Animal Microbiome

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Metagenomic insights into the resistome, mobilome, and virulome of dogs with diverse lifestyles

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Abstract

Background Dogs—whether pets, rural, or stray—exhibit distinct living styles that influence their fecal microbiota and resistomes, yet these dynamics remain underexplored. This study aimed to analyze and compare the fecal microbiota and resistomes of three groups of dogs (37 pets, 20 rural, and 25 stray dogs) in Shanghai, China.

Results Metagenomic analysis revealed substantial differences in fecal microbial composition and metabolic activities among the dog groups. Pet dogs displayed the lowest microbial diversity. Using Shapley Additive Explanations (SHAP), an interpretable machine learning approach, *Ligilactobacillus* emerged as the most diverse genus, with significantly higher SHAP values in stray dogs, suggesting enhanced adaptability to more variable and less controlled environments. Across all samples, 587 antibiotic resistance genes (ARGs) were identified, conferring resistance to 14 antibiotic classes. A striking observation was the detection of *mcr-1* in one pet dog, indicating a potential public health risk. The *floR* gene was identified as a key differentiator in resistance profiles, particularly in pet and rural dogs, likely due to antibiotic exposure in their environments.

Conclusion This study provides the first comprehensive assessment of fecal microbiota and resistome variations among dogs with different lifestyles, revealing a less resilient microbiome and heightened antimicrobial resistance in pet dogs, which could have public health implications.

Keywords Pet dogs, Rural dogs, Stray dogs, Fecal microbiota, Antimicrobial resistance

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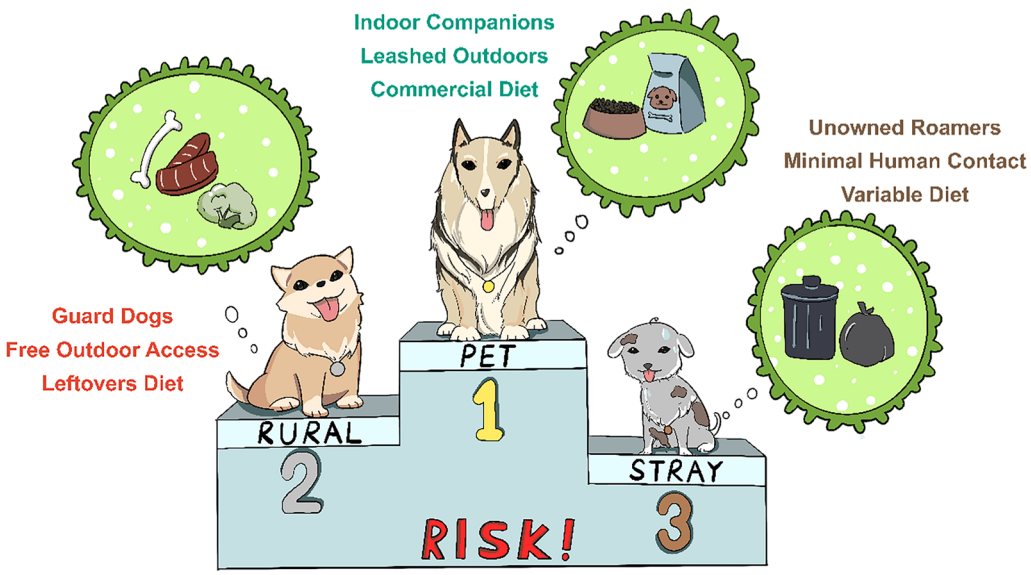
Graphical Abstract



Background

It is estimated that infections caused by antibiotic-resistant bacteria (ARB) have been associated with approximately 4.95 million human deaths in 2019 [1]. This alarming global burden prompted the World Health Organization (WHO) to recently prioritize combating antimicrobial resistance (AMR) as one of the most urgent human health issues [2]. ARB can acquire and transmit antibiotic resistance genes (ARGs) from various interfaces, such as animals and the environment, via horizontal gene transfer (HGT) [3, 4].

Studies have shown that food animals, wildlife, and companion animals serve as important reservoirs of ARGs [5–7]. However, most studies focus on food animals, as they account for two-thirds of total antibiotic use and can disseminate ARGs through the food chain [4, 5, 8]. Similar to food-producing animals, companion animals are also administered antimicrobial agents, including the highest priority critically important antimicrobials (HPCIA), like fluoroquinolones and third-generation cephalosporins[9]. Additionally, they often have close interactions with humans, engaging in close-contact behaviors such as kissing. Despite this, fewer studies have focused on AMR risk assessment in companion animals, even though critical ARGs and ARBs such as *mcr-1*, $bla_{\text{NDM-1}}$, and Methicillin-resistant *Staphylococcus aureus* (MRSA) have been identified in them [10–12]. Yang et al. found that ARG abundance in pet cats was higher than that in humans, and assessed the ARG risks in these animals [7]. They proposed that cat ownership could shape the resistome of the owner's feces, although the cat's resistance risk remains relatively low. Zhao et al. observed a strong correlation between macrolide ARGs in the fecal microbiota of dogs and humans, but emphasized only the key role of close relationships in this process without quantitatively assessing the risk of antibiotic resistance transmission [13]. These studies consider the owner and pet as a whole, overlooking the independent sources of ARGs in humans and pets. Recently, a cross-sectional study suggested that pets and humans might be two separate reservoirs, acquiring ARBs and ARGs from distinct sources [14], In this study, among only 9 of 299 families, both dogs and their owners carried $bla_{\text{CTX-M}}$ positive *E. coli* simultaneously, with only 1 of 9 families confirmed to have the same strain. This indicates that the occurrence of resistance between owners and dogs should not be solely attributed to close relationships, as it may be coincidental, although it can facilitate the exchange of resistance. When considering animals and hosts separately, there is currently a lack of completely understanding regarding the risk of ARGs' tranferabiltiy and pathogenicity. However, these studies primarily focus on companion animals as pets, lacking

comparisons with animals in other lifestyle contexts. The risk of ARG transmission in companion animals may differ depending on lifestyle, and understanding how various lifestyles impact fecal resistance risk can help clarify the role that close contact with humans plays in this process.

Therefore, we selected pet, stray, and rural dogs as research subjects. These dogs have completely different living conditions but are closely linked to human life. Pet dogs are fully integrated into human life with close, frequent contact, while stray dogs live independently, often relying on urban environments for survival with occasional indirect human interactions. Rural dogs fall somewhere in between, with moderate contact, often roaming freely but remaining associated with households. We employed a metagenomic approach to investigate the effects of the three lifestyles on the dog fecal microbiome and resistome. Our goal is to provide insights into the fecal microbiomes and resistomes of the three dog groups, while also assessing the risk of ARGs transmission among these groups.

Methods

Study design and sample collection

The three types of dogs included in this study have distinct characteristics: both pet and stray dogs live in urban areas, while rural dogs come from countryside regions [15]. Pet dogs have owners and live indoors, only allowed outside when on a leash, and their diet consists exclusively of commercial dog food. Stray dogs are unowned and roam freely outdoors in the city, with an unknown and highly variable diet. Rural dogs are kept by their owners to guard homes, with outdoor kennels in open yards, no leash restrictions, and free access to outdoor areas; they are typically fed their owners' leftovers. The pet dogs are kept for companionship and have lived with their owners for an extended period, followed by the rural dogs, while stray dogs only have occasional contact with humans.

In this study, we selected pet and rural dogs based on the following criteria: (1) no current acute illness; (2) no recent antibiotic use within the past 3 months. Our survey included data from 85 households, consisting of 55 urban and 30 rural families. We successfully obtained 57 anal swab samples from these households, including 37 from pet dogs and 20 from rural dogs. Additionally, 25 samples were collected from 40 stray dogs (Table S1).

Informed consent was obtained from all guardians of pet dogs and rural dogs participating in the study, and stray dog samples were collected with the local government's informed consent.

Samples were collected during November and December 2021. Samples from pet dogs were collected with the assistance of staff from a veterinary hospital. Samples from rural dogs were collected with the assistance from the local Animal Disease Prevention and Control Center staff. Stray dog samples were collected after the dogs were anesthetized, with anesthesia administered by the local public security department. The samples were stored in preservative solution (LifeGuard Soil Preservation Kit (catalog no: 12868–1000) from Qiagen) and temporarily placed in an insulated box containing ice packs. They were then transported to the laboratory within two hours and stored at − 80 °C.

DNA extraction and metagenomic sequencing

All samples were submitted to the Personal Biotechnology (Shanghai, China) for DNA extraction and metagenomic sequencing. Sequencing was performed using the IlluminaNovaSeq6000 high-throughput sequencing platform with 2 × 150 bp PE reads according to the Whole Genome Shotgun (WGS) strategy.

Total microbial genomic DNA were extracted using the OMEGA Mag-Bind Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's instructions, and stored at -20 °C before further assessment. The quantity and quality of extracted DNA were measured using a Qubit™ 4 Fluorometer, (WiFi: Q33238, Qubit™ Assay Tubes: Q32856; Qubit™ 1X dsDNA HS Assay Kit: Q33231) (Invitrogen, USA) and agarose gel electrophoresis. Genomic DNA was used to construct metagenome shotgun sequencing libraries with insert sizes of 400 bp by using the Illumina TruSeq Nano DNA LT Library Preparation Kit. Each library was sequenced by the Illumina NovaSeq platform (Illumina, USA) with a PE150 strategy at Personal Biotechnology Co., Ltd. (Shanghai, China).

Metagenome assembling and genomic bins

For comparability, all samples used in this study were processed using the same pipeline [16]. Quality control was conducted using Trimmomatic v0.39 [17]. Host (dog) genomic sequences were removed by alignment against host genomes using BWA-0.7.17[18] and SAMtools v1.18 [19]. The reference genomes for the host was *Canis familiaris* (GCA_000002285.4). The metagenome module of SPAdes v3.15.5 [20] was used for assembly. Qualified reads were submitted to MetaPhlAn2 [21] and EukDetect [22] for taxonomic profiling. All genomes were annotated using MetaGeneMark v3.38. MetaWrap v1.3 [23] including MetaBAT2, Maxbin2, Concoct, was used to bin genomes from contigs, and CheckM v1.1.6 [24] was performed to assess bin quality. The taxonomy of all bins was determined using GTDB-tk v2.3 with r214 database [25]. Metagenome assembly genomes were dereplicated using dRep v3.0.0 [26] with parameters set

to 99% ANI, > 50% completion, and < 10% contamination. After calling and aligning 120 bacterial conserved proteins from all dereplicated bins using GTDB-tk, phylogenetic analysis was performed on the filtered high-quality metagenome assembly genomes with IQ-TREE v2.0.6 [27].

Identification and analysis of ARGs, mobile genetic elements (MGEs), and virulence factors (VFs)

ARGs and MGEs were identified by calling genes from contigs using MetaGeneMark, followed by BLAST, searching with 80% identity and 70% coverage for data filtering. ARG identification, classification, and mechanism was referenced to the most recent version of ResFinder (updated in April 2023) [28]. MGE identification was referenced against MobileOG (updated in August 2022) [29], ISFinder (updated in October 2020) [30] and ICEberg 2.0 [31]. VFs identification was referenced against VFDB [32].

Assessment on risk of antimicrobial resistance and pathogenicity (RARP)

We followed and modified the MetaCompare pipeline [33] (https://github.com/minoh0201/MetaCompare) to assess antibiotic resistance and pathogenic risk of fecal microbiota in dogs. This was done by assessing the relative abundance of ARGs (proportions of ARG-associated contigs), ARGs with potential mobility risk (proportions of ARG-MGE-associated contigs) andVFGs with potential mobility risk (proportions of VFGs-MGE-associated contigs). Euclidean distances were calculated for the three risk factors to obtain a score for each.

$$score = \frac{1.0}{(2 + lg\sqrt{\left(0.01-\left(\frac{nARG}{nContigs}\right)\right)^2+\left(0.01-\left(\frac{nARG\_MGE}{nContigs}\right)\right)^2+\left(0.01-\left(\frac{nVFG\_MGE}{nContigs}\right)\right)^2})^2}$$

Statistical analysis and graphing

R and Python were used for microbial and ARG composition analyses, β-diversity analysis (PCoA), and permutation multivariate analysis (Adonis) to determine sample differences. Microeco packages [34] were used for Linear discriminant analysis Effect Size (LEfSe) and mapping. Significant box plots were generated using the ggpubr package and ggplot2 package [35]. Kruskal–Wallis and Mann–Whitney tests were performed using SPSS 26.0 to compare differences in the non-normal data and corrected using the Bonferroni method. A P value < 0.05 was considered statistically significant. The bacterial abundance is presented in the form of mean ± standard deviation. Venn diagrams and bar graphs were generated by the ggplot2 package and Prism 9.0.0. Correlation network analysis of bacteria and ARGs was conducted to infer potential hosts of ARGs. The Spearman's hierarchical correlation coefficients calculated using Python were subjected to construct the correlation network with $|\rho| > 0.8$ and P value < 0.01. The network was visualized using Gephi-0.10.1. Phylogenetic trees were produced using iTOL (https://itol.embl.de/). Antibiotic resistance and pathogenic risk scores were calculated using Python. The calculation formulas in Python are as follows:

df['distance'] = df.apply(lambda row: math.sqrt((0.01—row['fARG'])**2 + (0.01—row['fARG_MGE'])**2 + (0.01—row['fVFG_MGE'])**2), axis = 1).

df['score'] = df.apply(lambda row: 1.0 / ((2 + math.log10(row['distance']))**2), axis = 1).

Machine learning models for classifying bacterial species and ARGs among three groups of dogs

To validate the differences in microbial communities and ARGs among the three dog groups, we employed an interpretable machine learning method, SHappley Additive exPlanations (SHAP). SHAP values reinforced the differences identified by differential abundance analysis from LEfSe and Kruskal–Wallis and Mann–Whitney tests mentioned above. In brief, we applied several machine learning methods including Random Forest Classifier (RFC), Support Vector Machine (SVM) and Decision Tree (DT), using the ScikitLearn v1.5.1 (https://scikit-learn.org/) to predict the most distinct groups of bacteria or ARGs based on the abundance data of taxa and ARGs. Five-fold nested Cross-Validation (CV) was used for hyperparameter optimization. Model performance was assessed using accuracy, F1 score, precision, recall and Area Under the Curve (AUC) between folds. SHAP values were utilized to interpret predictions from all methods using the shap.TreeExplainer function of SHAP v0.46[36]. Feature contributions were extracted from each fold of the nested CV and consolidated into a consensus.

Results

Composition of fecal microbial communities in the three dog groups

According to MetaPhlAn2's analysis, sequenced reads from all samples were assigned to 12 different phyla at the phylum level. *Bacillota* had the highest abundance (42.3% ± 26.3%) among all samples, followed by *Pseudomonadota* (17.3% ± 21.4%), *Bacteroidota*

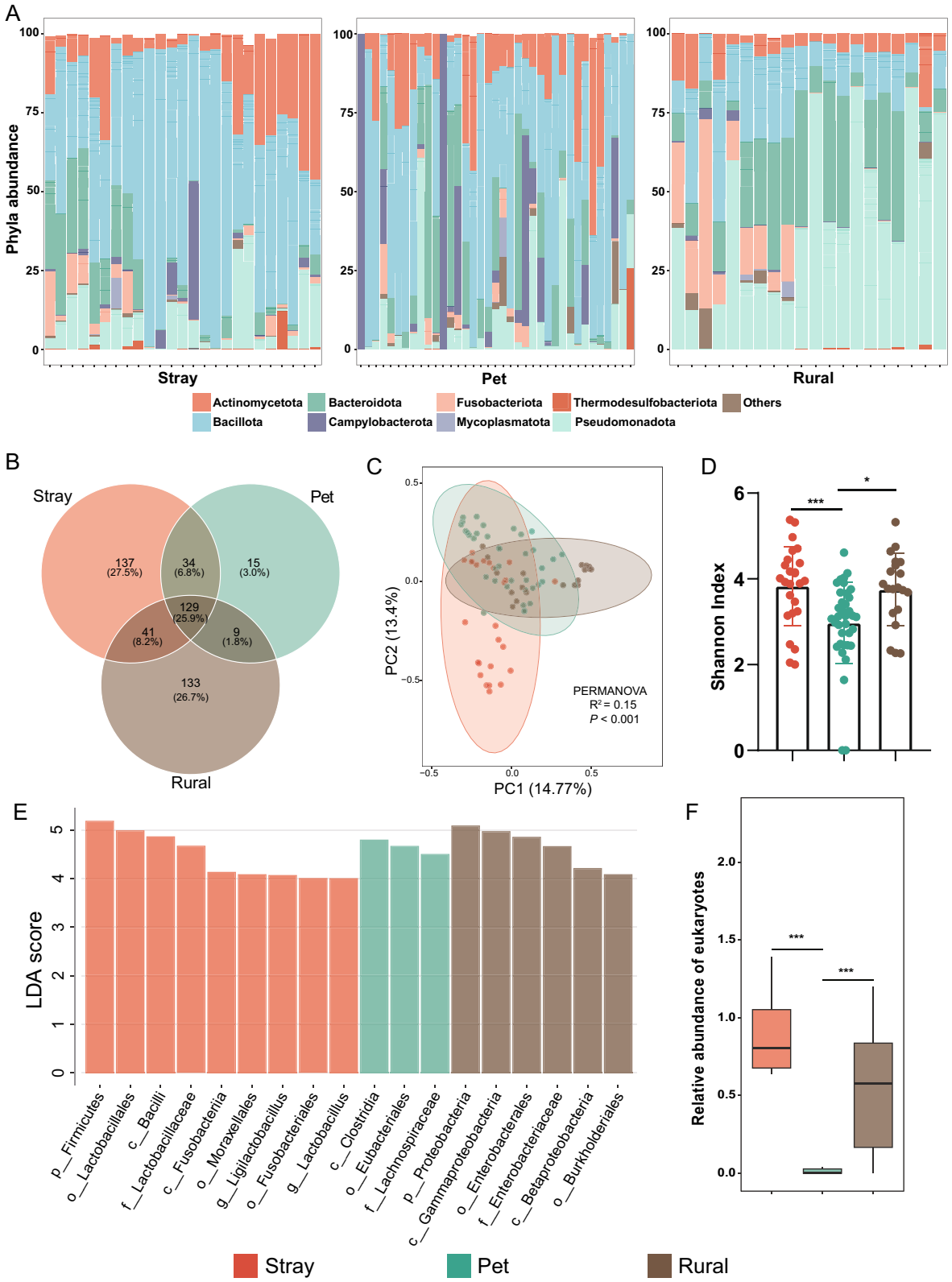


Fig. 1 Comparison of fecal microbiome in three dog groups. **A** Fecal bacterial composition at the phylum level. **B** Venn diagram of the fecal microbiota at the genus level. **C** Principal Coordinate Analysis (PCoA) of the fecal microbiota composition at the genus level based on the Bray–Curtis distance matrix. **D** The α-diversity (Shannon index), the error bars inside denote the mean with SD. **E** The LEfSe analysis of the fecal microbiota in three groups (LDA > 4). **F** Differences in fungal abundance among the three groups. Abbreviations: LEfSe, Linear discriminant analysis Effect Size

(14.5% ± 16.5%), and *Actinomycetota* (12.5% ± 13.4%) (Fig. S1A). The fecal bacterial composition at the phylum level showed considerable within-group variation. (Fig. 1A). Some low-abundance phyla, such as *Spirochaetota*, *Synergistota*, and *Verrucomicrobiota*, were detected exclusively in samples from stray dogs, while *Acidobacteriota* was found only in samples from rural dogs. The Archaeal phylum *Nitrososphaerota* was also identified in stray dogs, albeit with very low relative abundance (Table S2). The fecal bacterial composition of stray dogs and pet dogs was similar at the phylum level. *Bacillota* was the dominant bacterial phylum, accounting for 54.6% ± 22.0% in stray dogs and 47.1% ± 26.3% in pet dogs, significantly higher than in rural dogs (18.2% ± 18.6%) ($P < 0.001$). However, *Pseudomonadota* (41.4% ± 25.0%) were more abundant in rural dogs than in stray dogs (10.4% ± 9.7%) and pet dogs (9.4% ± 14.0%) ($P < 0.05$, Fig.S1B). In summary, *Actinomycetota, Bacteroidota, Bacillota*, and *Pseudomonadota* are the major divisions of the fecal microbiota in dogs, collectively accounting for approximately 90% or more of all bacterial phyla. At the class level, *Actinomycetota* is dominated by *Actinomycetia* and *Coriobacteriia*; *Bacteroidota* by *Bacteroidia*; *Bacillota* by *Bacilli* and *Clostridia*; and *Pseudomonadota* by *Gammaproteobacteria* (Table S3).

At the genus level, we identified a total of 498 genera, showing the number of unique and shared genera for each group (Fig. 1B, Table S4). We used Principal Coordinate Analysis (PCoA) to assess the similarity of the fecal microbial composition among dogs with different lifestyles (Fig. 1C). Rural dogs and pet dogs exhibited high similarity, whereas stray dogs shows a minimal overlap between the two groups, suggesting a potential difference in their composition. and displayed considerable within-group variability. The α-diversity indices (Fig. 1D), including observed genera and the Shannon index, showed that the diversity of fecal microbiota in pet dogs was lower than in rural dogs and stray dogs ($P < 0.01$), with no significant difference between rural and stray dogs ($P = 0.95$). We performed LEfSe analysis on microbial abundance profiles to identify the taxa associated with specific lifestyles (Fig. 1E). Across all dogs, 18 characteristic species (LDA > 4.0) were found: stray dogs had the most (n = 9), followed by rural dogs (n = 6) and pet dogs (n = 3).

Three fungi phyla were found in the dog feces: *Ascomycota, Basidiomycota,* and *Mucoromycota*. *Nematoda* was identified from only one stray dog feces (Fig. 1F, Table S5). *Ascomycota* and *Basidiomycota* were found in all dog groups, while *Mucoromycota* was found only in stray dogs and rural dogs. Comparing the eukaryotic abundances across the three dog groups, we found that stray dogs and rural dogs had higher eukaryotic abundance than pet dogs ($P < 0.001$).

Abundant ARGs in the fecal microbiota

A total of 587 ARGs were detected in the fecal microbiome and categorized into eight antimicrobial resistance mechanisms (Fig. 2A). The majority of ARGs were associated with target protection. PCoA analysis revealed that the abundance of fecal ARGs was more similar in rural and pet dogs, but distinct from that in stray dogs (Fig. 2B). The abundance of ARGs varied considerably among individuals within the stray dog group, mirroring the fecal microbial composition patterns across the three dog groups.

Rural dogs carried the highest number of ARGs (117 ± 66), followed by stray dogs (106 ± 30). Pet dogs had the lowest number (44 ± 20) (Fig.S2A). Despite the higher numbers of ARGs in stray and rural dogs, the relative abundance of all ARGs (represented by RPKM) averaged across stray dogs and rural dogs was lower than in pet dogs ($P < 0.001$, Fig.S2B). The abundance of resistance genes for aminoglycosides, beta-lactams, macrolides, tetracyclines, and multi-drug resistance in the feces of pet dogs was significantly higher than in the other two groups ($P < 0.01$, Fig. 2C). However, the distribution of specific ARGs varied among the three dog groups. ARGs conferring resistance to streptozotocin A was only found in stray dogs, while fosfomycin and glycopeptide resistance genes were found almost exclusively in rural dogs. Additionally, ARGs conferring resistance to polymyxin, such as *mcr-1*, *mcr-7*, and *mcr-10*, were found in both pet and rural dogs (Fig. 2C). An unexpectedly high relative abundance (RPKM = 165.12) of *mcr-1* was observed in one pet dog (M051) (Table S6).

(See figure on next page.)

Fig. 2 Comparison of ARGs among three dog groups. **A** Classification of ARGs referring to mechanisms. **B** Principal coordinate analysis (PCoA) based on Bray–Curtis distance in the composition of ARGs. **C** Comparison of relative abundance across antibiotic classes for all ARGs. **D** Co-occurrence network analysis of fecal microbial ARGs in dogs, with Spearman's correlation coefficient $|\rho| > 0.8$ and significant $P < 0.01$ correlation. The size of each node is proportional to the number of connections. **E** Co-occurrence network analysis of fecal microbial ARGs and genera in dogs, with Spearman's correlation coefficient $|\rho| > 0.8$ and significant $P < 0.01$ correlation. The size of each node is proportional to the number of connections

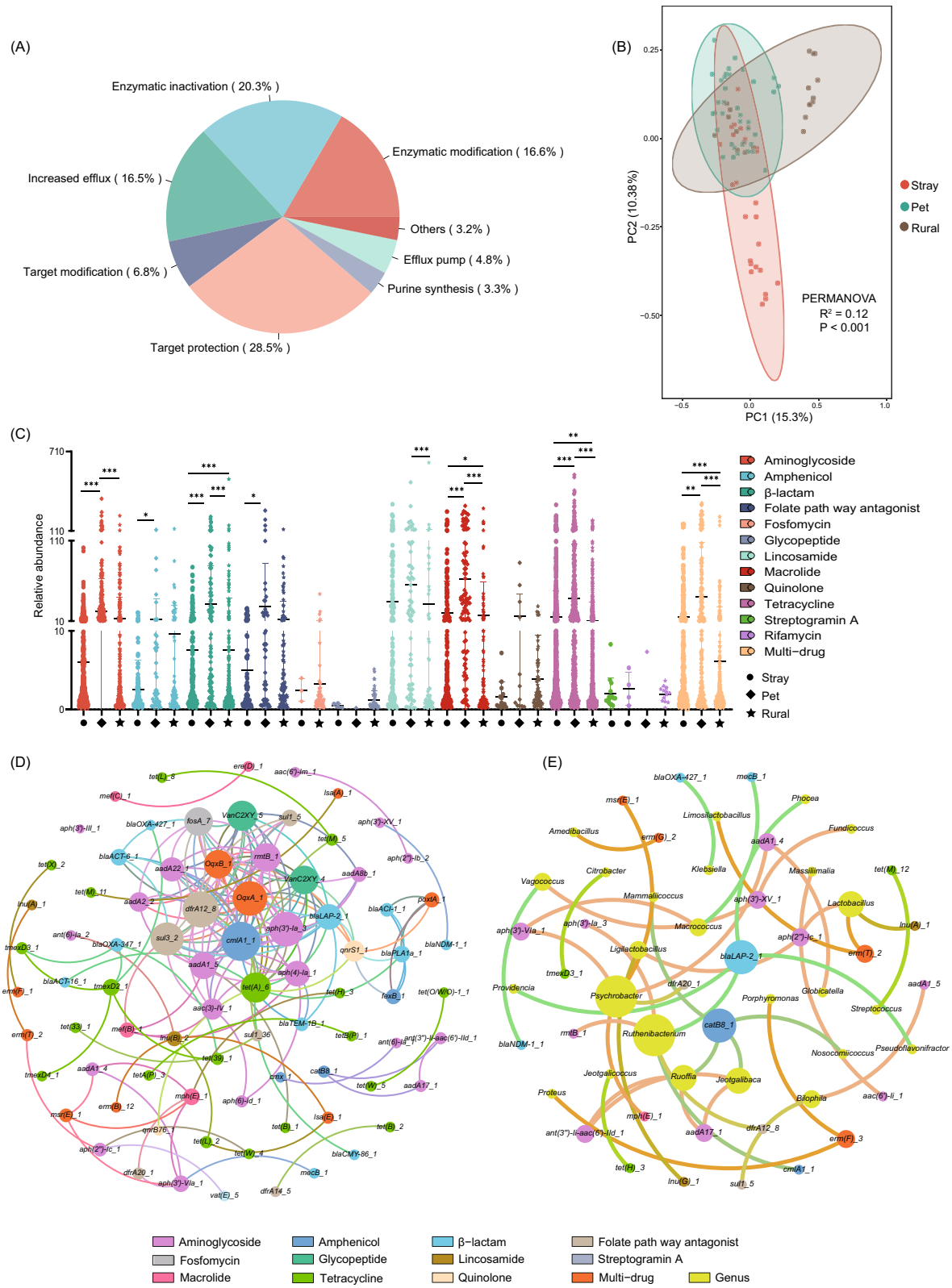


Fig. 2 (See legend on previous page.)

Co-occurrence network analyses of ARGs revealed high correlations among multiple classes of antibiotics and bacteria (Spearman's correlation coefficient $\rho > 0.8$, $P < 0.01$; Fig. 2D and Fig. 2E). Some ARGs, such as aminoglycosides resistance genes, *cmlA1*, bla_{LAP-2}, *sul1*, *sul3*, *dfrA*, *tet(A)*, *oqxA*, *oqxB*, and *qnrS1*, showed high correlations with multiple ARGs. *Psychrobacter*, *Ruthenibacterium*, *Lactobacillus*, *Ruoffia*, and *Jeotgalibaca* were highly correlated with at least three ARGs. The bla_{LAP-2} and *catB8* genes each exhibit a high correlation with four different ARGs. Additionally, six aminoglycoside resistance genes (*aph(3')*-VIa, *ant(3")-Ii-aac(6')-IId*, *aadA17*, *aph(2")-Ic*, *aph(3')-XV*, and *aadA1*) are each highly correlated with two distinct bacterial genera.

The correlation between MGEs and ARGs

We identified 1385 Insertion Sequences (ISs) belonging to 26 IS families. The number of ISs in pet dogs was significantly lower than in stray and rural dogs ($P < 0.001$, Fig.S3A). We compared the relative abundance of ISs among the three dog groups, finding significant differences between each other ($P < 0.001$, Fig. 3A). Additionally, we investigated the abundance of other mobile genetic elements in fecal microbiomes of three dog groups. Similar to ISs, we found that both integrative and conjugative elements (ICEs) and integrative and mobilizable elements (IMEs) were significantly more abundant in pet dogs than in the other groups ($P < 0.001$), while cis-mobilizable elements (CIMEs)

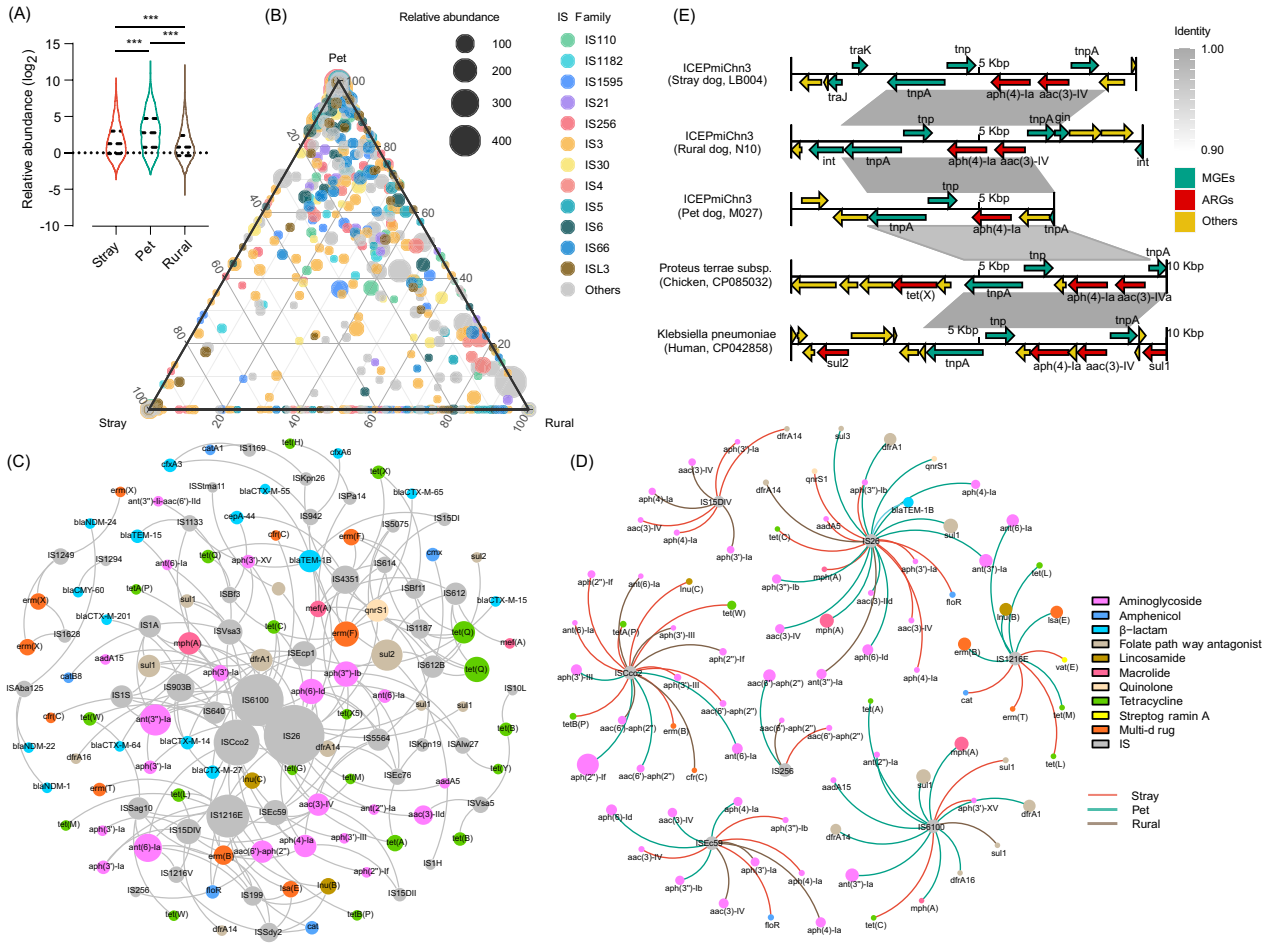


Fig. 3 The correlation between MGEs and ARGs. **A** Comparison of fecal T-ISs and T-ARGs in three dog groups. **B** Comparison of the dominance of IS families in three dog groups. The color of the bubbles represents the family of ISs, and the size of the bubbles represents the relative abundance of ISs. **C** Network analysis of ARGs and ISs for potential transmission risk. The size of each node is proportional to the number of connections and the color of the nodes represents the class of antibiotic (with gray representing IS). **D** Network analysis of dominant IS-ARGs in the feces of three dog groups. The color of the edges represents each dog group. The color of the nodes represents the classes of antibiotics (with gray representing IS), and the size of the nodes represents the relative abundance of ARGs. **E** The alignment of the representative ICE*Pmi*Chn3 ICEs (LB004, N10, M027) with transposons in other research on chicken (CP085032) and human (CP042858). Abbreviations: T-ARGs, Transfer-ARGs; T-ISs Transfer-ISs; ICE, Integrative and Conjugative Element

were only found in pet dogs and stray dogs ($P < 0.001$, Fig.S3B).

To evaluate the spread potential of ARGs, we analyzed Transfer-ARGs (T-ARGs) and Transfer-ISs (T-ISs), which are ARGs and ISs coexisting on the same contig within a distance of less than 5 kb upstream or downstream. We observed notable differences in both T-ARGs and T-ISs for pet dogs compared to the other two groups ($P < 0.001$, Fig.S3C). T-ISs and T-ARGs are over-represented in pet dogs, while rural dogs have the lowest number of T-ISs and the lowest relative abundance. Grouping IS families by relative abundance within each group, we observed that most high-abundance IS sequences were found in pet dogs, with a few IS families, such as IS*3* and IS*66,* accounting for the majority of sequences (Fig. 3B).

We then conducted a network analysis of T-ARGs and T-ISs (Fig. 3C and Fig. 3D) containing 89 ISs and 110 ARGs conferring to 10 classes of antibiotics. Among the ARGs, various ARGs pose potential transmission risks, especially those conferring aminoglycoside resistance, such as *aac(3)-IV*, *ant(3")-Ia*, etc. as well as the extended-spectrum Beta-lactamase (ESBL) genes, such as bla_{SHV}, bla_{TEM}, etc. We also identified the quinolone resistance gene *qnrS1*, which poses a potential transmission risk, despite quinolones being synthetic antibiotics.

Among the IS families found in our study, the IS*1595* family was the most dominant with ISs-ARGs. IS*Cco2* and IS*Sag10* were its main members, widely present in all groups. IS*Cco2* was associated with a wide range of ARGs, particularly aminoglycosides (96% of the IS-ARGs), while IS*Sag10* showed a strong association with phosphomycin ARGs (98% of the IS-ARGs). Additionally, IS*Kpn19* of the IS*Kra4* family was repeatedly identified around *qnrS1*. Notably, carbapenem resistance genes including $bla_{\text{NDM-1}}$, $bla_{\text{NDM-22,}}$ and $bla_{\text{NDM-24,}}$ were identified in multiple stray dog samples, often associated with IS*Aba125*. The gene $bla_{\text{CTX-M}}$ was mainly associated with IS*5* and IS*1380*, while the bla_{TEM} was widely linked to IS*5*, IS*3*, IS*200/*IS*600*, IS*6*, and IS*66*. Moreover, endemic IS families were observed in different dog groups. For instance, the IS*1* family was almost exclusively found in pet dogs and was associated with ARGs conferring resistance to tetracyclines, sulfonamides, and macrolides. The most abundant IS*6*-related gene, IS*Ec59*, found in ICE*Pmi*Chn3, was discovered in all three dog groups, exhibiting similar structures carrying aminoglycoside ARGs (*aac(3)-IV* and *aph(4)-Ia*) (Fig. 3E).

MAG-based analysis of antibiotic resistant bacteria in three dog groups

In this study, we performed metagenomic binning on individual swab samples, creating a set of Metagenome-Assembled Genomes (MAGs) with an estimated completeness ≥ 50% and an estimated contamination ≤ 10%. A total of 1,832 MAGs were assembled from anal swabs of stray dogs (n = 803), pet dogs (n = 509), and rural dogs (n = 520) (Table S7). These MAGs were classified into eleven phyla, with *Bacillota* (n = 1095), *Actinomycetota* (n = 162), *Pseudomonadota* (n = 185), *Bacteroidota* (n = 247), *Fusobacteriota* (n = 64), and *Campylobacterota* (n = 62) being the dominant (Fig. 4A). We compared the relative abundance of ARGs, ISs, and VFGs carried by MAGs in the three groups. Pet dogs had significantly higher abundances of ARGs and ISs compared to stray dogs and rural dogs. For VFGs, pet dogs showed highest abundance, while stray dogs had the lowest among the three groups (Fig. 4B).

Among the three dog groups, pet and stray dogs shared more similar phyla structures. Approximately 85% of MAGs in pet dog swabs were annotated to *Bacillota* (66.6%), *Actinomycetota* (10.4%), and *Bacteroidota* (10.2%). Similarly, these phyla accounted for 61.7%, 9.5%, and 13.4% of MAGs in stray dog. However, *Bacillota* (49.8%), *Pseudomonadota* (18.8%) and *Bacteroidota* (16.7%) were the most prevalent in the rural dogs. *Bacillota* is the most dominant phylum in all three dog groups (Fig. 4C). Among the six phyla, *Campylobacterota* carried the fewest ARGs (< 0.1 per MAG). The other five phyla-level bacteria have a high prevalence of tetracycline-resistant ARGs. Beta-lactam-resistant ARGs were more abundant in *Bacteroidota* and *Pseudomonadota*, while *Bacteroidota* carried a higher number of multidrug-resistant ARGs (> 0.1 per MAG) (Fig. 4D).

Of the 1832 MAGs, 407 carried 13 types of ARGs, with tetracycline, aminoglycoside, multidrug, Beta-lactam, and lincosamide resistance genes being the most frequently detected. In these 407 ARG-carrying MAGs, 263 might undergo HGT of ARGs due to the presence of IS family genes. Moreover, it's noteworthy that some bacteria exhibit both potential multi-antibiotic resistance and pathogenicity characteristics. 169 ARG-carrying MAGs revealed pathogenic potential by harboring various VFGs (called pathogenic antibiotic-resistant bacteria, PARB), spanning 30 different genera including *Escherichia* (25/169), *Peptacetobacter* (20/169), *Enterococcus* (13/169), *Blautia* (11/169), *Corynebacterium* (11/169), and others (Table S8). A wide range of PARB MAGs also carried ISs (134/169), which further facilitates the dissemination of ARGs and simultaneously poses significant threats to human health. Among these PARBs, 18 harbor at least 3 ARGs and 10 VFGs, belonging to *Escherichia, Klebsiella, Streptococcus, Enterobacter, Enterococcus*, *Proteus* and *Phocaeicola*. Particularly, *Escherichia coli* (7) and *Klebsiella pneumoniae* (3) MAG are the most common, both of which are important enterobacteriaceae pathogens. Important Beta-lactam resistance genes such

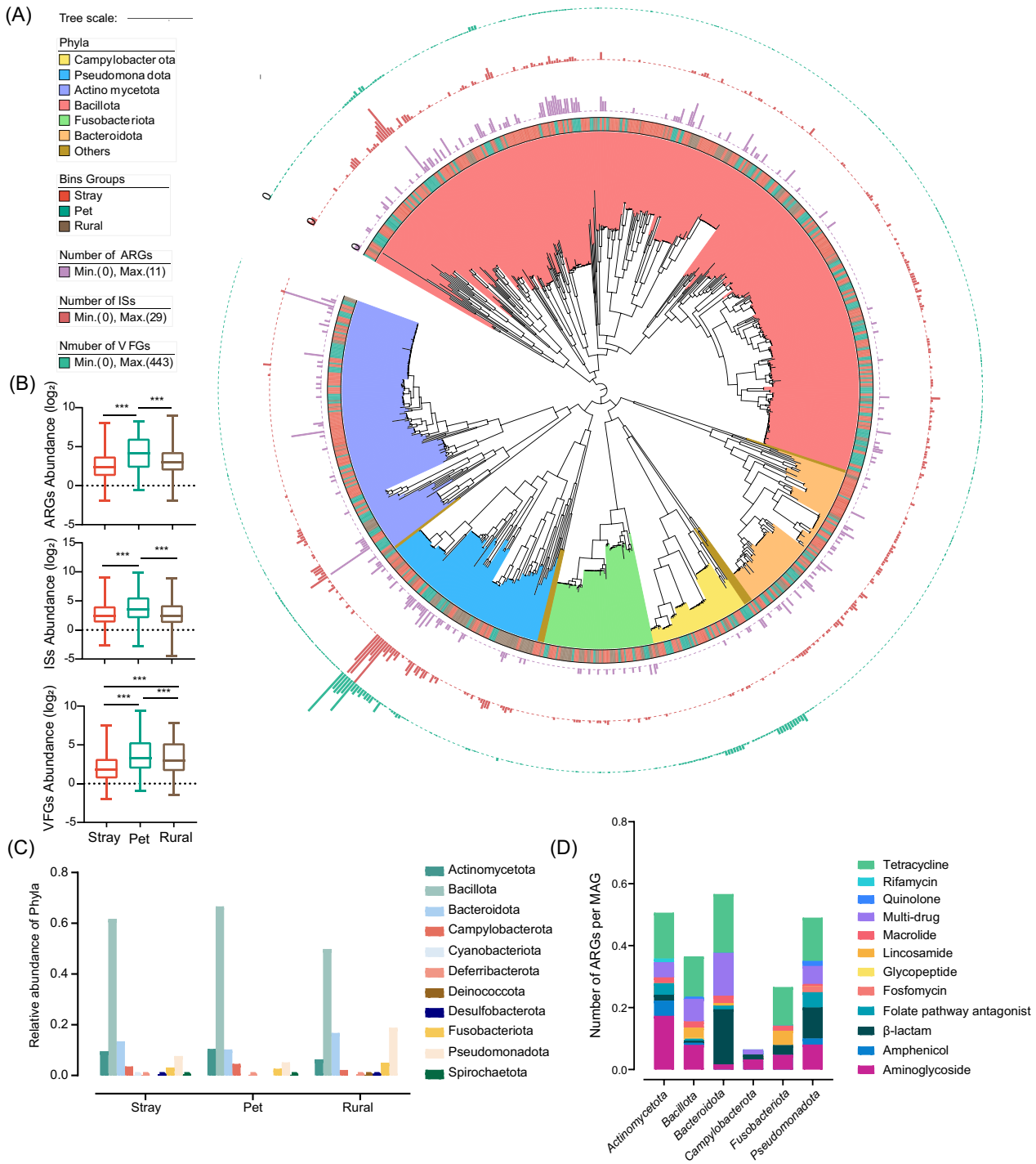


Fig. 4 Overview of MAGs in three dog groups. **A** Phylogenomic tree of dereplicated MAGs from all samples. Inner to outer rings represent phylum taxonomies, bin groups, and numbers of ARGs, ISs, and VFGs of each MAG. **B** Comparison of relative abundance of ARGs, ISs and VFGs of MAGs among three groups. **C** Percentage of phylum taxonomies in different dog groups. **D** Classification of antibiotics in MAGs associated with ARGs across various phyla from all samples

as bla_{NDM}, bla_{TEM}, and bla_{SHV} were also found in eight PARB MAGs belonging to *Klebsiella* (3), *Anaerobiospirillum* (3), *Corynebacterium* (1), and *Escherichia* (1), although they may not exhibit multiple resistance. Notably, the genes bla_{NDM-5} and $bla_{SHV-191}$ were present in a *Klebsiella pneumoniae* MAG from sample N09.

Differences in the shaping of the fecal microbiome and resistome in dogs living in various environments

We used machine learning methods to deeply explore the differences in the fecal microbial communities and ARG distributions among the three dog groups (pet, rural and stray dogs). In the microbial community analysis, the random forest model performed the best, achieving an average accuracy of 0.888 and an F1 value of 0.882 for the genus-level classification (Table S9, Fig. S4A). Among them, the distributions of the genera *Ligilactobacillus* and *Limosilactobacillus* were significantly different (Table S10, Fig. 5A). Notably, *Ligilactobacillus* was significantly more abundant in stray dogs than in other groups. For ARG classification, the random forest model performed the best, obtaining an average accuracy of 0.815, an F1 value of 0.808, a Precision value of 0.870, and a Recall value of 0.815 (Table S9, Fig.S4B). Further SHAP value analysis revealed important features predicted by the model, especially the high SHAP value of the *floR* gene in different dog populations, indicating that *floR* may be a key factor contributing to the differences in ARGs between different populations (Table S11, Fig. 5B).

We evaluated the risk of antibiotic resistance and pathogenicity (RARP) across all samples, assigning RARP scores to 82 dog samples. The RARP ranged from a minimum of 17.63 (M052 from pet dog) to a maximum of 34.69 (LGB1 from stray dog). To compare the level of RARP among the three groups, we categorized dogs into four risk levels: high-risk dogs (HRDs) with a RARP of 24 or more, upper-medium risk dogs (UMRDs) with a RARP between 22 and 24, medium-risk dogs (MRDs) with a RARP between 20 and 22, and low-risk dogs (LRDs) with a RARP of 20 or less as shown in Fig.S5. Among stray dogs, only one (4.0%) fell into the HRDs, one (4.0%) into the UMRDs, eight (32.0%) into the MRDs, and the remaining 15 (60.0%) into the LRDs. Most (55.0%) rural dogs fell into the LRDs with only three (15%) in the UMRDs and six (30.0%) in the MRDs. Surprisingly, the pet group had nine dogs (24.3%) in the HRDs, six (16.2%) in the UMRDs, 13 (35.1%) in the MRDs, and eight (21.6%) in the LRDs. Pet dogs had more than 40% in the UMRDs, much higher than the percentages for stray and rural dogs (Fig. 5C).

Discussion

Consistent with other dog fecal microbiome studies, five major phyla—*Bacillota*, *Bacteroidota*, *Pseudomonadota*, *Actinomycetota*, and *Fusobacteriota*—were observed in this study, with *Bacillota* and *Bacteroidota* contributing to over half of the microbiota [37, 38], although their proportions varied among the three dog groups. For instance, *Bacillota* was the most abundant in stray and pet dogs, while *Pseudomonadota* was in rural dogs. At the genus level, 498 bacterial genera were identified in the three dog groups, of which only 129 genera were shared among all three groups. Pet dogs had the lowest genera diversity; this aligns with the lowest Shannon index observed in the pet dog fecal microbiome. The abundance of fecal fungi was significantly higher in stray and rural dogs than pet dogs, as expected. There are many studies demonstrate both phylogeny and diet play important role in animals including dogs. [38, 39] Epidemiological information shows that pet dogs primarily consume commercial dog food, which is relatively simple, whereas the other two dog groups have more exposure to a diverse range of foods, resulting in a more varied and complex diet.

Through machine learning, investigating microbes reveals the distinguishing between different dog populations. Certain important lactic acid bacteria (LAB) genera in the dog feces may reflect gut health to some extent [40–42]. Several studies have shown that adding LAB can relieve diarrhea symptoms in dogs [42, 43], suggesting that dogs with low intestinal LAB have a harder time recovering. The low abundance of LAB in pet dogs could potentially make their health more fragile. The difference in LAB within the three dog groups may be a compensatory response to a more complex and difficult-to-digest diet. *Ligilactobacillus* and *Limosilactobacillus* provide

(See figure on next page.)

Fig. 5 Differences in fecal microbiota communities and distribution of drug resistance genes among three dog groups (pet dogs, rural dogs, and stray dogs) based on machine learning methods (SVM, random forest, decision tree) and Assessment on Risk of Antimicrobial Resistance and Pathogenicity (RARP). **A** The top 25 enriched genera in different dog groups, as deduced from machine learning-based methods and the Kruskal–Wallis rank test. The abundancne of genera within each group of dogs is shown on the left. The adjusted p-values, calculated using the Benjamini–Hochberg method, are shown in the middle, The mean SHAP values from the machine learning analysis are shown on the right. **B** The top 25 enriched ARGs in different dog groups identified using machine learning-based methods and the Kruskal–Wallis rank test. The abundancne of ARGs, the adjusted *p*-values of the Benjamini–Hochberg method, and the mean SHAP values from the machine learning are shown from left to right. **C** Percentages of RARP in three dog groups

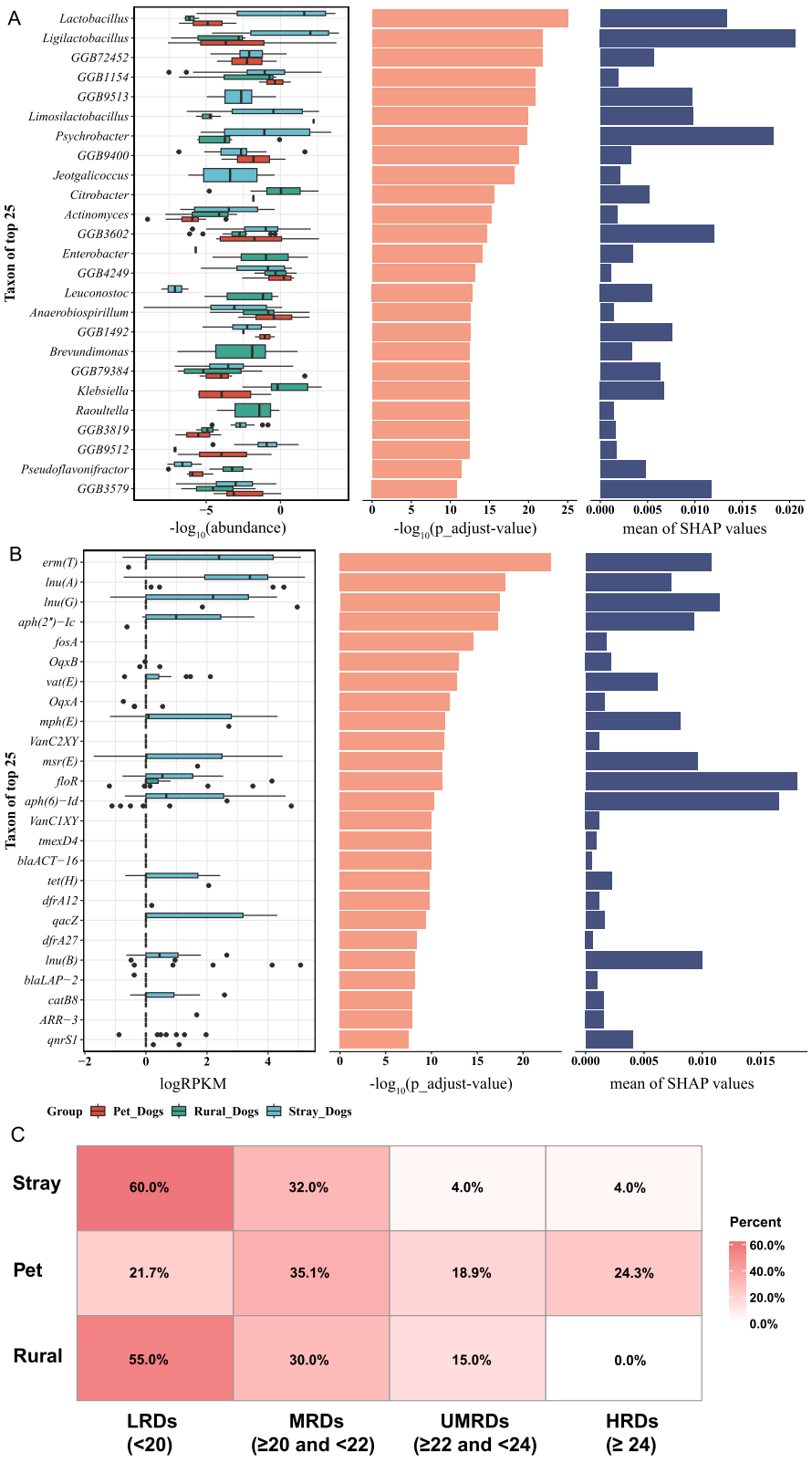


Fig. 5 (See legend on previous page.)

significant insights into how environmental and dietary factors affect the fecal microbiota. In addition, machine learning analysis indicates that *floR* shows significant differences among the three dog groups, suggesting that its distribution pattern across groups may be associated with specific resistance characteristics. Although *floR* may not independently serve as a reliable resistance biomarker, its notable differences among various dog populations provide important reference information for evaluating antibiotic resistance. Nevertheless, further research is needed to clarify the potential risks associated with *floR* and its transmission between humans and animals.

In our study, we identified a total of 587 ARGs targeting 14 classes of antibiotics, covering almost all clinically used antibiotics, and observed prevalent multidrug resistance (MDR) genes. Such abundant ARGs in dogs indicate that they can act as ARGs reservoirs. Rural and stray dogs exhibited a higher average number of ARGs compared to pet dogs, potentially due to increased exposure to the external environment, although their overall abundance was very low. Notably, in a pet dog sample, we observed a high level of *mcr-1*, which confers resistance to last-resort antibiotic polymyxin [44]. Network analysis suggested that various ARGs pose a potential transmission risk, with aminoglycoside resistance genes being the most commonly associated with ISs. Aminoglycoside resistance genes were also discovered to co-occur with multiple types of ARGs. It is striking that such co-occurring ARGs were observed in ICEs or transposons. For instance, the (SXT)/R391 family of ICEs was first identified in Proteus mirabilis from broilers in Shandong Province, China, in 2013. In our study, the most abundant member of this family, ICEPmiChn3, was also detected in the fecal [45]. Similar conformations of ICE*Pmi*Chn3 with those from chicken and humans fecal microbiota suggest their prevalent roles in the transmission of ARGs. The key recombinase encoded by *tnpA* in ICE*Pmi*Chn3 is homologous to those identified in IS*26* of the IS*6* family, which have been shown to possess strong transferable capabilities [46–48]. The high abundance and transferability of ARGs in dog fecal microbiota indicate a significant risk to human health.

In addition to the risk posed by abundant transferable ARGs in dog fecal microbiota, more VFGs were simultaneously discovered in 18 PARBs deduced from MAGs. Among these 18 PARBs, one *E. coli* and three *K. pneumoniae* MAGs carrying ISs and a large number of VFGs are of particular concern, as they also harbored important Beta-lactam resistance genes such as bla_{TEM}, bla_{SHV} and bla_{NDM}. Considering that both *K. pneumoniae* and *E. coli* are the most common nosocomial bacteria, their highly pathogenic features and resistance to clinically commonly used Beta-lactam antibiotics pose a risk of transmission. In the risk assessment, we found that pet dogs had the highest overall RARP. Epidemiological information shows that both pet dogs and rural dogs have more frequent contact with humans, but the RARP of rural dogs is closer to that of stray dogs than to that of pet dogs.

There is evidence that dog ownership has a measurable impact on the human microbiome. During the contact between humans and dogs, dog owners develop a skin microbiome that is more similar to that of their dogs [49]. Especially, the beneficial aspect of dog ownership on the human fecal microbiome has been observed. For instance, infants interacting with healthy dogs also develop a similar fecal microbiome and acquire a richer probiotic population [50, 51]. Due to ethical considerations, there have been no studies on the impact of diseased dogs on human microbiota, but they could potentially pose a risk of transmitting harmful factors to humans, including pathogenic bacteria, ARGs, and ARBs. Animal fecal contamination has been identified as a key regulatory factor for the resistome of children's fecal microbiota [52]. Although no antibiotics were administered to the dogs in the three months prior to the study, the effects of prior antimicrobial treatments may persist beyond this period. Compared to other groups, pet dogs are likely to have received antibiotic treatments at some point in their lives, which may be a significant factor contributing to the higher risk of antimicrobial resistance observed in this group [53]. The highest RARP observed in the pet dog from our study poses a potential public health threat as well.

This study has certain limitations. One potential limitation is that the pet dogs included were primarily of European or American breeds, whereas the rural dogs were predominantly Chinese breeds. This distinction introduces the possibility that genetic differences inherent to the host may have influenced our findings, potentially contributing to the considerable within-group variability observed in fecal microbiota composition. While the presence of ARGs like *mcr-1* and bla_{NDM} in our samples is alarming, further validation would be required to confirm their clinical significance. Additionally, given the challenges in collecting samples from stray dogs, we were unable to obtain more comprehensive epidemiological data for this group. Furthermore, because fecal samples

from pet owners were not included, the study is limited in its ability to fully investigate the potential effects of close human-pet contact, which may affect the applicability and interpretation of some results.

Conclusions

Our study conducted an analysis of the fecal microbiome and resistome of dogs with different living styles, highlighting differences in their microbial composition. Our study indicates that pet dogs exhibit lower diversity of fecal microbiota and a reduced presence of lactic acid bacteria, suggesting a less healthy gut microbiota compared to the other two groups. A reservoir of 587 ARGs conferring resistance to 14 classes of antibiotics was found in the dog feces. The highest RAPR was discovered in pet dogs, suggesting a potential public health risk.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42523-024-00364-x.

Additional file 1.

Additional file 2: Table S1: Details Information of sample collection. Table S2: Relative abundance of bacteria identified at the genus level for all samples (Contains Archaea). Table S3: Relative abundance of bacteria identified at the class level for all samples. Table S4: Relative abundance of bacteria identified at the genus level for all samples. Table S5: Distribution and relative abundance of fungal species in all samples. Table S6: Relative abundance of ARGs identified in all samples. Table S7. Completeness and taxonomy information of 1832 MAGs. Table S8: Information about 167 MAGs carrying both ARG and VFG, including the number of ARG, VFG and IS carried by each MAG. Table S9: The accuracy of all machine learning methods in predicting the most enriched genera or ARGs within the three dog groups. Table S10: The mean SHAP values for each genus, as deduced from mahine learning methods. Table S11. The mean SHAP values for each ARG, as deduced from mahine learning methods.

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Not applicable.

Author contributions
Nan Zhou, Weiye Chen and Luming Xia contributed equally to this work. YZZ and HLC conceived and designed the project. YWC, ZLC, and LMX collected samples. HLC and NZ analyzed metagenomic sequencing data. HLC, YZZ, NZ, WYC, and HJZ analyzed and interpreted the results. NZ and WYC created the figures and wrote the manuscript. XKG, HLC and YZZ revised the paper. All authors discussed and interpreted the data and contributed to the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive in National Genomics Data Center, China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA013605) under project number PRJCA020390.

Declarations

Ethics approval and consent to participate
Animal ethical approval was granted by the Ethics Committee of Shanghai Jiao Tong University School of Medicine (A-2021–014).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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