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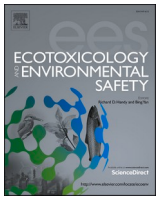

Metagenomic profiling of cecal microbiota and antibiotic resistome in rodents

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ABSTRACT

The rodent gut microbiota is a known reservoir of antimicrobial resistance, yet the distribution of antibiotic resistance genes (ARGs) within rodent cecal microbial communities and the specific bacterial species harboring these ARGs remain largely underexplored. This study employed high-throughput sequencing of 122 samples from five distinct rodent species to comprehensively profile the diversity and distribution of ARGs and to identify the bacterial hosts of these genes. A gene catalog of the rodent cecal microbiome was constructed, comprising 22,757,369 non-redundant genes. Analysis of the microbial composition and diversity revealed that *Bacillota* and *Bacteroidota* were the dominant bacterial phyla across different rodent species, with significant variations in species composition among the rodents. In total, 3703 putative antimicrobial resistance protein-coding genes were identified, corresponding to 392 unique ARG types classified into 32 resistance classes. The most enriched ARGs in the rodent cecal microbiome were associated with multidrug resistance, followed by glycopeptide and elfamycin antibiotics. Procrustes analysis demonstrated a correlation between the structure of the microbial community and the resistome. Metagenomic assembly-based host tracking indicated that most ARG-carrying contigs originated from the bacterial family *Oscillospiraceae*. Additionally, 130 ARGs showed significant correlations with mobile genetic elements. These findings provide new insights into the cecal microbiota and the prevalence of ARGs across five rodent species. Future research on a wider range of wild rodent species carrying ARGs will further elucidate the mechanisms underlying the transmission of antimicrobial resistance.

1. Introduction

The spread of antimicrobial resistance genes (ARGs) presents a significant public health challenge, threatening the effectiveness of antibiotic treatments (Theophilus and Taft, 2023). Antimicrobial resistance could have profound economic impacts, with costs projected to reach US $100 trillion by 2050 (Lange et al., 2018). Numerous ARGs have been identified, with nearly all antibiotics having corresponding resistance genes (Ventola, 2015). Environments such as soil, water, and animal waste are rich sources of diverse ARGs, contributing to the dissemination of natural resistome into bacterial populations through mechanisms like horizontal gene transfer (Smillie et al., 2011). The ability of bacteria to acquire ARGs via horizontal gene transfer is particularly concerning for public health (Pehrsson et al., 2013; Sun et al., 2019).

The gut microbiota, a diverse community of microorganisms in the gastrointestinal tract, includes eukaryotes, archaea, bacteria, and viruses, and plays a vital role in maintaining host health (Caballero-Flores et al., 2023; Flint et al., 2012; Martino et al., 2022). However, growing evidence shows that the gut microbiota can serve as a reservoir for antibiotic-resistant bacteria (ARB) and their associated ARGs (Hu et al., 2013; Wang et al., 2019; Xiao et al., 2016). Horizontal gene transfer of plasmids and transposons facilitates the exchange of ARGs among

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different species, particularly in microbial communities exposed to antibiotics (Martínez and Baquero, 2014). Bacteria harboring ARGs on mobile genetic elements (MGEs) are prevalent in many microbial communities, including those associated with humans and animals (Hu et al., 2016). Studies have demonstrated that gut bacteria carrying ARGs can be transmitted between animals and humans (Ding et al., 2022; Khezri et al., 2020). For instance, one study suggested that pet cats may influence the human gut resistome (Yang et al., 2023). Additionally, evidence links swine farming activities to alterations in the gut microbiota and resistome of nearby residents (Gao et al., 2024).

In recent years, wild animals have been recognized as vectors and secondary sources of ARB for both humans and animals (Manaia, 2017; Manaia et al., 2020). Studies have shown that the gut microbiomes of wild birds harbor a high diversity and abundance of ARGs (Cao et al., 2020; Rasmussen and Chua, 2023; Zhao et al., 2020). Microbiome analysis of feral swine, coyotes, domesticated cattle, and their surrounding environments reveals that wild animals harbor a higher abundance of antibiotic-resistant organisms than livestock (Lee et al., 2022). Despite not being directly exposed to antibiotics, wild animals, influenced by agriculture and environmental factors, still carry a significant number of ARGs (Jones et al., 2013; Wiethoelter et al., 2015) Rodentia, the largest Order of mammals, includes nearly 2600 species, representing 40 % of all mammal species (D'Elía et al., 2019). Rodents come into close contact with humans, particularly those living near human settlements, which increases their potential to contaminate the local environment with pathogenic organisms (Williams et al., 2018). Some rodent species such as the Norway rat *Rattus norvergicus* and the house mouse *Mus musculus* may enter homes and agricultural fields (Xu et al., 2015; Zhang et al., 2010), causing damage and spreading diseases (Gajda et al., 2017; Kosoy et al., 2015).

Some rodent species, such as *R. norvergicus* and *M. musculus*, carry ARB (Himsworth et al., 2014; Himsworth et al., 2016; Williams et al., 2018). Even wild, presumably antibiotic-naive rodent populations have also been found to carry ARB (Gilliver et al., 1999). However, the role of wild rodents in harboring ARGs is still not well understood. While carrying ARB isn't necessarily harmful, the risk arises when these bacteria are transmitted to human environments. Rodents living in landfills, sewage systems, or areas with poor sanitation, can acquire ARB from human waste. Antibiotic use in human medicine and agriculture induces selection pressure, leading to an increase in antibiotic resistance and an accumulation of ARGs in bacteria which can be released into the environment via wastewater and runoff (Hu et al., 2017), and rodents can encounter through contaminated water, soil, or waste. Once rodents carry ARB, they can transmit it to humans through direct contact, food contamination, or via other animals. The primary concern is the horizontal gene transfer of ARGs between bacteria in rodent and human microbiomes, perpetuating the spread of resistance (McInnes et al., 2020). In natural ecosystems, the interactions between ARGs and MGEs can promote the spread and exchange of resistance genes among microbial communities. The cecum, as a primary site of microbial activity in rodents, provides an ideal environment for these interactions. This study aims to investigate the extent of these interactions in the rodent cecum, offering new insights into the mechanisms of ARG dissemination in natural environments. To achieve this, metagenomic sequencing was conducted on the cecal contents of 122 individual rodents. The study first characterized the diversity and structure of the microbial communities in the rodent cecum and then assessed the antibiotic resistance profiles within these communities. Additionally, the study analyzed the transfer of ARGs between different bacterial species via MGEs within the rodent cecum.

2. Materials and methods

2.1. Trapping and sampling of wild rodents

A total of 94 wild rodents were collected for this study. *Lasiopodomys brandtii* (*L. brandtii*, $n = 43$) were captured in the wilderness of East Ujimqin County, Inner Mongolia Autonomous Region, China (N44°59′58" E116°6′19") in September 2018 and June 2019. *Spermophilus dauricus* (*S. dauricus*, $n = 27$) were captured in the Daqing region of Heilongjiang, China (N46°34′29″ E125°11′59″) in June 2018 and July 2020. *Apodemus agrarius* (*A. agrarius*, $n = 24$) were captured from two locations, 4 samples from Daqing, Heilongjiang (N46°34′29″ E125°11′59″) in August 2018, and 20 samples from Yueyang, Hunan (N29°28′35″ E112°46′29″) in August 2023. Detailed sample information is provided in Table S1a. Rodents were attracted using peanuts or sunflower seeds as bait. Traps were set after 6 pm and checked the following morning at 6 am to minimize captivity duration. The rodents were euthanized via cervical dislocation and dissected in the field, and their intestinal segments were collected and immediately transported to the laboratory under low-temperature anaerobic conditions. Cecal contents were collected under sterile conditions. All procedures were approved by Qingdao Agricultural University's Research Ethics Committee for the Care and Use of Laboratory Animals.

2.2. DNA extraction and metagenomic sequencing

The cecal contents from 94 wild rodents in this study were preserved at −80℃ until DNA extraction. DNA extraction was performed using the OMEGA Mag-Bind Soil DNA Kit (M5635–02, Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's instructions. The concentration and purity of the extracted DNA were assessed using the Qubit™ 4 Fluorometer and 1 % agarose gel electrophoresis, respectively. DNA samples meeting quality control criteria were utilized for library construction at a concentration of 10 nM. Sequencing libraries were prepared by Shanghai Personalbio Technology Co., Ltd., and sequenced using the Illumina HiSeq platform with paired-end (2 × 150) sequencing.

2.3. Data collection

The publicly available datasets from two rodent studies were obtained from the NCBI SRA database. These datasets included fecal metagenomes from 9 wild *Ictidomys tridecemlineatus* (*I. tridecemlineatus*), 10 wild *Mus musculus domesticus* (*M. m. domesticus*), and 9 laboratory *M. m. domesticus* y mice. Detailed sample information is provided in Table S1b. Metadata of the samples was retrieved from the respective research studies available in the NCBI BioSample database, identified by accession IDs PRJNA693524 (Regan et al., 2022) and PRJNA390686 (Rosshart et al., 2017).

2.4. Metagenome data analysis

The 94 samples collected in this study, along with 28 samples obtained from NCBI, underwent identical data processing procedures. The methods were as follows: Fastp (v0.23.0) (Chen et al., 2018) was used to obtain high-quality reads, which were then processed by Bowtie2 (2.5.0) (Langmead and Salzberg, 2012) to remove contamination from host genomic DNA sequences. Contigs were assembled using MEGAHIT (1.2.9) (Li et al., 2016). Gene prediction was performed with Prodigal (v2.6.1) (Hyatt et al., 2010), focusing on contigs longer than 500 bp. The predicted proteins were clustered using MMseqs2 (v7e284) (Steinegger and Söding, 2017) with parameters set at 90 % identity and 90 % overlap. Incomplete genes shorter than 100 bp were removed, and the remaining non-redundant genes were used for further analysis. Taxonomic assignment of microbial genes was conducted with BLASTN (v2.13.0) (Altschul et al., 1990), using the NCBI-NT database (v5.0, September 2023), which includes archaea, bacteria, fungi, and viruses. To unravel the functional profile of cecal microbiota in rodents, the non-redundant gene catalog underwent annotation using Carbohydrate-Active enZYmes (CAZys) classifications and Kyoto Encyclopedia of Genes and Genomes (KEGG). To quantify bacterial

abundance, clean reads (20 million per sample) were aligned to the gene catalog using Bowtie2 (v2.5.0) using default parameters. Mapped read counts for genes were normalized to Transcripts Per Kilobase of exon model per Million mapped reads (TPM).

2.5. Identification and annotation of ARGs and MGEs

ARGs were identified by aligning protein sequences of the genes to the Comprehensive Antibiotic Resistance Database (CARD, v3.2.7) using DIAMOND (v2.1.8.162) (Buchfink et al., 2015), with criteria set at >80 % sequence identity, >80 % query coverage, and an e-value of 1e-5. ARGs conferring resistance to two or more drug classes were categorized as multi-drug, excluding those conferring resistance to macrolide, lincosamide, and streptogramin antibiotics, which were grouped into the MLS. ARGs conferring resistance through a minimum of two mechanisms were categorized as multi-type mechanisms. MGEs were identified by aligning protein sequences of the genes against the MGE Database (Pärnänen et al., 2018) using BLASTN (v 2.13.0). Protein sequences (Open Reading Frame, ORFs) were annotated using the CARD database to identify ARGs, while their gene sequences were mapped to the NCBI NT database to annotate bacteria. If a gene was identified as an ARG and annotated to a bacterium, the bacterium was assumed to serve as the host of the ARGs. Due to the generation of the non-redundant gene catalog at 90 % identity of protein sequences, it is possible for multiple protein sequences to be annotated to the same type of resistance gene or microbial taxon. To assess the abundance of ARGs and MGEs, clean reads were aligned to the reference genes using Bowtie2 (v2.5.0) with default parameters, and the mapped read counts were normalized to TPM. To determine the prevalence of ARGs and MGEs, a threshold of TPM >100 was applied to identify their presence in a sample.

2.6. Statistical analyses

Rarefaction curves were generated based on gene profile using the vegan package (version 2.6–4). The six categories of CAZy were shown by the number of genes compared to the CAZy, and the relative abundance stack map was drawn based on the TPM profile of genes compared to the KEGG level B pathways. The Shannon's Diversity Index and bacterial species richness were calculated from the relative abundance of both taxonomic and functional genes. To assess β-diversity, Principal Component Analysis (PCA) was performed based on the Aitchison distance using the robCompositions package (version 2.4.1). Additionally, the Wilcoxon rank-sum test was used to assess the significant differences in diversity indices of the abundance of taxa among different rodent species.

Procrustes association analysis was performed based on the taxa and ARGs profiles using the 'procrustes' function in the 'vegan' package. Mantel tests were conducted based on family-level and ARGs profiles using the 'mantel_test' function in the 'LinkET' (version 0.7.4) R package, accessible at https://github.com/Hy4m/linkET. To identify the taxonomic assignment of the contigs harboring ARGs, Sankey plots were produced using the 'ggsankey' package (version 0.0.9), and gene arrow maps were generated using the 'gggenes' (version 0.4.1) package.

The co-abundance of ARGs and MGEs was analyzed based on ARGs and MGEs profiles using Spearman's correlation analysis with the 'corr. test' function from the psych package (version 2.4.6.26). Network graphs were visualized using the R package 'ggraph' (version 2.1.0). All other visualizations were produced using the ggplot2 package (version 3.3.6). Statistical analyses were conducted using R version 4.3.1 (Team, 2023).

3. Results

3.1. Construction of rodents' cecal microbiome gene catalog

A total of 47 million contigs, each with a minimum length of 500 base pairs (bp), were generated. These contigs had an average length of 1481 bp, with an average N50 value of 2450 bp (Table S2). A total of 89.3 million open reading frames (ORFs) were identified, of which 30.2 million (33.8 %) were predicted to be complete. Through clustering at 90 % nucleotide sequence identity, a non-redundant microbial gene catalog comprising 22,757,369 genes was constructed, with an average length of 626.4 bp (ranging from 102 bp to 128,319 bp). Rarefaction curve analysis revealed that gene richness gradually increased with the accumulation of sequencing samples, suggesting that the number of samples used in this study is limited, and further investigations should be conducted to supplement the rodent gene set (Fig. 1A). Within the nonredundant gene catalog, *L. brandtii* contributed 8.1 million (35.7 %) genes, *A. agrarius* provided 6.1 million (26.8 %) genes, and *S. dauricus* contributed 5.8 million (25.4 %) genes (Fig. 1B).

Out of 22,757,369 non-redundant genes, a total of 4852,339 genes were taxonomically classified as bacteria. These classified bacterial genes were distributed across 44 phyla, 544 families, and 2076 genera. At the phylum level, the majority of genes were *Bacillota* (48.4 %) and *Actinomycetota* (15.7 %), followed by *Pseudomonadota* (14.8 %) and *Bacteroidota* (13.3 %) (Fig. 1C). Meanwhile, 17,239,837 (75.8 %) genes could not be classified into any species. Among the classified genes, a small proportion were annotated as archaea (n = 7802, 0.1 %), viruses (n = 492,633, 8.9 %), and fungi (n = 164,758, 3.0 %), respectively (Fig. 1D).

Out of 22,757,369 genes, 9.81 % (2231,894) were annotated as CAZyme-encoding genes, spanning 415 CAZyme subclasses, including 16 auxiliary activities, 83 carbohydrate-binding modules, 19 carbohydrate esterases, 156 glycoside hydrolases, 105 glycosyltransferases, and 36 polysaccharide lyases. Glycosyltransferases and glycoside hydrolases were the most enriched enzymes (Fig. 1E; Table S3). Furthermore, approximately 41.16 % (9365,938) of protein-coding genes could be mapped to 9634 different KEGG orthologs. The top three abundant functional orthologs of the rodent cecal microbiome were B09182 (Protein families: genetic information processing), B09183 (Protein families: signaling and cellular processes), and B09101 (Carbohydrate metabolism) (Fig. 1F). Among the non-redundant proteins, 3703 were identified as antibiotic resistance protein-coding genes by aligning with CARD, belonging to 392 distinct ARGs (Table S4).

3.2. Characterization and comparison of cecal microbiota in different rodents

To gain a deeper understanding of the characteristics of rodent cecal microbiota, we conducted a comparative analysis of microbial community composition and diversity across various rodent species. Rarefaction curve analysis revealed that microbial community richness approaches a plateau with cumulative sequencing data, although there is a potential for discovering additional diversity (Fig. 2A). At the phylum level, *Bacillota* and *Bacteroidota* emerged as dominant phyla with the highest relative abundance. Interestingly, laboratory mice showed lower *Bacteroidota* abundance compared to the wild rodent species, while *Bacillota* exhibited the opposite trend (Fig. 2B, Fig. S1A). Furthermore, this study findings indicated that *Thermodesulfobacteriota* abundance was higher in *L. brandtii* and *A. agrarius* compared to others (p<0.05, Wilcoxon-rank sum test), while *Actinomycetota* abundance in *A. agrarius* was higher than in other species (p<0.05, Wilcoxon-rank sum test; Fig. S1B-C).

At the family level, differences in cecal microbial composition were observed across rodent species, particularly between wild rodents and laboratory mice (Fig. 2C). Specifically, *Lachnospiraceae* abundance was higher in laboratory mice compared to other species (p<0.001, Wilcoxon-rank sum test; Fig. S1D). Moreover, *Lactobacillaceae* relative abundance in *A. agrarius* and *M. m. domesticus* was higher than in other species (Wilcoxon-rank sum test, p<0.05; Fig. S1E), along with *Muribaculaceae* in *A. agrarius* and *L. brandtii* (p<0.05, Wilcoxon-rank sum test; Fig. S1F). Additionally, a substantial proportion of unidentified

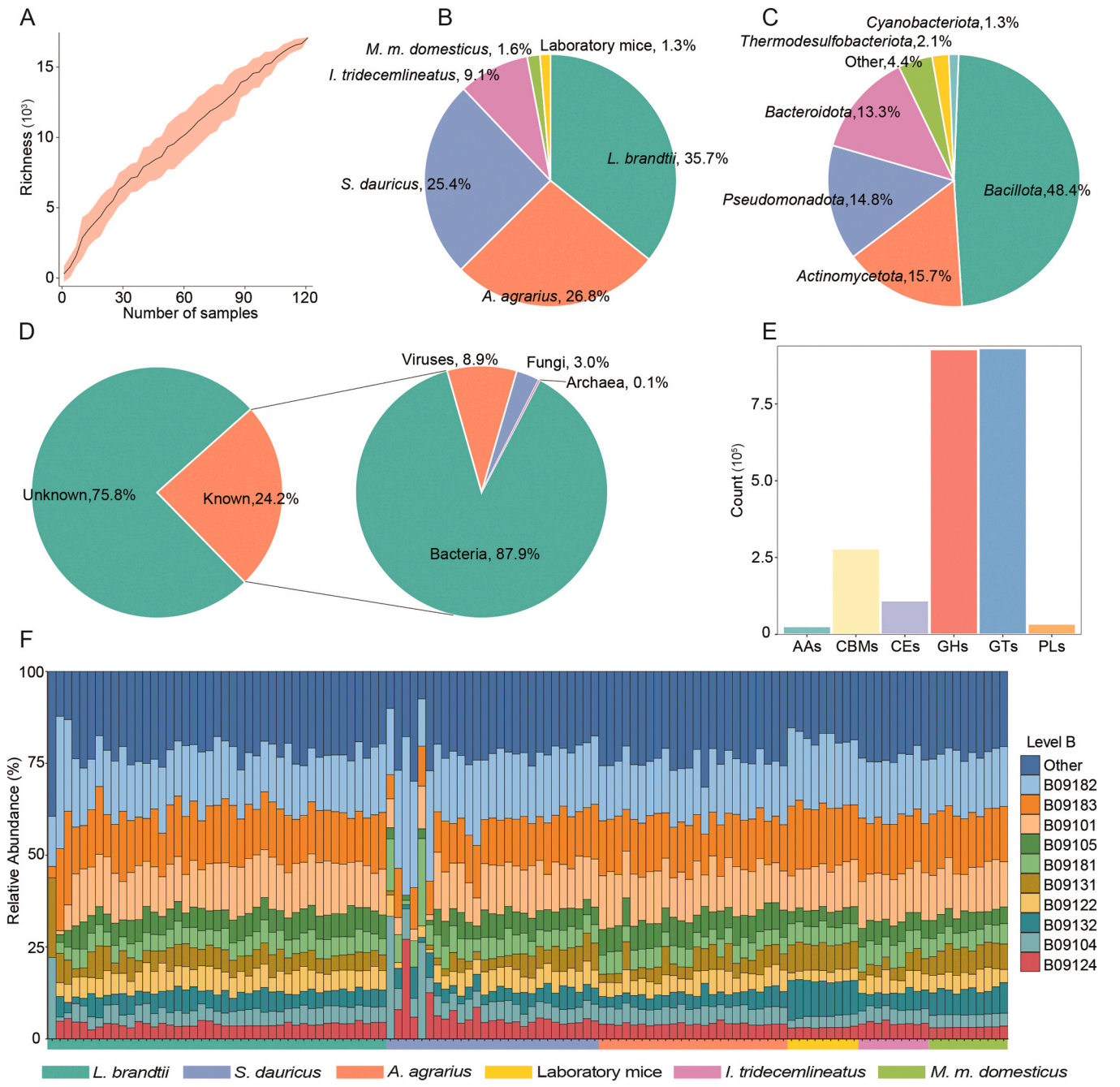

Fig. 1.. Composition and functional classification of 22,757,369 non-redundant gene catalog. A: Rarefaction curve analysis showing bacterial species richness. B: Pie chart illustrating the contribution of non-redundant genes from each rodent species. C: Pie chart displaying the composition of the cecal microbiota at the phylum level. D: Classification of non-redundant gene species annotation. E: Bar chart indicating the number of Carbohydrate-Active enZYmes (CAZymes) in each of the six categories. F: The 10 most abundant KEGG level B pathways across all tested samples, based on shotgun metagenomic sequencing data.

bacterial taxa were detected in rodent cecal microbiomes across various taxonomic levels, highlighting the need for further investigations.

Assessing diversity within microbial communities is crucial for understanding ecosystem health. In this study, the diversity of microbial communities across different rodent species was compared. In α-diversity analysis, the Shannon index revealed that microbial community diversity in laboratory was higher ($p<0.05$, Wilcoxon-rank sum test) than in other rodent species (Fig. 2D). The Richness index also showed differences among rodent species, with the lowest observed in *S. dauricus* and the highest in *M. m. domesticus* (Fig. 2E). PCA based on Aitchison distances illustrated differences in cecal microbial community structure among rodent species (Fig. 2F). The cecal microbiota structure of wild rodents differed markedly from that of laboratory mice counterparts. While variations in cecal microbiota were observed across different wild rodent species, there was some overlap. Intriguingly, wild rodents exhibited notable individual heterogeneity in their cecal microbiota, contrasting with the relatively uniform cecal microbiota observed in laboratory mice individuals.

3.3. *The cecal microbiota of rodents as a natural reservoir of ARGs*

Within the established gene catalog, a comprehensive set of 3703 putative proteins was identified as ARG-coding genes through alignment with CARD. These genes encompassed 392 distinct ARGs, including *tet*

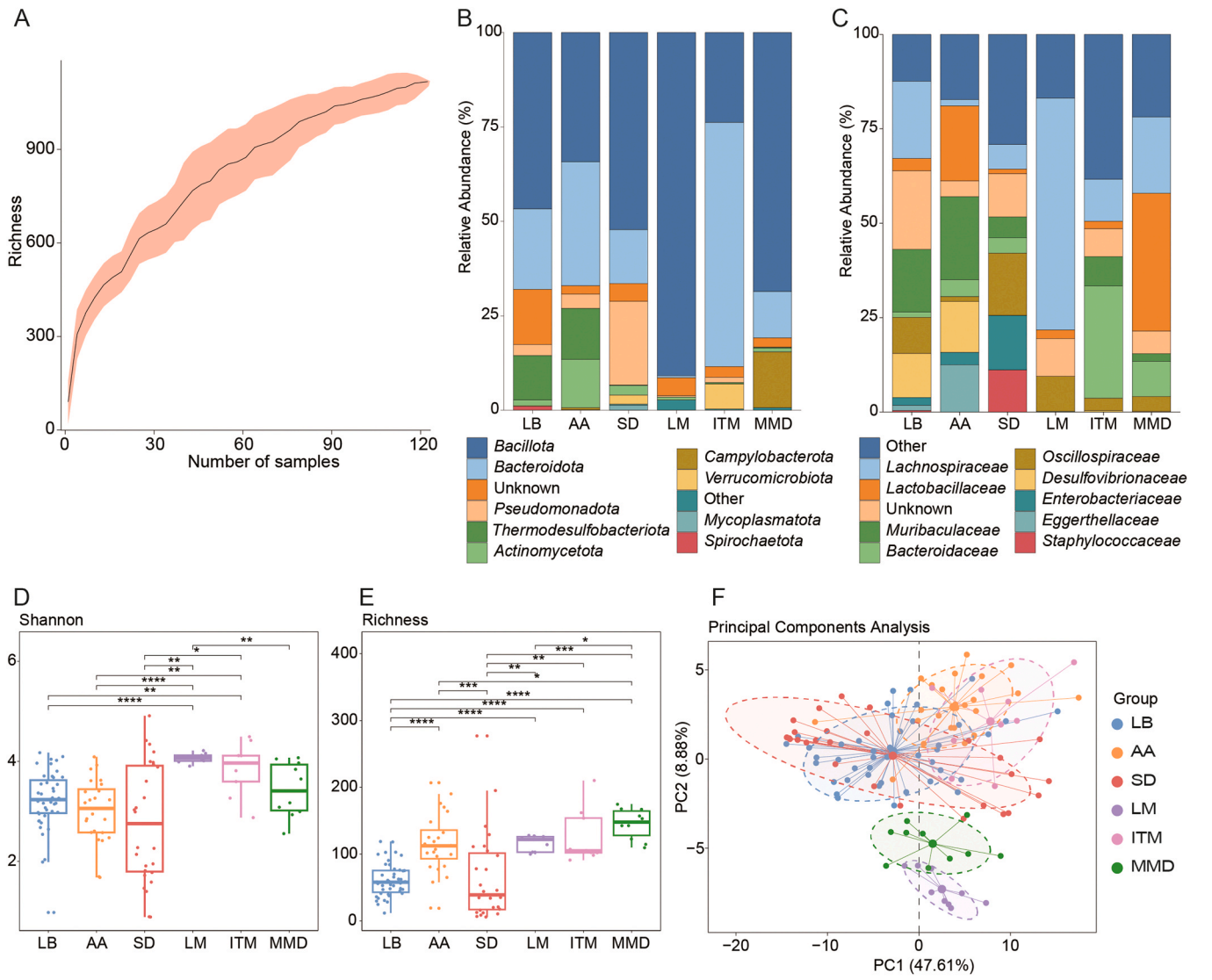

Fig. 2. Analysis of the composition and diversity in different rodent species. A: Rarefaction curve illustrating the relationship between sample number and richness. B-C: Stacked bar graphs depict the community composition of the cecal microbiota at both the phylum and family levels. D-E: Boxplots illustrating the species Shannon and Richness indices across all rodent species, respectively. Significance levels were determined using the Wilcoxon rank-sum test: *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$. F: The scatter plot exhibits β-diversity, representing the compositional variation of the cecal microbiome across all rodent species. LB: *Lasiopodomys brandtii*; AA: *Apodemus agrarius*; SD: *Spermophilus dauricus*; LM: laboratory *Mus musculus domesticus*; ITM: *Ictidomys tridecemlineatus*; MMD: *Mus musculus domesticus*.

(Q), *tet*(M), and *vanG*, which were associated with 32 resistance types (Table S4). Among the distinct ARGs, 217 (55.4 %) conferred resistance to a single drug class, such as peptide antibiotics (30, 7.7 %), glycopeptide antibiotics (27, 6.9 %), and tetracycline antibiotics (26, 6.6 %). Notably, 175 ARGs (44.6 %) exhibited resistance to at least two antibiotic classes. Within this subgroup, 0.8 % of ARGs demonstrated resistances to macrolide, lincosamide, and streptogramin antibiotics (MLS, Fig. 3A). Based on ARGs abundance, this study found that multi-drug resistance (31.0 %) was the predominant phenotype observed in the rodent cecal microbiome, followed by resistance to glycopeptide antibiotics (28.5 %) and elfamycin antibiotics (25.1 %) (Fig. 3B). Concerning resistance mechanisms, antibiotic target alteration (62.7 %) and multi-type mechanisms (27.2 %) emerged as the major contributors to ARG-mediated resistance, followed by antibiotic efflux (5.1 %) and antibiotic target protection (4.2 %) (Fig. 3C).

Fourteen ARGs were identified in over 80 % of the samples, indicating their widespread presence across the rodent cecal microbiome (Table S5). Particularly noteworthy is the detection of *Saur_rpoC_DAP*, *Cdif_EFTu_ELF*, *Ecol_EFTu_KIR*, *Efac_EFTu_GE2A*, *Cdif_rpoB_RIF* and *Cdif_rpoC_VAN* in all samples examined. These ARGs confer resistance to various drugs, a phenomenon often stemming from point mutations or sequence variations within specific regions of their DNA sequences. Conversely, a considerable portion of the ARGs (60.6 %) exhibited low prevalence, being present in fewer than 10 % of the samples. Noteworthy, *Cdif_rpoC_VAN*, *Cdif_rpoB_RIF*, *Cdif_EFTu_ELF*, *Efac_EFTu_GE2A*, and *Ecol_EFTu_KIR* were among the top five most abundant ARGs (Fig. 3D). Additionally, ARGs detected in at least 95 % of the samples were designated as core ARGs in this study, revealing nine such ARGs. These core ARGs primarily conferred resistance to elfamycin antibiotics through alterations in the antibiotic's target mechanism (Table S5). Furthermore, the investigation delved into the diversity and compositional differences in ARGs carried by various rodent species, alongside their corresponding drug-resistant phenotypes. Comparing the diversity and richness of ARGs among different rodent species revealed that *A. agrarius* harbored higher ($p<0.01$, Wilcoxon-rank sum test) diversity and richness of these genes compared to other rodent species. In contrast, *L. brandtii* exhibited the lowest levels of diversity and richness in its carried ARGs (Fig. 3E-F). Exploring unique and shared ARGs

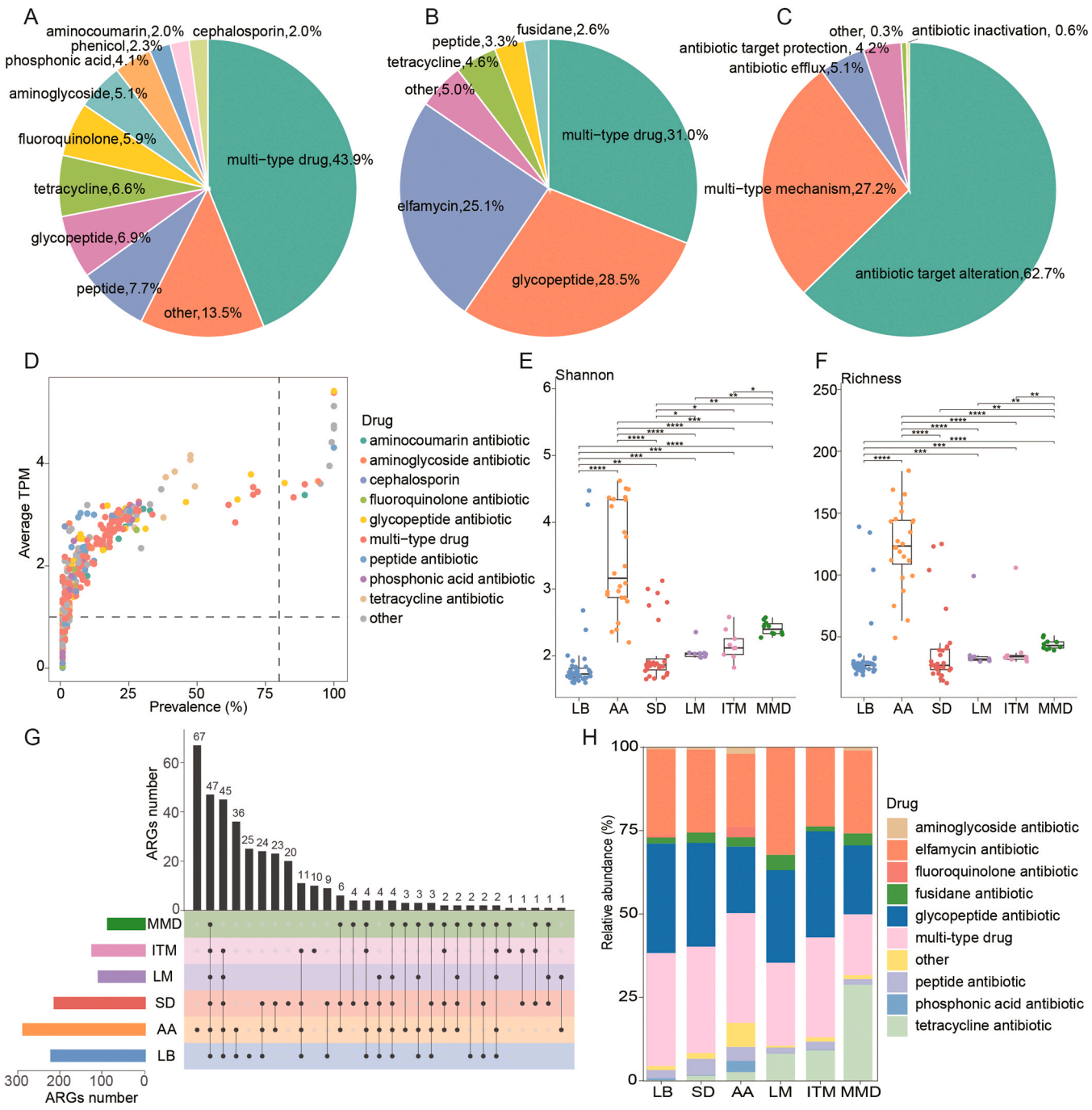

Fig. 3. Classification of ARGs and differences of ARGs carried by different rodents. A-B: Number and abundance of ARGs categorized by antimicrobial class. All ARGs were classified according to the antimicrobial they confer resistance to. Abbreviated names of antimicrobial classes are used throughout the figures, with corresponding full names provided in Table S3. C: Abundance of resistance mechanisms observed in 122 rodent samples. D: Prevalence and average relative abundance of the 382 ARGs detected in 122 rodents cecal samples. E-F: Shannon and Richness indices of the cecal microbiome ARGs in each rodent species. Statistical significance is denoted by asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. G: Upset plot illustrating shared and unique ARGs among each rodent species. H: Stacked bar chart showing differences in antibiotic classes carried by specific rodent species. LB: *Lasiopodomys brandtii*; AA: *Apodemus agrarius*; SD: *Spermophilus dauricus*; LM: laboratory *Mus musculus domesticus*; ITM: *Ictidomys tridecemlineatus*; MMD: *Mus musculus domesticus*.

among different hosts enhances our understanding of the diversity of ARGs. Among the five rodent species, 47 ARGs were commonly found, indicating widespread distribution among rodents (Fig. 3G). Only four ARGs were shared among the five wild rodent species. Regarding unique ARGs, *A. agrarius* carried the largest number, totaling 67 ARGs, followed by *L. brandtii*, *S. dauricus*, and *I. tridecemlineatus* species. Compositional analysis of the resistome suggested that multi-drug, glycopeptide, and elfamycin antibiotic phenotypes predominated within the ARGs profiles of the six rodent species, collectively comprising over 70 % of the relative abundance (Fig. 3H). Similar to the variations observed in the cecal microbiota, the compositional differences in the resistome were evident among different rodent populations. For instance, the tetracycline resistance phenotype was more prevalent in the *M. m. domesticus* compared to other rodents.

ARGs were broadly distributed among various bacteria, endowing their host bacteria with antibiotic resistance, and potentially leading to

the emergence of multiple drug resistance bacteria. In the present study, the host bacteria of ARGs were identified through the taxonomic assignment of the contigs harboring the ORFs of ARGs. A total of 3703 non-redundant ARG ORFs were distributed across 3450 contigs, with 3612 contigs annotated to bacteria (Table S4). Among these, bacteria from the phylum *Bacillota*, predominantly *Clostridia*, were the primary carriers of ARGs, accounting for 37.9 % of the total ARGs, followed by *Pseudomonadota* (Fig. S2). These bacteria, with the highest relative abundance in the wild rodent cecal microbiome, suggest potential implications for the health and ecology of rodents. Among the identified bacteria, *Oscillospiraceae* bacterium harbored the largest number of ARGs, with 145 ARG ORFs, followed by *Flavonifractor plautii* (85 ARG ORFs) and *Escherichia coli* (69 ARG ORFs) (Fig. S2; Table S4).

3.4. Cecal microbiota and mobilome related to resistome landscape

To better understand the impact of cecal microbiota composition on the resistome characteristics, this study investigated the correlation between the cecal microbial community and the antibiotic resistance profile. The results revealed a significant positive correlation between the Richness index of the cecal microbiota and those of the resistome (Spearman's correlation: R=0.44, p =5.3e-07; Fig. S3A), suggesting a

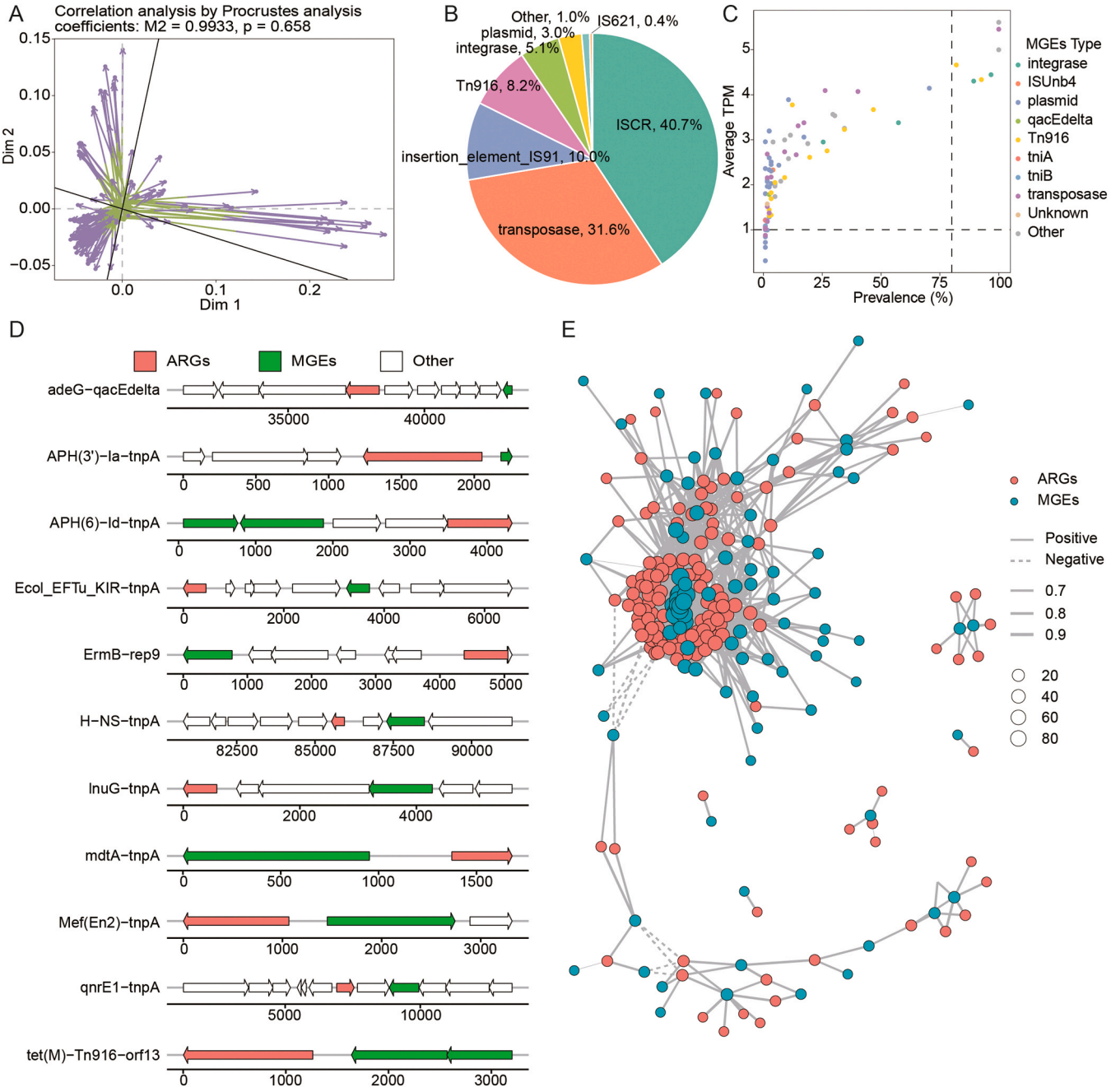

Fig. 4. Mobile genetic elements classification and their relationship with antimicrobial resistance genes. A. Procrustes correlation analysis exploring the correlation between cecal microbiota and resistome. B. Abundances of different types of MGEs across 122 rodents cecal samples. C: Prevalence and average relative abundance of MGEs identified in the 122 rodent cecal samples. D: Arrow diagrams illustrating specific combinations of ARGs and MGEs within the contigs. Right arrows denote genes on the forward strand, while left arrows indicate genes on the reverse strand. E: Network diagram depicting co-abundance patterns of ARGs and MGEs, with distinctly colored nodes representing different ARGs and MGEs. Solid lines signify positive relationships, dashed lines represent negative relationships, and the thickness of the lines reflects the strength of the correlation.

potential association between cecal microbiota composition and antibiotic resistance. Moreover, Procrustes association analysis unveiled a close correlation between the cecal microbiota and the resistance group (PROTEST, M^2=0.9933, p=0.658). It appeared that much of the difference in rodent cecal resistome could be attributed to differences in the microbiota (Fig. 4A). Mantel analysis demonstrated that the *Enterobacteriaceae* families exhibited significant correlations with resistance phenotypes to 10 different antimicrobial drugs (R>0.3, p<0.05, mantel test; Fig. S3D; Table S6), indicating their potential pivotal role in the observed shifts within the resistome composition.

MGEs are pivotal in facilitating the horizontal transfer of ARGs within and between bacterial cells. Understanding the distribution of MGEs in bacterial populations and their relationship with ARGs is crucial. A total of 1678 ORFs belonging to MGEs were identified from 122 metagenomes by aligning putative protein sequences of the gene catalog to the MGE database. These 1678 ORFs were associated with 87 MGEs and classified into 23 types, including ISCR, transposase, Tn916, and others (Table S7). The ISCR gene exhibited the highest abundance in the rodent cecal microbiome, constituting 40.7 % of the total abundance of 392s. Transposase genes, encompassing 19 MGEs, accounted for 31.6 % of the total abundance of MGEs. Additionally, 45 ORFs covering 31 MGEs were identified as plasmid genes. However, these plasmid genes comprised only 3.0 % of the total abundance of all MGEs (Fig. 4B). Furthermore, three MGEs, namely tnpA, ISCrsp1, and IS91, were found in all the samples examined (Fig. 4C; Table S8).

The analysis revealed significant positive correlations between the profiles of MGEs and ARGs as measured by both the Richness (Spearman's correlation: R=0.79, p =2.2e-16) and Shannon (Spearman's correlation: R=0.56, p<2.2e-16) indices (Fig. S3B-C). At the contig level, only 130 ARG-associated ORFs were found to co-occur with MGEs on the same contigs. Considering the gene mobility may influence ARG presence across sources and the potential importance of MGEs in developing effective surveillance systems (Ikhimiukor et al., 2022), we searched for ARGs within 5 kilobases (kb) of a MGE (Maciel-Guerra et al., 2023) and considered these MGE–ARG combinations to be potentially mobile ARGs. In total, 18 different MGE-ARG combinations (potentially mobile ARGs) were identified, featuring 15 unique ARGs (Fig. 4D, Table S9). This low co-occurrence rate may be attributed to the short contig length, given that the average length of bacterial genes is about 1000 bp (Koonin and Wolf, 2008), while the average length of contigs in the present study was only 1480 bp. Therefore, the relationships between ARGs and MGEs based solely on co-occurrence on the same contigs were likely underestimated. Consequently, the co-abundance relationships between ARGs and MGEs were explored, revealing significant correlations between the abundance of 144 ARGs and 25 MGEs (Spearman's correlation: $r \geq 0.6$, $p < 0.05$; Fig. 4E; Table S10). The analysis revealed that Transposase was associated with the most ARGs, while the five ARGs most associated with MGEs were AcrS, EC-13, evgA, emrY and mdtM. Additionally, only 4 MGEs exhibited negative correlations with ARGs, belonging to ISCR, TN916, and Transposase.

4. Discussion

Utilizing a constructed catalog of 22,757,369 non-redundant genes from metagenomic data of 122 rodents, this study characterized the rodent cecal microbial community, compiled an ARG catalog, explored the relationship between ARGs and MGEs, and identified the bacterial hosts of these ARGs. This comprehensive gene catalog provides a valuable reference for investigating the rodent cecal microbiome and advancing our understanding of antibiotic resistance mechanisms. With the establishment of this extensive gene catalog containing over 22 million nonredundant genes, only 5517,532 genes could be taxonomically classified, highlighting that a significant portion of the species in the rodent cecal microbiome remains uncharacterized. Coelho et al. (2022) also found that the number of unigenes in different species may be habitat-specific. Since this catalog is based on a limited number of rodent species, its generalizability to other rodent species may be limited. Additionally, various confounding factors, such as temporal stability, evolutionary differences, and DNA extraction protocols, are likely to influence the gut microbiota (Hugenholtz and de Vos, 2018; Laukens et al., 2016; Perlman, 2016). These factors may explain why the non-redundant genes found in the rodents captured in this study differ from those in publicly available rodent data. To better account for these confounders and reduce variability in rodent gut microbiota studies, it is essential to include a broader range of rodent species in future analyses.

The gut microbiota plays several essential roles in animal hosts, including the production of diverse metabolites and nutrients, and protection against pathogens and toxic compounds (Parfrey et al., 2018; Suzuki, 2017). Given that these functions significantly impact host health, variations in gut microbiota composition are crucial in shaping animal response to environmental changes (Alberdi et al., 2016). This study identified *Bacillota* and *Bacteroidota* as the dominant bacterial phyla in the rodent cecum The abundance of *Bacteroidota* in the cecum of laboratory mice was lower than in wild rodents. Since wild rodents have more complex diets, they rely on *Bacteroidota* to break down complex food molecules, which enhances host nutrition and immune regulation (Despres et al., 2016; Ulsemer et al., 2013). This likely explains why wild rodents have significantly higher levels of intestinal *Bacteroidota* compared to laboratory mice. Consistent with previous research (Bowerman et al., 2021), the *Lachnospiraceae*, *Oscillospiraceae*, and *Muribaculaceae* families were found to be widely distributed in the rodent cecum. These findings suggest that different rodent species may host distinct cecal microbiota compositions and diversity, influenced by diet, environment, or other factors, with potential implications for their overall health and physiology. The diversity analysis also revealed significant differences in the cecal microbiota between laboratory mice and wild rodents, likely driven by differences in dietary habits, living environments, and other ecological factors (McKenzie et al., 2017; Trevelline et al., 2019; Tsukayama et al., 2018; West et al., 2019).

Previous research has suggested that both humans and domestic livestock can serve as significant reservoirs of ARGs, with a large proportion of these genes in pathogenic bacteria originating from these sources (Liu et al., 2016). In this study, rodents were found to exhibit relatively high levels of ARGs compared to other animals like dairy calves (Liu et al., 2019), pigs (Zhou et al., 2022), and birds (Cao et al., 2020). Even without direct exposure to antibiotics, the rodents harbored a considerable prevalence of ARGs within their cecal microbiomes, indicating that the rodent cecum may act as a natural reservoir for these genes. Severe multiple antibiotic resistance was detected in rodents, with genes such as *Saur_rpoC_DAP*, *Cdif_EFTu_ELF*, *Ecol_EFTu_KIR*, *Efac_EFTu_GE2A*, *Cdif_rpoB_RIF* and *Cdif_rpoC_VAN* present in all samples. These genes confer resistance to a braod range of antibiotics, including peptide, elfamycin, glycopeptide, and the multi-type drugs. Resistance phenotypes were predominantly abundant in rodent cecal microbiome.

Elfamycins, which target the essential translation factor EF-Tu, represent an underexplored class of antibiotics that offer a novel approach to antibiotic therapy (Prezioso et al., 2017). The presence of these ARGs in rodents highlights the need for further investigation into their origins and potential transmission pathways to humans. Previous studies have shown that human activities have a profound impact on the environmental distribution of antimicrobials and ARGs (Di Cesare et al., 2024; Guan et al., 2022; Zhang et al., 2015). Although wild animals are not directly exposed to antibiotics (Jones et al., 2013; Wiethoelter et al., 2015), agricultural practices and environmental factors may explain the high prevalence of ARGs in the wild rodents studied here.

The emergence and spread of ARGs are primarily driven by the misuse and overuse of antibiotics (Wright, 2010). Bacteria acquire antibiotic resistance by carrying ARGs, and these bacteria can be transferred from the environment and animals to humans through various ways, such as wastewater (Hassoun-Kheir et al., 2020; Nguyen et al., 2021), airborne particles (McEachran et al., 2015; Song et al.,

2021), soil (Ondon et al., 2021), and feces (Chen et al., 2024), posing significant health risks to humans (Martínez, 2008).

Previous studies have shown that both urban and wild rodents carry numerous ARB (Williams et al., 2018). The most extensively studied bacteria within the resistome are enteric pathogens, often associated with food- and water-borne diseases (Stange et al., 2016; Wilson and Török, 2018). *Escherichia coli*, a symbiotic intestinal bacterium in animals, plays a crucial role in predicting the development and potential spread of antimicrobial resistance (Caruso, 2018; Silva et al., 2023). In this study, *E. coli* carried 69 ARGs, while the *Enterobacteriaceae* family contributed the most to the total number of resistance genes and exhibited significant correlations with resistance to 10 different antimicrobial drugs. These findings highlight the need for further research into the mechanisms of ARG transmission in wild rodents and their potential implications for human health. ARGs are often associated with MGEs, which facilitate horizontal gene transfer across different species, including pathogenic bacteria (Bag et al., 2019; Montassier et al., 2021). The data from this study suggest that certain MGEs play a significant role in promoting the horizontal transfer of ARGs among diverse bacterial species, with the t*npA* gene identified as the most dominant MGE, widely distributed across various bacterial taxa. Co-occurrence and co-abundance analyses revealed interactions between ARGs and MGEs, highlighting that these interactions may be key drivers of changes in the antibiotic resistance profile within the rodent cecum. These findings emphasize the need for future research to investigate the molecular mechanisms underlying these interactions and their impact on cecum microbial ecology.

5. Conclusion

By compiling over 22 million non-redundant genes from 122 rodents, this study established an extensive ARG catalog, investigated the relationship between ARGs and MGEs, and identified the bacterial hosts of these ARGs. This gene catalog serves as a valuable resource for further investigation into rodent cecal microbiome and antibiotic resistance mechanisms. The study also revealed significant differences in cecal microbiota composition between laboratory mice and wild rodents, likely driven by dietary habits, living environments, and ecological factors. Wild rodents exhibited higher levels of *Bacteroidota*, essential for breaking down complex food molecules, compared to laboratory mice. The detection of severe multidrug resistance in rodents underscores the urgent need for further research into the origins and potential transmission of ARGs to humans. Additionally, the study identified specific MGEs that facilitate the horizontal transfer of ARGs among bacterial species, highlighting the importance of further investigation into the molecular mechanisms underlying these interactions and their influence on cecal microbial ecology.

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CRediT authorship contribution statement

Kai-Meng Shang: Writing – original draft, Visualization, Software, Conceptualization. **Hany M. Elsheikha:** Writing – review & editing, Validation, Conceptualization. **He Ma:** Writing – review & editing, Supervision, Project administration. **Yong-Jie Wei:** Visualization, Formal analysis. **Ji-Xin Zhao:** Software, Data curation. **Ya Qin:** Investigation. **Jian-Ming Li:** Resources, Investigation. **Zi-Yu Zhao:** Methodology, Investigation. **Xiao-Xuan Zhang:** Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.117186.

Data availability

The data that has been used is confidential.

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