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Enterococcus faecalis provides protection during scavenging in carrion crow (*Corvus corone*)

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ABSTRACT

The gut microbiota significantly influences host physiology and provides essential ecosystem services. While diet can affect the composition of the gut microbiota, the gut microbiota can also help the host adapt to specific dietary habits. The carrion crow (*Corvus corone*), an urban facultative scavenger bird, hosts an abundance of pathogens due to its scavenging behavior. Despite this, carrion crows infrequently exhibit illness, a phenomenon related to their unique physiological adaptability. At present, however, the role of the gut microbiota remains incompletely understood. In this study, we performed a comparative analysis using 16S rRNA amplicon sequencing technology to assess colonic content in carrion crows and 16 other bird species with different diets in Beijing, China. Our findings revealed that the dominant gut microbiota in carrion crows was primarily composed of Proteobacteria (75.51%) and Firmicutes (22.37%). Significant differences were observed in the relative abundance of *Enterococcus faecalis* among groups, highlighting its potential as a biomarker of facultative scavenging behavior in carrion crows. Subsequently, *E. faecalis* isolated from carrion crows was transplanted into model mice to explore the protective effects of this bacterial community against *Salmonella enterica* infection. Results showed that *E. faecalis* down-regulated the expression of pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and interleukin 6 (IL-6), prevented *S. enterica* colonization, and regulated the composition of gut microbiota in mice, thereby modulating the host's immune regulatory capacity. Therefore, *E. faecalis* exerts immunoregulatory and anti-pathogenic functions in carrion crows engaged in scavenging behavior, offering a representative case of how the gut microbiota contributes to the protection of hosts with specialized diets.

Keywords: Carrion crow; Facultative scavenger; Gut microbiota; *Enterococcus faecalis*; 16S rRNA sequencing

INTRODUCTION

The gut microbiota plays a crucial role in maintaining host health by interacting with various physiological systems and providing valuable ecosystem services (Chen et al., 2023; Wang et al., 2024; Wu et al., 2022; Yang et al., 2022). Birds are the most highly specialized class of vertebrates among tetrapods, encompassing over 10 000 species inhabiting diverse habitats (Wu et al., 2021). While extensive research has been conducted on the gut microbiota of wild birds, the physiological role of gut bacteria remains insufficiently validated. The gut microbiota assists birds in digesting food sources indigestible by the host, enhances nutrient uptake, and contributes to detoxification and immune function regulation, thereby providing protection for the host (Berlow et al., 2020). The gut microbiota can indirectly interact with pathogens by modulating immune function, and directly interact with pathogens through competitive exclusion and production of antibacterial compounds (Grond et al., 2018; Svihus et al., 2013).

The life history characteristics of different bird species exhibit significant variations, resulting in distinct gut microbiota compositions. This composition is driven by both intrinsic host factors and external environmental factors (Hird et al., 2015), including migration behavior, flight ability, diet, mating system, lifespan, and physiological function (Bodawatta et al., 2021b; Theis et al., 2016; Trevelline et al., 2018). Compared to

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resident birds, migratory birds encounter a greater diversity of microorganisms that can colonize their gut (Zhang et al., 2021), with diet considered to be the most influential factor shaping gut microbiota structure (Carmody et al., 2015; Muegge et al., 2011). Bird diets are highly diverse and can be broadly classified as carnivorous, omnivorous, or herbivorous. They can also be further subdivided into various feeding habits, such as nectar-feeding hummingbirds, plant-eating pheasants, insect-eating flycatchers, omnivorous magpies, predatory eagles, obligate scavenging vultures, and facultative scavenging crows (Beasley et al., 2015; Videvall et al., 2018).

Hosts with different diets have specific digestive requirements, leading to the presence of specialized gut bacteria that are adapted to the host's particular diet (Bodawatta et al., 2021a). For example, the gut microbiota of herbivorous birds is usually dominated by members of the phylum Bacteroidetes, which can help break down polysaccharides, cellulose, and other complex polymers (Wienemann et al., 2011). The gut microbiota also aids herbivorous birds in detoxifying their food (Kohl et al., 2016). For example, the wild Japanese rock ptarmigan (*Lagopus muta japonica*) exhibits greater gut microbiota diversity and more unique bacterial community compared to captive individuals, potentially attributed to their consumption of toxin-containing plants (Ushida et al., 2016). Carnivorous birds also consume a wide range of foods, including carrion, marine invertebrates, fish, and reptiles, resulting in a gut microbiota composed primarily of Proteobacteria and Firmicutes (Grond et al., 2018). For instance, vultures can safely consume decaying animal carcasses, which is largely attributed to the higher competitive ability of *Clostridium* in their hindgut microbiota compared to other bacteria (Roggenbuck et al., 2014).

Crows belong to the genus *Corvus* in the family Corvidae of the order Passeriformes. They are renowned for their high intelligence and social behavior, often congregating in large groups numbering in the thousands (Ericson et al., 2005; Haring et al., 2012; Holzhaider et al., 2011). Among them, the omnivorous carrion crow (*Corvus corone*) is widespread across Eurasia (Kersten et al., 2022). These crows inhabit large groups and consume carrion, garbage, seeds, fruits, and various plants, playing a crucial ecological role through the consumption of decaying matter and agricultural waste (Kövér et al., 2019; Preininger et al., 2019). Due to their facultative scavenging behavior, carrion crows are exposed to a higher risk of pathogen invasions and can serve as potential vectors for the transmission of diseases and disease-causing bacteria, including influenza, West Nile fever, cholera, *Salmonella*, *Giardia*, and *Shigella* (Hassan et al., 2017; Malekian et al., 2021). Notably, carrion crows facilitate the transmission of *Escherichia coli* from landfill to cities (Anjum et al., 2021). Urban environments also provide favorable conditions for viral genomes to acquire new mutations, leading to the presence of pathogenic strains with higher viral loads and increased transmission efficiency. Compared to species that reside in non-human habitats, carrion crows encounter a broader array of zoonotic pathogens and exhibit a heightened susceptibility to spillover events affecting human populations (Nabi et al., 2021; Staley & Bonneaud, 2015).

Despite numerous studies revealing the role of gut microbiota in the specialized feeding habits of wildlife (Wang et al., 2023), our understanding of its involvement in the facultative scavenging behavior of carrion crows remains limited. It is speculated that carrion crows may harbor specific gut microbiota that not only contribute to their scavenging ability but also provide protection against pathogenic bacteria. In this study, we employed 16S rRNA amplicon sequencing technology to compare and analyze the gut microbiota of carrion crows from Beijing, China, alongside 16 other bird species with distinct dietary habits. Furthermore, we conducted microbiota transplantation experiments in conjunction with the potential biomarkers to investigate the role of the gut microbiota in immune regulation and anti-pathogen infection in carrion crows engaged in scavenging. The primary objective of this study was to unravel the complex interactions between the gut microbiota and host, thereby shedding light on the significance of the gut microbiota in dietary adaptation and defense against pathogenic invasions. The insights gained from this research hold crucial implications for advancing our comprehension of gut microbiota functionality and its potential application in various fields, including health management.

MATERIALS AND METHODS

Sample collection and ethics statement

All study procedures and collection of samples were approved by the Animal Ethics Committee of the Institute of Zoology (IOZ), Chinese Academy of Sciences (CAS) (approval number: IOZ-IACUC-2022-250). Animal welfare was given utmost consideration, and all animals were euthanized using humane methods to minimize any potential suffering.

Acquisition and preservation of sequencing samples

All wild animal sequencing samples utilized in this study were obtained from bird mortality incidents at Beijing Capital International Airport (N40°04′21″, E116°35′51″) from April to June 2020. Individual birds that were beyond recovery and nearing death were selected for sampling within 1 h of their demise in a Biosafety Level II Laboratory. The sampling procedures adhered to the experimental animal care and use guidelines of the Institute of Zoology, Chinese Academy of Sciences. In brief, the bird strike samples were transported to the Institute of Zoology, Chinese Academy of Sciences, for colonic content sampling, then rapidly frozen using liquid nitrogen and stored in a −80°C freezer for subsequent DNA extraction. A total of 48 colonic samples from 17 bird species were collected. Based on dietary preferences, the 17 bird species were divided into four groups for comparative analysis:

1. Omnivores (facultative scavenging birds): carrion crow (*Corvus corone*, Ccor, *n*=6), rook (*Corvus frugilegus*, Cfru, *n*=3), and black kite (*Milvus migrans*, Mmig, *n*=1).

2. Omnivores (non-scavenging birds): common pheasant (*Phasianus colchicus*, Pcol, *n*=2).

3. Carnivores (predatory birds): northern goshawk (*Accipiter gentilis*, Agen, *n*=1), eastern buzzard (*Buteo japonicus*, Bjap, *n*=1), steppe eagle (*Aquila nipalensis*, Anip, *n*=3), upland buzzard (*Buteo hemilasius*, Bhem, *n*=3), long-legged buzzard (*Buteo rufinus*, Bruf, *n*=2), common kestrel (*Falco tinnunculus*, Ftin, *n*=2), saker falcon (*Falco cherrug*, Fche, *n*=3), peregrine falcon (*Falco peregrinus*, Fper, *n*=4), tawny wood owl (*Strix aluco*, Salu, *n*=1), spotted owlet (*Athene brama*, Abra, *n*=3), and gray-faced buzzard (*Butastur indicus*, Bind, *n*=1).

4. Herbivores: rock pigeon (*Columba livia*, Cliv, *n*=6) and budgerigar (*Melopsittacus undulatus*, Mund, *n*=6).

Additionally, laboratory-collected fecal samples were obtained from the colon of euthanized experimental mice and immediately stored at −80°C for total DNA extraction and subsequent sequencing.

DNA extraction, library construction, and sequencing

Total genomic DNA was extracted from samples using the cetyltrimethylammonium bromide (CTAB) method (Agbagwa et al., 2012). The purity and integrity of DNA were analyzed by 1% agarose gel electrophoresis, while DNA concentration was accurately quantified by Qubit. Based on the concentration, DNA was diluted to 1 μg/μL using sterile water. The 16S rRNA genes spanning the 16S V3–V4 regions were amplified using specific primers for 16S V4: 515F-806R (F5'-AYTGGGGYDTAAAGNG-3', R5'-AYTGGGYDTAAAGNG-3') with the barcode (Wei et al., 2022). Polymerase chain reaction (PCR) was carried out with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs, USA), 0.2 μmol/L forward and reverse primers, and 10 ng of template DNA. An equal volume of 1× loading buffer, containing SYBR Green, was mixed with the PCR products. Subsequently, electrophoresis was conducted on a 2% agarose gel for detection.

Sequencing libraries were generated using a TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's recommendations, with index codes added. Library quality was assessed using a Qubit 2.0 Fluorometer (Thermo Scientific, USA) and the Agilent Bioanalyzer 2100 system (USA). Finally, the libraries were sequenced on the Illumina NovaSeq platform (USA), generating 250 bp paired-end (PE) reads.

Processing of sequencing data and species annotation

Illumina NovaSeq sequencing was conducted on cloacal samples from birds. The raw PE data generated from sequencing were subsequently merged. Tag quality control was conducted using QIIME (v.1.9.1, http://qiime.org/scripts/split_libraries_fastq.html) (Caporaso et al., 2010), with high-quality clean tags obtained after strict filtering. Chimeric sequences were removed by comparing the assembled tag sequences against a species annotation database using VSEARCH (https://github.com/torognes/vsearch/), thus generating "effective tag" data for subsequent analysis.

The UPARSE algorithm (UPARSE v.7.0.1001, http://www.drive5.com/uparse/) (Haas et al., 2011) was used to cluster the effective tags from each sample into operational taxonomic units (OTUs) according to a 97% sequence identity threshold. Representative sequences for each OTU were selected based on their frequency of occurrence, with the UPARSE algorithm identifying the most frequently occurring sequence for each OTU.

Species annotation of the OTU sequences was performed using the Mothur method (Edgar, 2013) with the SILVA138 SSUrRNA database (http://www.arb-silva.de/) (Wang et al., 2007) (threshold set at 0.8–1), obtaining taxonomic information at the kingdom, phylum, class, order, family, genus, and species levels. The community composition of each sample at these taxonomic levels was also calculated.

For the analysis of mouse gut microbiota, Illumina sequencing was conducted to obtain PE reads, which were then segregated by sample. Initially, the raw reads were subjected to quality control and filtering based on sequencing quality, then merged based on overlap to obtain optimized data after quality control splicing. Subsequently, sequence denoising methods, such as DADA2/Deblur, were used to process the optimized data to obtain amplicon sequence variant (ASV) representative sequences and abundance information for species annotation and analysis.

Sample complexity analysis (alpha diversity)

The Chao1, Shannon, and Simpson indices were calculated using QIIME (v.1.9.1). Differences in alpha diversity among groups were evaluated using R software (v.2.15.3), applying both parametric (Tukey test) and non-parametric (Wilcoxon test) analyses from the agricolae package.

Multi-sample comparison analysis (beta diversity)

Unweighted and weighted UniFrac distances were calculated based on the OTU data, followed by beta diversity analysis to compare the microbial community compositions among different groups. Principal coordinate analysis (PCoA) and non-metric multi-dimensional scaling (NMDS) were employed using Bray-Curtis dissimilarity values to assess differences in bacterial community structure between groups. Principal component analysis (PCA), PCoA, and NMDS plots were generated using R software (v.2.15.3). PCA utilized the ade4 and ggplot2 packages, PCoA utilized the WGCNA, stats, and ggplot2 packages, and NMDS utilized the vegan package. Differences in beta diversity indices among groups were analyzed using parametric (Tukey test) and non-parametric (Wilcoxon test) analyses with the agricolae package.

Linear discriminant analysis (LDA) effect size (LEfSe) analysis was performed using LEfSe software, with the LDA score threshold set to 4 by default. The Kruskal-Wallis rank-sum test was applied to detect differentially abundant species between groups. LDA was then used to reduce dimensionality and evaluate the effect size of differential species to obtain LDA scores. The default score was set to 4, and *P*-values of the Kruskal-Wallis rank-sum test were set to ≤0.05. Metastats analysis was conducted using R software, employing permutation tests for each taxonomic level (phylum, class, order, family, genus, and species) to obtain *P*-values. The Benjamini-Hochberg false discovery rate (FDR) was employed to correct *P*-values, yielding *Q*-values.

Isolation, identification, and cultivation of *Enterococcus faecalis*

The intestinal samples from carrion crows were cultured in 6.5% NaCl nutrient broth (Hopebio HB8551, China) at 45°C for 18–24 h for *Enterococcus* enrichment. Following enrichment, the culture was streaked onto *Enterococcus* selective agar plates (Hopebio HB7014-1, China) and incubated at 37°C for 24 h. Colonies of *E. faecalis* on the selective agar appeared purple-red with a smooth surface. Colonies suspected to be *E. faecalis* were selected for gram staining microscopy, with positive cells presenting as oval or circular chain-shaped. DNA was extracted from the isolated *Enterococcus* strains using a bacterial DNA extraction kit (Tiangen, China), and 16S rRNA identification was performed using PCR with primers 16S rRNA (F: AGAGTTTGA TCCTGGCTCAG and R: CGGTTACCTTGTTACGACTT) with a length of 1 500 bp (Drancourt et al., 2000). The PCR products were sequenced, with the resulting sequences compared against the NCBI database using BLAST. The growth curve of *E. faecalis* was established in a preliminary experiment to determine the concentration of the bacterial solution for subsequent experiments.

Cultivation of *Salmonella enterica*

Salmonella enterica strains were obtained from the bacterial collection maintained in our laboratory, and a single colony was picked and inoculated into Luria-Bertani (LB) medium for cultivation at 37°C in a bacterial culture incubator. The growth curve of *S. enterica* was established in a preliminary experiment to determine the concentration of the bacterial solution for subsequent experiments.

Experimental animals

All 6-week-old female C57BL/6 mice (*n*=60) were acclimatized to the new environment for 7 days prior to the start of the experiments. The mice were housed in groups of five per cage and provided with *ad libitum* access to commercial rodent chow and water throughout the experiment. A 12 h light-dark cycle was maintained during the feeding period. All animal experiments were performed under animal biosafety level 2 (ABSL2) conditions at the Institute of Zoology, Chinese Academy of Sciences.

Antibiotic treatment

Mice were treated with antibiotics using the antibiotic treatment (ATB) method, as described previously (Zhang et al., 2020). Briefly, a combination of ampicillin (1 mg/mL), streptomycin (5 mg/mL), vancomycin (0.25 mg/mL), and metronidazole (1 mg/mL) (Sigma-Aldrich, USA) was added to the sterile drinking water of mice for 3 days, with treatment stopped 1 day prior to microbial transplantation.

Microbial transplantation

Prior to microbiota transplantation, the *E. faecalis* and *S. enterica* strains were cultured until reaching the logarithmic growth phase. The bacterial cultures were then harvested through centrifugation (5 000 r/min, 5 min, 4°C), and resuspended at a concentration of 5×10^7 colony forming units (CFU)/mL. Sixty mice pre-treated with ATB were randomly divided into four groups (*n*=15/group) and received a daily oral gavage with the following treatments: Group 1, negative control (NC) group (sterile PBS, *n*=15, 100 μL/mouse); Group 2, Ef group (*E. faecalis* suspension, *n*=15, 100 μL/mouse); Group 3, Se group (*S. enterica* suspension, *n*=15, 100 μL/mouse); and Group 4, Ef+Se group (equal amounts of *E. faecalis* and *S. enterica* suspensions, *n*=15, 100 μL/mouse). Survival rate and body weight were monitored for 12 days after three consecutive days of treatment. Blood and intestinal samples were collected from five mice in each group on days 0, 6, and 12 post-gavage.

Enzyme-linked immunosorbent assay (ELISA) for cytokine concentrations

To determine cytokine levels, blood samples were allowed to clot at 4°C overnight, followed by centrifugation (12 000 r/min for 10 min at 4°C with a centrifugation radius of 8 cm) to separate the serum. The following cytokines were detected using Magnetic Luminex® Assay Multiplex kits (R&D Systems, USA; catalog number: LXSAMSM-16): Tumor Necrosis Factor-alpha (TNF-α), Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Interferon-gamma (IFN-γ).

Hematoxylin & eosin (H&E) staining of intestinal tissues

After euthanizing the mice, the abdominal cavity was opened, and overall intestinal condition was observed. Subsequently, 0.5–1.0 cm colon tissue samples were taken from below the cecum, fixed in 4% polyformaldehyde, and stored. Paraffin sections were prepared and stained with H&E for pathological examination of the mouse colon tissue.

RESULTS

Gut microbial diversity of birds

To assess gut microbial diversity in birds, we conducted 16S rRNA gene sequencing of the V3–V4 regions in 48 colorectal samples from 17 bird species. A total of 4 973 529 raw PE reads were obtained, yielding 3 119 725 effective tags after quality control, with an effective rate of 62.73% (Supplementary Table S1). These tags were clustered into OTUs based on 97% identity, resulting in the identification of 1 964 OTUs (Supplementary Table S2).

We first assessed within-group diversity using rarefaction (Supplementary Figure S1A) and rank-abundance curves (Supplementary Figure S1B). The rarefaction curve showed that the sequencing data reached a plateau, indicating sufficient sequencing depth, while the rank-abundance curve indicated that a few dominant species occupied a large proportion of microbial abundance in the carrion crow gut microbiota. Additionally, a species accumulation boxplot (Supplementary Figure S1C) was employed to evaluate species abundance, with its flatness suggesting sufficient sampling for data analysis.

We next conducted alpha-diversity analysis of each group of samples using the Shannon (Figure 1A), ACE (Figure 1B), and Chao1 indices (Figure 1C) to characterize bacterial abundance and diversity within the community at the OTU level (Supplementary Table S3). All three analyses indicated that alpha-diversity was significantly lower in the gut microbiota of the carrion crow compared to the other groups, suggesting lower diversity compared with other dietary birds, with a few dominant bacterial groups occupying the dominant ecological niche.

We then analyzed the relative abundance and proportions of the gut microbiota at the phylum (Figure 1D) and genus levels (Figure 1E) for each group of birds (Supplementary Tables S4, S5). At the phylum level, significant variation was observed in the gut microbiota composition among different species. However, in the scavenging *Corvus* species, carrion crows (Ccor) and rooks (Cfru) exhibited relatively similar gut microbiota composition, with Proteobacteria and Firmicutes as the predominant phyla. Specifically, the relative abundances of Proteobacteria and Firmicutes in the carrion crow gut were 75.51% and 22.37%, respectively, constituting the core gut microbiota and accounting for 97.88% of the total gut microbiota. Among the herbivorous birds, Firmicutes was identified as the dominant phylum in pigeons (Cliv) (80.61%) and eclectus parrots (Mund) (86.06%), while Bacteroidetes (67.12%) and Firmicutes (25.24%) were the dominant phyla in the omnivorous common pheasant (Pcol). Despite sharing a similar diet, the gut microbiota composition in carnivorous birds varied significantly. At the genus level, *Escherichia-Shigella* (74.73%) and *Enterococcus* (19.96%) were the dominant genera in the gut microbiota of the carrion crow and rook, which differed from other bird species.

Specific gut microbiota may affect scavenging behavior adaptation in carrion crows

To investigate the potential influence of specific gut microbiota on scavenging adaptation in carrion crows, we conducted comparative analysis of the gut microbiota among different dietary bird species. Both PCoA (PC1=15.63%, PC2=14.46%)

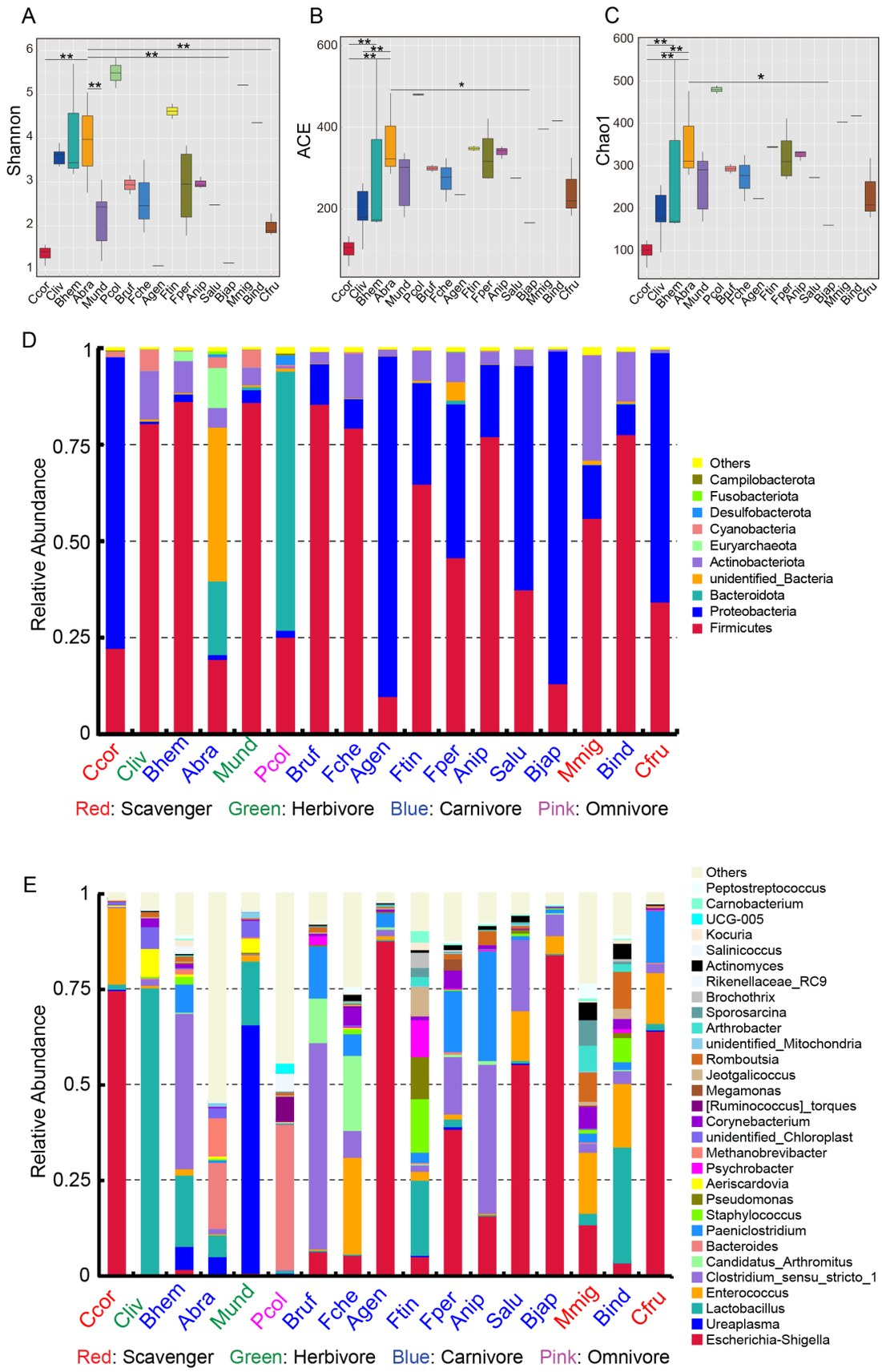

Figure 1 Gut microbial diversity of birds

A: Shannon index. B: ACE index. C: Chao1 index. Y-axis represents number of OTUs in the community. Non-parametric Wilcoxon tests were used for multiple group comparisons (ns: Not significant; *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001, respectively). D: Bar chart of relative species abundance at the phylum level. E: Bar chart of relative species abundance at the genus level. X-axis represents group name, y-axis represents relative abundance. “Others” refers to the sum of the relative abundance of all other phyla in the figure except for the legend.

(Figure 2A) and NMDS (Stress=0.159) (Figure 2B) clearly separated the carrion crow group from the other groups, indicating significant differences in gut microbiota composition among the 17 groups. Additionally, inter-group weighted (Figure 2C) and unweighted UniFrac beta diversity analysis (Figure 2D) revealed significant differences among groups. To identify species with differential abundance among groups, we employed LEfSe analysis to screen for significant species biomarkers. We conducted comparative analyses between the carrion crow and birds with different diets, as well as among birds with similar diets. Firstly, we selected five groups of birds with good reproducibility, including carrion crow (Ccor), pigeon (Cliv), Himalayan buzzard (Bhem), collared scops owl (Abra), and budgerigar (Mund), for LEfSe analysis (Figure 2E). Among them, Proteobacteria, Gammaproteobacteria, Enterobacterales, *Escherichia-Shigella*, Enterobacteriaceae, *Escherichia coli*, Enterococcaceae, *Enterococcus*, *Enterococcus faecalis*, *Hafnia-Obesumbacterium*, and Hafniaceae showed significant differences in abundance in carrion crow. Further evolutionary analysis (phylogenetic distribution) of these bacteria (Figure 2F) revealed that the microbial taxa with significant differences in abundance were mainly classified into two categories: *Enterococcaceae* and *Enterobacteriaceae*.

To compare similar scavenging corvids, we performed LEfSe analysis of carrion crows (Ccor) and rooks (Cfru) (Figure 2G) (default score of 4 and Kruskal-Wallis rank-sum test $P<0.05$). Compared to rooks, *E. faecalis* showed significantly different abundance in carrion crows. The *t*-test results confirmed the significant differences between the two groups, highlighting differential abundance of certain species ($P\leq0.05$) (Figure 2H). Further examination of the relative abundance of *E. faecalis* showed enrichment in all corvid species samples (Supplementary Figure S2A). Its abundance was also observed in all six groups (Figure 2I), further supporting its association with the gut microbiota of carrion crows.

To explore the dominant and closely interacting species in the gut microbiota of the carrion crow, we constructed co-occurrence networks to detect the community structure and function of the complex microbial environment (Supplementary Figure S2B). Results showed that *Enterococcus* and *Escherichia-Shigella* (Enterobacteriaceae family) were the two largest bacterial populations in the gut and exhibited high negative correlation. As *Escherichia-Shigella* are common opportunistic pathogens in the gut, we speculate that the high abundance of *Enterococcus* effectively inhibits the high abundance of *Escherichia-Shigella* in scavenging activities. Thus, these findings suggest that *Enterococcus faecalis* may serve as a biomarker associated with the promotion of scavenging behavior in carrion crows.

Protective effects of carrion crow-derived *E. faecalis* against *S. enterica* infection in mice

To investigate whether carrion crow-derived *E. faecalis* can provide protection against other pathogenic microorganisms, we isolated and cultured the *E. faecalis* strain with the highest abundance in the colon contents of carrion crows. Drug resistance testing showed that this strain exhibited resistance to metronidazole antibiotics (Figure 3A). PCoA analysis between the ATB group and Normal Feeding group demonstrated significant differences in the composition of the gut microbiota (Supplementary Figure S3A). Microbiota transplantation was then performed by orally administering mice with *E. faecalis* (Ef), *S. enterica* (Se), or a combination of both (Ef+Se) (Figure 3B). No mouse mortality was observed throughout the experiment. Regarding body weight, results showed that, compared to the PBS group, the Ef group experienced in increase in body weight, while the Se group exhibited a continuous decline in body weight until the end of the experiment. For mice in the Ef+Se group, the downward trend in weight ceased on day 9, with stabilization thereafter (Figure 3C). These findings suggest that *E. faecalis* may mitigate the weight loss induced by *S. enterica* infection, offering a certain protective effect.

To explore the potential mechanism by which *E. faecalis* provides protection against *S. enterica* infection, we evaluated the serum concentrations of various cytokines, including TNF-α, IFN-γ, IL-10, IL-6, and IL-17A, in each group. Compared to the NC group, the levels of TNF-α ($P<0.01$), IFN-γ ($P<0.05$), IL-10 ($P<0.05$), and IL-6 ($P<0.05$) were significantly increased in the serum of mice infected with *S. enterica* for 15 days, while the concentration of IL-17A showed a slight albeit non-significant increase. Compared to the Se group, the Ef+Se group showed a decrease in TNF-α, IFN-γ, IL-10, and IL-6, indicating that *E. faecalis* can down-regulate the expression of pro-inflammatory cytokines induced by *S. enterica* infection. In terms of anti-inflammatory cytokine IL-10, oral gavage with both *E. faecalis* and *S. enterica* alone resulted in an up-regulation in its expression, whereas a decreasing trend was observed when in combination. Additionally, oral gavage with *E. faecalis* alone had a certain inhibitory effect on the expression of pro-inflammatory cytokine IL-17A, whereas co-administration of *E. faecalis* and *S. enterica* increased the expression of IL-17A. Furthermore, histological examination revealed that the intestinal tissues of mice in the Se and Ef+Se groups displayed severe pathologies on day 3 post-infection, including congestion, inflammatory cell infiltration, and fibrosis. Furthermore, the Ef+Se group showed reduced intestinal inflammation on day 15 compared with the Se group (Figure 3E).

To explore changes in the gut microbiota composition of the host after microbiota transplantation, 16S rRNA gene sequencing was applied to analyze the colon contents of mice on day 15. Notably, PCA (Figure 4A) and NMDS (Stress=0.07) (Figure 4B) were employed to assess the differences in bacterial community structure between groups. NMDS analysis was also used to compare the four treatment groups on day 15 with the ATB and NC groups on day 0 (Supplementary Figure S3B). Results showed that the communities of each group were clearly separated, indicating significant differences in gut microbiota composition. Next, changes in the abundance of *Enterococcus* and *Salmonella* in these groups were characterized at the end of the experiment. Results showed that the relative abundance of *Enterococcus* was lower in the Ef+Se group than in the Ef group, indicating that *Salmonella* may inhibit *Enterococcus* colonization (Figure 4C). However, simultaneously, *Salmonella* was not detected in the Se or Ef+Se groups infected with *Salmonella* via oral gavage. This may be related to severe intestinal damage caused by *Salmonella* infection or the selection of sampling sites influencing these results.

Significant differences in gut microbiota composition were also observed among the four groups. Analysis of relative abundance at the genus level revealed significant differences

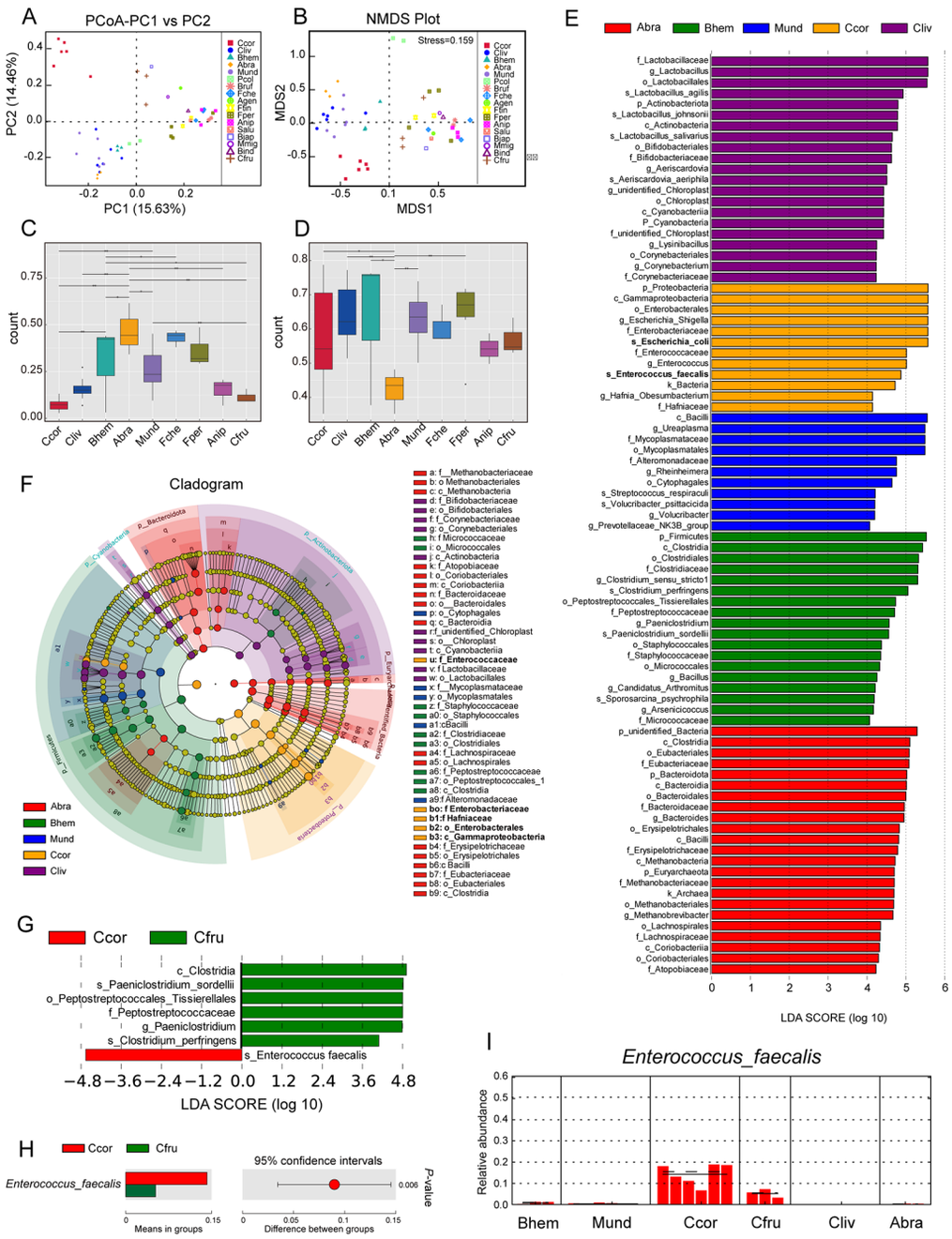

Figure 2 Analysis of biomarker among groups

A: PCoA among all groups. Horizontal coordinate indicates one principal component, vertical coordinate indicates another principal component, and percentage indicates the contribution of the principal component to sample variance. B: NMDS analysis among all groups (Stress=0.159). Each point in the graph represents a sample, and distance between points represents degree of difference. Each sample within the same group is represented by the same color. For stress values less than 0.2, NMDS accurately reflects the degree of differences between samples. C: Box plot based on weighted UniFrac beta diversity. Wilcoxon rank-sum test. D: Box plot based on unweighted UniFrac beta diversity. E: Bar chart of linear discriminant analysis (LDA) score distribution according to LEfSe differential analysis among Abra, Bhem, Mund Ccor, and Cliv groups. Bar chart displays microbial taxa with LDA scores greater than a set value (Wilcoxon rank-sum test, P<0.01; LDA score>4). F: Evolutionary tree according to LEfSe differential analysis among Abra, Bhem, Mund Ccor, and Cliv groups. Circles radiating from the center represent taxonomic levels from phylum to species (or subspecies), and each small circle at different taxonomic levels represents a classification at that level, with the diameter of the circle proportional to its relative abundance. Species with no significant difference are in yellow, while biomarker species are colored according to their corresponding groups. G: Bar chart of LDA score distribution according to LEfSe differential analysis among Ccor and Cfru groups. Bar chart displays microbial taxa with LDA scores greater than a set value (Wilcoxon rank-sum test, P<0.01; LDA score>4). H: Comparison of species abundance between groups using t-test. Left graph displays abundance of differentially abundant species between groups, with each bar representing mean abundance in each group. Right graph displays confidence of inter-group differences, with the P-value of the significance test for each differentially abundant species on the far right. I: Comparison of abundance of biomarker species with significant inter-group differences among Abra, Bhem, Mund Ccor, Cfru, and Cliv groups. Biomarker was identified through LEfSe differential analysis (Wilcoxon rank-sum test, P<0.01; LDA score>4).

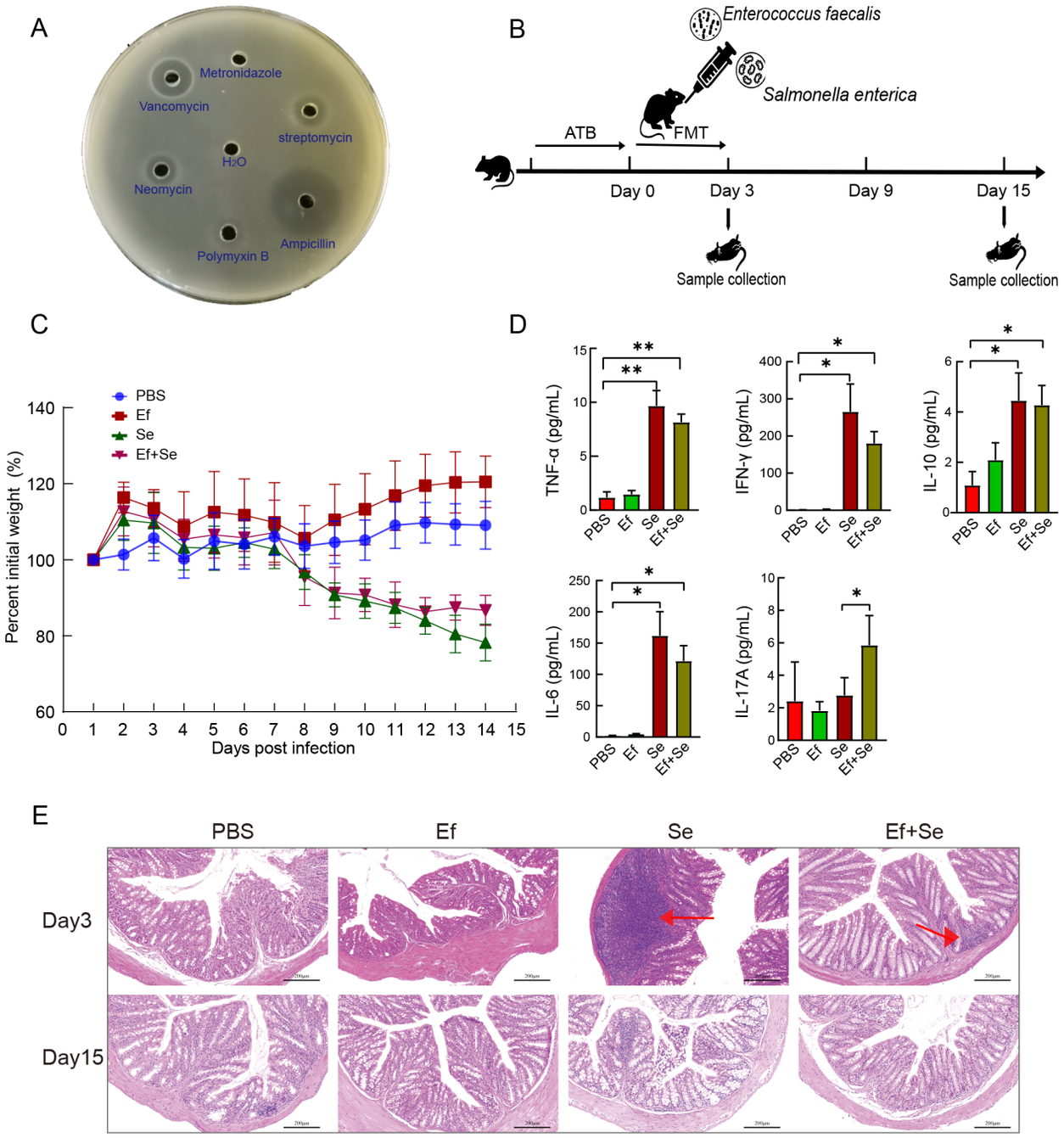

Figure 3 Gavage of carrion crow-derived *E. faecalis* enhances resistance to *S. enterica* infection in mice

A: Antibiotic resistance testing of *E. faecalis*. B: Experimental procedure: 60 mice were pretreated with ATB and randomly divided into four groups (n=15 mice/group). For the first three days, mice were gavaged with PBS, *E. faecalis* culture (5×10^7 CFU/mL), *S. enterica* culture (5×10^7 CFU/mL), or a mixture of *E. faecalis* (5×10^7 CFU/mL) and *S. enterica* culture (5×10^7 CFU/mL) to a total volume of 100 μL per day. Physiological status and body weight of mice were monitored daily for 15 days. C: Changes in mouse body weight. Data are presented as mean±SD. D: Cytokine concentrations. Multiple comparisons were done by ordinary one-way ANOVA (ns: Not significant; *: P<0.05; **: P<0.01; ***: P<0.001; ***: P<0.0001, respectively). E: Histological examination (H&E staining).

in bacterial genera among the groups (Figure 4D). Compared to the NC group, the Ef group showed an increase in the relative abundance of *Muribaculaceae*, while the relative abundance of *Lachnospiraceae*_NK4A136 decreased. Compared to the Se group, the Ef+Se group showed a slight increase in the abundance of *Muribaculaceae*. We also analyzed the abundance of *Muribaculaceae* in the four groups based on community abundance data (Supplementary Figure S3C). Compared to the control group, the Se group showed an increase in the abundance of *Lachnospiraceae*_NK4A136. Compared to the Se group, the Ef+Se group showed a slight decrease in the abundance of *Lachnospiraceae*_NK4A136 (Supplementary Figure S3D), indicating the opposite trends for these two bacterial groups. Other bacteria at different taxonomic levels also showed varying changes. These results indicate that *E. faecalis* can regulate the gut microbiota composition, thereby modulating anti-inflammatory and immune responses in animals.

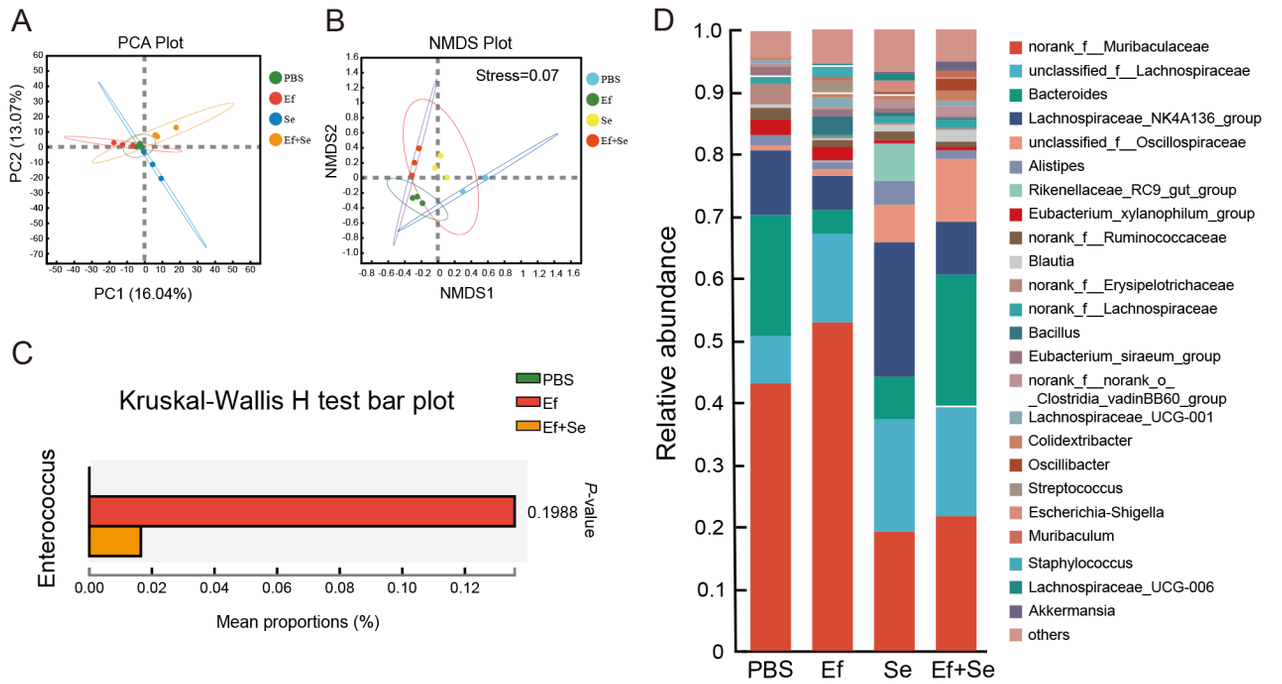

Figure 4 Analysis of microbial composition and abundance differences in different groups

A: PCA among all groups. Each point in the graph represents a sample, and distance between points represents degree of difference. B: NMDS analysis among all four groups (Stress=0.07). C: Abundance differences of *E. faecalis* among different groups (Kruskal-Wallis rank sum test). X-axis represents percentage value of a certain species abundance in each sample, and different colors represent different groups (ns: Not significant; *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001, respectively). D: Community bar chart showing proportion of each microbial taxa in each group. X-axis represents group name, and Y-axis represents proportion of microbial taxa in the group. Different colors of columns represent different microbial taxa, and lengths of columns represent proportion of microbial taxa.

DISCUSSION

Birds play crucial roles in ecosystems, attributed to their complex life histories, dietary diversity, and selective pressures from their aerial lifestyle. These elements shape the gut microbiota of birds, leading to low stability and high plasticity in response to environmental and dietary variations (Bodawatta et al., 2022; Waite & Taylor, 2015). Diet is the main intrinsic driving force influencing gut microbiota structure (Carmody et al., 2015). The gut microbiota of wild birds has garnered increasing attention due to its potential role as a source of many human and animal diseases, either through direct transmission or as zoonotic pathogen carriers (Lu et al., 2008; Sun et al., 2022). Of note, crows carry a multitude of parasites, viruses, and bacteria, positioning them as significant reservoir hosts and vectors of infection (Maeda et al., 2013). For example, crows demonstrate high susceptibility to the highly pathogenic H5 avian influenza virus, resulting in prolonged viral shedding and low-titer dissemination (Soda et al., 2020). Furthermore, crows infected with highly pathogenic avian influenza viruses VN/559, Hok/4, HK/810, or Tw/0502 exhibit significantly longer survival times than chickens, suggesting potentially stronger immune capabilities compared to domestic animals (Hiono et al., 2016).

Research on avian gut microbiota, particularly wild birds in their natural habitats, is limited, with a predominant focus on poultry due to the challenges posed by complex sampling methods and low DNA extraction rates under field conditions (Sun et al., 2022). Notably, significant variations in the richness and composition of the gut microbiota exist across different regions of the gastrointestinal tract, as seen in Passeriformes (Berlow et al., 2020). Furthermore, non-lethal methods, such as cloacal swabs and fecal samples, which serve as the primary source of avian gut microbiota research due to their ease of collection (Grond et al., 2018; Li et al., 2018), only reflect the microbial composition of the colon and do not accurately represent the microbial composition of different regions of the gut (Videvall et al., 2018; Youngblut et al., 2019). In contrast, samples from the avian colon, known for their high microbial abundance, high alpha diversity, and strong correlation with specific taxonomic groups, provide a more accurate reflection of the characteristics of the host's gut contents (Drovetski et al., 2018; Videvall et al., 2018). In this study, a specific time frame and geographic area were selected, leading to a constrained number of samples. The absence of obligate scavenger vultures in the vicinity of Beijing presented a challenge in the selection of control groups. To address this, we extensively utilized samples from deceased birds for the extraction of microbiota from various regions of the gut, aiming to enhance the representation of the gut microbiome. Despite these efforts, this study has several limitations in sample collection. Therefore, future research should focus on the collection of a broader array of samples to improve the depth of experimental analysis.

Bird gut microbiota typically features a predominance of bacterial phyla such as Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria. However, the proportions of these phyla can vary among different bird species (Grond et al., 2018). Within these microbial communities, certain species and populations play vital roles in maintaining the stability and functionality of the intestinal microbiome (Guinane & Cotter, 2013). In our study, carrion crows exhibited a low microbial diversity in the gut, as observed in other scavenging birds. For example, vultures show low gut microbiota diversity,

with a significant presence of *Clostridia* and *Fusobacteria*. Notably, both *Clostridia* and *Fusobacteria* are associated with the potential to induce illnesses in various animal species (Roggenbuck et al., 2014).

Our study revealed that the carrion crow gut microbiota was predominantly composed of the Bacteroidetes and Firmicutes phyla. Scavenging birds tend to accumulate higher levels of Bacteroidetes, with studies suggesting an increase in pathogenic bacteria alongside Bacteroidetes abundance (Rizzatti et al., 2017; Vester-Andersen et al., 2019). Various opportunistic pathogens, including *Campylobacter*, *Escherichia*, *Helicobacter*, *Leptospira*, *Salmonella*, and *Vibrio*, have been isolated from avian hosts (Grond et al., 2018; Stecher et al., 2013). For instance, research on red kites (*Milvus milvus*) found a significantly higher prevalence of *Salmonella sp*. in the gut microbiota of those consuming livestock carrion compared to those feeding on wild prey (Blanco, 2014). The phylum Firmicutes, which is primarily composed of gram-positive bacteria, generally coexists with the host, aside from a few pathogenic species such as *Chlamydophila psittaci*, *Clostridium botulinum*, and *Clostridium perfringens* (Wu et al., 2009). Evidence suggests that these bacteria may impact host weight and health through energy metabolism and immune regulation (Lopetuso et al., 2013). Studies have reported a positive correlation between the abundance of Firmicutes and both weight gain and immune function in domestic chickens (Xu et al., 2021). Commonly used probiotics, such as *Lactobacillus*, belong to the Firmicutes phylum (Suvorov, 2013). Notably, chickens fed with lactic acid bacteria (e.g., *Lactobacillus spp*.) or *Bacillus spp*. show immune system modulation and lower prevalence of pathogens such as *Clostridium* and avian influenza virus (Grant et al., 2018; Yitbarek et al., 2018). Short-chain fatty acids (SCFAs), fermentation by-products of Firmicutes, provide a direct energy source that can be absorbed by the intestinal wall of the host (Svihus et al., 2013), reducing harmful microbial infection through butyrate-producing bacteria such as *Butyricicoccus pullicaecorum* (Eeckhaut et al., 2016).

The biomarker identified in this study, *E. faecalis*, which also belongs to Firmicutes, is a gram-positive facultative anaerobe that can coexist in the gastrointestinal tract of various organisms. It has competitive advantages in food fermentation and intestinal colonization, leading to its widespread application in the fields of medicine and food engineering (de Almeida et al., 2018; Qiao et al., 2020). However, the genus *Enterococcus* includes strains that are potentially pathogenic and resistant to antibiotics, posing risks to the host's immune system upon infection (Fisher & Phillips, 2009). Consequently, we conducted a drug resistance assay on the isolated *E. faecalis*, which indicated that it did not possess characteristics of vancomycin-resistant enterococci (VRE).

Scavenging vultures possess a unique capacity to selectively enrich their gut microbial communities with beneficial symbionts, thereby naturally filtering out many common pathogens and lowering their infection risk (Roggenbuck et al., 2014). Crows may employ similar strategies to mitigate their risk of pathogen infection. Additionally, various studies have indicated that administering probiotics can effectively reduce the prevalence and abundance of specific avian pathogens, such as *Salmonella*, *Campylobacter*, *Clostridium*, *Escherichia-Shigella*, and avian influenza (Bodawatta et al., 2022; Eeckhaut et al., 2016; Reuben et al., 2019). *Enterococcus*, as a member of the gut microbiota, can contribute to the creation of an acidic anaerobic environment in the gut, thus inhibiting pathogenic bacteria while promoting the survival and growth of beneficial lactic acid-producing bacteria (Rivera-Chávez et al., 2016).

Based on co-occurrence network analysis, *Enterococcus* and *Shigella* were identified as the most abundant bacterial genera in the carrion crow gut and exhibited a strong negative correlation. This suggests that carrion crows may protect themselves by competitively excluding harmful bacteria, such as *Shigella*, from adhering to the gut surface through beneficial bacteria such as *E. faecalis*. To experimentally validate this hypothesis, we initially planned to establish an *E. faecalis* colonization model, followed by *S. enterica* infection. However, this approach would not have directly confirmed the competitive advantage of carrion crow-derived *E. faecalis* over other microbes. Hence, a gavage experiment using both *E. faecalis* and pathogenic bacteria simultaneously was conducted. The colonization effects of *E. faecalis* and *S. enterica* were investigated using the annotation results of microbial abundance. Interestingly, the relative abundance of *Enterococcus* was indeed higher in the group receiving only a single gavage of *E. faecalis* compared to the group receiving mixed gavage, highlighting the competitive advantage of *E. faecalis*. Conversely, in both the single *S. enterica* gavage group and the mixed gavage group, *S. enterica* annotations were absent. This may be because the pathogenic bacteria were gradually eliminated from the host as the stable composition of "core microbiota" as established during the later stages of the experiment (Thursby & Juge, 2017).

Our study focused on non-estrus and non-pregnant female mice for experimentation as we did not investigate the effects of sex on avian gut microbiota. This choice was primarily based on differences in immune responses between female and male mice, with female mice generally exhibiting stronger immune responses, potentially attributable to hormone regulation (Klein & Flanagan, 2016). Similar patterns have been observed in birds, with females showing higher antibody and cell-mediated immune responses to immune challenges (Pap et al., 2010). Therefore, choosing female mice allowed for easier observation of changes and differences in immune responses. During the microbiota transplantation experiment, changes in body weight were monitored as a physiological indicator. Results showed that the mice receiving *E. faecalis* alone displayed a notable increase in body weight compared to the control group, suggesting probiotic effects. Furthermore, compared to the *S. enterica* group, the mixed group demonstrated a slight suppression of the downward trend in body weight on day 9. This suppression may be related to the use of antibiotics in mice, potentially inducing adverse reactions when the symbiotic relationship between the gut microbiota and host is disrupted after antibiotic treatment (Lengfelder et al., 2019).

Our research also revealed that *S. enterica* infection can cause severe intestinal inflammation. Notably, we observed that when the two bacteria were co-administered, *E. faecalis* effectively decreased the expression of pro-inflammatory factors TNF-α, IFN-γ, and IL-6, in contrast to the group that only received *S. enterica*. Administration of *E. faecalis* alone resulted in the up-regulation of the anti-inflammatory factor IL-10, but this effect was not amplified in the mixed group. Additionally, in the mixed gavage group, an increase was observed in the expression of pro-inflammatory factor IL-17A.

These observations may be related to the composition and functional changes of the gut microbiota, causing different responses of *E. faecalis* to different inflammatory factors (Lengfelder et al., 2019). Given its high microbial density and direct interaction with numerous gut microbes, the colon is considered a primary site for immune activity within the intestinal tract (Brown & Esterházy, 2021). The colonic immune system plays a crucial role in regulating inflammatory reactions, antimicrobial defense, food tolerance, and immune responses (James et al., 2020). Thus, we utilized H&E staining to assess the status of colonic intestinal inflammation. Research has demonstrated that specific strains of *E. faecalis* can modulate inflammatory responses (Kao & Kline, 2019; Wang et al., 2014). Our study confirmed that *E. faecalis* can reduce intestinal inflammation caused by *S. enterica*.

Our findings further showed that *E. faecalis* effectively modulated the composition of the gut microbiota. Significant shifts were observed in the abundances of Muribaculaceae and Lachnospiraceae NK4A136, displaying divergent trends in different groups. Administration of *E. faecalis* alone led to an increase in the abundance of Muribaculaceae, while administration of *S. enterica* led to a significant decrease in abundance. This effect was mitigated when both strains were introduced together. The primary function of Muribaculaceae is to degrade various complex carbohydrates, and changes in its abundance may be related to the specific adaptability of Muribaculaceae to the mouse intestine (Seedorf et al., 2014). These findings emphasize the importance of selecting appropriate animal models in wildlife research to accurately represent the host's condition. This also represents a limitation of our research, as we recognize that the use of chickens may more closely mirror the ecological conditions and gut traits characteristic of scavenging crows. However, our understanding of immune systems and gut microbiomes in mice offers valuable insights into their immune responses and microbiota interactions. This allows for meaningful comparisons with prior studies, enhancing the reliability of our findings (Langille et al., 2014). Mice are widely used as model organisms in wildlife microbiota research, including giant pandas (Zhou et al., 2020). In our future study, chickens will be used as a model to investigate avian gut microbiota, which may provide more directly applicable insights into bird species.

CONCLUSIONS

In summary, our study demonstrated the protective role of *E. faecalis* in influencing the scavenging behavior of carrion crows, illustrating how certain gut microbes can enhance the host's adaptation to specialized diets. These findings have significant implications for the development and utilization of probiotics derived from wild animal sources, as well as providing insights into disease monitoring in urban resident birds. However, it is important to acknowledge that our study primarily focused on describing the association of *E. faecalis* within the microbiome of model animals. Further research is needed to investigate the effects of this bacterium on non-model wild bird hosts. Such research will be crucial for elucidating the precise role of the gut microbiota in shaping the dietary specialization of wild animals.

DATA AVAILABILITY

The data presented in the study have been deposited in the National Genomics Data Center (NGDC) under accession number PRJCA016388, Science Data Bank (DOI:10.57760/sciencedb.j00139.00122), and NCBI database (BioProjectID PRJNA1095565).

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

H.X.H. and B.H. contributed to the conception of the study. J.X., J.M.W., and Y.N.X. contributed significantly to the collection of samples and performed the experiments. S.Y.H. contributed significantly to analysis and manuscript preparation. B.W. and Q.X.Z. helped perform analyses with constructive discussions. B.H. performed data analyses and wrote the manuscript. All authors read and approved the final version of the manuscript.

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