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RESEARCH ARTICLE

A Resistome Profiling and Microbiome Analysis in Zoo Animals: Uncovering Hidden Threats to Public Health

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Antibiotic resistance genes (ARGs) and their associated resistomes pose significant threats to global public health, yet their dynamics in zoo animals remain poorly understood. This study addresses this gap by analyzing fecal samples from diverse zoo animals in Jiangsu, China. We identified 1,415 ARG subtypes, with tetracycline and multidrug resistance genes being most prevalent. Notably, resistome profiles clustered according to host dietary preferences: tetracycline resistance genes were abundant in herbivores, omnivores, and carnivores, while multidrug efflux genes were enriched in bamboo-feeding animals. Microbiome analysis showed distinct microbial community structures across different dietary groups. The correlation between microbial community structure and dietary preferences suggests that diet significantly influences ARG distribution. Furthermore, insertion sequences (ISs) and plasmid types likely play key roles in ARG transmission within the zoo environment. This study provides new insights into the fecal resistome in zoo animals, demonstrating the significant influence of diet and microbial community structure on ARG profiles. These findings have crucial implications for the prevention and management of multidrug-resistant bacteria in zoos, emphasizing the need for targeted interventions to mitigate ARG spread.

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INTRODUCTION

Antibiotics have long been essential tools in clinical treatment and animal husbandry for controlling bacterial infections (Yang et al., 2022). However, the widespread emergence and dissemination of antibiotic resistance genes (ARGs) due to the overuse and misuse of antibiotics have emerged as a pressing global public health concern. ARGs are now recognized as novel environmental pollutants, posing significant threats to both human and animal health. Many ARGs reside on mobile genetic elements (MGEs), such as transposons and conjugative elements (e.g., plasmids), which facilitate their efficient spread between bacterial cells, including from nonpathogenic and commensal species to pathogens (Acman et al., 2022). This horizontal gene transfer exacerbates the spread of resistance, rendering critical antibiotics ineffective.

Tigecycline, colistin, and carbapenems have traditionally been considered last-resort drugs for treating Enterobacterial infections. However, the increasing prevalence of plasmid-mediated carbapenem resistance genes (blaNDM and blaKPC), tigecycline resistance genes (tet(X) and tmexCD-toprJ), and colistin resistance genes (mcr-1) in animals has severely undermined the efficacy of these drugs in clinical settings (Shen et al., 2020; Dong et al., 2022; Li et al., 2023). This trend highlights the urgent need for comprehensive strategies to monitor and mitigate the spread of ARGs.

Previous studies have attempted to elucidate the occurrence and distribution of ARGs using various methods, including quantitative PCR (qPCR) and high-throughput quantitative polymerase chain reaction (HT-qPCR) (Zhu et al., 2013; Zhao et al., 2018). Metagenomics, defined as the high-throughput

sequencing of microbial communities, has revolutionized our understanding of bacterial diversity and function. This technology has been widely applied to identify novel biocatalysts, ARGs, virulence genes, and to detect pathogens (Charalampous et al., 2019; Lloyd-Price et al., 2019; Gu et al., 2021; Khan et al., 2025). Metagenomic sequencing has also been instrumental in monitoring the prevalence and diversity of ARGs in gut microbiomes and various environmental settings (Donia et al., 2014; Zhang et al., 2018). These studies have significantly enhanced our knowledge of ARG diversity and abundance.

Zoos are pivotal institutions for animal health research, conservation, and public health surveillance. They provide valuable insights into zoonotic infectious diseases and antimicrobial resistance (AMR) (Min et al., 2023). The emergence and spread of antibiotic resistance in zoos not only jeopardize the health of animals and humans but also threaten the long-term survival of endangered species. Human-animal interactions in zoos increase the risk of transmitting drug-resistant bacteria, making the study of ARGs in zoo environments crucial for public health security (Furlan et al., 2020). Despite this, research on ARGs in zoo animal feces remains limited (Min et al., 2023; Sealey et al., 2023).

Animal feces are recognized as significant reservoirs of ARGs, yet studies on ARGs in zoo animal feces are still scarce. Given the potential for ARGs to spread from animals to humans, understanding the resistome in zoo settings is essential. In this study, we aimed to comprehensively investigate the composition of microorganisms and ARGs in the feces of various zoo animals using metagenomic approaches. Our goal is to provide a theoretical basis for developing rational and effective control measures to mitigate the spread of antibiotic resistance in zoos and beyond.

MATERIALS AND METHODS

Sample collection: A total of 26 mixed fresh fecal samples of animals were collected in a zoo located in Jiangsu, China in September, 2022. Collected samples were kept in the box with ice packs for conveying to the lab immediately. Based on host dietary habits, 26 samples were divided into four groups, including herbivorous animal group (GA, n=10), omnivorous animal group (OA, n=8), carnivorous animal group (CA, n=3), bamboo-feeding animal group (BA, n=5). GA contained two fecal samples from white kangaroo (BDSF1-BDSF2), one from zebra (BMF), two from giraffe (GJLG-GJLM), one from gnu (JMF), two from deer (LF1-LF2), one from ostrich (TNF) and one from alpaca (YTF). OA was composed of three fecal samples from gibbon (CBYF1-CBYF3), two from guereza (HBYHF1-HBYHF2) and three from golden monkey (JSHF1-JSHF3). CA was consisted of one fecal sample from leopard (BZF), one from wolf (LFB1) and one from tiger (LHF). BA included three fecal samples from panda (DXMF1-DXMF3) and two from red panda (XXMF1-XXMF2). The detailed information of 26 samples was provided in Table S1.

DNA extraction, metagenomic sequencing and data processing: The total DNA of 26 fecal samples was extracted using a BOLAZ® Stool DNA Kit (DE0513, BOLAZ, Nanjing, China) according to the manufacturer's protocol. The quality and concentration of extracted DNA were detected by a NanoDrop 2000 microvolume spectrophotometer (Thermo Fisher Scientific, Waltham, USA), whereas the final concentration was determined by Qubit™ 4.0 (Invitrogen, CA, USA). The genomic DNA with OD260/OD280 ratios of 1.8 to 2.2 and a final concentration of ≥40 ng/μL was selected to perform metagenomic sequencing. Subsequently, qualified DNA samples (n=26) was subjected to construct DNA libraries and sequencing using Illumina Hiseq 2500 platform for generating 2×150bp paired-end reads. Raw reads were quality controlled, trimmed and filtered by fastp v0.23.2 (Chen et al., 2018). The information of raw data and clean data was summarized in Table S2. Quality-trimmed reads of metagenome samples were assembled using MEGAHIT v1.2.9 (Li et al., 2015).

Analysis of ARGs, ISs, plasmid types and microbial taxa: ARGs were identified and quantified using ARGs-OAP v2.0 based on the SARG database (Yin et al., 2018). In order to filter ARG-like sequences, the BLASTX alignment was performed using an e-value of 1e−7 and sequences with ≥70% identity and ≥70% coverage were identified and annotated (Yang et al., 2016; Yin et al., 2018). The ARG abundance was normalized by the length of ARG reference sequences with the number of cells from metagenomic datasets and was calculated as follows:

$$\text{Abundance}=\frac{\sum_{1}^{n}\frac{N_{ARG-like\,sequence}\times L_{reads}/L_{ARG\,reference\,sequence}}{\text{Cell number}}}{}$$

$$\text{Cell number}=\frac{\sum_{1}^{n}N_{16S\,sequence}\times L_{reads}/L_{16S\,sequence}}{\sum_{i=1}^{m}M_i\times a_i/A}$$

Here, n means the number of ARG sequences mapped to ARG reference sequences; NARG-like sequence means the number of ARG-like sequences annotated as one specific ARG reference sequence; Lreads means the read length (150 bp); LARG reference sequence means the correspondingly specific ARG reference sequence length; N16S sequence means the number of 16S rRNA gene sequences; L16S sequence means the 16S rRNA gene full length; m means the total taxa identified in the metagenome dataset depending on the information gathered from the hyper variable region; ai means the abundance of matched hypervariable sequences of taxon i from the metagenome dataset; A means the total abundance of matched hypervariable sequences of m taxa and Mi means the copy number of taxon i in CopyRighter database (Yang et al., 2016).

ISs and plasmid types were identified using Blastn (v2.13.0+) by employing the ISFinder and PlasmidFinder databases with a similarity threshold of 80% and a minimum query coverage of 70% (Carattoli et al., 2014; Siguier et al., 2006). Phylogenetic classifications of trimmed sequences were conducted

using Kraken2 on the default parameters based on the NCBI reference nucleotide database (RefSeq) to achieve microbial classifications at different taxonomic levels (Wood and Salzberg, 2014). The relative abundance of microbial taxa was estimated using Bracken (Lu et al., 2017).

Statistical analysis and visualization: Barplots depicting the relative abundance of resistomes and microbiomes were done by GraphPad Prism v9.0.0. The alpha diversities (shannon idex) of ARGs, microbial taxa, ISs and plasmid types were calculated by R Studio v4.1.2 with the vegan package (Wen et al., 2023) and boxplots were done using OriginPro 2021. Analysis of similarities (Anosim) of resistomes and microbiomes among four groups were analyzed on the website (https://www.omicshare.com). Principal coordinate analysis (PCoA) was conducted based on the Bray-Curtis distance. Procrustes analysis between resistomes and microbiomes, and the spearman correlation coefficient, were conducted on the omicshare platform. Linear discriminant analysis Effect Size (LEfSe) was analyzed in R Studio v4.1.2 with the microeco package. The network between ARGs and microbial taxa was visualized by Gephi v0.10.1 based on the spearman correlation coefficient. The line regression between ARGs and MGEs was analyzed using GraphPad Prism v9.0.0.

RESULTS

Basic Features of Metagenomic Sequences: Metagenomic short-read sequencing generated approximately 468 Gbp of raw data, with contributions from GA (37%), OA (27%), CA (11%), and BA (25%) samples, respectively. On average, each fecal sample produced approximately 18 Gbp of raw data, with GA, OA, CA, and BA samples yielding mean ± SD values of 17 ± 3 Gbp, 16 ± 4 Gbp, 17 ± 4 Gbp, and 23 ± 9 Gbp, respectively. A total of approximately 457 Gbp of clean data were obtained, averaging 60 million reads per fecal sample. These clean reads were utilized for subsequent analyses.

Characteristics of Animal Fecal Resistomes in the Zoo: A comprehensive analysis of animal fecal resistomes revealed the presence of 1415 known ARG subtypes encoding resistance to 25 antibiotic classes. Among these, 52 ARGs with a relative abundance ＞0.02 were identified as prominent subtypes for detailed analysis. The most prevalent ARGs conferred resistance to tetracycline, multidrug, macrolide, beta-lactam, polymyxin, bacitracin, aminoglycoside, and chloramphenicol (Fig. 1A). Notably, variations in ARG distribution patterns were observed among GA, OA, CA, and BA samples. Compared to BA samples, GA, OA, and CA samples exhibited significantly higher relative abundances of tetracycline resistance genes (*tet*) and macrolide resistance genes (*cfr, erm, llm, lnu, lsa, mef, mel,* and *vat*) (Fig. 1B). Conversely, BA samples had higher relative abundances of multidrug efflux genes (*acr, emr, mdf, mdt, mex, msb, sde*, and *tol*) and polymyxin resistance genes (arn, ept, pmr, and ros). Additionally, aminoglycoside resistance genes *(aac, aad, ant*, and *aph*) were more prevalent in OA and CA samples.

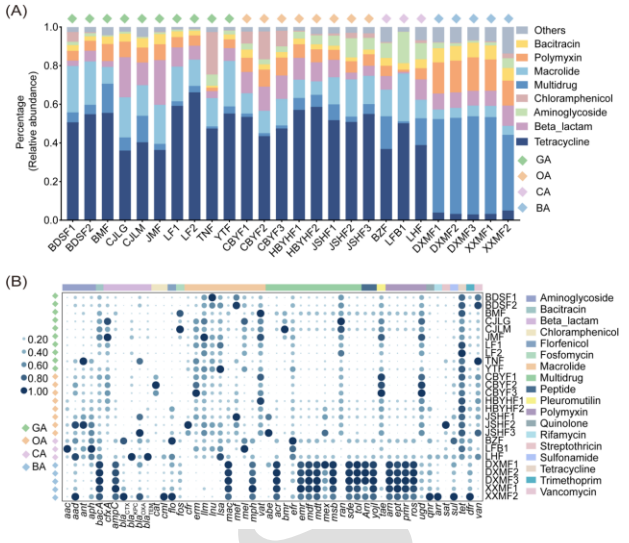

Fig. 1: Resistome Composition of Zoo Animal Fecal Samples. (A) Relative abundance of antimicrobial resistance types in each fecal sample. (B) Relative abundance of selected antimicrobial resistance gene (ARG) subtypes in each fecal sample.

Further screening for polymyxin-, carbapenem-, and tigecycline-resistant genes identified a total of 57 resistance genes, with *blaKPC-17*, *tet(X3)*, tet*(X4)*, and *tet(X2)* being notably abundant across all sample types. CA samples exhibited higher abundances of *mcr-1, bla_{KPC-16}, bla_{KPC-2}*, and *bla_{NDM-5}* compared to GA, OA, and BA samples, indicating a higher prevalence of these critical resistance genes in CA samples.

The diversity of ARG subtypes varied among the four groups, with ARG counts ranging from 532 to 1255 (Fig. 2A). A total of 321 shared ARGs conferring resistance to 21 antimicrobial types were identified across all groups, accounting for approximately 56%, 60%, 45%, and 26% of total ARGs in GA, OA, CA, and BA samples, respectively (Fig. 2A). BA samples exhibited a significantly higher number of unique ARGs compared to the other groups. Shannon diversity analysis revealed that ARG diversity was lower in GA and OA samples than in CA and BA samples (Fig. 2B). Principal Coordinates Analysis (PCoA) further demonstrated distinct clustering of fecal resistomes between GA/OA and CA/BA samples (Anosim, r=0.757, p=0.001, Fig. 2C), highlighting the influence of host dietary preferences on resistome profiles.

Characteristics of Animal Fecal Microbiomes in the Zoo: Analysis of 26 fecal microbial communities revealed the presence of 63 phyla, with Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria being the predominant phyla (Fig. 3A). Microbial community compositions varied significantly among the four groups at the phylum level. GA, OA, and CA samples had higher relative abundances of Firmicutes, Bacteroidetes, and Actinobacteria, while BA samples were dominated by Proteobacteria and Uroviricota. These differences were corroborated by LEfSe analysis (Fig. 4A). Additionally, GA and OA samples exhibited significantly higher Shannon diversity indices for microbial phyla compared to CA and BA samples (Fig. 4B). Further examination of microbial community compositions at the

genus and species levels revealed 2565 genera across the 26 fecal samples (Table S6). At the genus level, *Escherichia*, *Bacteroides*, *Faecalibacterium*, and *Clostridium* were the most abundant (Fig. 3B). Notably, GA and OA samples were dominated by *Bacteroides*, with additional high abundances of *Faecalibacterium* and *Treponema* in OA samples. CA samples were characterized by a high abundance of *Clostridium*, while BA samples were dominated by *Escherichia*. At the species level, *Escherichia coli* and *Faecalibacterium prausnitzii* were the most prevalent (Fig. 3C).

Principal Coordinates Analysis (PCoA) confirmed significant differences in microbial community compositions among the four groups at the phylum

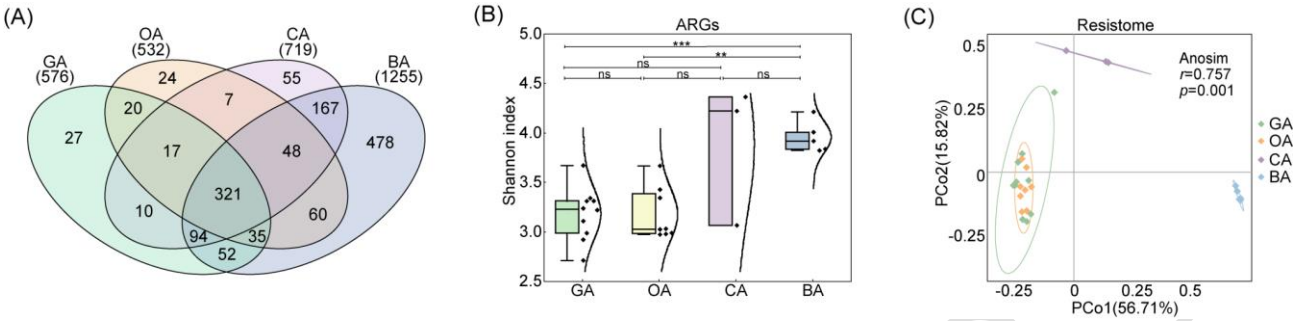

Fig. 2: Comparative Resistome Analysis of Zoo Animal Fecal Samples. (A) Shared and unique ARGs among GA (Grass-feeding animals), OA (Omnivorous animals), CA (Carnivorous animals), and BA (Bamboo-feeding animals). (B) Shannon diversity of ARGs in each group. (C) Principal Coordinates Analysis (PCoA) of ARGs among groups (Bray-Curtis distance; ANOSIM, r=0.757, p=0.001).

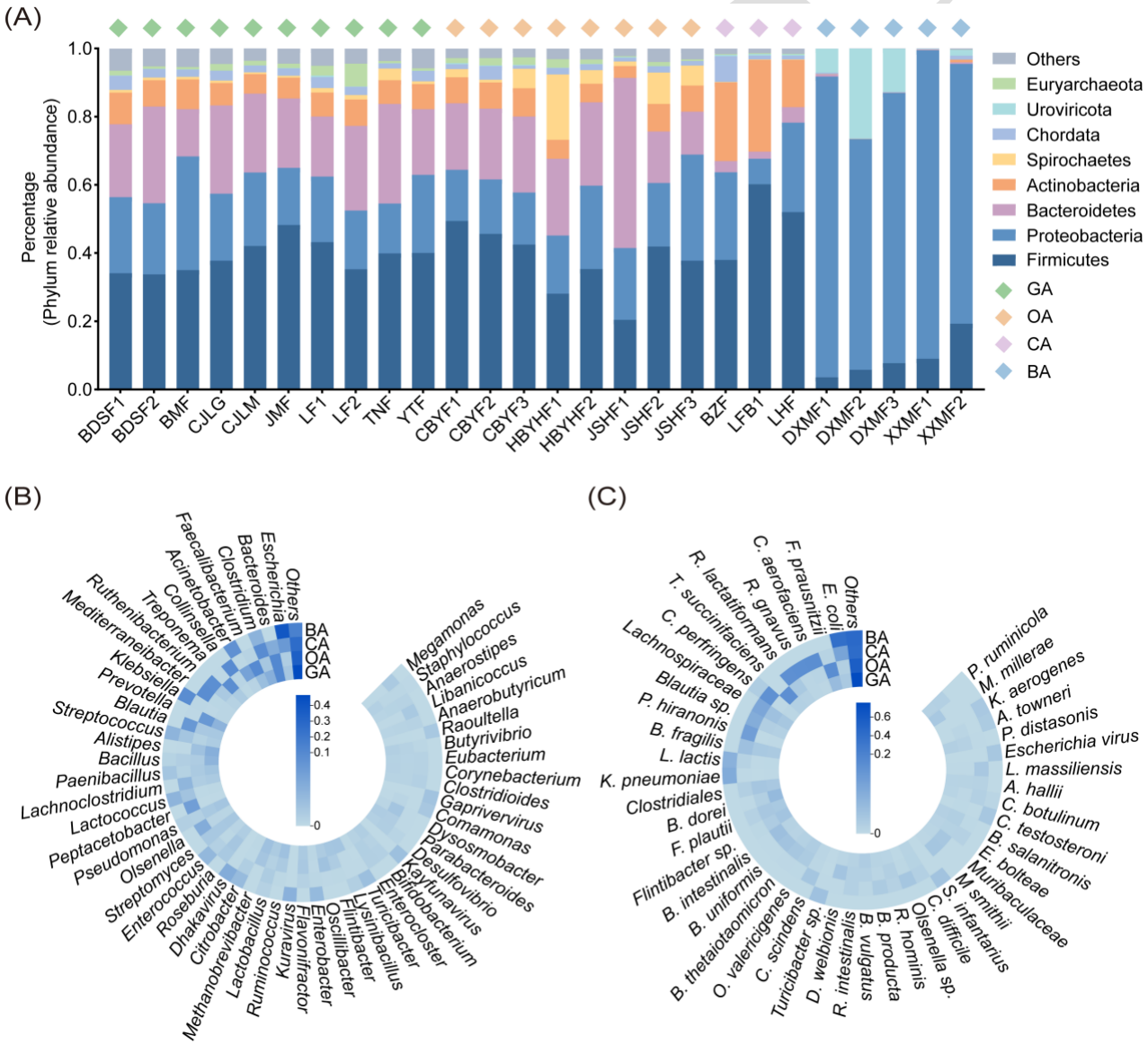

Fig. 3: Microbial Community Composition of Zoo Animal Fecal Samples. (A) Phylum-level composition. (B) Genus-level composition. (C) Species-level composition.

(Anosim, r=0.686, p=0.001), genus (Anosim, r=0.799, p=0.001), and species levels (Anosim, r=0.828, p=0.001) (Fig. 4C). These results underscore the strong association between fecal microbiomes and host dietary preferences, mirroring the patterns observed in fecal resistomes.

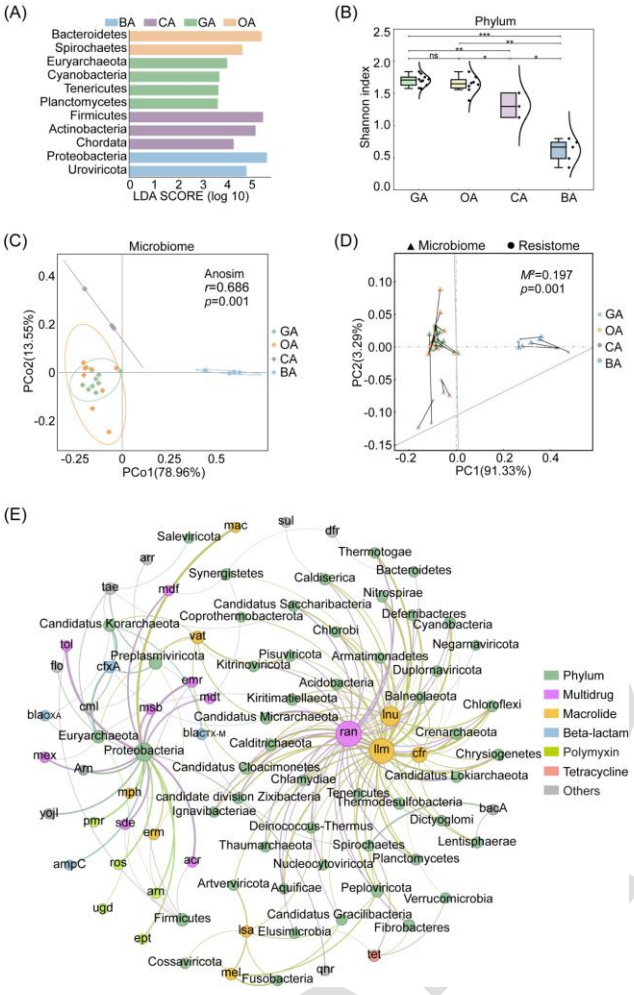

Fig. 4: Microbiome Comparison and Resistome-Microbiome Association in Zoo Animal Fecal Samples. (A) LEfSe analysis of microbial communities at the phylum level (LDA score > 3.5). (B) Shannon diversity of microbial communities at the phylum level. (C) Principal Coordinates Analysis (PCoA) of microbial communities at the phylum level (Bray-Curtis distance; ANOSIM, r=0.686, p=0.001). (D) Procrustes analysis showing correlation between fecal resistomes and microbiomes (M^2=0.197, p=0.001). (E) Network analysis of co-occurrence between 52 prominent ARG subtypes and microbial phyla (Nodes represent antimicrobial types and microbial phyla; edges indicate Spearman correlation, r>0.7, p<0.001).

Linkage Between Fecal Resistomes and Corresponding Microbiomes: Building on previous findings that resistome profiles in bird feces correlate with microbial taxonomic structures (Luo et al., 2022), we explored the relationship between fecal resistomes and microbiomes in zoo animals. Procrustes analysis revealed a significant correlation between microbial taxonomic structures (at the phylum level) and ARG profiles (M2=0.197, p=0.001, Fig. 4D). Network analysis based on Spearman correlation coefficients (r>0.7, p<0.001) identified co-occurrence patterns between 52 prominent ARG subtypes and microbial phyla (Fig. 4E). Notably, Proteobacteria were associated with multidrug efflux genes (*acr, emr, mdf, mdt, mex, msb, sde*, and *tol*) and polymyxin resistance genes (arn, ept, pmr, ugd, and ros), potentially explaining the high prevalence of these genes in BA samples (Fig. 1 and Fig. 3A). Additionally, macrolide resistance genes (*llm, lnu,* and *cfr*) were linked to multiple microbial phyla, suggesting that the high diversity of microbial phyla in GA and OA samples may contribute to the enrichment of these ARGs (Fig. 1 and Fig. 4B).

To elucidate the high abundance of tetracycline-resistant genes in GA, OA, and CA samples, we conducted network analysis based on Spearman correlation coefficients (r>0.7, p<0.001) between ARGs and predominant microbial genera. Tetracycline-resistant genes (*tet(A), tet(B), tet(L), tet(34)*, and *tet(37)*) were associated with *Faecalibacterium, Treponema*, and *Prevotella*, which were relatively abundant in GA and OA samples. This finding indicates that microbial community composition significantly influences the abundance of tetracycline-resistant genes in these samples, although the reason for their prevalence in CA samples remains unclear. Overall, our results highlight that microbial communities are a primary driver of ARG emergence and dissemination in the zoo environment.

Correlation of ARGs with ISs and Plasmid Types: To investigate the mobility of ARGs, we examined their correlations with insertion sequences (ISs) and plasmid types in animal fecal samples. Comparison of Shannon diversity indices revealed that CA and BA samples had higher IS diversity than GA and OA samples, mirroring the diversity patterns of ARGs (Fig. 5A). Conversely, the Shannon diversity of plasmid types in CA samples was significantly different from that in GA, OA, and BA samples, contrasting with ARG diversity (Fig. 5B).

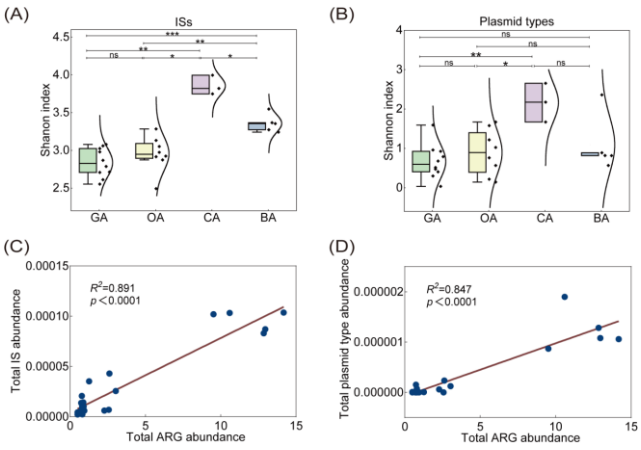

Fig. 5: Correlation of ARGs with Mobile Genetic Elements in Zoo Animal Fecal Samples. (A) Shannon diversity of insertion sequences (ISs) in each group. (B) Shannon diversity of plasmid types in each group. (C) Positive correlation between total ARG abundance and total IS abundance. (D) Positive correlation between total ARG abundance and total plasmid type abundance.

Linear regression analysis showed a positive correlation between total ARG abundance and both IS abundance (R^2=0.891, p<0.0001, Fig. 5C) and total plasmid type abundance (R^2=0.847, p<0.0001, Fig. 5D) in the 26 fecal samples. Network analysis based on Spearman correlation coefficients (r>0.8, p<0.001) further revealed strong associations between various

plasmid types (IncHI1, IncHI2, IncFI, IncFII, IncI, IncB/O/K/Z, IncX1, IncX3, IncR, IncY) and ARGs conferring resistance to multidrug, macrolide, beta-lactam, polymyxin, aminoglycoside and chloramphenicol. These findings suggest that ISs and specific plasmid types play significant roles in the transmission of ARGs among bacteria in the zoo environment.

DISCUSSION

Animal feces from zoo animals represent a significant reservoir of antibiotic resistance genes (ARGs), posing potential risks to both animal and human health. Despite this, the resistome of zoo animal origin remains underexplored (Min et al., 2023). Our investigation revealed a diverse array of resistance genes in animal fecal samples, indicating the complexity of ARG profiles in zoo animals. Intriguingly, our findings underscore substantial differences in resistome compositions among animals with distinct dietary preferences, particularly evident in the BA group, which exhibited a distinct ARG profile compared to other groups. Specifically, the BA group harbored a higher proportion of multidrug and polymyxin resistance genotypes, juxtaposed with a lower prevalence of macrolide and tetracycline-resistant genotypes. This observation is particularly concerning given the critical role of polymyxins in clinical settings for treating infections caused by multidrug-resistant (MDR) and extensively drug-resistant (XDR) Gram-negative bacteria (Jiang et al., 2020). The presence of polymyxin-resistant strains in zoo animals could potentially compromise clinical treatment efficacy, echoing findings by Liu et al. on the treatment failure risks associated with such resistance (Liu et al., 2016).

The high prevalence of multidrug and polymyxin resistance genes in the BA group may be attributed to their unique dietary habits and environmental exposures. For instance, bamboo-feeders (BA group) may have a diet that selects for specific gut microbiota capable of degrading complex plant materials, which in turn may harbor ARGs as a byproduct of microbial adaptation (Zhang et al., 2018).

Beyond ARGs, our analysis delved into the fecal microbiota of animals across different dietary groups. Utilizing Kraken for microbial species classification and quantifying each genus, we identified 63 bacterial phyla across 26 fecal microbial communities, with Firmicutes, Proteobacteria, and Bacteroidetes emerging as the dominant phyla. This finding aligns with previous studies (Min et al., 2023) while also revealing marked inter-group differences in microbial composition. Notably, microbial profiles within the same dietary group exhibited significant similarity, suggesting that diet exerts a profound influence on gut microbiota composition (Gupta et al., 2020). This dietary impact was not only evident at the phylum level but also manifested prominently at the genus and species levels, where substantial inter-group disparities were observed. For instance, the BA group's microbiota was characterized by a higher abundance of Proteobacteria, which may be linked to its unique dietary regimen. This phenomenon underscores the intricate relationship between diet and gut microbial ecology.

Recent studies have shown that diet can significantly shape the gut microbiota and its associated resistome. For example, a study by Turnbaugh et al. (2008) demonstrated that dietary changes can rapidly alter the composition of gut microbiota in humans, with potential implications for ARG profiles. Similarly, our findings suggest that the distinct dietary preferences of zoo animals may select for specific microbial communities that carry unique ARG profiles. This highlights the importance of considering diet as a key factor in managing ARG dissemination in zoo settings.

Moreover, our study unveiled a robust correlation between ARG composition and microbial communities at the phylum level, indicating that different bacterial species within the same phylum may share common ARGs. This finding suggests that ARG dissemination within specific phyla could be facilitated through horizontal gene transfer mechanisms, further complicating efforts to control ARG spread. Horizontal gene transfer has been identified as a major driver of ARG spread in microbial communities (Smillie et al., 2011), and our results support this notion by highlighting the interconnectedness of ARGs and microbial taxonomy.

Mobile genetic elements, such as plasmids and insertion sequences (ISs), are pivotal in ARG dissemination (Bahl et al., 2018). These elements can mediate the transfer of ARGs to other hosts, thereby exacerbating contamination risks (Li et al., 2023). Our results corroborate this by demonstrating a positive correlation between the abundance of resistance genes and that of ISs and plasmids, collectively termed the mobilome. Network analysis further revealed strong associations between various ARGs and specific plasmid types, highlighting the role of plasmids as key facilitators of ARG transmission. The high abundance of plasmids in zoo animal feces is particularly alarming, as it significantly heightens the risk of ARG transmission and poses a substantial burden on animal health.

Recent advancements in understanding the role of mobile genetic elements in ARG dissemination highlight the need for targeted interventions. For instance, studies have shown that certain environmental conditions can promote the transfer of ARGs via plasmids (Partridge et al., 2011).

In conclusion, our study highlights the need for further research and targeted interventions to address ARG dissemination in zoo settings. Given the intricate interplay between diet, microbiota, and ARG profiles, future research should focus on elucidating the underlying mechanisms driving these relationships. Additionally, monitoring and managing the mobilome in zoo environments could be a crucial strategy for curbing ARG spread. Further exploration of dietary modulation and its impact on gut microbiota and resistome dynamics may offer novel insights and potential mitigation strategies. For example, future studies may explore the potential effects of dietary interventions and probiotics on ARG profiles in zoo animals, based on our findings. This holistic approach will be essential in addressing the growing challenge of antibiotic resistance in zoo settings and beyond.

Conclusions: In this study, we performed a systematic analysis of the microbial composition, ARGs, insertion sequences, and plasmid types in the feces of zoo animals.

The results showed that ARGs in animal feces were diverse, and a variety of ARGs resistant to clinically important antibiotics were detected. In addition, mobile elements such as plasmids play an important role in mediating the spread of ARGs. These results suggest that we should strengthen the monitoring of ARGs in zoo animals and develop reasonable and effective control measures.

Data availability: The genome sequences in this study were deposited into the National Center for Biotechnology information under BioProject PRJNA1034944.

Declaration of competing interest: The authors declare no competing financial interest.

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