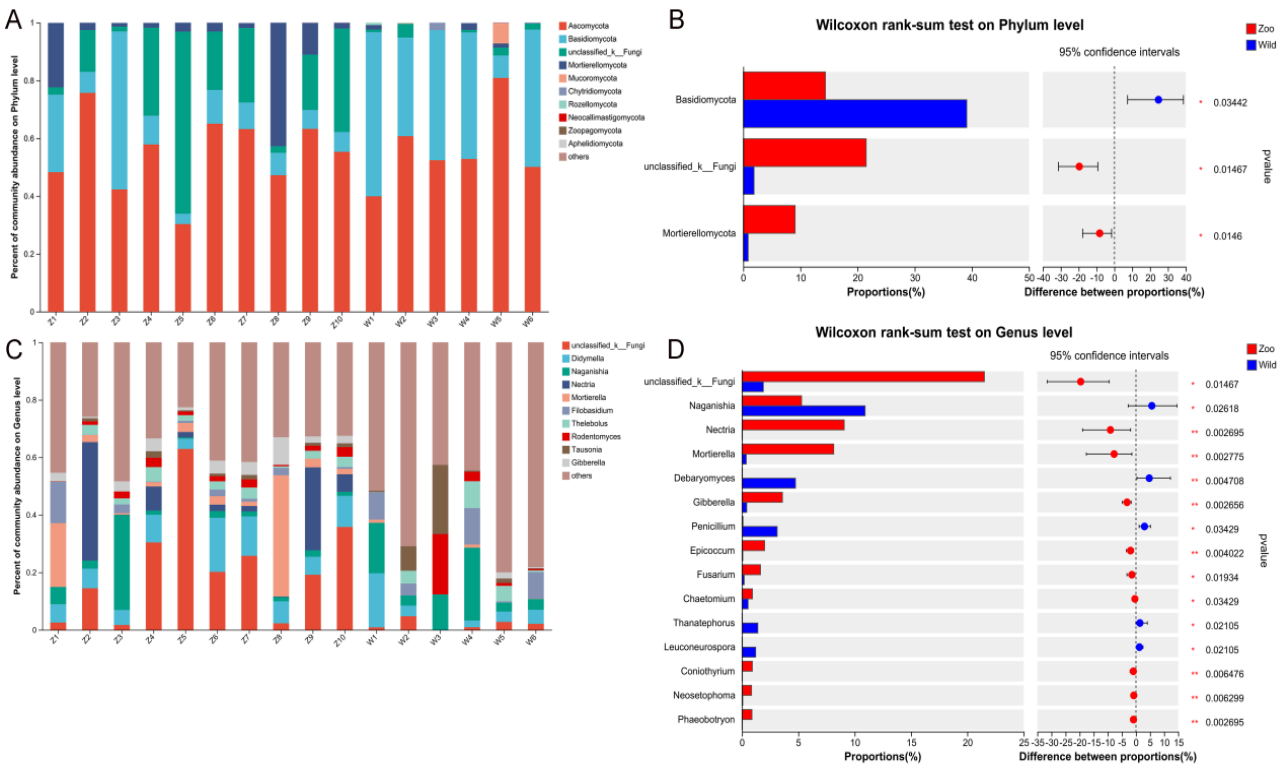


Fig. 2. The percent of community fungal richness of zoo and wild Himalayan vultures at the phylum (A) and genus (C) levels, along with the Wilcoxon rank-sum test results at the phylum (B) and genus (D) levels. Among them, unclassified_k_Fungi refers to sequences belonging to the kingdom Fungi that could not be annotated to any known phylum.

la, and *Filobasidium*, while the dominant genera in the zoo group samples were the genera *Naganishia*, *Didymella*, *Nectria*, and *Mortierella*. In addition, unclassified_k_Fungi and others were higher in both groups, proving that there are still many unclassified fungal genera and genera with very low levels of content. Wilcoxon rank sum test showed that *Nectria*, *Mortierella*, *Debaryomyces*, etc. were significantly different between the two groups(Fig. 2D, $p < 0.05$).

Diversity analysis. The alpha diversity index was calculated for each group to test for any differences in

alpha diversity between captive and zoo Himalayan vultures. According to the ACE index, the mean number of OTUs in the samples from the wild and zoo groups was 109.61 and 323.76, respectively, while the Chao1 index values were 109.2 and 323.65, respectively. The Shannon and Simpson indices were 3.179 and 0.0839 for the wild group samples and 3.2652 and 0.1305 for the zoo group, respectively (Table SIII). A test for inter-group differences in these four indices (Fig. 3, Wilcoxon rank sum test, $p < 0.05$) revealed that the zoo and captive groups demonstrated significant differences in

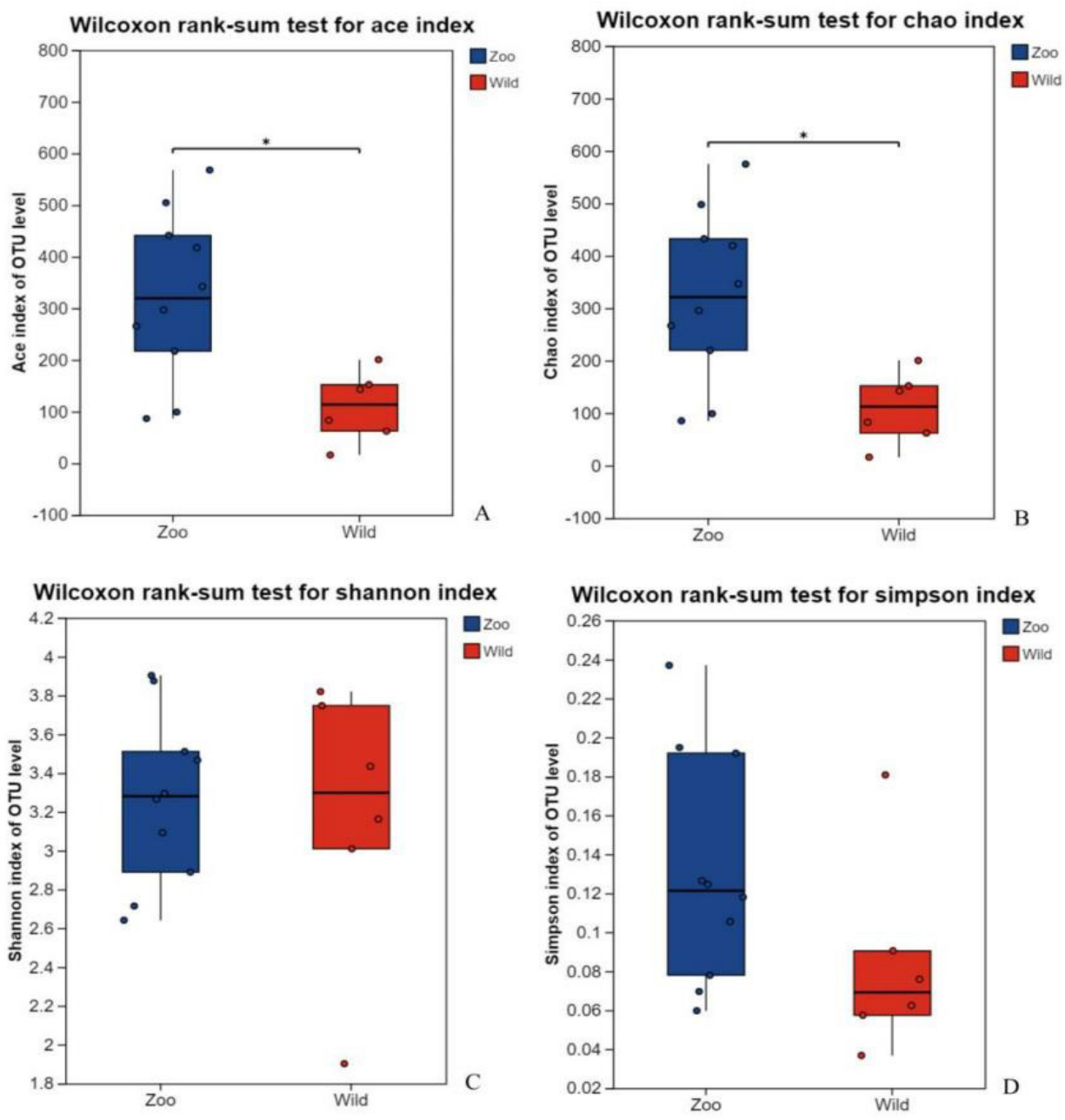


Fig. 3. Diversity index of fecal fungal communities in zoo and wild vultures. Ace, Chao1, Shannon, and Simpson indices were used to evaluate the alpha diversity of the fecal fungi.

the ACE and Chao1 indices, while exhibiting non-significant differences in the Shannon and Simpson indices. This suggests that the differences in the richness of intestinal fungi between wild and zoo Himalayan vultures were significant, whereas the evenness diversity was not significant.

The Shannon exponential sparse curve (Fig. 4) shows that the increase in the number of OTUs for each sample basically flattens out when the sequencing volume reaches 3,000, which indicates that more data volume will only produce a small number of OTUs, which also indicates that the amount of sequencing

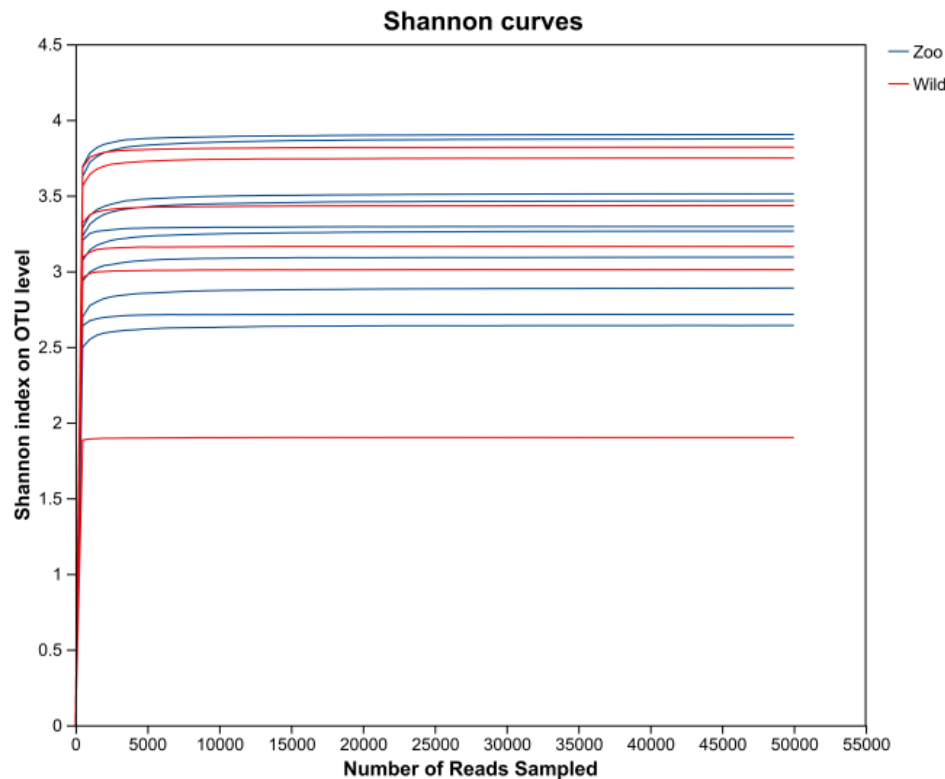


Fig. 4. The Shannon index rarefaction curve.

data for this experiment is reasonable and achieves the expected sequencing depth.

In order to further investigate the dynamics of Beta diversity of the intestinal fungal communities of wild and zoo Himalayan vultures, the differences between the two groups were observed by PCoA principal component analysis, where different colored dots represent samples from different subgroups, and when the closer the dots of the two samples are to each other indicates that the species composition of the two samples is more similar. The distance between the samples of the wild group and the zoo group in this study (Fig. 5A) verified that there were large differences in the intestinal fungal structure between captive and zoo Himalayan vultures. In addition, the mean variability (AVD) of wild and zoo Himalayan vultures was calculated as an evaluation index of fungal community stability (Fig. 5B), and it was found that the AVD value of the

wild group (0.697) was slightly higher than that of the zoo group (0.643), which indicated that the stability of the intestinal fungal community in the zoo group was slightly higher than that of the wild group.

Correlation analysis. To further understand the correlation of the Himalayan vulture gut fungal community at the genus level, we performed one-way correlation network analyses of the top 50 genera of taxonomic-level abundance in the captive and zoo Himalayan vulture fungal groups, respectively, using Spearman's correlation coefficient, and found that there were differences in the strength of the genus-level fungal correlations within the two groups. In the analysis of the top 50 genus-level abundances, there were 136 positive and 59 negative correlations for zoo vulture gut fungi (Fig. 6A), whereas there were 48 positive and 37 negative correlations for captive vulture gut fungi (Fig. 6B).

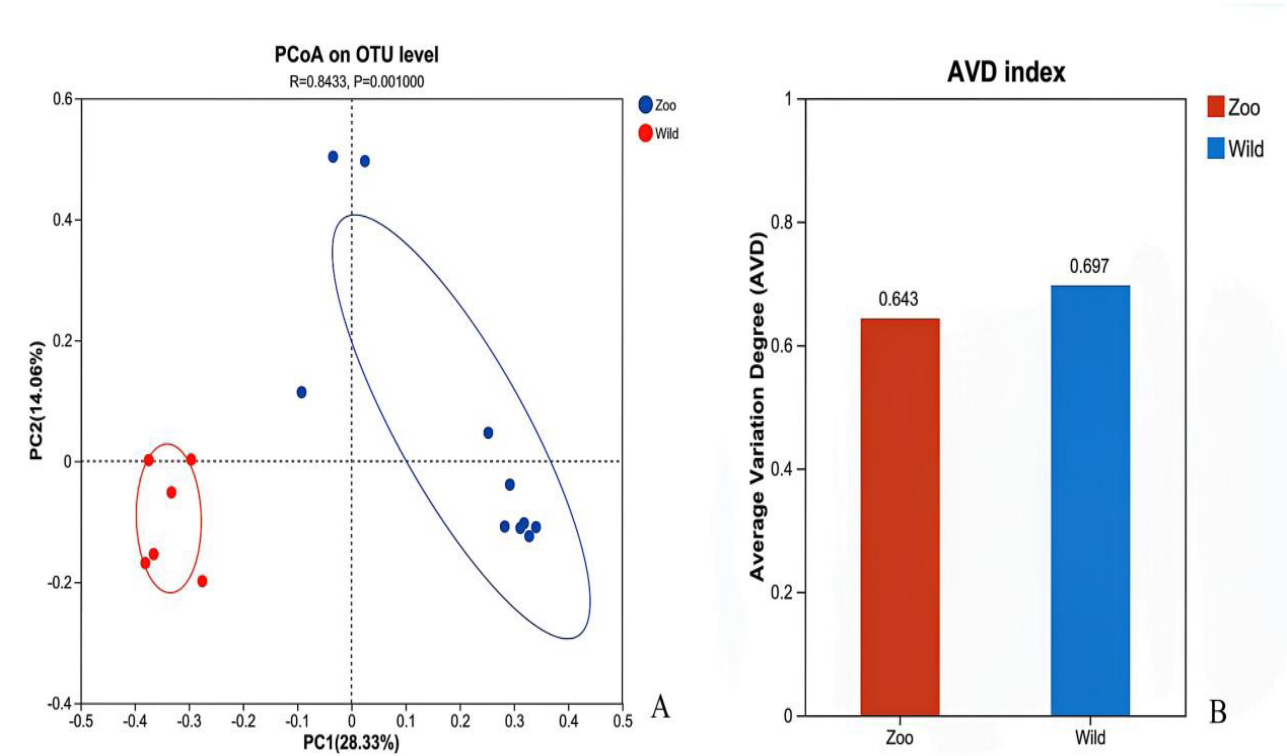


Fig. 5. Beta diversity of intestinal fungal communities in zoo and wild Himalayan Vultures. A) The results of PCoA analyses of OTUs; B) average variation degree of the two groups.

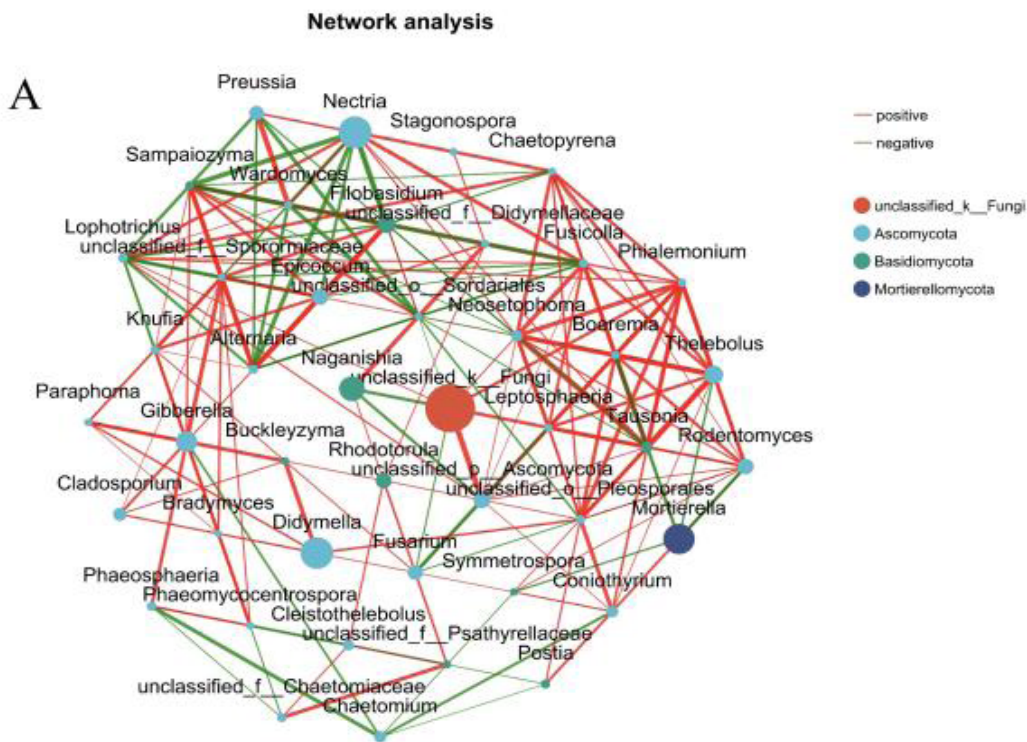


Fig. 6 A. Univariate correlation network analysis of the top 50 fungi at the genus level in A) zoo and B) wild Himalayan Vultures.

Predictive functional profiling of microbial communities. To further clarify the ecofunctional taxa of fungi in the gut of Himalayan vultures, the trophic taxa of fungi in the gut of Himalayan vultures were analyzed using the FUNGuild database. The results (Fig. 8, Table SIV) showed that there were nine tro-

phic types in the gut of Himalayan vultures, including Pathogen-Saprotroph-Symbiotroph, Pathotroph, and Pathotroph-Saprotroph, Pathotroph-Saprotroph-Symbiotroph, Pathotroph-Symbiotroph, Saprotroph, Saprotroph-Pathotroph-Symbiotroph, Saprotroph-Symbiotroph, and Symbiotroph.

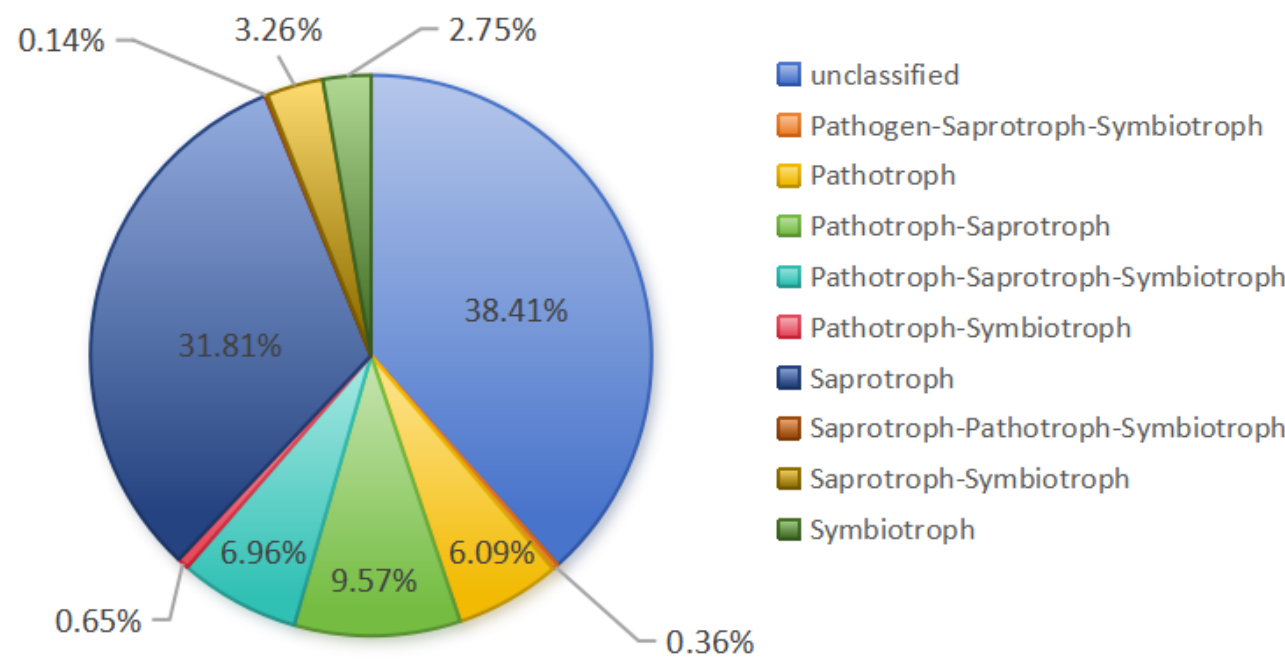


Fig. 8. Trophic Mode of intestinal fungi in Himalayan Vultures.

The results of FUNGuild’s ecofunctional classification showed a total of 78 functional taxa, excluding those with undefined functional roles. Fig. 9A shows the top 16 functional taxa at the abundance level, and the rest of the species ranked after the top 16 in abundance are collectively referred to as Others. The dominant functional taxa, as shown in Fig. 9A, were mainly Undefined Saprotroph (21.81%), Plant Pathogen (4.71%), Fungal Parasite-Plant Pathogen-Plant Saprotroph (2.61%), and Animal Pathogen-Plant Pathogen-Undefined Saprotroph (2.17%), Fungal Parasite-Undefined Saprotroph (1.67%), Dung Saprotroph-Plant Saprotroph (1.59%), Dung Saprotroph-Plant Saprotroph-Wood Saprotroph (1.59%), Endophyte-Litter Saprotroph-Soil Saprotroph-Undefined Saprotroph (1.52%), Ectomycorrhizal (1.45%), Wood Saprotroph (1.45%), Undefined Saprotroph-Wood Saprotroph

(0.94%), in addition to Animal Pathogen-Dung Saprotroph-Endophyte-Epiphyte-Plant Saprotroph-Wood Saprotroph, Animal Pathogen- Endophyte-Lichen Parasite-Plant Pathogen-Soil Saprotroph-Wood Saprotroph, Animal Pathogen-Undefined Saprotroph, Dung Saprotroph, Plant Pathogen-Wood Saprotroph all accounted for 0.87%.

As shown in Fig. 9B, the intestinal fungi of wild Himalayan vultures and zoo Himalayan vultures had a total of four significantly different functional taxa ($p < 0.05$), including Dung Saprotroph-Plant, Saprotroph-Wood Saprotroph, Endophyte-Litter Saprotroph-Soil Saprotroph-Undefined Saprotroph, Animal Pathogen-Endophyte-Fungal Parasite-Lichen Parasite-Plant Pathogen-Wood Saprotroph, Undefined Saprotroph.



Fig. 9. Functional classification of fungal communities in Himalayan Vultures. A) Fungal function classification of zoo and wild vultures by the FUNGuild tool; B) Wilcoxon rank-sum test between the two groups with 95% confidence.

Discussion

As scavengers of highland ecosystems, Himalayan vultures have long been exposed to complex microbial environments due to their unique scavenging habits. Although recent macrogenomic studies have revealed that gut microbes play an important role in assisting host adaptation to scavenging ecology, the function of fungi as the second largest microbial group has long been overlooked, a limitation that may hinder a comprehensive understanding of host-microbe symbiotic mechanisms. Notably, in artificial captive environments, the interaction of multiple stressors, such as feed standardization, antibiotic exposure, and spatial constraints, may significantly reconfigure the community patterns of their gut fungi, which in turn affects the ecological adaptations of the host. To systematically reveal these differences, this study used 6 fecal samples of wild Himalayan vultures and 10 samples of captive individuals from Xining Wild Animal Park to analyze the composition, structure, and functional differentiation of the gut fungal communities between the wild and captive populations by ITS high-through-

put sequencing combined with bioinformatics analysis. This study is the first to investigate the intestinal fungal community of Himalayan vultures, which fills the gap of previous studies on scavenging birds, helps us to understand the differences between the intestinal microbial communities of wild and captive Himalayan vultures, and lays the foundation for further research on their intestinal flora.

Fungal flora accounts for a small percentage of the total intestinal flora but has an important role in the maintenance of homeostatic balance in the gut (Liu et al. 2025). In this study, it was found that the fungi of the Himalayan vulture consist of 417 genera in 11 phyla, 36 orders, 91 orders, and 204 families. Among them, Ascomycota and Basidiomycota are the dominant groups of intestinal fungi in wild vultures, and the dominant groups of intestinal fungi in captive vultures are also mainly Ascomycota and Basidiomycota. This finding is consistent with the fungal community composition observed in Baikal Teal (*Sibirionetta formosa*) (Sakda et al. 2023), Hooded cranes (*Grus monacha*) (Wu et al. 2022), and broiler chicken (Robinson et al. 2022). Furthermore, at the phylum level, the relative abundance

of Basidiomycota in the intestinal fungal community of wild Himalayan vultures was significantly higher than that of captive individuals ($p < 0.05$), suggesting that environmental differences may influence the distribution of fungal taxa through pathways such as food source or antibiotic exposure. Ascomycota are major cellulolytic fungi that secrete cellulases and hemicellulases to break down complex polysaccharides (Sun et al. 2022; Zhang et al. 2022a). Fungi of the Ascomycota phylum are widely involved in the degradation of complex organic matter such as lignin and cellulose in nature (Atiweh et al. 2022). Wild Himalayan vultures feed on carrion, and their intestines may be enriched with lignin-degrading associated Basidiomycota fungi through the ingestion of plant residues from animal remains, thus enhancing host adaptation to carrion feeding. In contrast, captive individuals suffer from a lack of such components in their diets, resulting in a decrease in the richness of the associated bacterial flora. At the genus level, *Naganishia*, and *Didymella* were the dominant fungal genera in captive and wild Himalayan vultures. In addition, the relative abundance of *Naganishia* in the gut of wild Himalayan vultures was significantly higher than that of captive individuals ($p < 0.05$), whereas the difference in the relative abundance of *Didymella* did not reach statistical significance between the two groups. *Naganishia* belongs to the yeast group of fungi in the phylum Basidiomycota, which is widely distributed in natural environments and is especially enriched in humus-rich habitats (Glushakova and Kachalkin 2023). Captive feeds are dominated by refined meat and lack complex carbon sources such as plant fibers, which may lead to a decrease in the abundance of *Naganishia* due to competitive nutritional disadvantages. *Didymella* belongs to the Ascomycota, which is a common endophytic and saprophytic fungus (Zhang et al. 2022c), and its relative abundance did not differ significantly between the wild and captive populations, which is possibly related to its ability to utilize a variety of carbon sources, such as monosaccharides, proteins, and other nutrients.

Regarding alpha diversity indices, fungal species richness (ACE and Chao1 indices) was significantly higher in captive Himalayan vultures than in the wild group, suggesting that the number of fungal species in the gut of captive individuals was higher. Despite the greater number of species in the captive group, the Shannon and Simpson indices did not change significantly, suggesting that the additional species were mostly low-abundance taxa and community evenness

was not significantly affected. This may be due to the introduction of new fungal sources by artificial feeds or heterologous fungi in the captive environment. In contrast, most of the fungi introduced by captivity were temporary colonizers that did not form stable ecological niches and had limited contribution to the community evenness. Most existing studies have demonstrated that captivity reduces microbial richness. For instance, Oriental White Storks (*Ciconia boyciana*) have been found to exhibit a higher diversity of intestinal flora compared to captive populations (Wu et al. 2021), but fungal richness was paradoxically higher in the present study. In the future, it will be necessary to see if these foods introduce new fungal types by analyzing the ingredients in the feeds. In the meantime, studies could explore whether increasing dietary diversity or supplementing with probiotics may restore native beneficial fungi in wild-type environments (Martínez-Mota et al. 2022; Dallas and Warne 2023). Principal component analysis of Beta diversity likewise showed highly significant differences in gut fungal communities between the two groups ($p < 0.01$), again suggesting that changes in the environment have a greater effect on the structure of the intestinal fungi of Himalayan vultures. In addition by calculating the average degree of variation (AVD) between wild and zoo Himalayan vultures, it was found that the AVD value of the wild group (0.697) was slightly higher than that of the zoo group (0.643), indicating that the stability of the intestinal fungal community in the zoo group was slightly higher than that in the wild group (Xun et al. 2021). Correlation network analysis revealed that the intestinal fungal network of captive Himalayan vultures was more complex (higher number of both positive and negative correlation edges) than that of wild Himalayan vultures, and all positive correlations were found after combining the two groups. It is hypothesized that this may be due to the fact that captive birds often consume a single artificial diet, forcing the fungal community to adapt to limited resources through synergism (positive correlation) and competition (negative correlation). In contrast, wild Himalayan vultures consume a variety of food items, and the fungal community can reduce direct competition through functional redundancy or ecological niche differentiation, thus simplifying the mutualistic pattern. The combined all-positive correlation may reflect basal metabolic demands shared by both groups, driving synchronized changes in the fungi and masking group-specific interactions.

In contrast to the findings of most studies (Yang et al. 2018; Guo et al. 2019), the present study reveals a more diverse intestinal fungal community in captive Himalayan vultures and more complex interactions between different fungi. This phenomenon is in line with the findings that the microbial diversity of wild lemurs (*Lemur catta*) was not significantly higher than that of captive populations (Bornbusch et al. 2022), and with the findings that three insectivorous bat species (*Rhinolophus ferrumequinum*, *Vespertilio sinensis*, and *Hipposideros armiger*) with elevated gut flora diversity in captive individuals are highly consistent with the findings reported by Xiao et al. (2019). Together, the above evidence suggests that the traditional binary division between wild and captive is difficult to fully elucidate the complex mechanisms by which environmental factors influence host-microbe interactions. Specifically, multiple factors such as feed refinement in captive management (Liu et al. 2024b), antibiotic exposure (Campbell et al. 2020), environmental noise (Berlow et al. 2022), and physical space constraints (Xie et al. 2016) may jointly alter the composition and interaction patterns of fungal communities. This finding suggests that future research needs to move beyond simple habitat type comparisons to a multidimensional system of analysis, such as analyzing feed composition, tracking the effects of antibiotics, and monitoring fungal changes over time, in order to clarify how captivity specifically affects the intestinal flora of Himalayan vultures.

Fungal trophic taxa in Himalayan vultures were analyzed using the FUNGuild database, and the results showed that there were nine trophic types of intestinal fungi in Himalayan vultures, with pathotrophic and saprotrophic types predominating. The zoo captive Himalayan vultures were analyzed in Saprotroph, Endophyte-Saprotroph, Pathogen(Animal, Plant)-Endophyte-Parasite-Saprotroph, and had a significantly higher relative abundance of functional groups than wild Himalayan vultures. In contrast, the relative abundance of functional groups in the Undefined Saprotroph was significantly higher in wild Himalayan vultures than in captive Himalayan vultures. It is hypothesized that the captive environment, such as spatial restriction and artificial light, may lead to changes in host immune status and promote colonization by endophytes and animal pathogens. Wild Himalayan vultures feed on natural carrion, insects, and occasional plant remains, with complex sources of humus such as animal offal, bone, and hair, spawning more meta-

bolic adaptations of the Undefined Saprotroph, which contribute to the catabolism of indigestible dietary fibers and nutrient redistribution (Borruso et al. 2021; Li et al. 2022).

The gut microorganisms of birds mainly come from their living environment, and environmental changes can affect their activities, foraging, and growth, as well as directly change the composition of the gut flora (Yao et al. 2023; Zhang et al. 2024). Compared with wild birds, captive birds have less space to move around, have a single diet, and are exposed to antibiotics, etc. These changes can affect the species of intestinal fungi and even jeopardize health. This study applies macrogenomic sequencing technology to the study of fungal communities in Himalayan vultures, revealing significant differences in gut fungi between wild and captive Himalayan vultures. Furthermore, captive environments pose risks for pathogenic fungal proliferation.

Availability of data and material

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive in National Genomics Data Center, China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA024322) that are publicly accessible at <https://ngdc.cncb.ac.cn/gsa>.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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Supplementary materials are available on the journal's website.