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Antimicrobial resistance characteristics and phylogenetic relationships of pleuromutilin-resistant Enterococcus isolates from different environmental samples along a laying hen production chain

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ABSTRACT

Antimicrobial resistance in the laying hen production industry has become a serious public health problem. The antimicrobial resistance and phylogenetic relationships of the common conditional pathogen *Enterococcus* along the laying hen production chain have not been systematically clarified. 105 *Enterococcus* isolates were obtained from 115 environmental samples (air, dust, feces, flies, sewage, and soil) collected along the laying hen production chain (breeding chicken, chick, young chicken, and commercial laying hen). These *Enterococcus* isolates exhibited resistance to some clinically relevant antibiotics, such as tetracycline (92.4%), streptomycin (92.4%), and erythromycin (91.4%), and all strains had multidrug resistance phenotypes. Whole genome sequencing characterized 29 acquired antibiotic resistance genes (ARGs) that conferred resistance to 11 classes of antibiotics in 51 pleuromutilin-resistant *Enterococcus* isolates, and *lsa(E)*, which mediates resistance to pleuromutilins, always co-occurred with *lnu(B)*. Alignments with the Mobile Genetic Elements database identified four transposons (Tn554, Tn558, Tn6261, and Tn6674) with several ARGs (*erm(A)*, *ant(9)-la*, *fex(A)*, and *optrA*) that mediated resistance to many clinically important antibiotics. Moreover, we identified two new transposons that carried ARGs in the Tn554 family designated as Tn7508 and Tn7492. A complementary approach based on conventional multi-locus sequence typing and whole genome single nucleotide polymorphism analysis showed that phylogenetically related pleuromutilin-resistant *Enterococcus* isolates were widely distributed in various environments on different production farms. Our results indicate that environmental contamination by antimicrobial-resistant *Enterococcus* requires greater attention, and they highlight the risk of pleuromutilin-resistant *Enterococcus* and

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ARGs disseminating along the laying hen production chain, thereby warranting effective disinfection.

Introduction

The laying hen production industry is a crucial component of the food supply and the associated environment is closely related to humans. The extensive use of antibiotics and the confined production environment in laying hen farms can readily lead to the emergence and spread of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs), thereby reducing the effectiveness of antibiotic treatment and potentially promoting bacterial infections in humans (File et al., 2019; Li et al., 2015; Skowron et al., 2016). Studies conducted around the world have demonstrated that the livestock production environment can be an important reservoir for antimicrobial resistance and ARB can spread among humans, environments, and animals to cause medical and ecological problems (Forsberg et al., 2012; Hu et al., 2017; O'Dea et al., 2019; Van Boeckel et al., 2015; Wang et al., 2021; Zhu et al., 2021). In the animal husbandry process, *Enterococcus* can also contaminate animal-derived food and endanger public health (Torres et al., 2018). Moreover, *Enterococcus* strains can convert from medium priority pathogens into high priority clinical pathogens due to their inherent antimicrobial resistance, capacity to acquire and transmit the determinants of antimicrobial resistance, and flexible genomes (Arias and Murray, 2012). Some animal-derived *Enterococcus* strains may not pose risks to humans but their high genetic plasticity means that they can act as donors of ARGs to other pathogenic *Enterococcus* strains and Gram-positive bacteria, which limits the treatment choices for infections (Garcia-Solache and Rice, 2019; Hammerum, 2012). Thus, it is necessary to determine the distribution and antimicrobial resistance profiles of *Enterococcus* strains in various environments, such as in livestock production and hospitals.

Pleuromutilins comprise a class of antibiotics that selectively inhibit bacterial translation by binding to the highly conserved peptidyl transferase center of the bacterial ribosome, and they have potent activities against Gram-positive and fastidious Gram-negative pathogens, as well as mycoplasma and intracellular organisms such as *Chlamydia* spp. and *Legionella pneumophila* (Davidovich et al., 2007; Paukner and Riedl, 2017). Two pleuromutilin derivatives comprising tiamulin and valnemulin have been successfully utilized as antibiotics for respiratory tract and intestinal infectious diseases in poultry for decades (Tang et al., 2012; van Duijkeren et al., 2014). The activity spectrums of these two antibiotics are similar, but valnemulin is more active than tiamulin and it has been widely used in veterinary practice (Koutsoumanis et al., 2021; Yang et al., 2014; Zhao et al., 2014). A pleuromutilin derivative called retapamulin was approved for the treatment of topical methicillin-resistant *Staphylococcus aureus* (MRSA) infections in 2007 (Jones et al., 2006). Lefamulin is also a pleuromutilin derivative and it has targeted antibacterial activity against the most prevalent community-acquired bacterial pneumonia pathogens (File et al., 2019). However, resistance to pleuromutilin has increasingly been reported in Gram-positive bacteria in recent years (Crowe-McAuliffe et al., 2021). Moreover, pleuromutilin-resistant *Enterococcus* strains typically exhibit multidrug resistance (MDR, i.e., not susceptible to three or more classes of antibiotics), with resistance to pleuromutilin, lincosamide, and streptogramin A (PLS_A) antimicrobial agents (Schwarz et al., 2016). However, little information is available regarding the resistance of *Enterococcus* strains to pleuromutilin during laying hen production. The ARG profiles and details about the transmission of ARGs by antibiotic-resistant *Enterococcus* strains in laying hen production environments also need further elucidation. Thus, in the present study, we determined the antimicrobial resistance profiles of *Enterococcus* isolates along a laying hen production chain in Sichuan, China. The chain contained four farms in different production stages (breeding chicken, chick, young chicken, and commercial laying hen). The breeding chicken farm was responsible for the production of breeding eggs and the eggs were transported to the chick farm for hatching. After hatching, the one-day-old chicks were vaccinated and given antibiotics, and then transferred to the young chicken farm for about four months to grow into young chickens. Finally, the young chickens were transferred to the commercial laying hen farm to start laying eggs. The use of antibiotics was discontinued before the egg-laying period when health care mainly involved using herbs in order to reduce antibiotic residues in eggs. In total, 105 *Enterococcus* isolates were obtained from air, dust, feces, flies, sewage, and soil samples collected from the four farms. The *Enterococcus* isolates were tested to determine their susceptibility to 14 clinically important antibiotics. In addition, 51 pleuromutilin-resistant *Enterococcus* isolates were analyzed by whole genome sequencing (WGS) to assess the acquired ARGs, ARGs transferred by mobile genetic elements (MGEs), and phylogenetic relationships of pleuromutilin-resistant *Enterococcus* isolates. We suggest that the laying hen production environment is an important reservoir for antimicrobial-resistant *Enterococcus* strains. In addition, phylogenetically related pleuromutilin-resistant *Enterococcus* isolates and MGEs that carried ARGs were widely distributed in different environmental samples and production farms. The results obtained in this study will hopefully provide a basis and reference for the control of ARB and ARGs in layer hen breeding to improve public health standards.

1. Materials and methods

1.1. *Experimental design and sampling*

To identify the potential sources of *Enterococcus* contamination in the production environment, air, dust, feces, flies, sewage, and soil samples were collected from each laying

hen farm along a laying hen production chain in Sichuan, which is an important province for livestock and poultry breeding in China. Air samples were collected with an Anderson Class VI air sampler (S6, Sennon, China). Samples of flies were collected by using sticky fly boards, where 30 to 40 flies were collected per sample. Each air, dust, feces, sewage, and soil sample was a mixture of three parallel samples from the same location. Details of the sampling sites are presented in Appendix A Fig. S1. In total, 115 samples were collected, with 28 from the breeding chicken farm, 28 from the chick farm, 30 from the young chicken farm, and 29 from the commercial laying hen farm. The samples were stored on ice and immediately transported to the laboratory.

1.2. *Bacterial isolation and identification*

After arriving at the laboratory, each sample was mixed at 2000 r/min with a vortex shaker (VM800, JOANLAB, China) for 30 min to disperse it uniformly. An appropriate amount of each sample was then pre-enriched with 10 mL of fresh brain heart infusion (BHI, Land Bridge, China) broth and incubated for 18 hr at 37°C with shaking (180 r/min). The resultant bacterial culture was plated onto Pfizer Selective *Enterococcus* Agar medium (Land Bridge, China) and incubated overnight at 37°C. A single brown colony with a round and smooth surface was selected from each plate and treated as a presumptive *Enterococcus* isolate. Presumptive *Enterococcus* isolates were determined by analyzing the entire 16S rDNA gene via Sanger sequencing (Tsingke, China). All identified pure bacterial strains were stored at −20°C in BHI broth containing 25% glycerol for further analysis.

1.3. *Antimicrobial susceptibility testing*

All *Enterococcus* isolates ($n = 105$) were subjected to antimicrobial susceptibility test using the Kirby–Bauer disk diffusion method or microbroth dilution method. Fourteen antimicrobial agents comprising gentamycin (GEN, 120 μg), fosfomycin (FOS, 200 μg), clindamycin (CLI, 10 μg), linezolid (LZD, 30 μg), chloramphenicol (CHL, 30 μg), vancomycin (VAN, 30 μg), erythromycin (ERY, 15 μg), florfenicol (FFC, 30 μg), streptomycin (STR, 300 μg), tetracycline (TET, 30 μg), levofloxacin (LEV, 5 μg), teicoplanin (TEC, 30 μg), rifampin (RIF, 5 μg), and the pleuromutilin derivative valnemulin (VAL, microbroth dilution method) were tested in the assays. All antimicrobial susceptibility discs were obtained from Thermo Fisher Technology Co. Ltd, China and valnemulin was obtained from Tsingke Biotechnology Co. Ltd, China. Results were interpreted based on the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018, 2019). *Staphylococcus aureus* ATCC 25923 (Kirby–Bauer disk diffusion) and *Enterococcus faecalis* ATCC 29212 (microbroth dilution method) were used for quality control in assays. Resistance to valnemulin was determined using the standard microbroth dilution method. Briefly, single bacterial colonies were cultured in Mueller Hinton Broth (MHB, Land Bridge, China) at 37°C and 180 r/min for 8–12 hr. Subsequently, two-fold dilution of valnemulin in MHB were mixed with an equal volume of bacterial suspensions in MHB containing approximately 1.5×10^6 colony-forming units/mL in a sterile 96-well microtiter plate. The plate was incubated for 18 hr at 37°C and the minimum inhibitory concentration (MIC) was then quantified. MIC were defined as the lowest concentrations of antibiotics that resulted in no visible growth of bacteria. There is no recommended resistant cutoff for valnemulin in *Enterococcus*, so the cutoff for another veterinary pleuromutilin antibiotic called tiamulin was referenced in this study. The resistant cutoff for tiamulin is 32 μg/mL (CLSI, 2018), and many studies have shown that valnemulin has a lower MIC compared with tiamulin (Koutsoumanis et al., 2021). Thus, resistance to valnemulin was determined when the MIC was greater than or equal to 16 μg/mL in this study.

1.4. *WGS and analysis of pleuromutilin-resistant* Enterococcus *isolates*

Pleuromutilin-resistant *Enterococcus* isolates (excluding isolates with similar resistance, $n = 51$) were subjected to WGS to systematically characterize the ARG profiles of isolates in the samples from the layer breeding enterprise and to infer their phylogenetic relationships. Briefly, bacterial genomic DNA was extracted using a genomic DNA kit (Tiangen, China). WGS was performed using the Illumina Hiseq X Ten platform (Novogene, China). Sequencing reads were assembled into contigs using SPAdes v.3.13.0 (Bankevich et al., 2012). QUAST v.5.0.2 was used to evaluate the quality of contigs (Gurevich et al., 2013). BUSCO v.5.3.2 was used to evaluate the completeness of the genomes (Simao et al., 2015). The presence of acquired ARGs was identified using the ResFinder web server v.4.1 with 90% identity (Zankari et al., 2012). The genetic environments where the pleuromutilin resistance genes were located were analyzed and genetic mapping was then performed using easyfig v2.2.5 (Sullivan et al., 2011). After downloading the MGE database v1.0.2, alignment of similar sequences was performed with BLAST (Johansson et al., 2021).

1.5. *Analyses of pleuromutilin-resistant* Enterococcus *isolates based on multi-locus sequence typing (MLST) and single nucleotide polymorphisms (SNPs)*

The phylogenetic relationships among pleuromutilin-resistant *Enterococcus* isolates were examined to investigate the possibility of bacterial transmission between different farms. Using the WGS data, the ST types of 24 *E. faecalis* isolates and 24 *Enterococcus faecium* (*E. faecium*) isolates were identified using the MLST web server v.2.0 (Larsen et al., 2012). Genomes of *Enterococcus* isolates that did not match the corresponding ST type were uploaded to pubMLST to obtain new ST sequence numbers (Jolley et al., 2018). PHYLOViZ v 2.0 (which is available for data analysis and the visualization of sequence-based typing methods and associated epidemiological and population data) was used to visualize the phylogenetic data (Nascimento et al., 2017). Phylogenetic analysis of *E. faecalis* ($n = 24$) and *E. faecium* ($n = 24$) genomes was performed based on SNPs using the CSI Phylogeny

Table 1 – Isolation rates of Enterococcus isolates from different breeding farms and different sample types.

Sample	Breeding chicken	Chick	Young chicken	Commercial laying hen	Total
Air	80.00% (4/5)	80.00% (4/5)	80.00% (4/5)	100.00% (5/5)	85.00% (17/20)
Dust	100.00% (5/5)	80.00% (4/5)	100.00% (5/5)	100.00% (5/5)	95.00% (19/20)
Feces	100.00% (5/5)	100.00% (5/5)	100.00% (5/5)	100.00% (5/5)	100.00% (20/20)
Flies	100.00% (5/5)	60.00% (3/5)	80.00% (4/5)	100.00% (5/5)	85.00% (17/20)
Sewage	100.00% (3/3)	100.00% (3/3)	100.00% (5/5)	100.00% (4/4)	100.00% (15/15)
Soil	80.00% (4/5)	100.00% (5/5)	100.00% (5/5)	60.00% (3/5)	85.00% (17/20)
Total	92.86% (26/28)	85.71% (24/28)	93.33% (28/30)	93.10% (27/29)	91.30% (105/115)

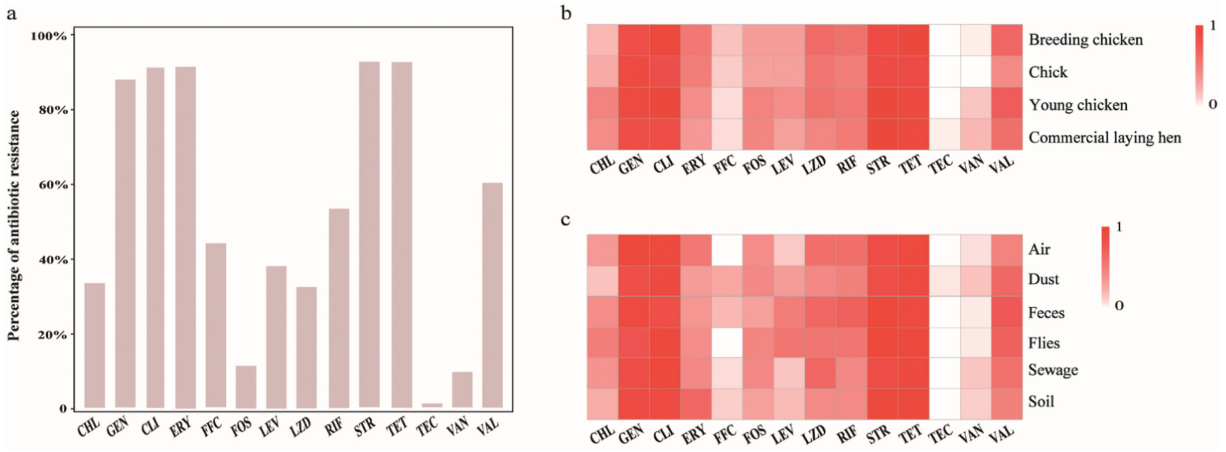

Fig. 1 – Antibiotic susceptibility profiles of *Enterococcus* isolates. (a) Bar plots showing the percentages of *Enterococcus* isolates with resistance to a series of antibiotics ($n = 14$). Heatmap depicting the percentages of *Enterococcus* isolates from different production farms (b) and different environmental samples (c) with resistance against a series of antibiotics.

web server v.1.4 (Kaas et al., 2014). Phylogenetic trees were visualized with iTol (Letunic and Bork, 2021).

2. Results

2.1. *Antimicrobial susceptibility of* Enterococcus *isolates in different environmental samples and production farms*

In total, 105 *Enterococcus* strains were identified (Table 1). The isolation rates ranged from 60% to 100%, thereby indicating that environments such as feces and sewage on large-scale layer farms were important sources of *Enterococcus*. Antimicrobial susceptibility tests were conducted for 105 *Enterococcus* isolates and the results are shown in Fig. 1. Among the 105 *Enterococcus* isolates, 92.4% exhibited resistance to TET and STR, while 91.4% were resistant to ERY and CLI (Fig. 1a). In addition, 60% of the *Enterococcus* isolates were resistant to VAL. Importantly, 32.4% of the isolates were resistant to LZD, 11.4% were resistant to FOS, and 9.5% were resistant to VAN. Resistance to these three antibiotics is a serious concern because they are important in clinics for treating infections associated with MRSA. All *Enterococcus* isolates were resistant to more than three classes of antibiotics. The resistance phenotypes were similar for *Enterococcus* isolates from different production farms (Fig. 1b) and for *Enterococcus* isolates from different environmental samples (Fig. 1c).

2.2. *ARG profiles of pleuromutilin-resistant* Enterococcus *isolates*

WGS was conducted to characterize the antimicrobial resistance determinants in pleuromutilin-resistant *Enterococcus* isolates ($n = 51$). All genomic data were well assembled with completeness > 98.4% and suitable for the subsequent analyses. According to the ResFinder results, 29 ARGs conferred resistance to antibiotics comprising aminoglycosides (*aph(6″)-Id*, *aph(2″)-Ia*, *aac(6′)-aph (2″)*, *aac(6′)-I*, *aac(6′)-Iid*,*ant(6)-Ia*, *ant(9)-Ia*, *aph(3′)-III*, *str*, and *aadD*), glycopeptides (*vanC2* and *vanXY*), oxazolidinones (*optrA* and *poxtA*), amphenicols (*fexA*, *fexB*, *poxtA*, *optrA*, and *cat*), streptogramin a (*lsa(A)*, *lsa(E)*, *vat(D)*, and *vat(E)*), streptogramin b (*erm(A)*, *erm(B)*, and *msr(C)*), lincosamide (*lsa(A)*, *lsa(E)*, *lnu(B)*, *erm(A)*, and *erm(B)*), macrolides (*erm(A)*, *erm(B)*, *mefA*, and *msr(C)*), tetracyclines (*tet(L)*, *tet(M)*, *tet(O)*, and *poxtA*), pleuromutilin (*lsa(E)*), quinolone (*pcrC*), and trimethoprim (*dfrG*) in pleuromutilin-resistant *Enterococcus* isolates, where *lsa(E)* and *lnu(B)* ($n = 51$), *aph(3′)-III* ($n = 50$), *erm(B)* ($n = 49$), *tet(L)* ($n = 48$), *tet(M)* ($n = 47$) and *ant(6)-Ia* ($n = 44$) were the most frequently observed ARGs (Fig. 2). Five types of genetic environments

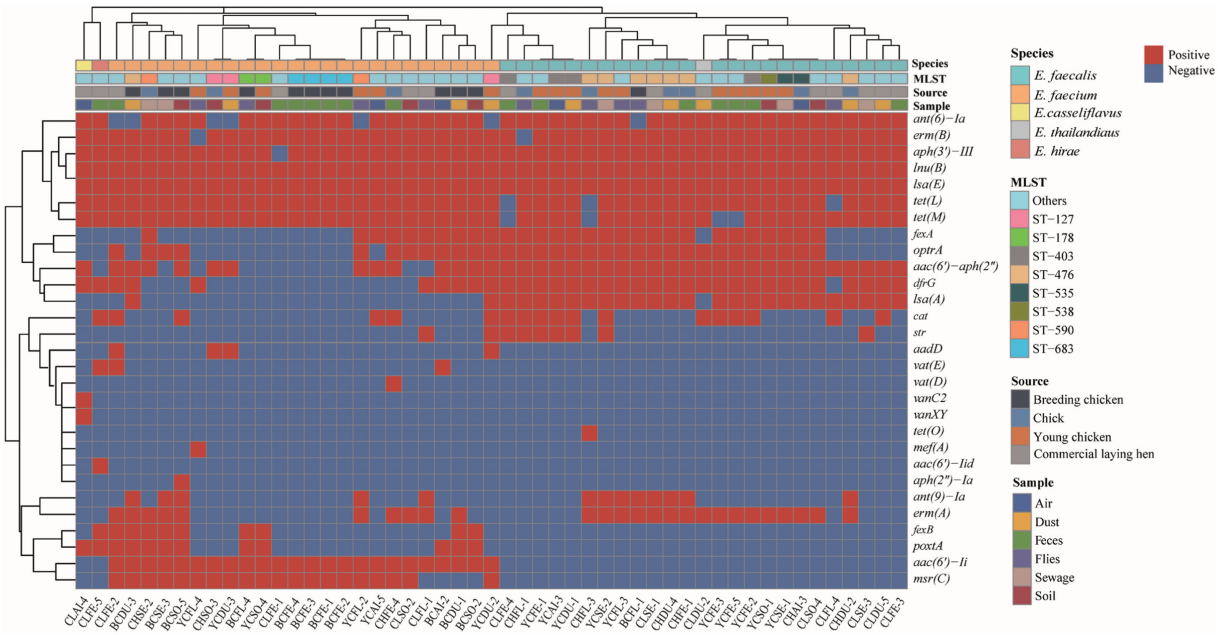

Fig. 2 – Heatmap depicting the distribution of antibiotic resistance genes (ARGs, *n* = 29) detected in bacterial genomes of *Enterococcus* isolates (*n* = 51). ARGs (rows) and bacterial genomes (columns) were both clustered based on the Euclidean distances measured using the pheatmap package (v 1.0.12) in R. Multi-locus sequence typing (MLST) was implemented to characterize the ST types of isolates and less frequently observed ST types were clustered into "Others".

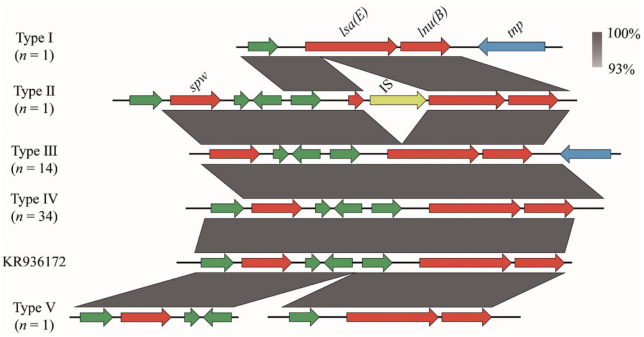

Fig. 3 – Genetic environment of pleuromutilin-resistant gene *lsa(E)*. Genes are indicated as arrows and the arrowhead shows the direction of transcription. Antimicrobial resistance genes (*lsa(E)*, *lnu(B)*, and *spw*) are shown in red, transposase genes (*tnp*) are displayed in blue, insertion sequence is displayed in yellow, and other genes are displayed in green. Darker shading indicates higher nucleotide sequence identity.

were found for 51 pleuromutilin-resistant *Enterococcus* strains. The *lsa(E)* gene always co-occurred with *lnu(B)* (Fig. 3). The *spw* gene was generally present upstream of *lsa(E)* (*n* = 50). The transposase gene, *tnp*, was found downstream of *lnu(B)* in 15 pleuromutilin-resistant *Enterococcus* isolates. In addition, an insertion sequence (IS) was found in the *lsa(E)* of one *Enterococcus* isolates. Some *Enterococcus* isolates from different samples collected on the same farm (CLSO-4, CLFL-4, CLSE-3, CLDU-5, and CLFE-3) had similar ARGs profiles. It should be noted that *poxtA* (which increases the MIC or resistance to oxazolidinones, phenicols, and tetracyclines) and/or *optrA* (responsible for combined resistance to oxazolidinones and phenicols) (Schwarz et al., 2021) were identified in 37 (72.5%) strains.

2.3. *MGE profiles of pleuromutilin-resistant* Enterococcus *isolates*

MGE database alignment was performed to analyze horizontal gene transfer, which is one of the main processes that accelerate the development and enrichment of antimicrobial resistance in the environment (Andersson and Hughes, 2014; Heuer and Smalla, 2007). Four transposons (Tn554 (*n* = 12), Tn558 (*n* = 5), Tn6261 (*n* = 1) and Tn6674 (*n* = 7)) that carry ARGs were found in 51 pleuromutilin-resistant *Enterococcus* isolates (Fig. 4a). Tn554 carries *erm(A)* and *ant(9)-la*, and it was found in pleuromutilin-resistant *Enterococcus* isolates from four production farms and several environmental samples at the same breeding farm (such as dust, flies, sewage and soil from the breeding chicken farm). The BLAST results showed that all of the Tn554 fragments in pleuromutilin-resistant *Enterococcus* isolates shared 100% identity. Tn558 carries *fex(A)* and Tn6674 carries *optrA*, *fex(A)*, *erm(A)*, and *ant(9)-la*, and they were also found on multiple production farms and in various sample types. Moreover, we found two new transposons that carry ARGs in the Tn554 family, designated as Tn7508 and Tn7492 by the transposon nomenclature center (https://transposon.lstmed.ac.uk/) (Fig. 4b) (Tansirichaiya et al., 2019). Tn7508 carries *fexA*, *optrA*, *erm(A)*, and insertion sequence ISBce13. Tn7492 carries *fexA*, *optrA*, and insertion sequence ISBce13.

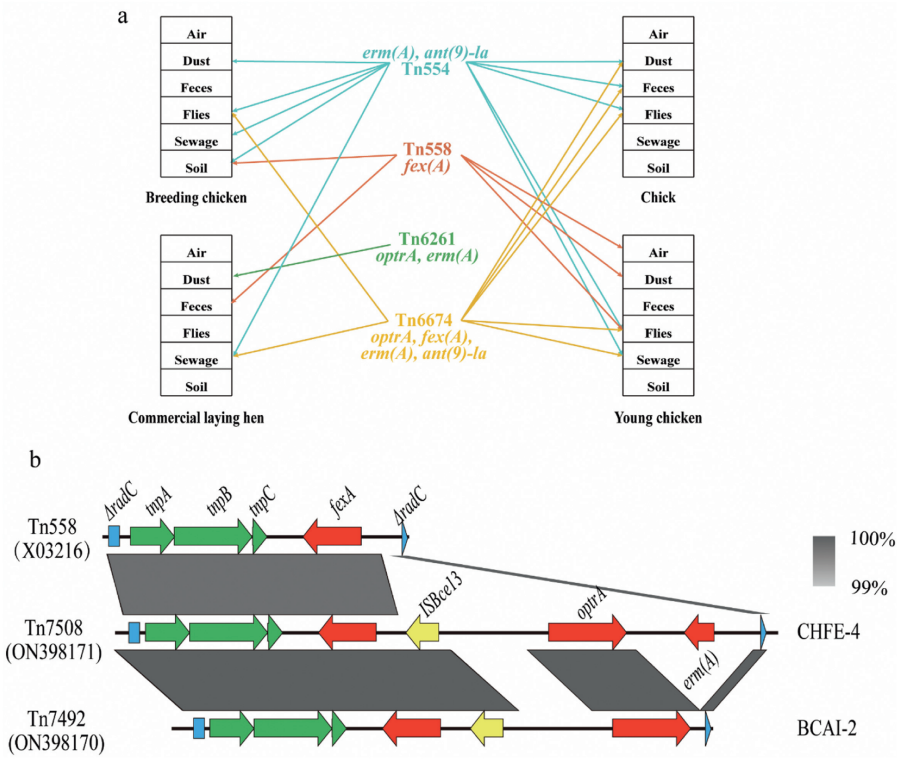

Fig. 4 – (a) Distribution of transposons carrying ARGs. (b) Organization of Tn7508 and Tn7492 compared with Tn558. Genes are indicated as arrows and the arrowhead shows the direction of transcription. Genes (*tnpA, tnpB* and *tnpC*) that encode transposition functions are shown in green, resistance genes are displayed in red, insertion sequences are displayed in yellow, and other genes are displayed in blue. Darker shading indicates higher nucleotide sequence identity.

2.4. *Molecular typing of pleuromutilin-resistant* Enterococcus *isolates from different farms and environmental sample types*

MLST was conducted to determine the genetic correlations among pleuromutilin-resistant *Enterococcus* isolates from four production farms and six environmental samples based on the WGS results. We found 18 ST types in 24 *E. faecium* and 12 ST types in 24 *E. faecalis*. ST2240 ($n = 4$) was the most frequently observed ST type for *E. faecium* and ST476 ($n = 7$) was the most frequently observed ST type for *E. faecalis* (Fig. 5, **Appendix A Tables S1** and **S2**). *E. faecium* ST2237, ST2240, and ST2241 were newly discovered ST types. Moreover, multiple strains with the same ST type were isolated from production farms in different breeding stages and different environmental samples. ST476 was present in samples of dust, flies, and feces from the chick breeding farm, and was also present in samples from the other three farms. ST403 was present in samples of air, dust, and feces from the young chicken farm and in feces from the commercial laying hen farm. ST1681 was present in the chick farm and young chicken farm.

2.5. *SNP phylogenetic analyses of pleuromutilin-resistant* Enterococcus *isolates*

The genomes of pleuromutilin-resistant *Enterococcus* isolates ($n = 51$) were used to infer the phylogenetic distances among isolates (Fig. 6). The results showed that the pleuromutilin-resistant *Enterococcus* isolates from soil, dust, and air samples collected on the breeding chicken farm (BCSO-2, BCDU-1, BCDU-3, and BCAI-2) were more closely related. The pleuromutilin-resistant *Enterococcus* isolates from samples of flies and dust collected on the chick farm (CHFL-3 and CHDU-2) were more closely related. Similarly, the isolates from flies and feces samples collected on the commercial laying hen farm (CLFL-1 and CLFE-1) were more closely related. In addition, many of the isolates from different farms were clustered, such as CLFE-2, YCSO-4, and BCFL-4. Overall, the results were highly consistent with those obtained by MLST analysis, where genetically related pleuromutilin-resistant *Enterococcus* isolates were found in production farms at different breeding stages, thereby suggesting that genetically related pleuromutilin-resistant *Enterococcus* strains were widely distributed in different types of environments in the laying hen production chain.

3. Discussion

Few detailed studies have investigated the antimicrobial resistance of *Enterococcus* isolates in the laying hen production chain. In this study, we found that *Enterococcus* isolates were widely distributed in various environmental samples from laying hen production farms in different breeding stages in Sichuan, China. The overall high isolation rate of *Enterococcus* (91.3%, 105/115) is consistent with previous findings, where

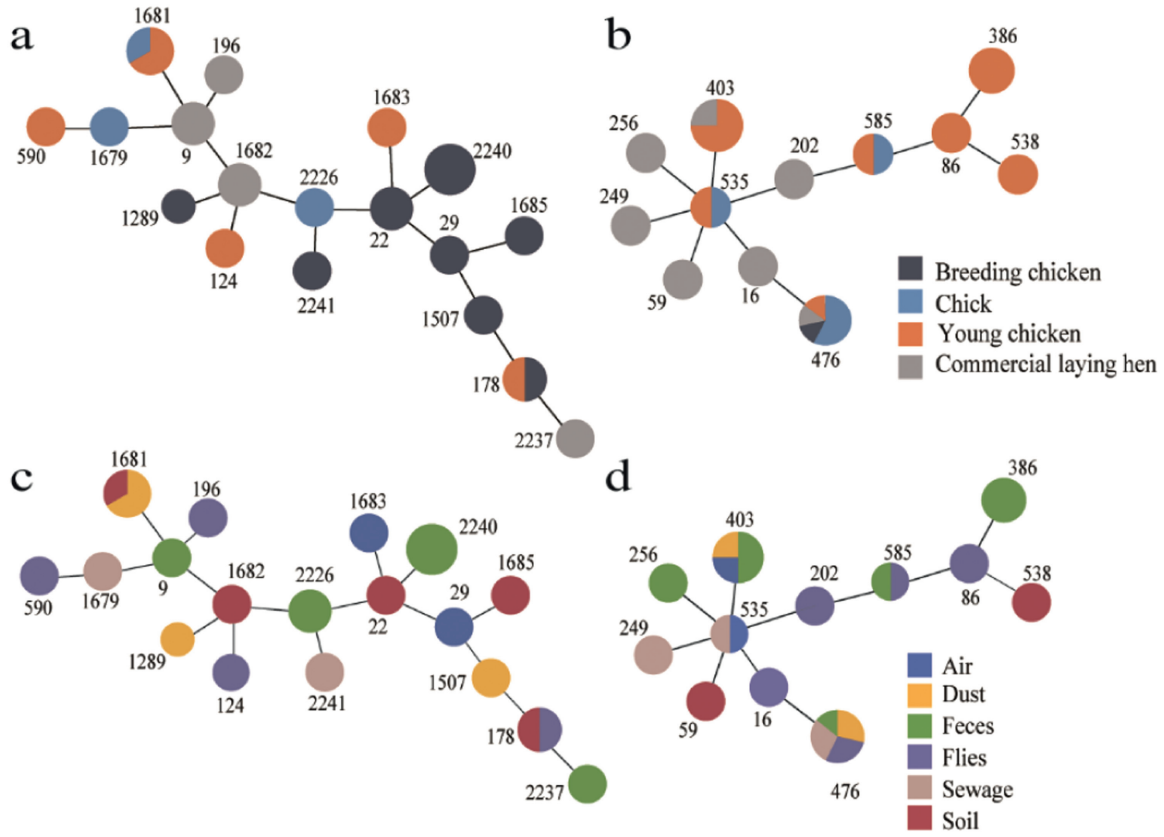

Fig. 5 – Multi-locus sequence typing results for *E. faecium* isolates (a, c) and *E. faecalis* isolates (b, d). Different colors correspond to different breeding farms or sample types, and the size of each circle represents the number of isolates that belong to a specific ST type.

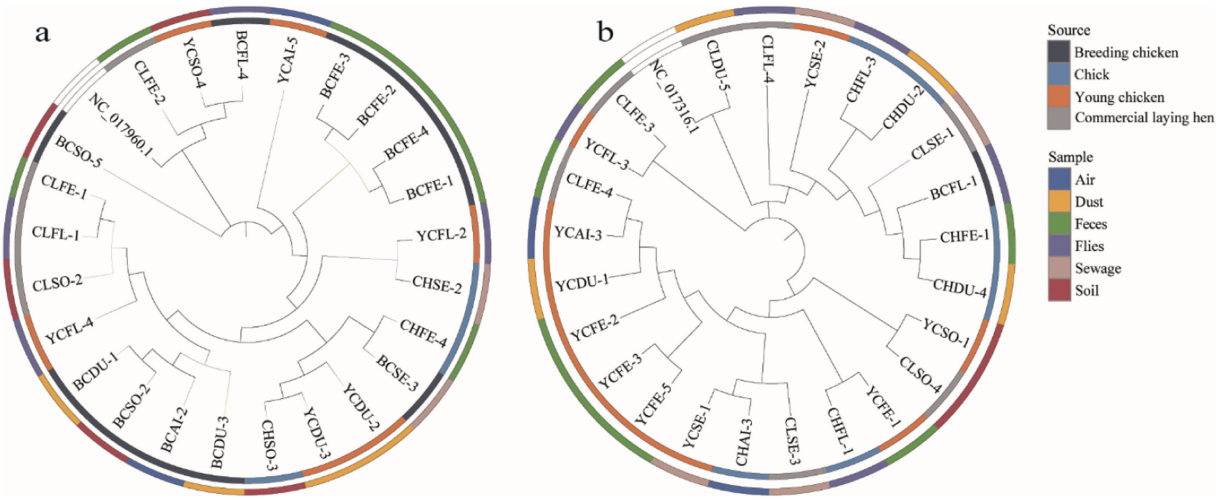

Fig. 6 – Phylogenetic analysis based on single nucleotide polymorphisms in *E. faecium* (a) and *E. faecalis* (b) genomes.

Enterococcus isolates were detected in 72.2% of farms in Tunisia and 97% of rivers and stream samples (Ben Said et al., 2016; Cho et al., 2020). Consistent with previous surveys of animal-derived food and human infection, *E. faecalis* ($n = 60$) was the most prevalent *Enterococcus* species in the present study, followed by *E. faecium* ($n = 42$) (Top et al., 2007; Tyson et al., 2018).

The main difficulty when treating infections caused by pathogens is simultaneous resistance to several antibiotics. All *Enterococcus* isolates from laying hen production farms in the present study exhibited the MDR phenotype. We used 16 μg/mL as the cutoff for valnemulin based on the cutoff in the microbroth dilution method for tiamulin in the CLSI

guidelines, and previously reported results. The ResFinder results showed that all 51 pleuromutilin-resistant *Enterococcus* isolates were positive for the pleuromutilin-resistant gene *lsa(E)*. Thus, the cutoff appeared to be reasonable. The antimicrobial resistance of *Enterococcus* isolates from the breeding chicken farm was similar to that of isolates from other farms after antibiotics were no longer applied (Fig. 1**b**), thereby indicating that the short-term break in antibiotics had no significant effect on antimicrobial resistance. Other approaches need to be applied to reduce antimicrobial resistance during egg production, such as extending the antibiotic break period. Vancomycin-resistant *Enterococcus* (VRE) is a major challenge for clinical treatment, and reports of VRE have been increasing in recent years (Arias and Murray, 2012; Bender et al., 2018). It should be noted that the proportion of VRE isolates in the present study was as high as 9.5%. Linezolid is one of the last lines of defense against infections and an antibiotic for the treatment of VRE approved by the US Food and Drug Administration (Zahedi Bialvaei et al., 2017). The proportion of linezolid-resistant *Enterococcus* isolates in the present study was 32.4%. Host-switching events are frequent between humans and animals as well as among animals (Haag et al., 2019), and thus greater attention should be paid to these antimicrobial-resistant *Enterococcus* strains.

Few studies have documented the ARG profiles of antimicrobial-resistant *Enterococcus* isolates from different breeding stages and environments in laying hen production farms. Based on WGS analysis, we characterized 29 ARGs that conferred resistance to 11 classes of antibiotics in pleuromutilin-resistant *Enterococcus* isolates ($n = 51$) (Fig. 2). A study has shown that the high genetic plasticity and environmental adaptability of *Enterococcus* strains contribute to the widespread transmission of ARGs (Fiore et al., 2019). *lsa(E)* encodes a Lsa-type ATP binding cassette-F (ABC-F) protein that confers combined resistance to PLS_A antimicrobials, which play important roles in the prevention and treatment of bacterial diseases in both humans and animals (Yang et al., 2020). *lsa(E)* has been identified in various Gram-positive bacteria of animal origin, including *Staphylococcus, Streptococcus, Enterococcus*, and *Erysipelothrix* (Bojarska et al., 2016; Deng et al., 2017; Si et al., 2015; Zhang et al., 2015). In particular, it has been shown that *lsa(E)* can be transferred among strains with plasmids and the presence of *lsa(E)* may facilitate the persistence and dissemination of multi-resistant plasmid in MRSA (Li et al., 2013). In this study, *lsa(E)* and *lnu(B)*, which mediates resistance to lincosamide by encoding lincosamide nucleotide transferases (Fessler et al., 2018), co-occurred in all pleuromutilin-resistant *Enterococcus* isolates (Fig. 3), which is consistent with previous studies of the *lsa(E)* genetic environment where *lsa(E)* and *lnu(B)* were located close to each other, and they were often inherited and transferred together (Berbel et al., 2019; Zhi et al., 2021). The *spw* gene that mediates spectinomycin resistance was found upstream of *lsa(E)* in 50 pleuromutilin-resistant *Enterococcus* isolates, and the coexistence of *lsa(E)* and various ARGs has been shown in many studies (Si et al., 2015; Wang et al., 2015a), which is a great potential safety hazard. In addition, the similarity of the ARG clusters containing *lsa(E)* in *Enterococcus* and MRSA has been documented (Li et al., 2014). These findings indicate that *lsa(E)* is often located in multi-resistance gene clusters and it can be transferred between strains, and thus continuous monitoring of *lsa(E)* in Gram-positive bacteria is required. Moreover, it should be noted that 64.7% of the pleuromutilin-resistant *Enterococcus* isolates carried *optrA* and/or *poxtA*. The *optrA* encodes an ABC-F protein that mediates resistance to phenicols and oxazolidinones by target protection (Yoon et al., 2020). The protein encoded by *poxtA* shares 32% similarity with *optrA*, and thus they share the structural characteristics of the F family of the typical ABC protein superfamily, which increase the MIC or resistance to oxazolidinone, phenicols, and tetracyclines through ribosomal protection (Antonelli et al., 2018; Schwarz et al., 2021). Moreover, 87.5% (28/32) of the *optrA*-positive pleuromutilin-resistant *Enterococcus* isolates in our study co-carried phenicol exporter gene *fexA*, which is consistent with a previous report that *optrA*-positive isolates co-carried *fexA* with a high frequency (85.7%, 30/35) in *Enterococcus*, and the presence of both *optrA* and *fexA* in the same isolate has also been described in *Enterococcus* isolates from humans and animals in China (Cai et al., 2015; Wang et al., 2015b). In addition, *Enterococcus* isolates that carried multiple ARGs were found in different environmental samples from the laying hen production farm in the present study, such as samples of sewage, feces, and flies. According to previous studies, ARB and intracellular ARGs are likely to spread from farms into surrounding streams and agricultural soils and then spread further into the human environment (Fang et al., 2018). Therefore, the spread of ARB and transmission of ARGs between farms and the surrounding environment as well as between the living environment and human medical environment requires greater attention. Among the 51 pleuromutilin-resistant *Enterococcus* isolates in this study, only one isolate (CLAI-4) was found to carry vancomycin resistance genes *vanC2* and *vanXY* because sensitivity to vancomycin in *Enterococcus* is also associated with sensitivity to pleuromutilin due to "collateral sensitivity" (Li et al., 2022a).

We also found four transposons that carry several important ARGs (*erm(A), ant(9)-la, fex(A)*, and *optrA*) in 51 pleuromutilin-resistant *Enterococcus* isolates, and three of these transposons were widely distributed in *Enterococcus* isolates from different farms and environments (Fig 4a), thereby agreeing with previous reports that transposons may play roles in the spread of ARGs across farms and samples (Zhang et al., 2020, 2021). According to the BLAST alignment results, two new transposons that carry ARGs in the Tn554 family designated as Tn7508 and Tn7492 were identified (Fig. 4b). These two transposon elements carry various ARGs such as *optrA* and *fexA*. Previous study have suggested that animals and humans constitute overlapping antimicrobial resistance reservoirs (Wegener, 2003), and thus the presence of ARGs in animal-derived food can affect human health. Our results highlight the important roles of *Enterococcus* strains as reservoirs of ARGs.

The different environments on laying hen production farms could be significant vehicles for the transmission of ARB or even diseases (Li et al., 2022b; Zhu et al., 2021), so we also examined the phylogenetic relationships among pleuromutilin-resistant *Enterococcus* isolates from different farms and environmental samples. The MLST and SNP results both indicated that phylogenetically related pleuromutilin-resistant *Enterococcus* isolates were widely distributed in

various samples from different production farms, which suggests that pleuromutilin-resistant *Enterococcus* strains may be transmitted in different environments and production farms. In particular, *E. faecalis* ST476 was detected in several samples from four farms (Fig. 5, Appendix A Table S2). The SNP results also showed that these *E. faecalis* ST476 strains were closely related (Fig. 6). Thus, greater attention should be paid to cross-contamination between these sites in the breeding process. More importantly, it should be noted that *E. faecalis* ST16, a hospital-related clone (Larsen et al., 2010; Olsen et al., 2012), was found in this study, which suggests that there is a connection between poultry-derived *Enterococcus* and hospital-related *Enterococcus*. These findings suggest that feces, sewage, flies, air, dust, and soil may play important roles in bacterial transmission and the spread of antimicrobial resistance. These environmental sources are common in laying hen farms and thus they are relatively less well regulated (e.g., by disinfection) than others. Therefore, greater attention should be focused on the ARB and ARGs carried in these environmental sources during livestock breeding.

In this study, we systematically assessed the distribution of *Enterococcus* strains and their resistance profiles by collecting different types of environment samples along a laying hen production chain in Sichuan, China. Based on MLST and SNP analyses, we determined the genetic relatedness among pleuromutilin-resistant *Enterococcus* isolates from laying hen production farms. Our results showed that the laying hen production industry was a reservoir for antibiotic-resistant *Enterococcus* isolates, and genetically related pleuromutilin-resistant *Enterococcus* isolates were widely distributed in different environmental samples collected from different production farms, thereby suggesting the occurrence of ARB transmission in the laying hen production chain. The high genetic plasticity of *Enterococcus* strains also allows them to carry and transfer many ARGs. Thus, continuous surveillance of antimicrobial resistance in the laying hen production industry is imperative in order to prevent the introduction of last-resort antimicrobial resistance into the food chain and clinical environment. National or global epidemiological studies are needed to fully assess the risk associated with antimicrobial resistance from laying hen production industries. In addition, improving the immunity of poultry or using alternatives to antibiotics in production is strongly encouraged to prevent the spread of ARB and antimicrobial resistance.

4. Conclusion

Enterococcus isolates were widely distributed in samples from different breeding stages and environments in layer farms. The layer breeding farms were reservoirs for ARGs, and the *Enterococcus* isolates exhibited resistance to a wide array of clinically important antibiotics, including fosfomycin, linezolid, and vancomycin. In addition, we found that pleuromutilin-resistant *Enterococcus* isolates carried several important ARGs. Importantly, we demonstrated the coexistence of phylogenetically related pleuromutilin-resistant *Enterococcus* isolates in different farms and environments. The layer breeding system plays a vital role in the food supply system, and our findings suggest that antibiotic resistance in *Enterococcus* strains should be monitored in the layer breeding environment.

Data availability

The nucleotide sequences of bacterial genomes of pleuromutilin-resistant *Enterococcus* isolates ($n = 51$) were deposited in the NCBI database and are publicly available under accession numbers PRJNA833257, PRJNA833259, PRJNA836296, PRJNA836294, and PRJNA836293. The nucleotide sequences of Tn7508 and Tn7492 were also deposited in the NCBI database and are publicly available under accession numbers ON398171 and ON398170, respectively.

Declaration of Competing Interest

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

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Appendix A Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2023.01.012.

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