Viral metagenomics reveals diverse viruses and antibiotic resistance genes in fecal samples of swine from farms in Shandong province

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Viral metagenomics reveals diverse viruses and antibiotic resistance genes in fecal samples of swine from farms in Shandong province

Abstract

Almost all research on the swine gut microbiome has focused on bacteria. However, these studies paid little attention to the swine gut virome. Here, metagenomic sequencing was employed to characterize the gut viromes and reservoir of antibiotic resistance genes (ARGs) in 16 pig farms which are distributed in 16 different prefecture-level cities in Shandong province. We identified the community composition of the gut viromes, and the top 50 viruses, and showed the patterns of the virus in 16 pig farms at the phylum, genus, and species levels. A total of 191 ARGs potentially conferring resistance to 17 classes of antibiotics were detected in the 16 pig farms. These 16 samples exhibited a superior abundance and more widespread distribution of genes associated with tetracycline, aminoglycosides, and macrolide-lincosamide-streptogramin (MLS) than other ARGs. Our research provides a universal overview of the prevalence and expression of virus and ARGs in pig gut microbiota.

***Keywords*:** Viral metagenomics; ARGs; Pig; Antibiotics; Gut microbiota.

Study contribution

This study provides the first comprehensive analysis of the fecal virome and antibiotic resistance genes (ARGs) in pigs from 16 farms in Shandong province using viral metagenomics. The findings reveal the diversity and composition of pig gut viruses and identify a high abundance of ARGs, particularly those conferring resistance to tetracyclines, aminoglycosides, and MLS antibiotics. The results offer important insights into the role of the virome in pig gut health and highlight the need for monitoring and managing ARGs in livestock to mitigate potential risks to public health.

Introduction

A gut microbiome is comprised of bacteria, viruses, fungi, and other microorganisms. Bacteria make up 95 % of the total microbial flora, and they have received the majority of attention in studies.[1, 2] However, viruses are often overlooked when it comes to maintaining the health and function of the host. Emerging research indicates that the intestinal virome plays a role in controlling metabolism, growth, and disease advancement by interacting with the accompanying bacteriome and the host itself.[3, 4] Many porcine enteroviruses, a group of highly contagious pathogens, have been reported to cause severe diseases. For example, porcine deltacoronavirus (PDCoV), an emerging swine enteric virus that causes severe diarrhea in pigs, has caused significant economic losses for pork producers worldwide.[5] There are three enteric viruses—porcine teschovirus, sapelovirus, and enterovirus —that can affect pigs and wild boars globally.[6] Thus, studying the swine gut virome has significant implications

for understanding disease onset and progression.

Antibiotic resistance is a global public health threat responsible for 700 000 deaths annually and is projected to cause 10 million deaths by 2050 if left unchecked.[7] Animal gut microbiomes have been found to harbor a greater variety of ARGs than human gut microbiomes, largely due to more extensive use of antibiotics in food animals.[8] Bacteria possess a wide range of ARGs, with most originating from the environment or from antibiotic producers via horizontal gene transfer (HGT).[9, 10] Sites with dense microbial communities, such as the guts of humans and animals, may exhibit higher rates of ARGs transfer via mobile genetic elements.[11] Although such transmission may complicate human infection treatment, comprehensive metagenomic studies of ARGs diversity and abundance in food-producing animals remain limited.[12]

A previous study showed that many genetic sequences from human and animal gut viromes lack evident homology to known viral genomes.[13] This highlights our limited knowledge of the viral reservoir and suggests that many unidentified viruses circulate among humans and both wild and domestic animals. Given the vast diversity of viral sequences, viral metagenomics should prioritize disease outbreaks and samples from key species.[14] As a powerful tool, viral metagenomics aids in detecting both known and highly divergent viruses. Advances in high-throughput sequencing technologies have greatly expanded our understanding of the microbiome, including viral components. Tools such as VirSorter[15] and VirFinder[16] significantly improve the analysis of metagenomic data by enabling the identification of viral sequences through nucleotide or translated protein homologies to known viruses. In this study, we

performed metagenomic sequencing to explore the viral community and ARGs in 16 pig farms located in different prefecture-level cities in Shandong province, aiming to provide a more detailed picture of the virome and ARGs composition and activity in the pig gut environment.

Materials and methods

Ethical statement

This study did not involve direct experimentation on animals. Instead, it analyzed microorganisms and metabolites present in pig feces, which were used solely as research material. No procedures were performed on live animals, and there was no impact on their welfare. Therefore, ethical approval was not required.

Sample collection

Pig fecal samples were collected from 16 pig farms distributed across prefecture-level cities in Shandong province (**Table S1**). Briefly, approximately 10 g of each fecal sample was collected and stored in RNAlater (Invitrogen, AM7020), suspended in the stabilizer solution, and mixed thoroughly. Samples were then immediately transported on ice to the laboratory for nucleic acid extraction.

DNA extraction, library construction, and metagenomic sequencing

Total genomic DNA was extracted from fecal samples using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's instructions. DNA concentration and purity were assessed using a TBS-380 fluorometer and NanoDrop2000 spectrophotometer, respectively. DNA quality was verified by electrophoresis on a 1 % agarose gel. A paired-end library was constructed from DNA fragmented to an average size of approximately 400 bp using a Covaris M220 (Gene Company Limited, China). Paired-end sequencing was performed on the DNBSEQ-T7 platform at Wefind Biotechnology Co., Ltd. (Wuhan, China) using the DNBSEQ-T7RS Reagent Kit (FCL PE150) V2.0, following the manufacturer's instructions (https://www.mgi-tech.com/products/reagents_info/43/). Sequencing results were deposited in the National Center for Biotechnology Information (NCBI) database (Accession Number: PRJNA796172).

Sequence quality control and genome assembly

High-quality clean reads were obtained by removing adapter sequences, trimming, and filtering out low-quality reads using *fastp*[17] (https://github.com/OpenGene/fastp, version 0.20.0) on the Majorbio Cloud platform (cloud.majorbio.com). Clean reads were mapped to the pig reference genome using Burrows-Wheeler Aligner[18] (http://bio-bwa.sourceforge.net, version 0.7.9a) to remove host-origin reads. The remaining reads were assembled into contigs using MEGAHIT, which uses succinct de Bruijn graphs. Contigs longer than 500 bp were selected for further analysis.

Gene prediction, taxonomy, and functional annotation

Prodigal was used to identify open reading frames (ORFs) in contigs.[19] The predicted ORFs with length being or over 200 bp were retrieved and translated into amino acid sequences using the NCBI translation table[20] (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/index.cgi?chapter=tgencodes#SG1). The CD-HIT (http://www.bioinformatics.org/cd-hit/, version 4.6.1) was used to construct a non-redundant gene catalog[21] with 95 % sequence identity and 90% coverage. Reads after quality control were mapped to the non-redundant gene catalog with 95 % identity using bowtie2[22] (https://bowtie-bio.sourceforge.net/bowtie2/index.shtml), and gene abundance in each sample was evaluated.

Representative sequences of non-redundant gene catalog were annotated based on the NCBI NR database using blastp as implemented in DIAMOND v0.9.19[23] (http://www.diamondsearch.org/index.php, version 0.8.35) for taxonomic annotations. Cluster of orthologous groups of proteins annotation for the representative sequences was conducted by using Diamond[23] (http://www.diamondsearch.org/index.php, version 0.8.35) against eggNOG database (version 4.5.1) with an e-value cutoff of 1e-5. The KEGG enrichment analysis was performed by using Diamond[24] (http://www.diamondsearch.org/index.php, version 0.8.35) against the Kyoto Encyclopedia of Genes and Genomes database[25] (http://www.genome.jp/keeg/, version 94.2) with an e-value cutoff of 1e-5. Antibiotic resistance genes annotation was conducted by using Diamond[23] (http://www.diamondsearch.org/index.php, version

0.8.35) against SARG2.0 database[26] (http://smile.hku.hk/SARGs.) with an e-value cutoff of 1e-5.

Statistical analysis

A significance threshold of $P < 0.05$ was used for all statistical tests. GraphPad Prism (version 6.0) was used to generate stacked bar charts. Heatmaps were created using the vegan and heatmap packages in R.

Results

Alpha and beta diversities of fecal viruses in 16 pig farms

To evaluate differences in intestinal viruses across the groups, alpha diversity indices (Shannon, Simpson, and inverse Simpson) were analyzed. Higher Shannon index values indicate greater diversity, whereas higher Simpson values suggest lower diversity. The DZ group exhibited the highest Shannon index, although no statistically significant differences were observed ($P > 0.05$) (**Figure 1A**). The Simpson and inverse Simpson indices followed trends similar to the richness index, with higher values in the DZ and J groups. In contrast, the RZ group had the lowest Simpson and inverse Simpson values, suggesting relatively low diversity in that group. Overall, variation in viral communities across different farms in Shandong province appeared small.

Subsequently, analysis of similarities was conducted to assess community similarity among the 16 pig farms. The results indicated distinct viral signatures at the

phylum (**Figure 1B**), genus (**Figure 1C**), and species (**Figure 1D**) levels.

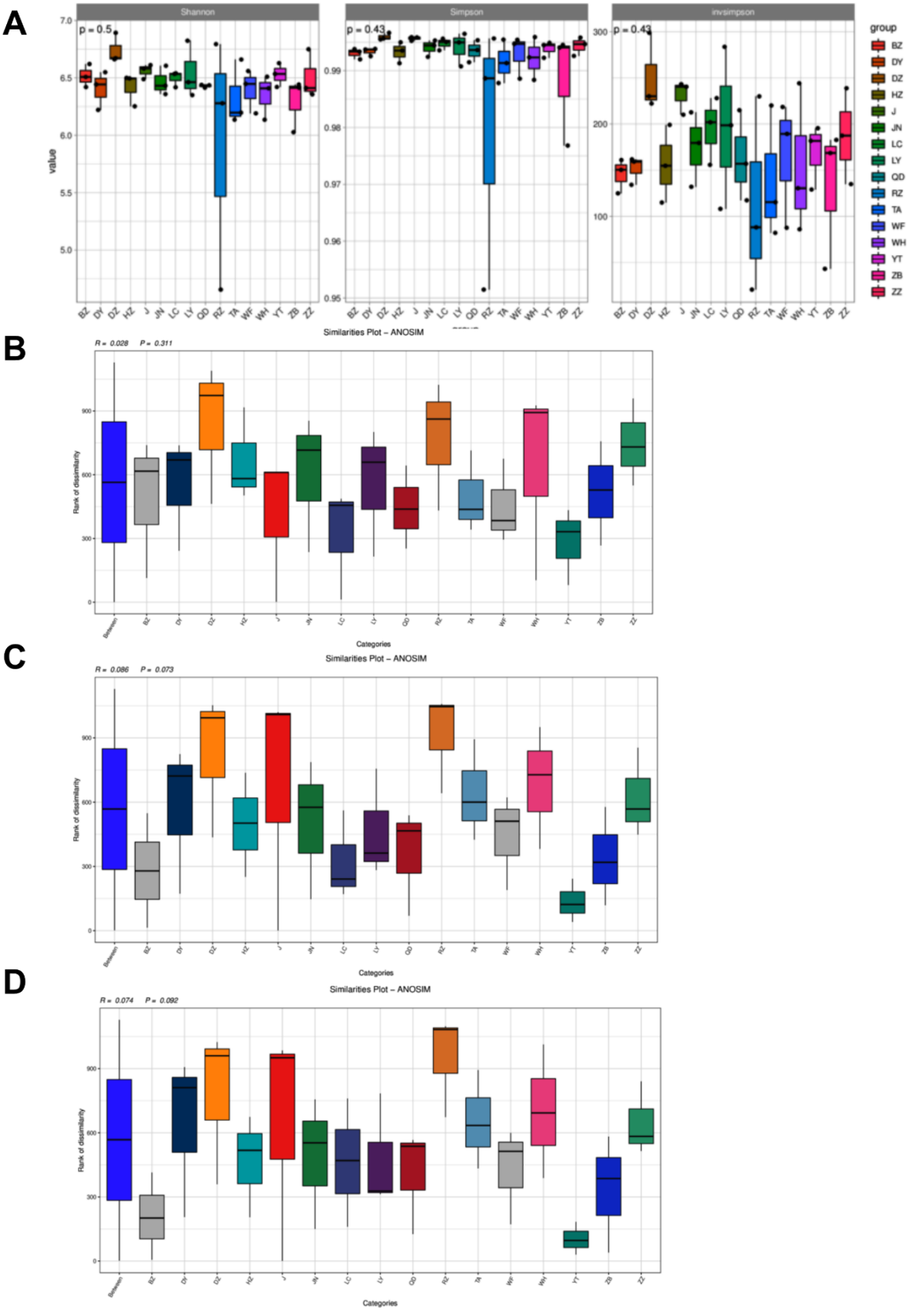

Figure 1. Characterization of gut virome in 16 pig farms. Richness indices Shannon (A), Simpson (B), inverse Simpson (C) and analysis of similarities of intestinal viruses at the phylum, genus, and species levels (D) are shown.

Community composition of the gut viromes in 16 pig farms at the phylum, genus, and species levels

Figure 2 displays the gut virome composition at the phylum, genus, and species levels. At the phylum level (**Figure 2A**), the dominant viral taxa included Uroviricota, Unclassified Viruses, Nucleocytoviricota, Phixviricota, Preplasmiviricota, and Hofneiviricota, with Uroviricota accounting for more than 90 % in all groups. The relative abundance of these phyla remained relatively stable across all farms. At the genus level (**Figure 2B**), viruses from the *Siphoviridae* family dominated across groups. Other genera varied notably by group. For instance, Tequatrovirus was notably elevated in the J group, representing 22.56 %, while Astrithrvirus reached 17.68 % in the RZ group.

At the species level (**Figure 2C**), *Siphoviridae* sp. predominated across all groups. The overall composition was consistent among most groups. In the J group, over 8 % of all viruses were identified as bacteriophages and streptococci. In the RZ group, *Pectobacterium phage DU_PP_III* and *Podoviridae_sp_ctdc61* were the most frequently annotated viruses, while in the WH group, *s_Bacteriophage* sp was most common.

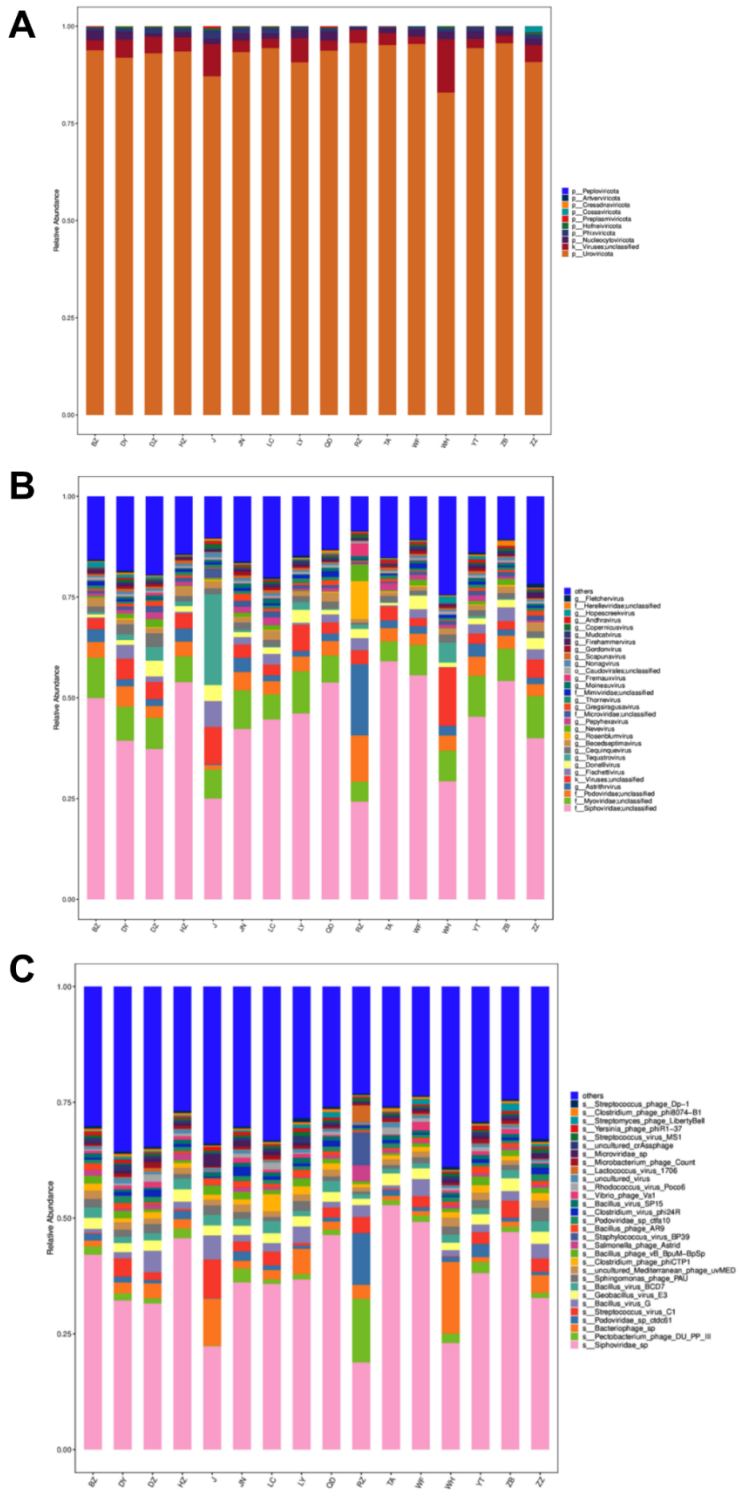

Figure 2. Community composition of the gut viromes in 16 pig farms at the phylum (A), genus (B), and species levels (C).

The core and farm-associated viromes

The top 50 viruses at the phylum, genus, and species levels are shown in the heatmap (**Figure 3**), reflecting viral distribution patterns across farms. At the phylum level (**Figure 3A**), Cossaviricota had notably higher expression in the ZZ group. Uroviricota levels were higher in the RZ group. Hofneiviricota and Cressdnaviricota were more abundant in the DY and DZ groups.

At the genus level (**Figure 3B**), overall differences were apparent among groups. *Felixounavirus*, *Nitunavirus*, and *Obolenskvirus* were particularly abundant in the TA group. The RZ group showed higher abundances of *Nevevirus*, *Podoviridae*, *Fremauxvirus*, *Astrithrvirus*, and *Rosenblumvirus*. The LC group exhibited elevated levels of *Krischvirus*, *Teubervirus*, and *Chopinvirus*.

At the species level (**Figure 3C**), eight viral species—including *Streptococcus virus MS1*, *Salmonella phage Astrid*, *Lactococcus virus 1706*, *Deep-sea thermophilic phage D6E*, *Staphylococcus virus BP39*, *Staphylococcus virus GRCS*, *Pectobacterium phage DU_PP_III*, and *Podoviridae_sp_ctdc61*—were uniquely abundant in the RZ group. In the BZ and LC groups, *Tetrasphaera virus TJE1* and *Lactococcus virus P087* were more highly expressed.

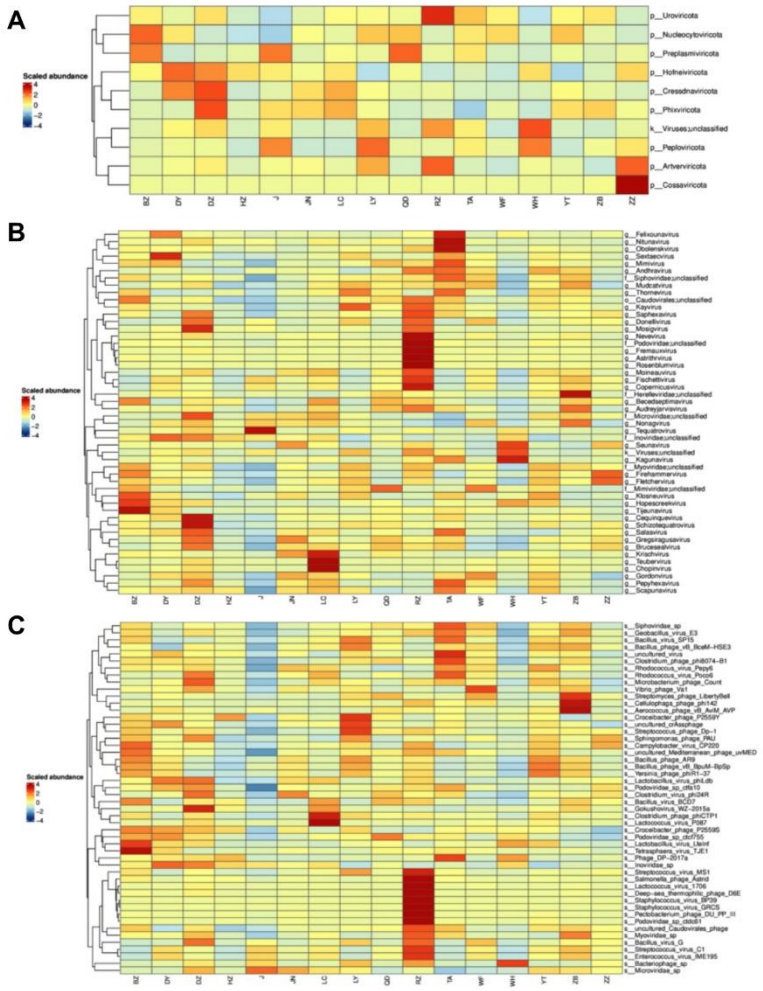

Figure 3. Heatmap showing abundances of top 50 viruses in the phylum (A), genus (B), and species levels (C).

Furthermore, the virus genus features, and key viruses, in each group were identified by using the linear discriminant analysis (LDA) effect size (LEfSe). The abundances of these features are visualized and exhibited in **Figure 4**. There were one characteristic viral genera in each group of BZ, RZ, WF, and ZZ in the genus level (**Figure 4A**). In the species level (**Figure 4B**), there were 4, 1, 1, 1 and 1 characteristic virus in the DY(Dongying), DZ, J(Jinan), JN(Jining), and ZZ(Zaozhuang) groups, respectively.

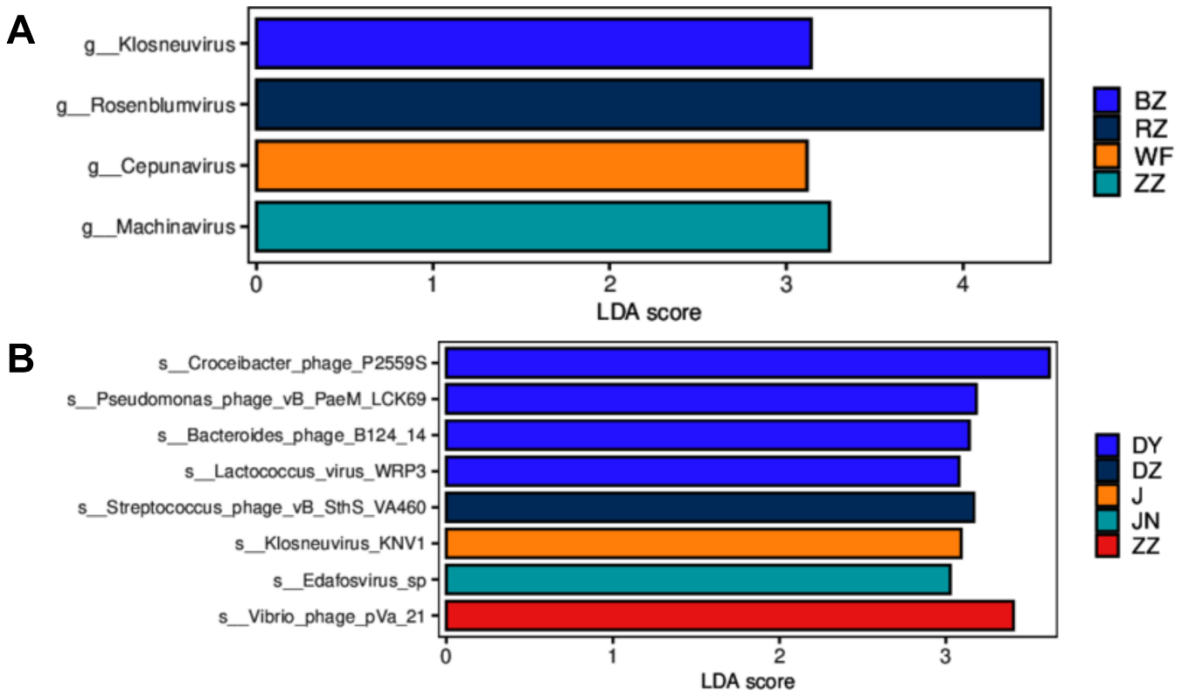

Figure 4. Characteristic viruses at genus (A) and species levels (B) were identified by the LEfSe algorithm (LDA score of > 3). LEfSe: linear discriminant analysis effect size.

Antibiotic resistance genes in 16 pig farms

Principal coordinates analysis (PCoA) revealed clear associations between gut resistome profiles and geographical location (**Figure 5A**). A total of 191 ARGs associated with 17 antibiotic classes were detected across the 16 farms (**Tables S2** and **S3**). Genes conferring resistance to tetracyclines, aminoglycosides, and macrolide-lincosamide-streptogramin (MLS) were particularly abundant and widely distributed (**Figure 5B**).

The top 30 most abundant ARGs varied significantly between farms (**Figure 5C**). The WH(Weihai), HZ(Heze), TA(Tai'an), and RZ(Zaozhuang) farms had high relative abundances of *aph3-III*, *aadE*, *tetW*, and *tet44*, suggesting similar resistome profiles. Likewise, LY(Linyi), BZ(Binzhou), ZZ(Zaozhuang), and YT(Yantai) farms showed high levels of *tetW*, *tetQ*, *ermF*, and *aadE*.

Furthermore, a wide distribution of ARGs with transfer potential was observed across the 16 farms (**Figure 5D**). The ARGs families with the most genes included tetracycline, multidrug, polymyxin, and beta-lactam. The HZ, TA, and WH farms had the highest ARGs abundances. In contrast, the J and JN groups exhibited low ARGs abundances. Notably, *cmeA* and *cmeB* were most abundant in the DZ(Dezhou) group, while *vanG* and *vanX* were uniquely highly expressed in the WH group.

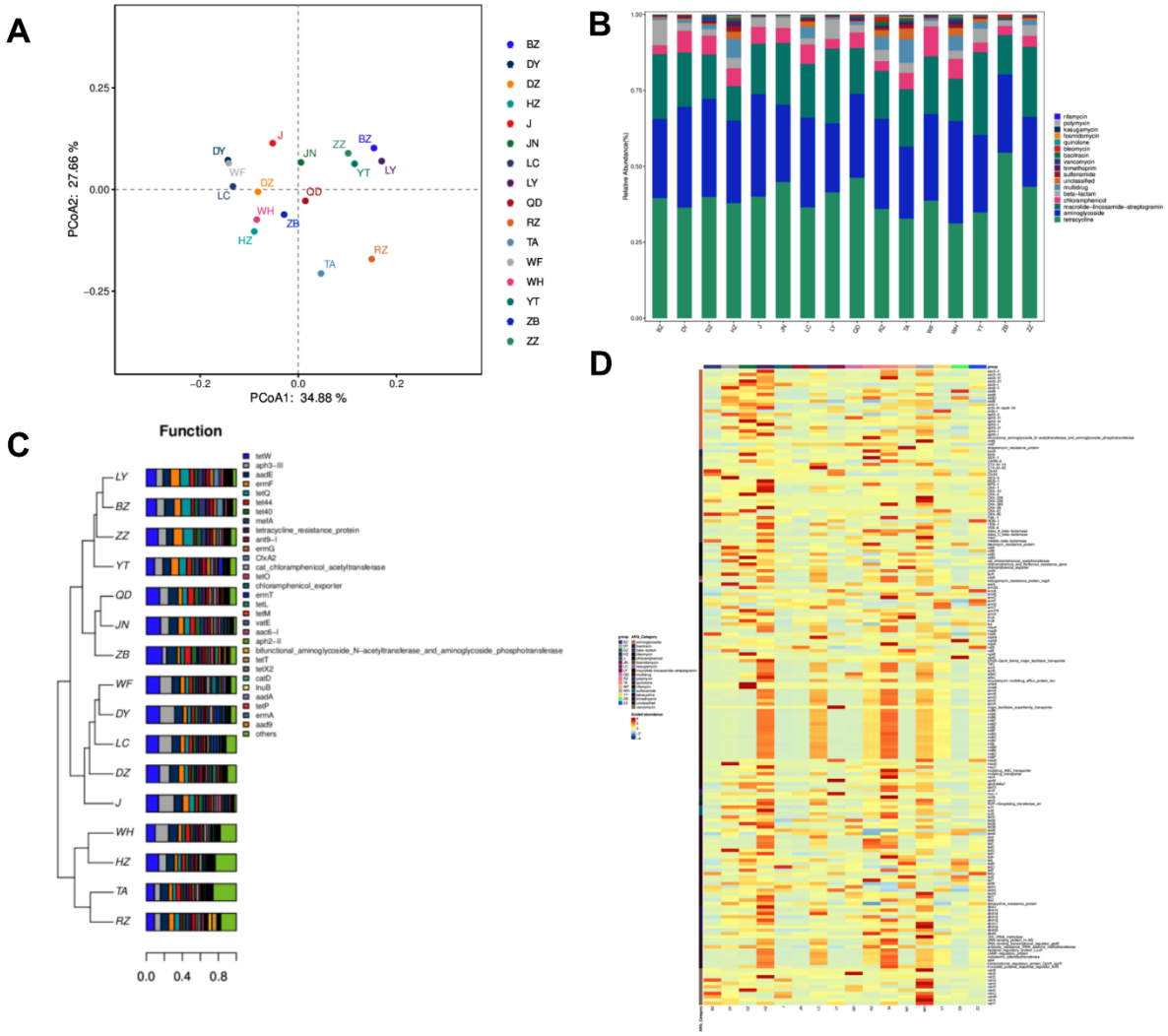

Figure 5. Classification and PCoA of detected ARGs. (A) PCoA analysis showing profiles of gut resistors in 16 pig farms. (B) Barplot showing the distribution of antibiotic classes. (C) Phylogenies and abundance of top 30 ARGs identified in the pig gut. (D) Heatmap showing abundances of ARGs. PCoA: Principal coordinates analysis, ARGs: antibiotic resistance genes.

Discussion

In recent years, increasing attention has been paid to human and animal enteric viruses. According to existing literature, swine enteric viruses have mainly been studied in the context of disease models.[27, 28] However, enteric viruses play essential roles in intestinal homeostasis and physiological function as key components of the gut microbiome. Among these, bacteriophages—being a significant part of the virome—interact with host bacteria in complex ways. On one hand, they regulate bacterial communities through lytic infection, thereby affecting the abundance of specific bacterial taxa and influencing the overall gut microbiome composition and functionality. Other viruses within the virome may also influence the gut microbiome. For example, some viruses that infect eukaryotic cells in the gut can affect host immune responses and intestinal barrier integrity. These host-virus interactions may indirectly impact bacterial communities, given that immune status and barrier function are crucial for microbiome stability. Despite these important roles, few studies have examined the swine gut virome in depth.[29]

In this study, fecal samples were collected from 16 pig farms in Shandong province to provide an extensive overview of the swine gut virome. This information allows for a better understanding of how swine gut viromes vary by region. Alpha diversity metrics (Shannon and Simpson indices) indicated that viral community variation among farms was generally low. The major viral phyla—*Uroviricota*, *Nucleocytoviricota*, *Phixviricota*, *Preplasmiviricota*, and *Hofneiviricota*—were consistently abundant across farms. These viruses likely colonize the swine gut early in life and persist through various

production stages (e.g., lactation, nursery, growing, finishing), suggesting they play a stabilizing role in the enteric virome.[30]

Nonetheless, differences in virome composition were observed between farms. For instance, *Cossaviricota* was more highly expressed in the ZZ group; *Uroviricota* was elevated in RZ; and DY and DZ had higher abundances of *Hofneiviricota* and *Cressdnaviricota*. These differences may be related to localized antibiotic use practices.

Our findings also highlight the presence and expression of numerous ARGs in the pig gut microbiomes across the 16 farms. The variability in ARGs abundance could be attributed to differences in antibiotic usage. While antibiotics are used to maintain health and prevent disease, they can also disrupt gut bacterial populations and facilitate the emergence of resistance.[31, 32] Previous studies have shown that ARGs in pigs can enter the food chain and potentially be transmitted to humans.[33] In this study, a total of 191 ARGs were identified, falling into 17 classes (**Table S2**). Among these, genes conferring resistance to tetracyclines, aminoglycosides, and MLS were the most prevalent—consistent with earlier studies reporting high levels of tetracycline resistance in human, poultry, and pig gut microbiomes.[11] For example, Wang et al. found tetracycline resistance to be the most prevalent in samples from humans (0.48 %), chickens (0.32 %), and pigs (0.48 %).[32] Our study also revealed farm-specific differences in ARGs expression. HZ, TA, and WH farms exhibited particularly high ARGs abundance, while J and JN farms showed lower levels. Factors beyond antibiotics—such as diet, environmental pollutants (e.g., heavy metals), and

disinfectant use—may also contribute to differences in ARGs profiles.[32]

Conclusion

This study investigated the characteristics of the enteric virome in 16 pig farms across prefecture-level cities in Shandong province using metagenomic techniques. The community composition of the gut virome was characterized at the phylum, genus, and species levels. The top 50 viruses at each taxonomic level were identified, revealing patterns of viral distribution across the different farms. Additionally, a total of 191 antibiotic resistance genes (ARGs), potentially conferring resistance to 17 classes of antibiotics, were detected. A wide range of ARGs abundances was observed among the 16 farms, particularly for those conferring resistance to tetracyclines, aminoglycosides, and MLS antibiotics. In the future, a comprehensive understanding of resistance gene transcription across time and geography in human and food-animal guts will be essential. Continuous monitoring of both human and pig gut microbiomes is recommended to evaluate the risk of horizontal transfer of highly expressed resistance genes.

Data availability

Sequence data associated with this project have been deposited in the NCBI Short Read Archive database (Accession Number: PRJNA796172).

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Conflicts of interest

The authors declare no competing interests.

Author contributions

Conceptualization: H Guo

Data curation: H Guo, C Su

Formal analysis: L Pan

Funding acquisition: H Guo

Investigation: L Jv

Methodology: C Su

Project administration: H Guo

Resources: H Guo

Software: C Wang

Supervision: H Guo

Visualization: Z Su

Writing-original draft: H Guo

Writing-review and editing: C Su, L Pan, H Guo

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