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Core and variable antimicrobial resistance genes in the gut microbiomes of Chinese and European pigs

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ABSTRACT

Monitoring the prevalence of antimicrobial resistance genes (ARGs) is vital for addressing the global crisis of antibiotic-resistant bacterial infections. Despite its importance, the characterization of ARGs and microbiome structures, as well as the identification of indicators for routine ARG monitoring in pig farms, are still lacking, particularly concerning variations in antimicrobial exposure in different countries or regions. Here, metagenomics and random forest machine learning were used to elucidate the ARG profiles, microbiome structures, and ARG contamination indicators in pig manure under different antimicrobial pressures between China and Europe. Results showed that Chinese pigs exposed to high-level antimicrobials exhibited higher total and plasmid-mediated ARG abundances compared to those in European pigs (P<0.05). *ANT(6)-Ib*, *APH(3')-IIIa*, and *tet*(40) were identified as shared core ARGs between the two pig populations. Furthermore, the core ARGs identified in pig populations were correlated with those found in human populations within the same geographical regions. *Lactobacillus* and *Prevotella* were identified as the dominant genera in the core microbiomes of Chinese and European pigs, respectively. Forty ARG markers and 43 biomarkers were able to differentiate between the Chinese and European pig manure samples with accuracies of 100% and 98.7%, respectively. Indicators for assessing ARG contamination in Chinese and European pigs also achieved high accuracy (r=0.72–0.88). *Escherichia flexneri* in both Chinese and European pig populations carried between 21 and 37 ARGs. The results of this study emphasize the importance of global collaboration in reducing antimicrobial resistance risk and provide validated indicators for evaluating the risk of ARG contamination in pig farms.

Keywords: Metagenomic; Pig manure; Antimicrobial pressure; Antimicrobial resistance genes; Microbiome

INTRODUCTION

The World Health Organization (WHO) identifies antimicrobial resistance (AMR) as a significant global threat to human and animal health (He et al., 2016). Projections for 2030 estimate a 200% increase in global antimicrobial consumption compared to the period from 2000 to 2015, with approximately 70% of this consumption anticipated to be for growth promotion and disease prevention in animal agriculture (Klein et al., 2018). Under antimicrobial pressure, antimicrobial resistance genes (ARGs) can disseminate across bacterial communities in different habitats via horizontal gene transfer (HGT), facilitated by mobile genetic elements (MGEs) such as plasmids, integrons, and insertion sequences (ISs) (Zhao et al., 2020). Bacteria, especially pathogenic bacteria, are the main factors involved in ARG transmission (Han et al., 2018). Under selective antimicrobial pressure, non-drug-resistant pathogens exhibit a propensity to acquire ARGs, while drug-resistant pathogens can persist and proliferate across various

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habitats (Liang et al., 2020). For example, *Escherichia coli*, a zoonotic pathogen, exhibits extensive multidrug resistance and can thrive and circulate in many different environments (Wang et al., 2017; Zhang et al., 2017).

To address the public health implications of ARGs, the European Union (EU) prohibited the use of antimicrobials as growth promoters in 2006. Similarly, China banned the inclusion of antibiotic additives in feed in 2020 (Ma et al., 2021). Pig manure is recognized as a substantial repository of ARGs (Duan et al., 2019), and its ARG and microbiome structures have been extensively studied. For example, ARGs have been identified in pigs and poultry from nine European countries, revealing compositional differences between these two animal groups (Munk et al., 2018). Additionally, studies have explored the microbiome and ARG structures in pigs from various industrialized feedlots in China (Wang et al., 2019), while a reference gene catalog for the pig gut microbiome has been established based on pig manure samples from France, Denmark, and China (Xiao et al., 2016). Although antimicrobial usage on pig farms is subject to regional and national variation, most previous studies have not compared ARG and microbiome structures in pigs across different countries or regions. A comprehensive understanding of ARGs and microbiome distributions in swine at a country/regional scale could more effectively assist policymakers in developing strategies to prevent and control ARG transmission.

Monitoring ARGs is an essential step in addressing AMR. ARGs originating from pig manure present a potential threat to human health worldwide (He et al., 2016). Various techniques, such as metagenomic analysis and quantitative polymerase chain reaction (qPCR), have been employed to assess the risk of ARG dissemination in pig farms and more extensive ARG contamination (Fang et al., 2018; Li et al., 2020; Pu et al., 2018; Yang et al., 2022b). Although metagenomics can offer comprehensive insights, its prohibitive costs limit its applicability for routine ARG surveillance in pig farms; therefore, qPCR remains the preferred approach. Previous studies have identified indicators for total ARG abundance in the pig gut microbiome, although they relied on a limited number of samples (16–24) (Joyce et al., 2019; Qian et al., 2018; Zhao et al., 2018). Furthermore, a recent extensive metagenomic analysis based on 425 Chinese pig manure samples identified 10 highly accurate indicators of AMR in pigs (Zhou et al., 2022). Nevertheless, these indicators do not adequately capture the variations in ARG profiles between Chinese and European pigs, indicating the pressing need for a targeted ARG subset that can assess regional ARG contamination and variation (Liu et al., 2019). Analytical tools used in prior studies for identifying indicative microbiome/ARG features have primarily relied on linear discriminant analysis (LDA) (Goecks et al., 2010). In contrast, the random forest (RF) machine learning algorithm excels in classification, prediction, and variable selection tasks, with an error rate much lower than that of LDA (Verikas et al., 2011). Recently, the application of RF has expanded to include the prediction of clinical diseases, prediction of drug resistance phenotypes of bacteria, and detection and prediction of global sewage AMR (Hendriksen et al., 2019; Parisot et al., 2018; Su et al., 2019).

In the current study, we utilized a publicly available reference gene catalogue for pigs, as well as pig metagenomic data from our laboratory, including well-characterized patterns of antimicrobial usage (Tong et al., 2022; Xiao et al., 2016). We aimed to: (i) decipher the association between the microbiome and ARG in pig manure under different antimicrobial pressure (different antimicrobial monitoring policies) based on metagenomics; (ii) identify a specific subset of ARG indicators in pig farms via the RF algorithm; and (iii) explore potential associations between ARGs and hosts through metagenomic assembled genomes (MAGs) obtained from samples.

MATERIALS AND METHODS

Sample collection

Publicly available pig manure metagenomic datasets from China, Denmark, and France were collected, including PRJEB11755 (*n*=287) and PRJNA750156 (*n*=35), the latter of which was provided by our laboratory. The patterns of antimicrobial use in these pigs have been well-characterized in a previous study (Supplementary Table S1) (Xiao et al., 2016). Based on national and regional policies governing antimicrobial regulation, pigs from France and Denmark were grouped as European, while pigs from China with high antimicrobial exposure were classified as Chinese. Additionally, metagenomic datasets from healthy individuals in Denmark (PRJEB4336, *n*=140) and China (PRJNA422434, *n*=109; PRJNA750156, *n*=4) were collected for comparative analysis of ARG profiles with pigs from their corresponding regions (Supplementary Table S2).

Metagenomic analysis

Raw sequence reads were filtered, then quality trimmed using Sickle (quality scores<20 or length<20 bp) (Joshi & Fass, 2011). Clean reads were *de novo* assembled based on *k*-mer size using CLC Genomics Workbench (v.10.0.1, CLC Bio, Denmark). Open reading frames (ORFs) of the assembled contigs were predicted using Prodigal (v.2.6.3) (Hyatt et al., 2010). ARG-like ORFs were searched against the Comprehensive Antibiotic Resistance Database (CARD) using BLASTN with an E-value threshold of $\leq 1\times10^{-5}$. Only ARG-like ORFs with an identity ≥80% and query coverage ≥70% were retained. The coverage of these ARG-like ORFs was determined by mapping them to the contigs with a minimum length coverage of 95% at 95% similarity using CLC Genomics Workbench. The number of bacterial cells in each metagenome dataset was generated using ARGs-OAP (v.2.0) (Yin et al., 2018). ARG abundance was normalized to the number of cells (copies/cell) in each metagenome dataset, calculated as follows (Yin et al., 2018):

$$\text{Coverage} = \sum_{1}^{n} \frac{N \times 150/L}{C} \quad (1)$$

Where N represents the number of reads mapped to ARG-like ORFs, 150 signifies the read length, L is the length of the target ARG-like ORF sequence, n denotes the number of ARG-like ORFs, and C is the bacterial cell number per metagenome dataset.

Plasmids, integrons, and ISs were identified using PlasmidFinder (Carattoli & Hasman, 2020), INTEGRALL database (Moura et al., 2009), and ISfinder (Siguier et al., 2006), respectively (accessed on 6 July 2020). The methods used for the normalization and calculation of MGE abundance were consistent with those used for the ARGs. Plasmid, chromosomal, and unclassified sequences from ARG-carrying contigs (ACCs) were predicted using PlasFlow (v.1.1.0)

(default parameters) (Krawczyk et al., 2018). The composition and relative abundance of the microbiome community were profiled using MetaPhlAn3 (v.3.0.2) (Beghini et al., 2021).

Metagenomic binning

MAGs were produced with the Binning_refiner module (parameter: --metabat2) in MetaWRAP (Song & Thomas, 2017; Uritskiy et al., 2018). Estimates of MAG completeness and contamination were performed using CheckM (v.1.1.2), with only those MAGs with completeness ≥80% and contamination ≤10% retained (Parks et al., 2015). The dRep program (v.3.2.2) was used to dereplicate the retained MAGs in both the Chinese and European pigs (parameters: -sa 0.99 and -pa 0.95) (Olm et al., 2017). Taxonomic affiliation for each MAG was performed using GTDB-Tk (v.1.7.0) based on the Genome Taxonomic Database (GTDB) with default parameters. Genomes were classified as novel strains if they exhibited an average nucleotide identity (ANI) of less than 99%, and as novel species based on an ANI of less than 95% or if no ANI was output by GTDB-Tk (Glendinning et al., 2020). Of note, *Shigella flexneri* was renamed as *Escherichia flexneri* as proposed in GTDB taxonomy (Sanford et al., 2021). BLASTN was used to screen the MAGs for acquired ARGs against the CARD database. PhyloPhlAn (v.3.0.2) was used to reconstruct the phylogenetic trees with 400 universal markers (Asnicar et al., 2020). The phylogenetic tree was visualized using the "ggtreeExtra" package (Xu et al., 2021).

Core resistome and microbiome

To identify ARGs consistently present in pig manure samples across China and Europe, the core resistome was defined based on occurrence in at least 95% of the metagenome datasets (Munk et al., 2018). Genera present in fewer than 95% of the metagenome datasets were excluded from the core microbiome. Changing trends in the total abundance of the core resistome were analyzed in both pig and human samples originating from the corresponding countries or regions.

ARG markers, biomarkers, and indicators

To identify the main discriminant features of ARGs (ARG markers) and microbiomes (biomarkers) between Chinese and European pigs, the relative abundances of ARGs and bacterial taxa (phylum, class, order, family, genus, and species level) were classified in the pig manure samples using the "randomForest" package (v.4.7-1.1) (Liaw & Wiener, 2002). Cross-validation was performed to select significant features, while varImpPlot was used to illustrate the importance of features in the classification. The "rfPermute" package (v.2.5.1) was used to assess the significance of important features, while the "ggplot2" package (v.3.3.6) was used to visualize important features and cross-validation curves. In the RF models, the significant features served as indicators for ARG contamination on pig farms. The "linkET" package (v.0.0.5) was used to estimate the importance and interpretability of the significant features in the ARG profiles of both the Chinese and European pigs to facilitate identification of key indicators.

To evaluate the ability of indicators to predict ARG abundance in Chinese and European pig manure, abundance matrices of resistance genes were downloaded from Chinese and European pig manure studies (Munk et al., 2018; Zhou et al., 2022). To reduce data noise, the low-antibiotic exposure group was excluded from the ARG abundance matrix of Chinese pigs. The abundance of ARGs within each sample was summed at the ARG subtype level. Spearman correlation analysis was then performed to assess the relationship between the abundance of indicators and total ARG abundance. The ARGs included in the selected indicators but not detected in the downloaded dataset were excluded from prediction analysis.

Statistical analysis

Richness and Shannon diversity indices were computed using the "picante" package. Principal component analysis (PCA) and permutational multivariate analysis (Adonis) were performed to reflect the variation in ARG profiles and bacterial community between Chinese and European pigs using the "vegan" package. Spearman correlation analysis was used to explore the correlations among core ARGs, ARG markers, and indicators with ARG profiles. The "ggplot2" package was used to construct violin plots, line charts, stacked plots, and scatter diagrams. Pie charts and Sankey diagrams were established using the "scatterpie" and "networkD3" packages, respectively. Nonparametric Kruskal-Wallis and Mann-Whitney tests were performed to compare differences in data, with $P<0.05$ considered statistically significant following false discovery rate (FDR) corrections.

RESULTS

ARG and microbiome response in Chinese and European pigs

We detected 19 ARG types and 291 ARG subtypes in the Chinese and European pigs (Supplementary Table S3). Both ARG abundance and diversity were significantly higher ($P<0.05$) in Chinese pigs than in European pigs (Supplementary Figure S1A). Notably, ARG abundance ranged from 1.57 to 7.73 copies/cell in Chinese pigs and from 0.31 to 2.36 copies/cell in European pigs. Similar to ARG distribution, MGE abundance and diversity were higher in the Chinese pigs ($P<0.05$; Supplementary Figure S1B), with MGE abundance ranging from 0.33 to 9.76 copies/cell in Chinese pigs and from 0.03 to 6.88 copies/cell in European pigs. ARG abundance on the chromosomes was markedly higher than that on the plasmids in both Chinese and European pigs ($P<0.05$; Figure 1A; Supplementary Table S4). Furthermore, ARG abundance on the plasmids was significantly higher in Chinese pigs than in European pigs ($P<0.05$). Genes conferring resistance to beta-lactam (45.4%–68.7%), diaminopyrimidine (56.7%–74.2%), and phenicol (65.2%–68.8%) were more prevalent on plasmids, while genes conferring resistance to fluoroquinolone (60.5%–76.2%), nucleoside (60.0%–86.0%), and peptide (72.4%–74.3%) were more frequently encoded on chromosomes in Chinese and European pigs (Figure 1B). Moreover, PCA showed that Chinese and European pigs could be well separated based on ARG subtype composition and bacterial structure (genera) (Adonis, $P<0.05$; Figure 1C, D). Aminoglycoside, tetracycline, and macrolide-lincosamide-streptogramin (MLS) were the dominant ARG types in Chinese pigs, while beta-lactam, tetracycline, and MLS were the dominant ARG types in European pigs (Supplementary Figure S1C). Bacteroidetes and Firmicutes were the dominant phyla in all samples (Supplementary Figure S1D).

Core resistome and microbiome in Chinese and European pigs

To identify the core ARGs and microbial species in the

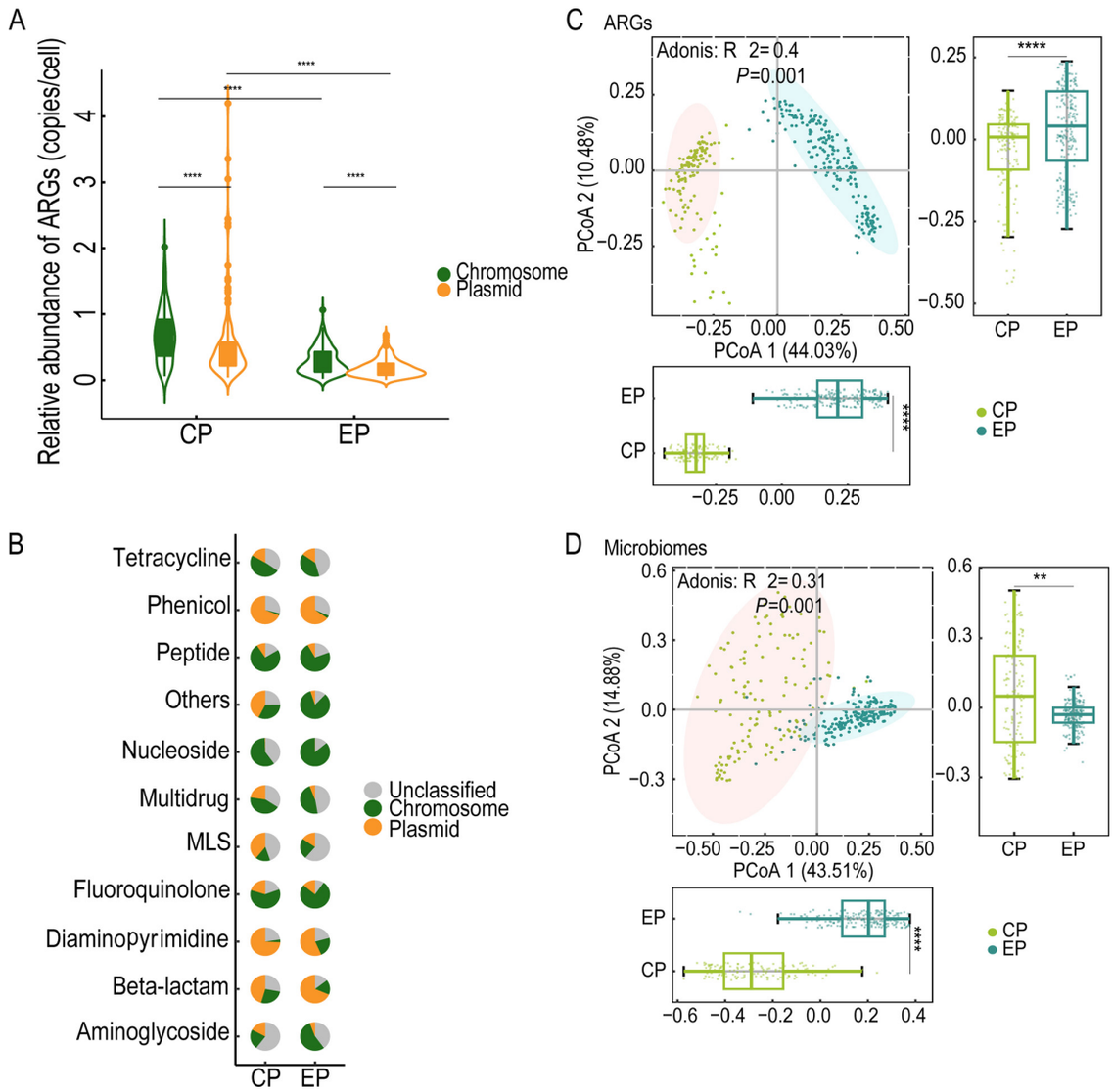

Figure 1 Location of ACCs and differences in ARG subtypes and microbiomes between groups

A: Abundance of ACCs located on plasmids and chromosomes (Mann-Whitney tests, *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001). B: Percentages of various ARG types located on plasmids, chromosomes, and unclassified sequences. MLS: macrolide-lincosamide-streptogramin. C, D: PCA and Adonis analysis based on ARGs (C) and microbiomes (D) in Chinese and European pigs. Ellipses were drawn with a 0.95 probability. CP: Chinese pig; EP: European pig.

Chinese and European pigs, we analyzed ARGs and species consistently present in at least 95% of samples across the different locations. In total, seven core ARGs were identified in Chinese pigs, including *ACI-1*, *ANT(6)-Ib*, *APH(3')-IIIa*, *CfxA2*, *ErmB*, *lsaE*, and *tet*(40), and four core ARGs were identified in European pigs, including *ANT(6)-Ib*, *APH(3')-IIIa*, *lnuC*, and *tet*(40) (Figure 2A, B). Although these core ARGs constituted a relatively small proportion of the overall ARG types, they were highly abundant, representing 53.5% and 48.0% of total ARG abundance in Chinese and European pigs, respectively (Figure 2B; Supplementary Figure S2A). A robust correlation existed between core resistome and total ARG abundance in European pigs (Spearman ρ=0.91, P<0.001; Figure 2C), while a significant but moderate correlation was observed in Chinese pigs (Spearman ρ=0.52, P<0.001; Figure 2C).

To elucidate the genetic composition of the core resistome, we assessed the presence of the ARGs on either plasmid or chromosomal contigs, as well as their co-occurrence with bacterial genera (Figure 2D; Supplementary Figure S2B). Excluding unclassified categories, a higher proportion of core ARGs targeting beta-lactam (62.3%) and MLS (98.0%) was found on the plasmids, while a higher proportion of core ARGs targeting tetracycline (100%), aminoglycoside (86.8%), and multidrug (80.1%) was found on the chromosomes in Chinese pigs. In contrast, in European pigs, aminoglycoside and tetracycline were more often found on chromosomes, while MLS was relatively evenly distributed on both plasmids (51.9%) and chromosomes (48.1%). The hosts for the core resistome were identified based on the recovered MAGs. Notably, Bacteroidota and Firmicutes_A served as the principal hosts for the core resistome in both the Chinese and European pig populations.

Interestingly, the observed trend in the abundance of the core resistome in Chinese pigs closely paralleled that in the Chinese human population, with the exception of *APH(3')−IIIa*. Similarly, the observed trend in the abundance of the core resistome in European pigs closely paralleled that in the European human population, with the exception of *tet*(40) (Figure 2F). Notably, these core antibiotic resistance genes were detected at a lower frequency in human samples

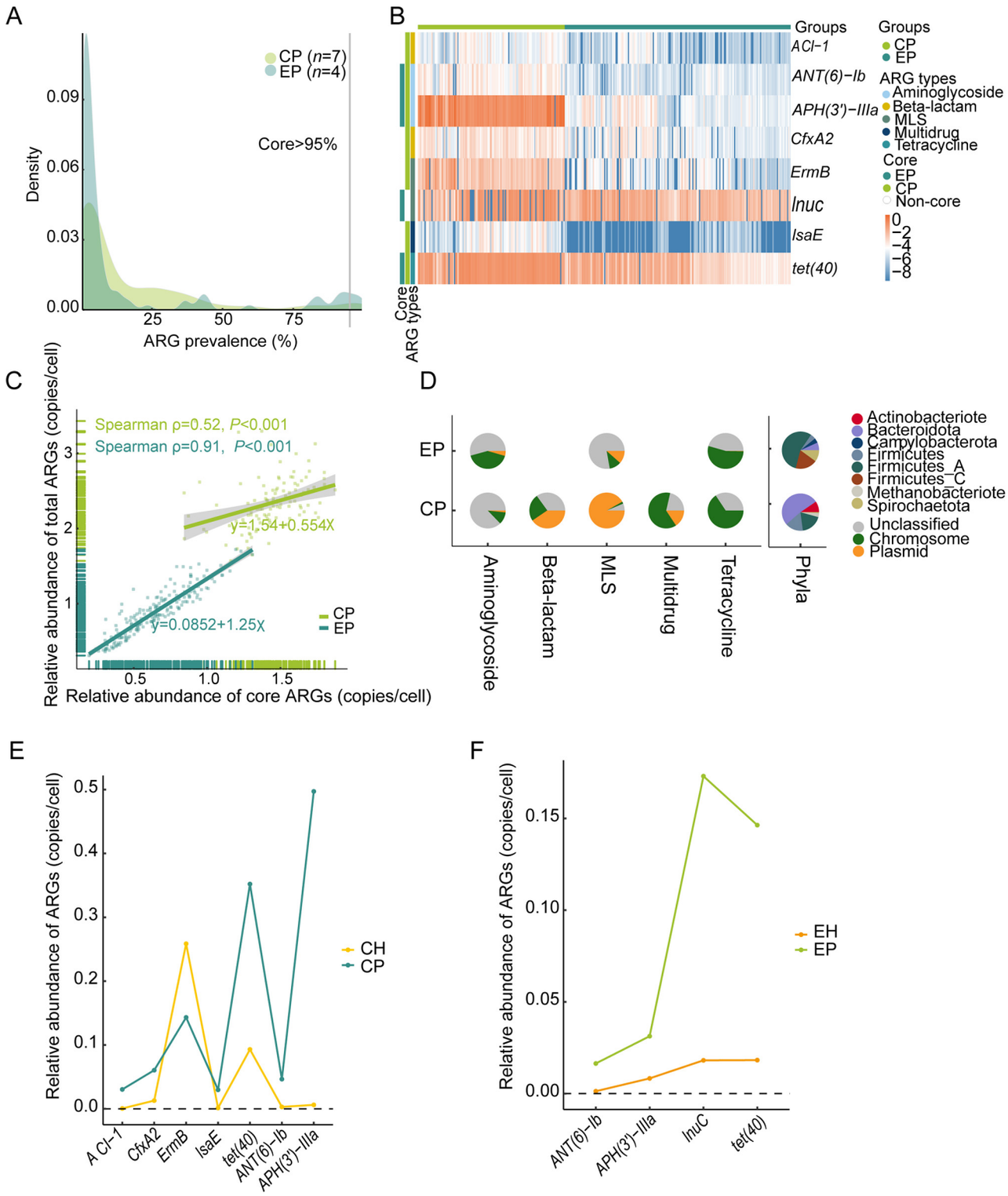

Figure 2 Core resistome of Chinese and European pigs

A: Distribution of ARG subtype prevalence in all manure samples from Chinese and European pigs. Gray lines show defined cutoffs. B: Quantitative profile of core resistome (coverage, ×/cell, $\log_2$ transferred). C: Correlation analysis of total ARG abundance and core resistome abundance. D: Proportion of locations and potential hosts in core resistome. E, F: Abundance of core ARGs in pigs and humans from China (E) and Europe, respectively. Core ARGs were identified in pigs from China and Europe, respectively.

(Supplementary Figure S2C, D).

Using the same thresholds established for the core resistome, we identified 11 core microbiomes (Supplementary Figure S3A), including seven from European pigs and three from Chinese pigs. *Lactobacillus* and *Prevotella* emerged as the dominant genera in the core microbiomes of Chinese and European pigs, respectively, while *Prevotella_sp_P3_122* was shared between both (Supplementary Figure S3C). The core microbiomes were present in high abundance in samples from the corresponding groups (Supplementary Figure S3B, C). No ARGs were detected in the core microbiomes.

Potential drivers for distinguishing Chinese and European pigs

Using RF machine learning, we identified key ARGs and microbial species that may serve as potential discriminators between Chinese and European pigs. Ten-fold cross-validation, repeated five times, was employed to evaluate the importance of each ARG subtype. The cross-validation error rate reached stability when the 40 most significant ARG subtypes, out of a total of 291, were included in the analysis; these 40 ARGs were consequently designated as marker ARGs (Figure 3A). Not all ARG subtypes contributed equally to the distinction between Chinese and European pigs, with *APH(3')-IIIa*, *fexA*, *optrA*, *SAT-4*, and *ErmB* emerging as the most important variables distinguishing between Chinese and European pigs (P<0.05; Figure 3A; Supplementary Table S5). The initial classification model demonstrated exceptional accuracy in differentiating between Chinese and European pigs, with an error rate of 0% and a recall rate of 100% (Supplementary Table S5). To simplify the classification model, the top 40 important ARG subtypes were selected for modeling instead of all ARG subtypes in the original dataset. The simplified classification model showed that the two pig populations could be accurately distinguished based on the 40 most important ARG subtypes, maintaining an error rate of 0% and a recall rate of 100% (Adonis, P<0.05; Figure 3B; Supplementary Table S6). These marker ARGs were highly abundant in both pig populations (Figure 3B, C), contributing 79.3% and 72.0% of the total ARG abundance in Chinese and European pigs, respectively (Supplementary Figure S4). Thus, these marker ARGs could potentially serve as indicators of ARG contamination in both pig populations (Spearman ρ=0.96, P<0.05) (Figure 3D).

RF analysis was used to identify Chinese and European pigs at the phylum, class, order, family, genus, and species levels. Our model showed 98.7% accuracy at the family level, the highest among all taxonomic levels (Supplementary Table S7). Ten-fold cross-validation, repeated five times, was also carried out to evaluate the importance of indicator bacterial families. The cross-validation error curve stabilized when the 43 (43/80) most relevant families were used (Supplementary Figure S5A). The contributions of bacterial families to differentiating between Chinese and European pigs are reported in Supplementary Table S7. Similarly, upon simplification of the classification model, Chinese and European pigs could be accurately differentiated, exhibiting recall rates of 97.4% and 98.4%, respectively, with an error rate of 1.30% (Adonis, P<0.05; Supplementary Figure S5B; Supplementary Table S8). Therefore, these findings indicate that the 43 most relevant families could be used as biomarkers to distinguish between Chinese and European pigs. Notably, these biomarkers accounted for 99.3% and 99.8% of the total microbiome abundance in Chinese and European pigs, respectively (Supplementary Figure S5C).

Indicators for ARG contamination in Chinese and European pigs

To identify the ARGs that affected dissimilarities in ARG profiles between Chinese and European pigs, multiple regression models were used to determine the correlation between total ARG abundance of Chinese and European pigs and the identified ARG markers (Figure 4A). Analysis revealed that the ARG markers accounted for 73.7% and 76.3% of the variation in ARG profiles of the Chinese and European pigs, respectively. *ErmB*, *AAC(6')-Ie-APH(2'')-Ia*, *APH(3')-Ia*, *sul3*, and *tet*(A) were the most significant variables in predicting the variance in total ARG abundance in Chinese pigs, with importance values exceeding 7 (P<0.05; Figure 4A; Supplementary Table S9), while *tet*(40), *ErmF*, *tet*(X10), *CfxA6*, and *ErmG* were the most significant predictors of variance in European pigs, with importance values exceeding 13 (P<0.05; Figure 4A; Supplementary Table S10). Thus, these significantly important ARG markers (P<0.05) could serve as indicators of total ARG abundance in both pig populations, as corroborated by Spearman correlation analysis (Spearman ρ=0.72, P<0.05, Chinese pigs, Figure 4B; Spearman ρ=0.88, P<0.05, European pigs, Figure 4B). Notably, these indicators accounted for 41.2% and 68.5% of the total ARG abundance in Chinese and European pigs, respectively (Supplementary Figure S6A). Regarding proportions of indicators, 25.0% in Chinese pigs and 23.0% in European pigs were assigned to plasmids (Figure 4C). Among the ARG classes, aminoglycoside (57.6%) was dominant in Chinese pigs, while MLS (46.5%) was dominant in European pigs. Furthermore, hosts for the ARG indicators were identified across nine bacterial phyla. In the Chinese pig population, Spirochaetota and Bacteroidota constituted the dominant host phyla, with respective proportions of 25.5% and 20.2%, while *E. flexneri* (phylum Proteobacteria) harbored the greatest number of ARG indicators. In contrast, in the European pig population, Bacteroidota and Firmicutes_A emerged as the principal hosts, accounting for 40.2% and 38.2% of the host range, respectively, with *Streptococcus alactolyticus* (phylum Cyanobacteria) identified as a potential host for the ARG indicator *tet*(X10) (Figure 4C; Supplementary Figure S6B).

To corroborate the predictive capacity of the selected ARGs in regard to total ARG abundance in both the Chinese and European pig populations, validation datasets were employed, specifically ARG datasets from nine European countries and an expanded gene catalog for Chinese pigs (Munk et al., 2018; Zhou et al., 2022). In the Chinese validation dataset, only 12 out of 18 indicators (i.e., *AAC(6')-Ie-APH(2'')-Ia*, *aadA2*, *APH(3')-Ia*, *dfrA12*, *emr*B, *Erm*B, *flo*R, *OXA-347*, *qacH*, *QnrS1*, *sul*1, and *sul*3) were found to be predictive of total ARG abundance, with a strong correlation observed between their abundance and total ARG abundance (Spearman ρ=0.61–0.80, P<0.001; Supplementary Figure S7A). In the European validation dataset, only five out of 13 indicators (*CfxA2*, *CfxA6*, *lnuC*, *tet*(40), and *ACI-1*) were identified, with a strong correlation observed between their abundance and total ARG abundance (Spearman ρ=0.89, P<0.001; Supplementary Figure S7B).

MAGs recovered from Chinese and European pigs

To investigate the microbiome structure, including uncultured taxa, we reconstructed 25 466 MAGs from 322 manure samples collected from Chinese and European pigs. Subsequent to dereplication and stringent quality control measures (completeness ≥80%, contamination ≤10%), 782 MAGs from Chinese pigs and 1 245 MAGs from European pigs were retained for downstream analysis (Supplementary Figure S8A; Supplementary Table S11). Based on taxonomic assignment using GTDB-Tk, we identified 18 phyla among the 2 027 retained MAGs (Supplementary Table S12). Firmicutes_A (n=338/387) and Bacteroidota (n=197/452) were the dominant phyla in Chinese and European pigs. Notably, Planctomycetota was endemic to Chinese pigs, while

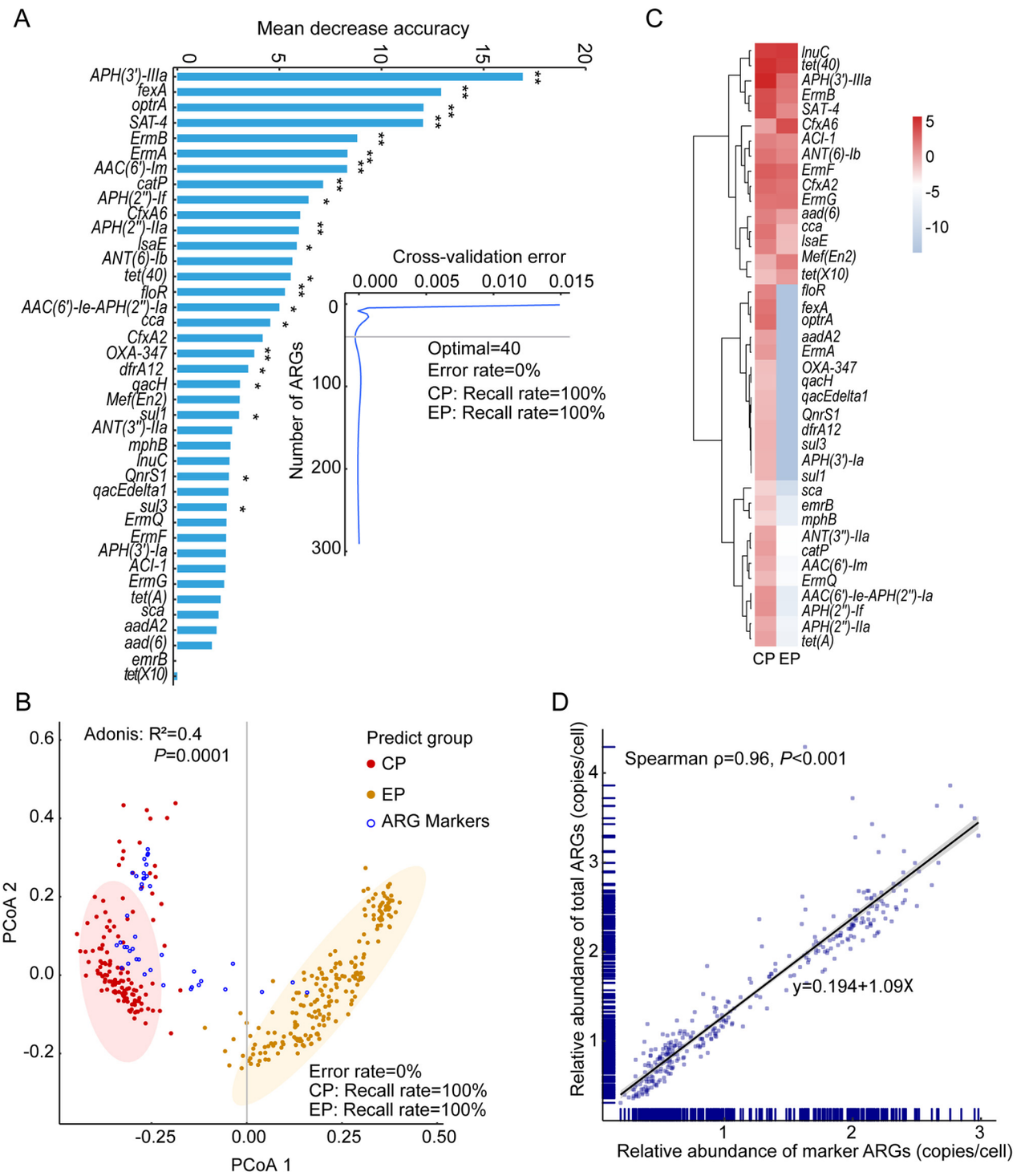

Figure 3 ARG markers between Chinese and European pigs

A: Top 40 ARG subtypes identified by RF classification of relative abundance of ARGs in Chinese and European pigs, ranked in descending order of importance to the accuracy of the model. Inset represents ten-fold cross-validation error for number of input ARGs that differentiate Chinese and European pigs. Higher mean decrease accuracy implies more important predictors. Significance levels: *: P<0.05; **: P<0.01. B: PCA and Adonis analysis were based on 40 ARG markers in Chinese and European pigs. C: Quantitative profile of 40 ARG markers in Chinese and European pigs (coverage, ×/cell, $\log_2$ transferred). cca: *Campylobacter_coli*_chloramphenicol_acetyltransferase, sca: *Streptococcus_suis*_chloramphenicol_acetyltransferase. D: Correlation analysis of total ARG abundance and ARG marker abundance.

Thermoplasmatota and Deferribacterota were endemic to European pigs. Among the bacterial genomes, 294 species-level MAGs from Chinese pigs and 373 species-level MAGs from European pigs were inferred as novel species, predominantly assigned to Firmicutes_A (Figure 5A; Supplementary Table S13). Among the relatively rare archaeal genomes, four putative new species (4/9) were identified in the Chinese pigs, whereas no new archaeal species were identified in the European pigs. Among the retained MAGs, 176 MAGs carried ARGs, including 104 MAGs (104/782) from Chinese pigs belonging to 10 phyla and 72 MAGs (72/1 245) from European pigs belonging to nine phyla (Supplementary

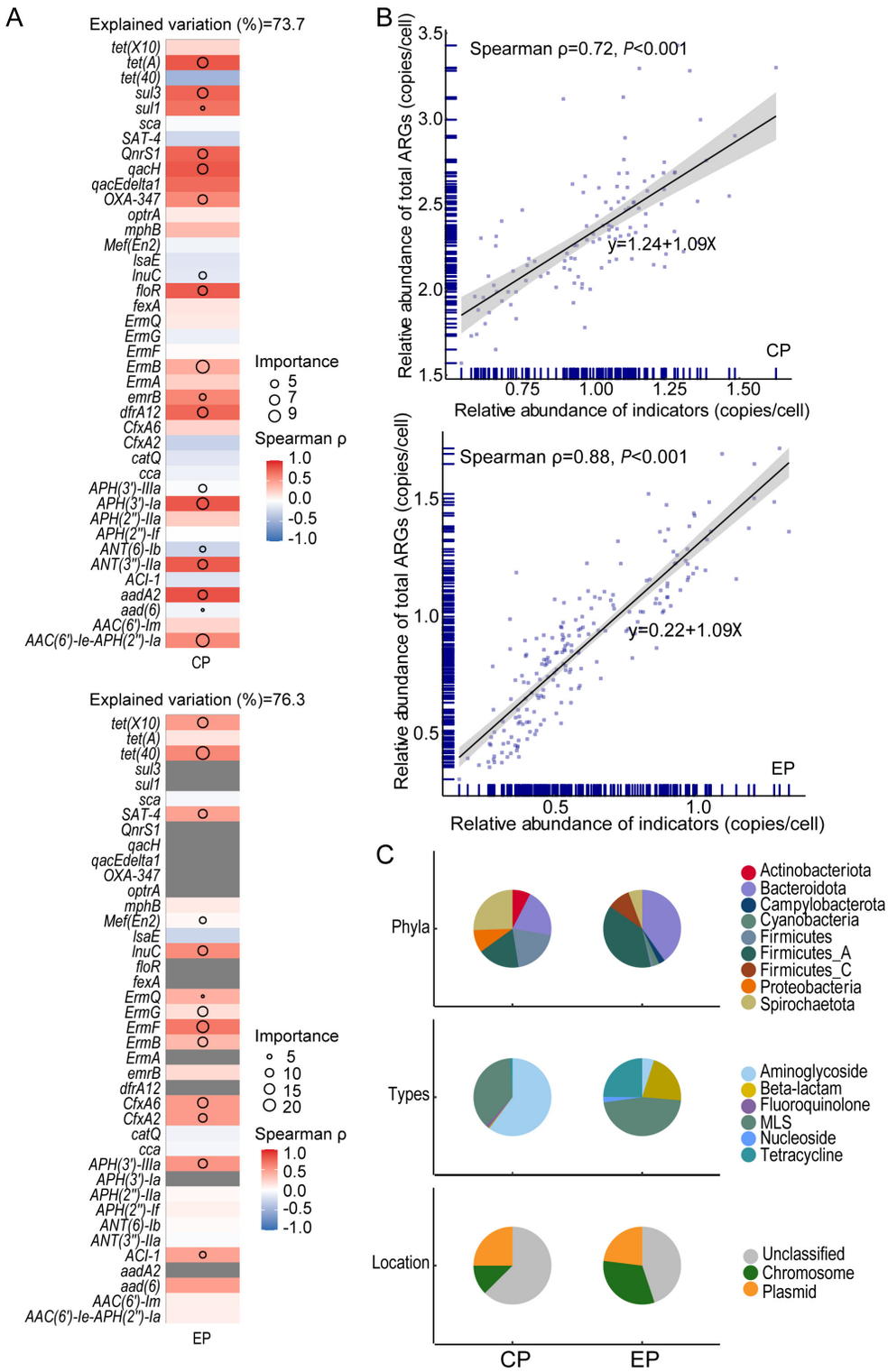

Figure 4 Indicators of ARG contamination in Chinese and European pigs

A: Potential contributions of ARG markers to ARG profiles and best multiple regression model for ARG markers in Chinese and European pigs, respectively. Proportion of explained variation was calculated via multiple regression modeling and variance decomposition analysis. Circle size represents importance of variable (P<0.05), with significantly important variable defined as indicators. Color represents Spearman correlation coefficient. cca: *Campylobacter_coli*_chloramphenicol_acetyltransferase, sca: *Streptococcus_suis*_chloramphenicol_acetyltransferase. B: Correlation analysis of total ARG abundance and indicator ARG abundance. C: Proportion of potential hosts, ARG types, and locations in indicator ARGs.

Figure S8B, C and Table S14). Firmicutes_A was the dominant phylum hosting ARGs in both populations, with 28 MAGs from Chinese pigs and 27 MAGs from European pigs. Phylogenetic analysis revealed a conspicuous presence of ARGs carried by *E. flexneri* (Proteobacteria) in the Chinese and European pigs (Figure 5A). Specifically, 47 ARGs were detected across 17 *E. flexneri* genomes, with the number of carried ARGs ranging from 22 to 35 in Chinese pigs and from

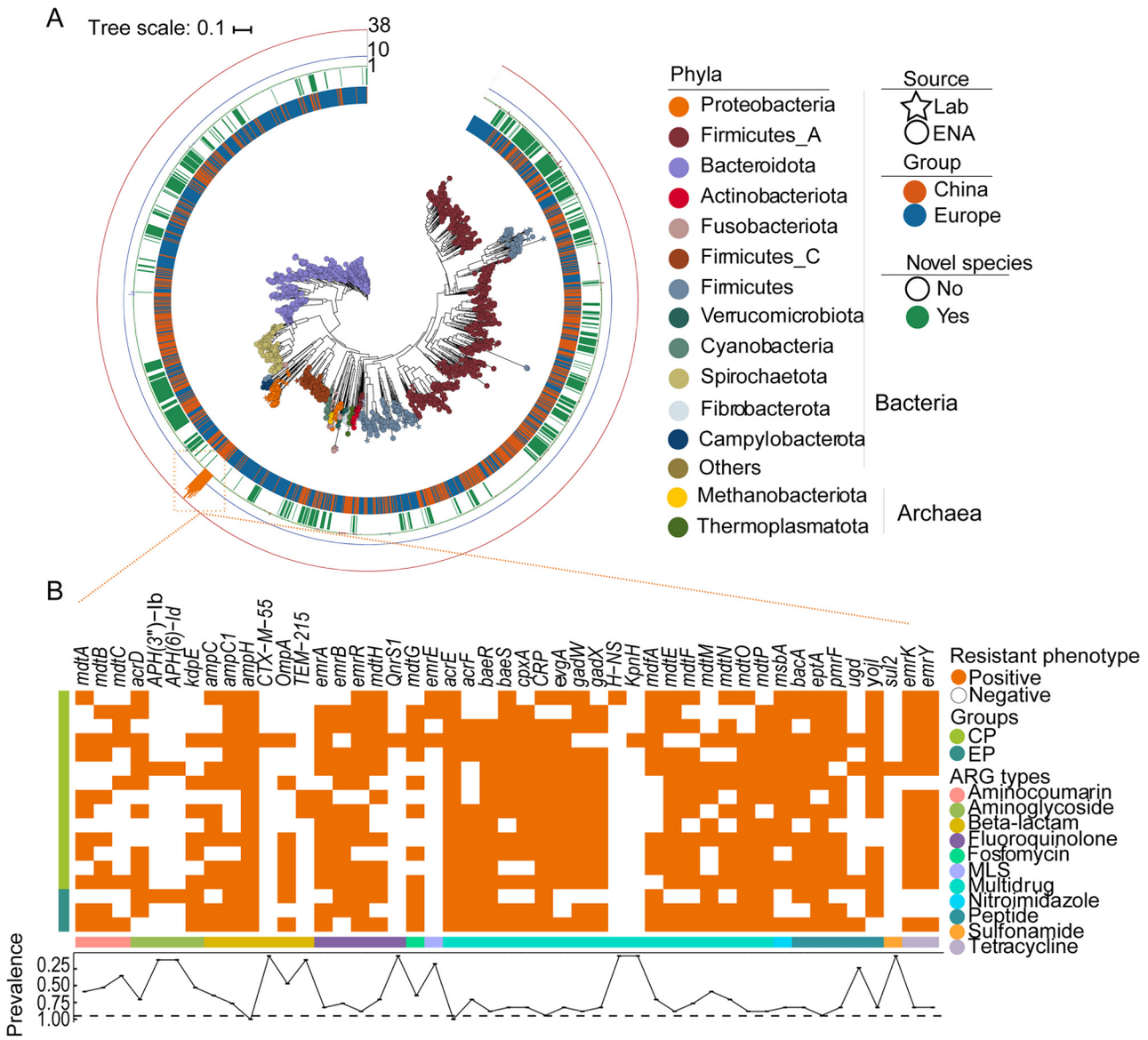

Figure 5 Metagenome-assembled nonredundant genomes (2 027) from pig manure microbiomes (322)

A: Phylogenetic tree of 2 015 bacterial and 12 archaeal species. From inner to outer rings: colors and shapes in first ring represent phyla and source, respectively; second ring represents groups; third ring represents novel species; and fourth ring represents number of ARGs carried by each species. B: ARGs detected in *E. flexneri* genomes. Line chart illustrates ARG prevalence in each *E. flexneri* genome.

21 to 37 in European pigs (Figure 5B). Notably, *acrE* (conferring multidrug resistance) and *ampH* (conferring beta-lactam resistance) occurred in all *E. flexneri* genomes. Multidrug resistance genes were highly prevalent in the *E. flexneri* genomes (18/47).

DISCUSSION

The association between the abundance of ARGs, MGEs, and microbiome structure was strongly influenced by the degree of antibiotic utilization. Our results showed that Chinese pigs exhibited greater diversity and abundance of ARGs and MGEs compared to European pigs. Previous studies have indicated that total levels of AMR are correlated with the use of antimicrobial agents in livestock and geographic regions with similar usage patterns display comparable resistance profiles (Munk et al., 2018). The abundance of ARGs on plasmids was much higher in Chinese pigs than in European pigs. Under selective antibiotic pressure, plasmids can indicate potential mobility and may effectively contribute to the spread of antibiotic resistance (Zhao et al., 2020). Recent studies have reported a significant association between ARG and MGE abundance (Li et al., 2022; Liu et al., 2018; Xie et al., 2022). Consequently, the higher levels of antimicrobial exposure in Chinese pigs, particularly in the absence of rigorous monitoring, may pose a greater risk to public health than in European pigs, where antibiotic use is more tightly regulated. Although ARGs were primarily located on chromosomes across all breeding modes (excluding those unclassified), the HGT mechanism can incorporate ARGs into chromosomes. Furthermore, free DNA released from the chromosomes can then be captured by other bacterial cells, thereby contributing to the spread of resistance (Croucher et al., 2016).

To determine potential response targets of ARGs and microbiomes in pigs under different antimicrobial exposure, we identified core ARGs in both Chinese and European pig populations. Of note, only eight core ARGs were identified in the two populations, implying that the majority of ARGs were

metastatic rather than constitutive in nature (Zhou et al., 2022). *ANT(6)−Ib*, *APH(3')−IIIa*, and *tet*(40) were identified as shared core ARGs across the different antimicrobial exposure regimes. These shared core ARGs were associated with commonly used classes of antibiotics, such as aminoglycosides and tetracyclines, which are the most widely used antibiotics for therapeutic and growth promotion in livestock worldwide (He et al., 2020). Notably, a correlation was found in the abundance of identified core ARGs between pig and human populations within corresponding geographic regions. Previous research has indicated that ARGs can be present in pigs even in the absence of antibiotic administration, highlighting the risk of ARG transmission from animal feces to both environmental reservoirs and human populations (Joyce et al., 2019). Moreover, bacteria harboring ARGs in manure have the potential for dissemination via multiple vectors, including rivers, groundwater, air dust, and agricultural products, thereby exacerbating ARG contamination on both regional and global scales (Gao et al., 2020a, 2020b; Smillie et al., 2011).

Our study revealed marked dissimilarities in both the ARG profiles and microbiome structures between the Chinese and European pigs. Using high-resolution machine learning models, we identified specific ARG markers and biomarker taxa, capable of distinguishing between these two pig populations. Antimicrobial exposure is known to significantly affect gut bacterial composition (Looft et al., 2014; Sun et al., 2020). As ARGs and microbiomes in pig manure are subject to distinct antibiotic exposure profiles, these factors provide a basis for tracking ARG contamination in pig farming operations. The environmental persistence and transmission of ARGs further support the need to establish ARG indicators for monitoring in pig farms (Li et al., 2019). Future ARG monitoring strategies could thus be based on recognized ARG indicators (Ishii, 2020). In this context, we identified specific high-resolution indicators of ARG contamination in Chinese and European pigs, pinpointing Spirochaetota, Bacteroidota, and Firmicutes_A as potential preferred hosts. Notably, *tet*(X10), which confers resistance to tigecycline, emerged as a significant indicator of ARG contamination in European pigs. Despite the EU's 2006 ban on the use of antibiotics as growth promoters, tetracycline usage in European livestock remained high at 41.2% between 2015 and 2017, compared to 31.2% in Asia (Fang et al., 2020). Furthermore, studies have reported higher detection rates of *tet*(X) variants in Europe than in Asia (Zhang et al., 2021). Antibiotic resistant bacteria may serve as potential vectors for the transfer of ARGs between environmental reservoirs and human or animal hosts when they persist across different geographical locations (Nnadozie & Odume, 2019). Therefore, the identification of indicators of ARG contamination and their respective hosts may provide a potential avenue for tracing the sources of ARG contamination in the environment and assessing ARG prevalence in pig farms.

The recovery of genomes from metagenomic datasets facilitates detailed strain classification and analysis of characteristics related to ARGs carried by bacteria (Almeida et al., 2021). In the current study, we examined the features of ARGs carried by the microbiome in both Chinese and European pigs through the examination of recovered medium-quality MAGs. Results showed that Firmicutes_A and Bacteroidota were prevalent in pig manure samples from both regions, consistent with previous research on pig and chicken gene catalogs showing Firmicutes_A and Bacteroidota dominance (Chen et al., 2021; Feng et al., 2021). Furthermore, Firmicutes_A was identified as the dominant host of ARGs in both Chinese and European pigs, in accordance with previous studies (Wang et al., 2021). Notably, *E. flexneri*, belonging to the phylum Proteobacteria, was found to harbor an extensive array of ARGs (ranging from 21 to 37), primarily constituting multidrug resistance genes, in both Chinese and European pigs. The average number of ARGs carried by animal-derived *E. coli* in China has risen from 8.05 to 16.85 over the last five decades, leading to excessive and extensive use of antimicrobial agents (Yang et al., 2022a). The indiscriminate use of antimicrobials has been linked to elevated levels of multidrug resistance in *E. coli* released into the environment near pig farms (Park et al., 2017). Of note, E. flexneri in European pigs, despite being subject to monitored antimicrobial administration, harbored ARG counts comparable to those in Chinese pigs, which are under unmonitored regimes. This underscores the role of *E. flexneri* as a zoonotic pathogen and a critical vector for ARG transmission (Zhang et al., 2020), as well as the urgent need for effective wastewater treatment strategies in pig farms worldwide (Cheng et al., 2020). The WHO has emphasized the necessity for a globally coordinated effort to address the escalating issue of AMR (Antimicrobial Resistance Collaborators, 2022).

In this study, we utilized metagenomic analyses and RF models to identify indicators of ARG contamination in both Chinese and European pig populations. However, we acknowledge that further improvements in analytical accuracy and an expanded sample set are required for a more comprehensive evaluation of ARG contamination within the pig industry. Consequently, the results presented here may contain some degree of uncertainty.

CONCLUSIONS

We identified ARGs and microbiome structures in pig manure across different countries and regions, revealing variations determined by antimicrobial usage.. Elevated antimicrobial pressure was found to promote ARG enrichment and potential migration. Aminoglycoside and tetracycline resistance genes were stable across the Chinese and European pig manure samples. Notably, the abundance of core ARGs identified in pigs may have implications for similar changes in humans. The core microbiome in pig manure exhibited geographical dependence. Utilizing high-resolution machine learning models, we identified specific ARG markers and biomarker taxa capable of discriminating between Chinese and European pig manure samples. We additionally identified indicators for accurately assessing AMR burden in both Chinese and European pig populations. MAG-based analysis demonstrated no specific host-association resistance pattern under different antimicrobial pressures and identified *E. flexneri* as exhibiting a multiple resistance pattern. These results emphasize the importance of collective global action aimed at reducing AMR risk and provide a reference subset of indicator ARGs for future routine AMR assessments in pig farms.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

C.H.T.: Visualization, Writing - Original draft preparation. Z.P.H. and L.D.: Data curation and Investigation. D.Y.X. and R.N.Z.: Conceptualization and Methodology. Z.L.Z. and W.G.X.: Conceived the study. All authors read and approved the final version of the manuscript.

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