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Food pathogens and antimicrobial resistance

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Food pathogens and antimicrobial resistance

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Editorial: Food pathogens and antimicrobial resistance

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Editorial on the Research Topic
Food pathogens and antimicrobial resistance

The issue of antimicrobial resistance (AMR) of microorganisms is prevalent worldwide (Neely and Holder, 1999). The overuse of antimicrobials in agricultural animals for food production has been proposed as the leading cause of the proliferation of antimicrobial-resistant pathogens (Minarini et al., 2020). Raw meat, cooked food products, and raw milk are often contaminated by food-borne pathogens, many of which are resistant to a variety of antimicrobials (Chao et al., 2007). The exchange of genetic information among food-borne pathogens is a major factor in the development of antimicrobial resistance. Therefore, monitoring antimicrobial-resistant at different nodes in the food chain is particularly important to understand the spread of antimicrobial-resistant (Di Ciccio, 2021).

In this Research Topic, nine original research articles and one review have been published. Hu et al. comprehensively reviewed the characteristics, epidemiology, pathogenic mechanisms, zoonotic potential, antimicrobial resistance, diagnosis, alternative control measures, and vaccine development of avian pathogenic *E. coli* (APEC).

Research has shown that intestinal microbiota plays a critical role in maintaining the integrity of gut barrier, and specific microorganisms in digestive tracts can aid in the treatment of gastrointestinal diseases, thereby reducing the reliance of antimicrobials and other drugs (Gresse et al., 2017). Using a model of Porcine epidemic diarrhea virus (PEDV)-infected LC and Large-white piglets established by Li et al., the authors analyzed differences in intestinal microbial diversity, community composition, and intestinal metabolites between PEDV-infected and healthy control piglets. This study offers a theoretical foundation for utilizing intestinal core microorganisms in the digestive system of PEDV-infected pigs, to address the issue of piglet diarrhea that arises from PEDV infection.

Bacterial AMR is usually regulated by genes. The research conducted by Tang et al. demonstrated that the plasmid-borne *cfr* gene facilitates multidrug resistance (MDR), which subsequently leads to *cfr*-positive *E. coli* exhibiting MDR. Moreover, the study revealed that *cfr* can form a circular intermediate of IS26-*cfr* during transmission, confirming IS26's significant role in the dissemination of the multidrug resistance gene *cfr*.

In the study of Chen et al., the genes that play a key role in multiple drug resistance in Bacillus cereus are *hblA, hblC, hblD, nheA,* and *nheB*. Lai et al.'s research indicate that the AMR of *Salmonella* is primarily attributed to the significant impact of the plasmid-mediated quinolone resistance gene *pmqr*, the β-lactam resistance gene bla_{Tm}, and the mutation of the quinolone resistance-determination region (QRDR) that contains the *pmqr* gene.

Ji et al. sequenced the complete genome of 322 *Listeria monocytogenes* strains isolated from food and discovered that these strains carry the drug resistance genes *aacA4, etM, Tets*, and *dfrG*. Furthermore, the researchers discovered a novel premature stop codon in the *inlA* gene, leading to a better comprehension of the genomic diversity of *Listeria monocytogenes*.

Buberg et al. studied the survival rate, colonization characteristics and conjugation ability of ESC-resistant *E. coli* isolates through a static *in vitro* digestion model (INFOGEST). The findings demonstrate that the strains are capable of surviving and reproducing within the *in vitro* digestion model. Furthermore, the strains exhibit the capability to adhere to and invade colon cells post-digestion, with a higher degree of adhesion to colon cells observed than cell invasion. This study demonstrated the survival and colonization ability of *E. coli* strains resistant to spectrum cephalosporin-resistant.

Krüger et al. discovered that the presence of mobile genetic elements encoding resistance and virulence genes results in variations in gene expression. Multiple drug resistance may be related to the existence of mobile genetic elements. *Salmonella* Infantis and *Salmonella* Heidelberg serotypes showing multiple drug resistance have been found to carry the $bla_{\mathrm{TEM-Ib}}$ and $bla_{\mathrm{CTX-M-65}}$ genes, which are related to mobile genetic elements.

The research conducted by Ma et al. investigated the prevalence of *Arcobacter* spp. in Shenzhen, China, as well as identifying its virulence and AMR through whole genome sequencing (WGS). This study discovered that antimicrobial resistant gene counts varied between different strains, and certain strains carried multiple drug resistance genes. Additionally, it was found that corresponding drug-resistant strains exhibited specific drug-resistance genes.

Finally, the results of Wang et al.'s study showed that the phage PaVOA effectively kills *Pseudomonas aeruginosa* within a short period using a rabbit skin infection model from New Zealand. Therefore, phage cocktail therapy represents a new approach to treating traumatic skin infections caused by MDR *Pseudomonas aeruginosa*.

In summary, this article Research Topic provides valuable insights into the epidemiological distribution and antimicrobial resistance of foodborne pathogens across various nodes of the food supply chain, aiding readers in understanding this important topic. This Research Topic of articles has garnered considerable attention from the appropriate practitioners, with over 11,000 views and a total of 2,315 downloads.

Author contributions

YW wrote the initial draft of the manuscript while JZ, ZP, XW, and PP provided substantial critical feedback and editing. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Prevalence and antimicrobial-resistant characterization of *Bacillus cereus* isolated from ready-to-eat rice products in Eastern China

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Bacillus cereus is a major food-borne bacterial pathogen in the world, which can cause diarrhea and emetic syndrome. This study aimed to reveal the quantitative prevalence of *B. cereus* in ready-to-eat (RTE) rice products in Eastern China and to gain essential information on the characteristics of *B. cereus* isolates. A total of 91 out of the 1071 samples were positive for *B. cereus*. The contamination level of *B. cereus* in 0.5 % of RTE rice product samples outnumbered 10^3 CFU/g. The number of *B. cereus* attained 10^5–10^6 CFU/g in one sample. The distribution patterns of virulence genes in *B. cereus* isolates were identified. 84.6% of the *B. cereus* isolates had at least one enterotoxin or emetic toxin gene. The predominant pattern was XXV. 9.9% of isolates belonged to it and possessed one enterotoxin gene *entFM*. The occurrence rate of *hblACD* and *nheABC* was 36.3% and 47.3%, respectively. Antimicrobial susceptibility tests revealed a high resistance rate toward penicillin, and 23.1% of the isolates were multi-drug resistant. *B. cereus* isolates were genotyped by using ERIC-PCR. 89 genotypes were determined. The Hunter Gaston Discriminatory Index (HGDI) attained 0.9995. Relationships analysis revealed that Group A *B. cereus* isolates tended to carry *hblA*, *hblC*, *hblD*, *nheA*, *nheB*, and show resistance to penicillin/trimethoprim/sulfamethoxazole. This study was useful for updating the knowledge of the contamination status of *B. cereus* in RTE rice products in China.

KEYWORDS

***Bacillus cereus*, quantitative prevalence, rice products, virulence gene, antimicrobial resistance, ERIC-PCR**

Introduction

Bacillus cereus is a major causative agent of food poisoning outbreaks worldwide. It causes two types of food-borne illnesses, including diarrheal and emetic syndrome. The diarrheal variant is characterized by abdominal pain and watery diarrhea, and is usually linked to the intake of enterotoxin-producing *B. cereus* vegetative cells (e.g., Nhe, Hbl, and CytK). The emetic type, characterized by vomiting symptoms, is caused by ingestion of cereulide, a toxic peptide released by *B. cereus* emetic strains (Senesi and Ghelardi, 2010; Paudyal et al., 2018; Rouzeau-Szynalski et al., 2020; Yue et al., 2020, 2021).

Bacillus cereus can persist in a variety of natural environments, including soil and plants, due to its strong survival ability. The endospore resistance to multiple stresses, the formation of biofilms (Glasset et al., 2021; Li et al., 2022a), and even withstanding most cleaning and decontamination processes at food processing steps also help its survival in the environment (Merzougui et al., 2014; Ramarao et al., 2015). *B. cereus* is frequently found in processed products/prepared food items, according to data on the prevalence of *Bacillus* in food and animals in the European Union (European Food Safety Authority, and European Centre for Disease Prevention, and Control., 2017). Rice is a very popular food around the world and is often used as a raw material for the preparation of diet food dishes in many countries. During the cultivation, harvesting, and handling process, rice might be contaminated with vegetative cells and endospores of *B. cereus* (Vasiee et al., 2016; Kindle et al., 2019; Rodrigo et al., 2021). Although vegetative cells can be killed during some cooking processes, such as heating, however, cereulide and endospores generally survive due to high-stress resistance. Under normal conditions, endospores can germinate and become vegetative cells (Rouzeau-Szynalski et al., 2020; Tsugukuni et al., 2020). The possible safety risk of food-borne pathogens in ready-to-eat (RTE) foods is increasingly gaining public attention, because no additional sterilization steps, during cooking, baking, or pasteurization, are normally conducted before the consumption (Chon et al., 2015; Yu et al., 2019; Martelli et al., 2021). Although the contamination status of *B. cereus* in some kinds of food including dairy products, infant foods, aquatic products (Zhang et al., 2017, 2020; Gao et al., 2018; Zhao et al., 2020), etc. has been revealed, the data for *B. cereus* distribution in rice, especially in RTE rice products was still sparse in China. In this study, we investigated the quantitative prevalence of *B. cereus* in RTE rice products sampled in Zhejiang Province, located in Eastern China. The virulence gene distribution profiles, genotyping and antimicrobial susceptibility of these isolates were also studied.

Materials and methods

Sampling and isolation of *Bacillus cereus*

A total of 1071 RTE rice product samples were collected from 11 cities covering the whole Zhejiang Province, Eastern China, during 2017−2019. The samples included 756 boiled rice dishes, 91 boiled rice noodles, 105 fried rice noodles, 33 sticky rice rolls, 64 boiled sticky rice dishes, and 22 fried rice cakes. Quantitative detection of *B. cereus* in each sample was performed by using the direct plating method (Liu et al., 2021; Yue et al., 2021; Anwar et al., 2022). Briefly, Twenty-five grams of each sample was suspended in 225 mL of PBS and subsequently homogenized for 2 mins. The homogenate was 10-fold serially diluted in sterilized normal saline. The dilutions were spread on Mannitol-Egg-Yolk-Polymyxin (MYP) agars separately in duplicate. Plates were incubated at 30°C for 24 h. Five presumptive colonies with typical morphology on each plate were selected for further identification. Suspected colonies were then identified using Gram staining and the VITEK2 compact system (BioMerieux, France), followed by rhizoid growth and parasporal crystal formation tests to differentiate *B. cereus* from *Bacillus thuringiensis* and *Bacillus mycoides*. GB/T 4789.14-2014 (Ministry of Health of the People's Republic of China, 2014) was used to calculate *B. cereus* numbers. One *B. cereus* isolate from each positive sample was stored for further characterization.

Detection of virulence genes

Genomic DNA was extracted from *B. cereus* by using a bacterial DNA extraction Kit (Omega, United States), according to manufacturer's instructions. The primers and PCR protocol for eleven virulence genes were used as previously described *ces* (Ehling-Schulz et al., 2005), *hblA* (Zhou et al., 2008), *hblC*, *hblD*, *nheA*, *nheB*, *nheC* (Melnick et al., 2012), *bceT* (in't Veld et al., 2001), *cytK1*, *cytK2* (Guinebretiere et al., 2006), and *entFM* (Ngamwongsatit et al., 2008). Individual PCR reaction (25 μL) contain 50 ng of DNA template, 0.5 μL of each primer (10 μM), 0.125 U of Taq polymerase (TaKaRa, Japan), 2.5 μL of 10 × PCR buffer (Mg^{2+} free), 1.5 μL of $MgCl_2$ (25 mM), and 2 μL of dNTP Mixture (2.5 mM). The amplicon was analyzed with 1% agarose gel. The gels were visualized by a UV Imaging System. A 100 bp DNA ladder (TaKaRa, Japan) was used as a DNA marker.

Antimicrobial susceptibility tests

Antimicrobial susceptibility assay of *B. cereus* isolates was tested by using the broth micro-dilution minimum inhibitory

concentrations (MICs) method according to the standard Clinical and Laboratory Standard Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2015). After 18 h of cultivation on nutrient agar at 37°C, *B. cereus* was suspended in 0.85 per cent (w/v) NaCl solution to 1.0 MCF, followed by dilution with Mueller-Hinton broth to the final concentration of 1∼2 × 10^5 CFU/mL. Each 100 μL of the bacterial inoculum was added to 96-well plates containing antibiotics and incubated at 37°C for 20 h. Twelve antimicrobials from different classes were employed, including imipenem (1−64 μg/mL), penicillin (0.06−8 μg/mL), chloramphenicol (2−128 μg/mL), ceftriaxone (4−128 μg/mL), vancomycin (1−128 μg/mL), amikacin (8−128 μg/mL), erythrocin (0.25−32 μg/mL), tetracycline (2−32 μg/mL), ciprofloxacin (0.5−16 μg/mL), clindamycin (0.12−16 μg/mL), trimethoprim/sulfamethoxazole (0.5/9.5−16/304 μg/mL), and rifampin (0.5−8 μg/mL). The MIC results were analyzed based on the breakpoints for *Bacillus* species as per CLSI guidelines (Clinical and Laboratory Standards Institute, 2015). The breakpoint for ceftriaxone was from CLSI documents M45-A2 (Clinical and Laboratory Standards Institute, 2010). The isolates resistant to three or more types of antimicrobial classified into different antimicrobial categories were defined as multi-drug resistant (Li et al., 2022b). *Staphylococcus aureus* ATCC 29213 was used as a positive control.

ERIC-PCR analysis

All 91 *B. cereus* isolates were genotyped by ERIC-PCR using the following primers ERIC-1: 5′-ATGTAAGCTCCTGGGGATTCAC-3′ and ERIC-2: 5′-AAGTAAGTGACTGGGGTGAGCG-3′ (Dorneles et al., 2012; Dorneles et al., 2014). The PCR mixture (25 μL) was 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 0.3 mM of each dNTP, 1 U of Taq DNA polymerase (Takara, Dalian, China), 0.4 μM of each primer and 75 ng of DNA template. PCR reaction was carried out as follows: 95°C for 3 min, 35 cycles of 94°C for 30 s, 46°C for 40 s, 72°C for 3 min and a final incubation at 72°C for 10 min. Amplicons size was analyzed by 2.0% agarose gel. The gels were visualized by a UV Imaging System. A 100 bp DNA ladder (TaKaRa, Japan) was used as a marker. A 100% of similarity in bands pattern was defined as an ERIC-PCR genotype according to previous report (Magyar et al., 2019).

Genetic typing analysis

The software BioNumerics 7.6 (Applied Maths, Sint-Martens-Latem, Belgium) was applied to estimate the band size of ERIC-PCR amplicons and analyze the genotypes. Clustering analysis was based on the Dice similarity coefficient and the unweighted pair group method with arithmetic mean (UPGMA). The Hunter and Gaston Diversity Index (HGDI) was calculated to evaluate the discriminatory capability of ERIC-PCR (Shi et al., 2021). Isolates that share 100% similarity of amplicon bands pattern were grouped into one genotype.

Statistical analysis

Chi-square analysis was performed using the SPSS v 21.0 software package to determine if a significant difference existed in the prevalence distribution of *B. cereus* in different RTE rice products. The *p*-Value of <0.05 was used as a significance level. Relationships between genotype groups and virulence gene distribution, and antibiotic resistance profiles were analyzed by carrying out Pearson's chi-square test and Fisher's exact test with the Bonferroni correction.

Results and discussion

Quantitative prevalence of *Bacillus cereus* in ready-to-eat rice product

The prevalence of *B. cereus* in 1071 RTE rice product samples examined in this study was described in the **Table 1**. *B. cereus* was detected in 8.49% (91/1076) of all samples collected, out of which 65/91 (71.4%) were from boiled rice dishes, 6/91 (6.6%) were from boiled rice noodles, 13/91 (14.3%) were from fried rice noodles, 2/91 (2.2%) were from sticky rice roll, 3/91 (3.3%) were from boiled sticky rice dishes and 2/91 (2.2%) were from fried rice cake. According to previously published data, there are significant variances in the detection rate of *B. cereus* in various types of food samples from different regions of the world (Wang et al., 2019; Xu et al., 2020; Qiu et al., 2021; Shi et al., 2021; Wu et al., 2021). The total occurrence rate of *B. cereus* in our study was similar to a previous study in which *B. cereus* was isolated from dairy products, rice and flour products in China (Zhao et al., 2020). The prevalence of *B. cerous* in our study was lower than in a previous study isolated from artisan cheeses made in Mexico and powdered food products in Switzerland (Heini et al., 2018; Adame-Gomez et al., 2020). There was no statistically significant difference ($p > 0.05$) in the prevalence of *B. cereus* across the six types of rice products in our study. *B. cereus* is an opportunistic pathogen found in food. Ingestion of 10^5−10^8 vegetative cells or 8 μg of emetic toxin per kg of body weight may lead to gastroenteritis or/and vomiting syndrome in adults (Paananen et al., 2002; Schoeni and Wong, 2005). According to our findings, the number of *B. cereus* detected in 1.0 % fried rice noodles and 0.8% boiled rice meal samples varied from 10^3 to 10^6 CFU/g. Food poisoning can occur after consuming a specific amount of these highly contaminated meals (Zeng et al., 2021, 2022).

TABLE 1 Quantitative prevalence of *Bacillus cereus* in ready-to-eat rice product.

Samples	No.	Positive	*B. cereus* level (CFU/g)					
			$10-10^2$	10^2-10^3	10^3-10^4	10^4-10^5	10^5-10^6	ND (<10)
Boiled rice dishes	756	65 (8.6%)	56 (7.4%)	3 (0.4%)	4 (0.5%)	1 (0.1%)	1 (0.1%)	691 (91.4%)
Boiled rice noodles	91	6 (6.6%)	5 (5.5%)	1 (1.1%)	0 (0%)	0 (0%)	0 (0%)	85 (93.4%)
Fried rice noodles	105	13 (12.4%)	9 (8.6%)	3 (2.9%)	1 (1.0%)	0 (0%)	0 (0%)	92 (87.6%)
Sticky rice roll	33	2 (6.1%)	2 (6.1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	31 (93.9%)
Boiled sticky rice dishes	64	3 (4.7%)	2 (3.1%)	1 (1.6%)	0 (0%)	0 (0%)	0 (0%)	61 (95.3 %)
Fried rice cake	22	2 (9.1%)	2 (9.1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	20 (90.9%)
Total	1071	91 (8.5%)	76 (7.1%)	8 (0.7%)	5 (0.5%)	1 (0.1%)	1 (0.1%)	980 (91.5%)

ND, not detected.

A number of safety criteria for *B. cereus* in RTE meals have been developed. In Canada and the United Kingdom, an acceptable threshold of 10^4 CFU/g is recommended. In South Korea, Australia, and New Zealand, a lower permissible threshold (10^3 CFU/g) is adopted (Nsw Food Authority, 2009; Health Canada, 2010; Chon et al., 2015). In our study, 91.5% of the samples had less than 10^3 CFU/g of *B. cereus*. However, 0.5% of the samples of RTE rice products had more than 10^3 CFU/g of *B. cereus*, which is more than the acceptable level in some countries. Although *B. cereus* in 91.5% of the samples was <10 CFU/g in our study, *B. cereus* in 0.5 % of RTE rice product samples outnumbered 10^3 CFU/g that could exceed the acceptable level of some countries.

Virulence gene profile of *Bacillus cereus* isolates

For many years, scientists have been studying the molecular mechanisms of *B. cereus* virulence. The diarrheal and emetic syndromes have been linked to several virulence factors including, secreted hemolysin BL (Hbl), necrotic enterotoxin (CytK), non-hemolytic enterotoxin (Nhe), enterotoxin FM (EntFM), BceT, and emetic toxin cereulide (Granum and Lund, 1997; Ehling-Schulz et al., 2005; Schoeni and Wong, 2005; Senesi and Ghelardi, 2010). Hbl or Nhe can promote fluid accumulation in ligated rabbit ileal loops due to their hemolytic, dermonecrotic, and vascular permeability activities (Schoeni and Wong, 2005; Griffiths and Schraft, 2017). Both of these enterotoxins comprise the tripartite complex. Three components are required for their maximal biological activity: proteins B, L1 and L2 in Hbl, and proteins A, B, and C in Nhe. Toxin activity has not been detected in any individual components (Arora, 2021; Sornchuer et al., 2022). The genes encoding Hbl and Nhe components are *hblA*, *hblC*, *hblD*, as well as *nheA*, *nheB*, and *nheC*, are located on two different operons (Sastalla et al., 2013). BceT, EntFM, CytK are all single-protein enterotoxins. BceT has cytotoxic, vascular permeability activities and can cause fluid accumulation in ligated mouse ileal loops (Agata et al., 1995). The necrotic enterotoxin CytK, which presents highly cytotoxic, necrotic and hemolytic activities, was initially incriminated in a severe gastroenteritis outbreak causing three patients' death in France (Lund et al., 2000; Alonzo et al., 2015).

In this study, the distribution of associated encoding genes of the above toxins in *B. cereus* isolates was investigated. 84.6% of the *B. cereus* isolates had at least one enterotoxin or emetic toxin gene. A total of 31 distribution patterns of virulence genes were determined in our study (**Table 2**). The predominant one was XXV, 9.9% of isolates belonged to it and possessed only one enterotoxin gene *entFM*. The *nheABC* genes were present in 47.3% of the isolates, this frequency was lower than in *B. cereus* isolates from various food source samples and clinical isolates associated with foodborne outbreaks in previous studies (Kim et al., 2011; Glasset et al., 2016; Zhang et al., 2017). The occurrence rate of *hblACD* was 36.3%, which is similar to the previous reports isolated from milk products (Hwang and Park, 2015; Zhang et al., 2017), and it is lower than that of ready-to-eat foods, including vegetables, infant rice flour, rice, and grain-based foods (Chon et al., 2015; Hwang and Park, 2015; Zhang et al., 2017). The coexistence of *hblACD* and *nheABC* was found in 24/91 (26.4%) isolates. Six isolates (6.6%) were found to possess all enterotoxin encoding genes detected in this study.

Two distinct variants of CytK have been reported: CytK1 and CytK2. CytK1 is more harmful than CytK2. Although CytK2 proteins are hemolytic and toxic to Vero cells and human intestinal Caco-2 cells, their toxicity was only around 20% CytK1 (Fagerlund et al., 2004). Furthermore, CytK1 has been linked to major *B. cereus* outbreaks (Fagerlund et al., 2004; Guinebretiere et al., 2006). According to our findings, 33.0% of *B. cereus* isolates had either *cytK1* or *cytK2*. CytK1 was found in one strain, accounting for 3.3% of all *cytK*-positive isolates. A previous study also observed this significant variation in *cytK1* and *cytK2* detection rates in *B. cereus* isolates from Chinese infant meals (Zhang et al., 2017). Foodborne *B. cereus* isolates may be slightly mild when producing diarrhea, according to the

TABLE 2 Virulence genes distribution profile of bacillus cereus isolates.

Patterns	*ces*	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*cytK1*	*cytK2*	*bceT*	*entFM*	No. of strains (%)
I	+	−	−	−	−	−	−	−	−	−	−	1 (1.1%)
II	+	−	−	−	−	−	−	−	−	−	+	1 (1.1%)
III	+	−	−	−	+	+	+	−	+	−	−	1 (1.1%)
IV	+	−	−	−	+	−	+	−	−	−	+	1 (1.1%)
V	−	+	+	+	−	−	−	−	−	+	−	3 (3.3%)
VI	−	+	+	−	−	−	−	−	−	−	+	1 (1.1%)
VII	−	+	+	+	−	−	−	−	−	+	+	2 (2.2%)
VIII	−	+	+	+	−	−	−	−	+	−	+	2 (2.2%)
IX	−	+	+	+	−	−	−	−	+	+	+	2 (2.2%)
X	−	−	−	−	+	+	+	−	−	−	+	6 (6.6%)
XI	−	−	−	−	+	+	+	−	−	−	−	3 (3.3%)
XII	−	−	−	−	+	+	+	−	+	−	−	2 (2.2%)
XIII	−	−	−	−	+	+	+	−	−	+	−	3 (3.3%)
XIV	−	−	−	−	+	+	+	−	−	+	+	1 (1.1%)
XV	−	−	−	−	+	+	+	−	+	−	+	1 (1.1%)
XVI	−	−	−	−	+	+	+	−	+	+	+	1 (1.1%)
XVII	−	+	−	+	+	+	+	−	−	−	+	1 (1.1%)
XVIII	−	+	+	+	+	+	+	−	−	+	+	7 (7.7%)
XIX	−	+	+	+	+	+	+	−	−	+	−	2 (2.2%)
XX	−	+	+	+	+	+	+	−	−	−	−	3 (3.3%)
XXI	−	+	+	+	+	+	+	−	+	+	−	4 (4.4%)
XXII	−	+	+	+	+	+	+	−	+	+	+	6 (6.6%)
XXIII	−	+	+	+	+	+	+	−	+	−	−	2 (2.2%)
XXIV	−	−	−	−	+	−	−	−	+	−	−	1 (1.1%)
XXV	−	−	−	−	−	−	−	−	−	−	+	9 (9.9%)
XXVI	−	−	−	−	−	−	−	−	+	−	−	1 (1.1%)
XXVII	−	−	−	−	−	−	−	−	+	−	+	4 (4.4%)
XXVIII	−	−	+	+	−	−	−	−	−	−	+	1 (1.1%)
XXIX	−	−	−	−	−	−	−	−	−	+	−	2 (2.2%)
XXX	−	−	−	−	−	−	−	−	+	+	+	2 (2.2%)
XXXI	−	−	−	−	−	−	−	+	−	−	+	1 (1.1%)

TABLE 3 Antibiotic susceptibility of *Bacillus cereus* isolates.

Antimicrobial class	Antimicrobial agents	MIC (μg/ml) Interpretive Criteria			No. of isolates (%)		
		Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
Penicillins	Penicillin	≤0.12	–	≥0.25	2 (2.2)	0 (0)	89 (97.8)
Carbapenems	Imipenem	≤4	8	≥16	91 (100)	0 (0)	0 (0)
Phenicols	Chloramphenicol	≤8	16	≥32	82 (90.1)	9 (9.9)	0 (0)
Cephems	Ceftriaxone	≤8	16–32	≥64	5 (5.5)	49 (53.8)	37 (40.7)
Glycopeptides	Vancomycin	≤4	–	–	91 (100)	0 (0)	0 (0)
Aminoglycosides	Amikacin	≤16	32	≥64	91 (100)	0 (0)	0 (0)
Macrolides	Erythromycin	≤0.5	1–4	≥8	71 (78.0)	20 (22.0)	0 (0)
Tetracyclines	Tetracycline	≤4	8	≥16	87 (95.6)	0 (0)	4 (4.4)
Quinolones	Ciprofloxacin	≤1	2	≥4	89 (97.8)	2 (2.2)	0 (0)
Lincosamides	Clindamycin	≤0.5	1–2	≥4	35 (38.5)	53 (58.2)	3 (3.3)
Folate Pathway Inhibitors	Trimethoprim/Sulfamethoxazole	≤2/38	–	≥4/76	14 (15.4)	0 (0)	77 (84.6)
Ansamycins	Rifampin	≤1	2	≥4	91 (100)	0 (0)	0 (0)

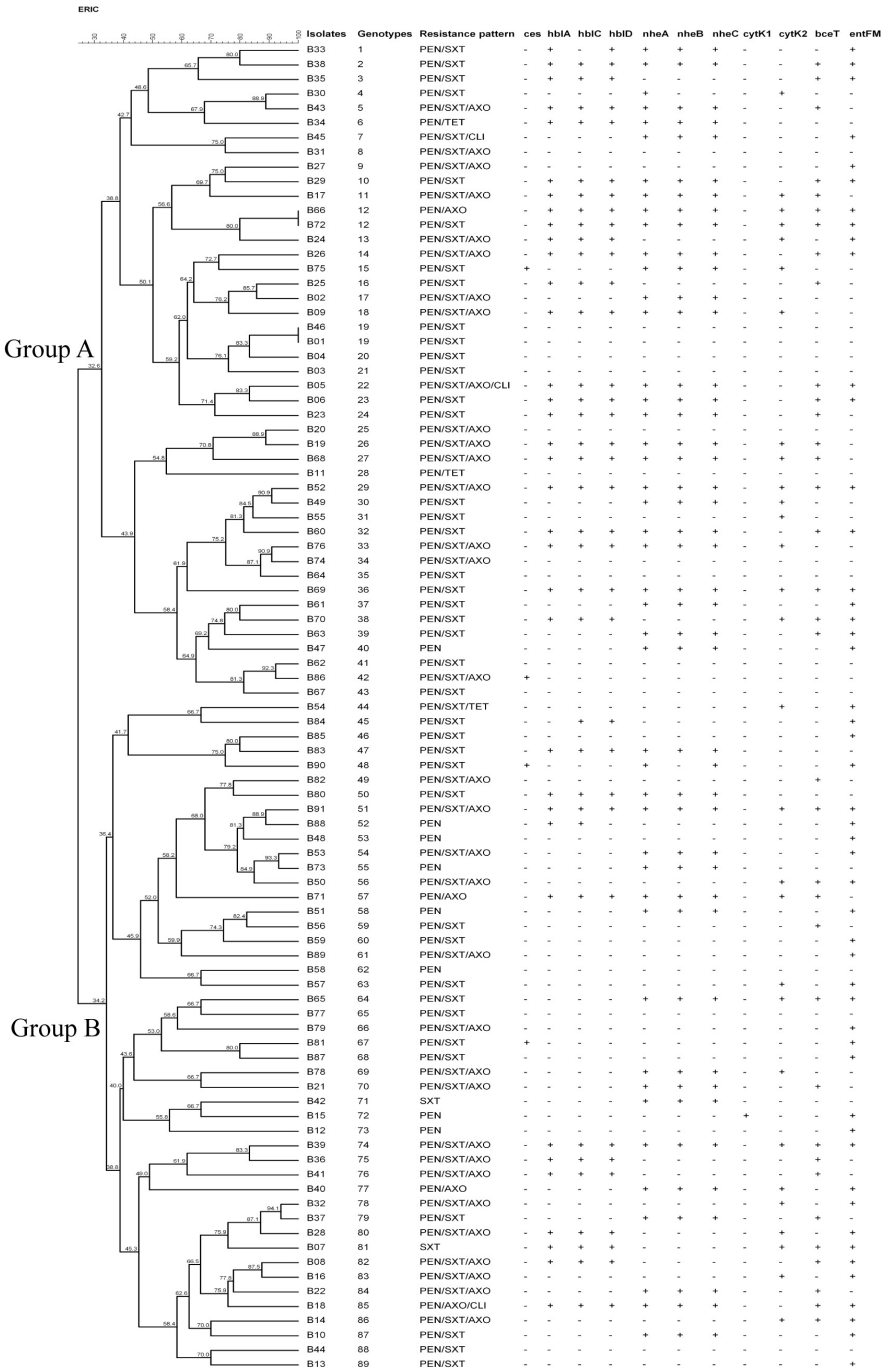

FIGURE 1
Characteristics of *Bacillus cereus* isolated from ready-to-eat rice products. The UPGMA tree was constructed by using BioNumerics 7.6. PEN, Penicillin; SXT, Trimethoprim/Sulfamethoxazole; AXO, Ceftriaxone; TET, Tetracycline; CLI, Clindamycin.

researchers. Meanwhile, in China, there was a risk of a *B. cereus* outbreak driven by a *cytK1*-positive strain. The *ces* gene for emetic toxin cereulide production was found in 4.4% of *B. cereus* isolates, consistent with the fact that *B. cereus* with *ces* was rarely isolated from food and environmental materials (Arslan et al., 2014; Chon et al., 2015; Zhang et al., 2017).

Prevalence of antimicrobial resistance

The antimicrobial susceptibility profile of the *B. cereus* isolates is shown in **Table 3**. Various susceptibility patterns against 12 types of antibiotics were exhibited. All isolates were susceptible to vancomycin, amikacin, imipenem, and

rifampin. 90.1%, 78.0%, 95.6%, and 97.8% of the isolates showed susceptibility to chloramphenicol, erythromycin, tetracycline, and ciprofloxacin, respectively. 97.8% of isolates were resistant to penicillin, consistent with published reports that *B. cereus* isolates from either clinical or food sources were mostly resistant to penicillins (Park et al., 2009; Merzougui et al., 2014; Zhang et al., 2017). A high rate of antimicrobial resistance (84.6% isolates) was also detected against trimethoprim/sulfamethoxazole. All *B. cereus* isolates were classified into eight antibiotic resistance patterns (**Figure 1**). Resistance to penicillin/trimethoprim/sulfamethoxazole was the most common in our study. 21 isolates (23.1%) were multidrug resistance, with 85.7% were resistant to penicillin/ceftriaxone/trimethoprim/sulfamethoxazole.

ERIC-PCR genotyping

All 91 *B. cereus* isolates were fingerprinted and assigned genotypes by using ERIC-PCR. The size of amplicons bands ranged approximately from 100 bp to 2000 bp. Each isolate produces 3-12 DNA bands. Considering 100% similarity in band pattern as a cut-off criteria, 89 genotypes were obtained, as a PCR-mediated fingerprinting typing approach. ERIC-PCR is more straightforward and rapid than PFGE and ribotyping (Dorneles et al., 2014; Magyar et al., 2019). It was initially applied to *B. cereus* for genetic discrimination by PO-REN Hsueh et al. (1999). However, a low number of *B. cereus* strains and low genetic diversity made it insufficient to evaluate the discriminatory capability of ERIC-PCR for *B. cereus*. In subsequent reports, ERIC-PCR was utilized to distinguish the strains of different species in the *Bacillus* genus (Shangkuan et al., 2000). According to our results, the calculated Hunter Gaston Discriminatory Index (HGDI) of ERIC-PCR on *B. cereus* genotyping attained 0.9995 using the optimal PCR system, demonstrating a high discriminatory capability.

Cluster analysis was performed based on UPGMA (**Figure 1**). Two major genotype groups (Group A and Group B) were defined in our study. 49.5% (45/91) of the isolates belonged to Group A, and 50.5% of the isolates belonged to Group B. Relationships analysis results between genotype groups and virulence gene distribution, antimicrobial resistance profiles demonstrated that there was no association between genotype groups and *nheC* ($\chi^2 = 3.167$, $p = 0.075$), *bceT* ($\chi^2 = 3.167$, $p = 0.075$), *cytK1* ($\chi^2 = 0.000$, $p = 0.987$), *cytK2* ($\chi^2 = 0.088$, $p = 0.767$), and *ces* ($\chi^2 = 0.239$, $p = 1.000$). Meanwhile, Group A *B. cereus* tended to carry *hblA* ($\chi^2 = 6.018$, $p = 0.014$), *hblC* ($\chi^2 = 4.09$, $p = 0.043$), *hblD* ($\chi^2 = 4.967$, $p = 0.026$), *nheA* ($\chi^2 = 3.963$, $p = 0.046$), and *nheB* ($\chi^2 = 3.957$, $p = 0.047$), and to be resistant to penicillin/trimethoprim/sulfamethoxazole ($\chi^2 = 4.643$, $p = 0.031$). Considering a limitation of ERIC-PCR as to the repetitive capabilities, genotypic diversity analysis of *B. cereus* based on the more reproducible methods, i.e., MLST and genomic sequencing (Nguyen and Tallent, 2019; Shen et al., 2021; Zhang et al., 2020) might be more informative in the future study.

Conclusion

Overall, an initial investigation was conducted of the quantitative prevalence and characterization of *B. cereus* isolated from ready-to-eat rice products in Zhejiang Province, Eastern China. A relatively high level of contamination was detected in ready-to-eat rice products, posing a risk of food poisoning and significant public health concern. Differences in the detection rate between the enterotoxin genes and emetic toxin genes revealed that *B. cereus* in ready-to-eat rice products was able to cause diarrhea and lead to food poisoning. *B. cereus* isolates presented high genetic diversity using ERIC-PCR with an HGDI of 0.9995. According to genetic relationships analysis, genotype Group A *B. cereus* isolates tended to carry *hblA*, *hblC*, *hblD*, *nheA*, *nheB*, and show resistance to penicillin/trimethoprim/sulfamethoxazole. This study provided essential data for addressing the microbial safety of ready-to-eat rice products in China (Peng et al., 2022), accordingly, might improve the appropriate safety criteria and policy.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

Author contributions

MY did the conceptualization, wrote, reviewed, and edited the manuscript, and carried out the project administration and funding acquisition. JC, JZ, and LZ investigated the data. HC and ZZ validated the data. JC and JZ carried out the data analysis. JZ wrote the original draft preparation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.964823/full#supplementary-material

SUPPLEMENTARY TABLE 1
The complete meta-data information for isolates in this study.

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Genomic characterization of multidrug-resistance gene *cfr* in *Escherichia coli* recovered from food animals in Eastern China

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The plasmid-borne *cfr* gene, mediating multiple drug resistance (MDR), has been observed in many Gram-positive bacteria. The prevalence of *cfr* and its co-occurrence with additional antimicrobial resistance (AMR) determinants in *Escherichia coli* is an ongoing issue. Additionally, the prevalence and transfer mechanism of the *cfr* gene remain partially investigated. Here, eight *cfr*-positive *E. coli* strains were screened using PCR from an extensive collection of *E. coli* (n =2,165) strains isolated from pigs and chickens in 2021 in China, with a prevalence rate of 0.37%. All of them were MDR and resistant to florfenicol and tetracycline. These strains can transfer the *cfr* gene to *E. coli* J53 by conjugation (1.05×10^{-1} – 1.01×10^{-6}). Moreover, the IncX4 plasmid p727A3-62K-cfr (62,717bp) harboring *cfr* in strain EC727A3 was confirmed using Oxford Nanopore Technology. The unknown type plasmid p737A1-27K-cfr (27,742bp) harboring *cfr* in strain EC737A1 was also identified. Notably, it was verified by PCR that three of the eight *E. coli* strains were able to form the *cfr*-IS*26* circular intermediate. It was 2,365bp in length in strains EC727A3 and ECJHZ21-173, and 2,022bp in length in EC737A1. Collectively, this study demonstrated that IS*26* plays a vital role in transmitting the MDR gene *cfr* in *E. coli via* conjugation and provided updated knowledge regarding *cfr* in *E. coli* in Eastern China.

KEYWORDS

***Escherichia coli*, florfenicol, cfr, antimicrobial resistance, circular intermediate**

Introduction

Antimicrobial resistance (AMR) is a serious threat to global public health. The capability of bacteria to acquire and transfer antibiotic resistance and virulence genes is dangerous and urgently crucial to both human and animal health. The multidrug-resistance (MDR) gene *cfr* encodes 23S rRNA methylase, which is resistant to five classes of antimicrobials, including phenols, lincosamides, oxazolidinones, pleuromutilin, and

streptomycin A class antibiotics (PhLOPSA phenotype) (Kehrenberg et al., 2005; Long et al., 2006), and has decreased susceptibility to the 16-membered macrolides spiramycin, and josamycin (Smith and Mankin, 2008). For the first time, the discovery of multiple AMR gene *cfr* in *Staphylococcus bovis* isolates has attracted attention in a global sense (Schwarz et al., 2000). Insertion sequences and transposons are associated with the spread of *cfr* in Gram-negative and Gram-positive bacteria, including but not limited to, *Enterococcus*, *Bacillus*, *Jeotgalicoccus*, *Macrococcus*, *Pasteurella multocida*, *Vibrio diabolicus*, *Escherichia coli*, *Streptococcus*, and *Proteus vulgaris* (Dai et al., 2010; Wang et al., 2011, 2012a,b, 2013; Chen et al., 2020a,b; Liu et al., 2022), considering that *cfr* is usually located on plasmids containing related insertion sequences and transposons (Shen et al., 2013; Partridge et al., 2018).

Based on published articles to date, a total of 112 strains of *E. coli* containing the MDR gene *cfr* have been identified in various provinces of China; the primary source of these *E. coli* strains are pigs, which may be related to the overuse of florfenicol for disease prevention and treatment in pig farms (Wang et al., 2012a, 2018; Zhang et al., 2014, 2015, 2016; Liu et al., 2017; Ma et al., 2021; Tang et al., 2021). For example, it coexists with the extended-spectrum-β-lactamase gene $bla_{CTX\text{-}M\text{-}14b}$, tigecycline resistance gene *tet*(X4), colistin resistance gene *mcr-1*, and florfenicol resistance gene *floR* (Zhang et al., 2015; Ma et al., 2021; Tang et al., 2021). These plasmids carrying the *cfr* gene in *E. coli* belong to the plasmid replicon type, including IncX4, IncA/C, IncF14: A-: B-, IncN-IncX1 (Zhang et al., 2014; Sun et al., 2015; Wang et al., 2018; Tang et al., 2021), of which, IncX4 plasmids are frequently detected in China (Wang et al., 2018). However, few studies have investigated the mechanisms of transmission of the MDR gene *cfr* in *E. coli*.

In this study, the prevalence and characteristics of *E. coli cfr*-positive strains in food animals were investigated. All *cfr*-positive strains were further sequenced by Illumina or Nanopore platforms, and the *cfr*-harboring plasmids were also identified and characterized. It was confirmed that circular intermediate and conjugation transfer promoted the transfer of the *cfr* gene. Our study highlights the severe threat posed by *cfr*-carrying *E. coli* to public health and provides new insight on its role in dissemination.

Materials and methods

Screening of the *cfr* gene

From May to December 2021, 2,103 *E. coli* strains were isolated from 11 cities in Zhejiang, including Hangzhou, Jinhua, Jiaxing, Qvzhou, Ningbo, Taizhou, Shaoxing, Zhoushan, Lishui, Wenzhou and Huzhou, including 1,186 strains from pigs, 904 from strains in chickens and 13 strains from ducks. Thirty-six *E. coli* strains were isolated from Jiangxi Province, 25 *E. coli* strains were isolated from Hunan Province, and one was isolated from Anhui Province (Table 1). PCR screening of isolated strains was performed to obtain the prevalence of the *cfr* gene in the above *E. coli* isolates with primer sequences (F: GTGAAGCTCTAGCCAACCGTC; R: GCAGCGTCAATAT CAATCCC), as described previously (Osman et al., 2019).

Antimicrobial Susceptibility Test

Escherichia coli was inoculated on Luria-Bertani (LB) agar medium for pure culture, according to the micro-dilution method recommended in the M100-S31 document of the American Committee for Clinical Laboratory Standardization (CLSI) (Humphries et al., 2021; Tang et al., 2022b). The antimicrobial susceptibility of *E. coli* to 13 tested antibiotics were, ampicillin (2–128 μg/ml), amoxicillin-clavulanate acid (4/2–128/64 μg/ml), cefotaxime (0.06–64 μg/ml), meropenem (0.5–16 μg/ml), amikacin (2–64 μg/ml), gentamicin (0.25–32 μg/ml), colistin (0.125–8 μg/ml), ceftiofur (0.25–32 μg/ml), ciprofloxacin (0.06–8 μg/ml), trimethoprim-sulfamethoxazole (0.5/9.5–16/304 μg/ml), tetracycline (0.25–64 μg/ml), tigecycline (0.25–32 μg/ml), and florfenicol (2–128 μg/ml). *E. coli* ATCC 25922 served as quality control bacteria.

Whole-genome sequencing

To further understand the genetic background of themultiple AMR gene *cfr* in *E. coli*, a genomic DNA extraction kit (Generay, Shanghai, China) was used to extract bacterial genomic DNA from all *cfr* positive strains for whole-genome sequencing (WGS). An Illumina sequencing library was generated using the NEXTflex DNA sequencing kit (Bioo Scientific, Austin, United States). Illumina paired-end sequencing was performed using the HiSeq-PE150 strategy, and the readings were filtered using fastp v0.12. Clean data were reconstructed using CLC Genomic Workbench 12.0. Prototypical strains were simultaneously whole-genome sequenced on the Oxford Nanopore GridION platform (Oxford, United Kingdom). The above genomic DNA library was prepared using the SQKLSK109 kit (Oxford Nanopore Technologies, Oxford, United Kingdom). Guppy v3.2.4 was used for base invocation and removal of adapter sequences. Sequences were assembled from scratch using a mixture of short and long reads from the Unicycler

TABLE 1 Strain information for screening the *cfr* gene.

Province	Animal	Number
Zhejiang	Pig	1,186
	Chicken	904
	Duck	13
Jiangxi	Duck	36
Hunan	Chicken	25
Anhui	Pig	1
Total	–	2,165

v0.4.4 pipeline (Wick et al., 2017). The reconstruction of plasmids from next generation sequence pair-end datasets was performed by PLACNETw (Vielva et al., 2017).

Antimicrobial resistance gene, virulence gene, phylogenetic tree and plasmid analysis

Acquired AMR genes and chromosomal mutations were predicted using ResFinder 4.1[1] with a percentage identification threshold of 90% and a minimum coverage length of 60%. The virulence genes were predicted using VirulenceFinder 2.0.[2] Plasmid replicon type identification using PlasmidFinder 2.1[3] with a percentage identification threshold of 95% and percentage coverage length of 60%. Multilocus sequence typing (MLST) was performed using MLST 2.0.[4] Phylogenetic analysis of genomes and plasmids based on maximum likelihood was performed using kSNP3 (Gardner et al., 2015). Easyfig 2.2.3 was used to compare the gene–environment (Sullivan et al., 2011). BRIG was used to plot circles of multiple plasmids for comparison (Alikhan et al., 2011).

Conjugation transfer assay

The *E. coli* strain J53 was selected as the recipient strain, and *cfr*-positive *E. coli* was selected as the donor strain. Florfenicol and sodium azide were added for the selection. First, we determined that *cfr*-positive *E. coli* could not be grown on LB plates containing 100 mg/l sodium azide, and J53 could no longer be grown on LB plates containing10 mg/l florfenicol. The method of conjugation transfer was mentioned in previous reports (Xu et al., 2021; Tang et al., 2022a). The donor bacteria and recipient bacteria were inoculated into LB broth and cultured on a shaker for 4–6 h. One milliliter of the bacterial solution was taken for centrifugation, and the donor and recipient bacteria were added to the LB plate overnight at 37°C. After gradient dilution with PBS, they were inoculated onto LB square plates containing 10 mg/l florfenicol and 100 mg/l sodium azide. Finally, single colonies that grew after mating were identified *via* PCR to exclude false-positive cases.

Detection of IS*26*-mediated circularization with a *cfr*-containing gene

To verify the circularization potential of the IS*26* flanking fragments in a plasmid, a pair of primers were designed and amplified by PCR to observe whether they could form the circular intermediate of *cfr*-IS*26*. The primers used to identify the *cfr*-IS26 circular intermediate are shown (F: GTTGCCTGGTG TAAATGATTC; R: CTGCTAAGAGCTTGATATTC). The size of the *cfr*-IS*26* circular intermediate was determined by Sanger sequencing.

Results

Antimicrobial susceptibility test of *E. coli* carrying the *cfr* gene

Eight *cfr*-positive isolates were identified from 2,165 *E. coli* isolates (1,187 from pigs, 929 from chickens, and 49 from ducks), and the prevalence was 0.37% (Table 2). Seven of the *cfr*-positive strains were isolated from pigs, and one strain was isolated from chicken. The AST results of eight positive *E. coli* isolates showed that all strains were resistant to ampicillin, amoxicillin-clavulanic acid, tetracycline, and florfenicol (Figure 1; Supplementary Table 1). All the strains were sensitive to colistin, meropenem, tigecycline, and amikacin.

Molecular characterization and conjugative transfer of *cfr*-positive isolates

The contigs carrying *cfr* gene assembled by the second generation sequence are between 1 and 3 Kb in length (Supplementary Figure 1). The *cfr*-harboring *E. coli* strains isolated from chicken and pig belonged to different branches. Among the strains ECJHZ21-040, ECJHZ21-049, and ECNBZ21-038 were clustered together. Additionally, ECQZJ21-074 belonged to independent lineages, and there were differences between them and in the seven strains mentioned above (Figure 1). The eight *E. coli* isolates had distinct sequence types (STs) with ST641, ST2179, ST4434, ST88, ST349, ST10562, ST48, and ST209, indicating that *cfr* was widely distributed in *E. coli* with different genetic backgrounds.

A total of 49 types of AMR determinants within 10 classes of antibiotics were detected (Figure 2A). In addition, there were two florfenicol genes (*cfr*, *floR*), three tetracycline genes (*tet*(A), *tet*(B), and *tet*(M)), 10 β-lactam genes ($bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-150}}$, $bla_{\text{TEM-1A}}$, $bla_{\text{TEM-1B}}$, $bla_{\text{OXA-10}}$, $bla_{\text{TEM-1C}}$, $bla_{\text{OXA-20}}$, $bla_{\text{OXA-135}}$, $bla_{\text{TEM-32}}$, $bla_{\text{OXA-1}}$), two quinolone genes (*qnr*S1, *qnr*S2), two rifamycin genes (*ARR*-2, *ARR*-3), three macrolide genes (*mph*(A), *mdf*(A), and *erm*(B)), one lincosamide gene (*Inu*(F)), six folate pathway antagonist genes (*sul*1, *sul*2, *sul*3, *dfrA*12, *dfrA*17, *dfrA*19), 14 aminoglycoside genes (*aad*A2b, *aph(4)-Ia*, *aac(3)-IV*, *aadA*2, *aph(3′)-Ia*, *aph(3″)-Ib*, *aac(3)-IId*, *aph(6)-Id*, *aad*A5, *aph(3′)-IIa*, *aad*A1, *aac(6′)-Ib-cr*, *aad*A22, *aad*A24) and some additional AMR determinants (Figure 2A). The virulence genes of the strains included *terC*, *traT*, *gad*, *lpfA*, *ompT*, *sitA*, *astA*, *hra*, etc. (Figure 2B). Among them, *astA* is a virulence gene encoding heat-stable enterotoxin of enteroaggregative *E. coli*, which may produce related toxins with the possibility of pathogenicity. Importantly, strain EC727A3

1 https://cge.cbs.dtu.dk/services/ResFinder/
2 http://cge.cbs.dty.dk/service/VirulenceFinder/
3 https://cge.cbs.dtu.dk/services/PlasmidFinder/
4 https://cge.cbs.dtu.dk/services/MLST/

TABLE 2 *cfr*-positive *E. coli* isolates in this study.

Strains	Source	Animal	City	Plasmid	Accession number
ECJHZ21-040	Feces	Pig	Jinhua	–	JAMYDT000000000
ECJHZ21-049	Feces	Pig	Jinhua	–	JAMYDS000000000
ECNBZ21-038	Feces	Pig	Ningbo	–	JAMYDR000000000
ECNBZ21-177	Feces	Pig	Ningbo	–	JAMYDQ000000000
ECJHZ21-173	Feces	Pig	Jinhua	–	JAMYDP000000000
ECQZJ21-074	Feces	Chicken	Qvzhou	–	JAMYDO000000000
EC727A3	Feces	Pig	Hangzhou	p727A3-62K-cfr	CP100062-CP100071
EC737A1	Gut	Pig	Zhoushan	p737A1-27K-cfr	CP100005-CP100012
ECJHZ21-058	Feces	Pig	Jinhua	–	JAMYDT000000001

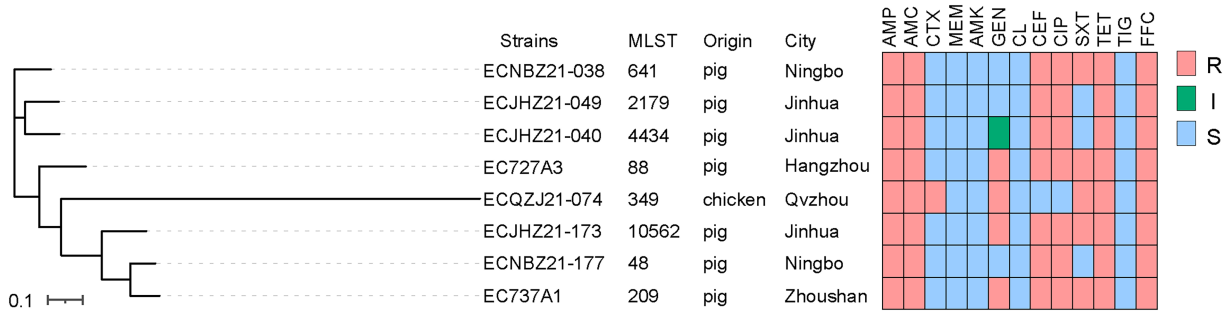

FIGURE 1
The phylogenetic tree and AMR of *E. coli* isolates. 'S' represents susceptibility, 'I' represents intermediate, and 'R' represents resistance. Penicillins: AMP, ampicillin; AMC, amoxicillin-clavulanic acid; Cephalosporins: CTX, cefotaxime; CEF, ceftiofur; Carbapenems: MEM, meropenem; Aminoglycosides: AMK, amikacin; GEN, gentamicin; Polypeptides: CL, colistin; Quinolones: CIP, ciprofloxacin; Sulfonamides: SXT, trimethoprim-sulfamethoxazole; Tetracyclines: TET, tetracycline; TIG, tigecycline; Chloramphenicol: FFC, florfenicol.

contains the virulence genes *stx*2A and *stx*2B that produce Shiga toxin, which may cause self-limiting diarrhoeal disease and sometimes bloody diarrhea as well as complications such as hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Fitzpatrick, 1999; Launders et al., 2016; Mcfarland et al., 2017). Plasmid replicons include 19 types such as IncFIC(FII), IncN, IncFIA(HI1), IncFIB(K), ColE10, IncR, Col156, IncQ1, Col440II, IncFII(29), p0111, IncFII(pCoo), IncFII, IncY, IncX1, IncHI2A, IncHI2, IncX4, and IncFIB. The plasmid types of the eight isolates remained genetically diverse (Figure 2C).

The conjugation transfer assay demonstrated that all transconjugants from *cfr*-positive *E. coli* strains and *E. coli* J53 could grow normally on LB plates containing 100 mg/l sodium azide and 10 mg/l florfenicol. Further, PCR confirmed that the transconjugant contained the *cfr* gene, which indicated that the conjugative transfer experiment was successful, with a transfer frequency of 1.05×10^{-1}–1.01×10^{-6}.

Genetic environment of the *cfr*-positive isolates.

Two isolates were randomly selected from the eight *cfr*-positive strains for nanopore sequencing to obtain their complete genome sequences. To understand how *cfr* is transmitted, the genetic background of the *cfr* gene was further investigated. The *cfr* gene was located on the IncX4-type plasmid p727A3-62K-cfr (CP100066) in strain EC727A3. The length of p727A3-62K-cfr was 62,717 bp, and the GC content was 44% (Figure 3A). Moreover, p727A3-62K-cfr had high similarity with another *cfr*-carrying plasmid and had the highest homology with plasmid pSD11 (KM212169.1, 37,672 bp) from porcine *E. coli* strain 8ZG6D (65% query coverage and 99.99% identity). The collinear comparison showed that p727A3-62K-cfr and pSD11 had two different gene arrangements. The 12,647 bp region had high homology with the sequence containing the *tet*(M) gene in pNT1N31-93k (CP075482, 93,332 bp), and there was an insertion sequence IS*1* upstream of *tet*(M) compared with pNT1N31-93k (Figure 3B). The other 10,831 bp region had a higher homology to a part of pSCZE4 (CP051226, 60,732 bp), and this sequence had three more IS*91* insertion sequences in the same direction than pSCZE4 (Figure 3B).

The *cfr* gene of strain EC737A1 was located on plasmid p737A1-27K-cfr (CP100008). The length of p737A1-27K-cfr was 27,742 bp, and the GC content was 43% (Figure 4A). Plasmid p737A1-27K-cfr had a high degree of homology (100% query coverage and 100% recognition) with plasmid unnamed4 (CP037908.1, 28,519 bp). The collinear comparison showed that a 777 bp region containing the IS*1* mobile element was inserted into the plasmid p737A1-27K-cfr to form unnamed4. However, the

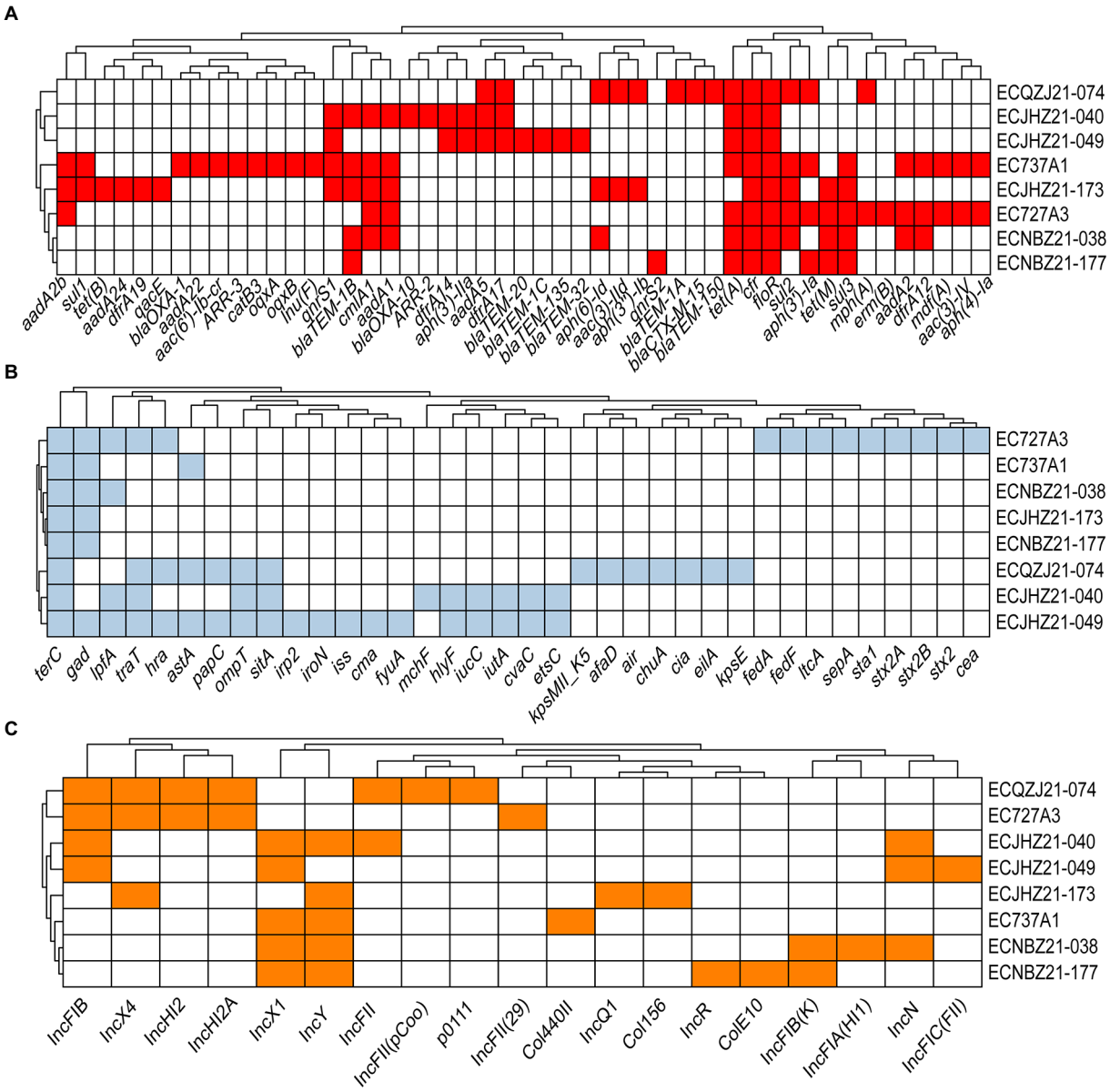

FIGURE 2
The AMR genes, plasmid replicons, and virulence genes in *cfr*-positive *E. coli*. **(A)** Acquired AMR gene in *cfr*-positive *E. coli*. Red indicates the AMR gene. **(B)** Virulence gene in *cfr*-positive *E. coli*. Light blue represents the presence of the virulence gene, and white represents the absence of the virulence gene. *terC*, tellurium ion resistance; *gad*, glutamate decarboxylase; *lpfA*, long polar fimbriae; *traT*, outer membrane protein complement resistance; *hra*, heat-resistant agglutinin; *astA*, EAST-1 heat-stable toxin; *papC*, outer membrane usher P fimbriae; *ompT*, outer membrane protease; *sitA*, iron transport; *irp2*, high molecular weight protein 2 non-ribosomal peptide synthetase; *iroN*, enterobactin siderophore receptor; *iss*, increased serum survival; *cma*, colicin M; *fyuA*, siderophore receptor; *mchF*, ABC transporter; hlyF, hemolysin F; *iucC*, aerobactin synthetase; *iutA*, ferric aerobactin receptor; *cvaC*, microcin C; *etsC*, putative type I secretion outer membrane; *kpsMII_K5*, polysialic acid transport; *afaD*, afimbrial adhesion; *air*, enteroaggregative immunoglobulin; *chuA*, outer membrane hemin receptor; *cia*, colicin ia; *eilA*, salmonella HilA homolog; *kpsE*, capsule polysaccharide export inner-membrane; *fedA*, fimbrial protein F107 subunit A; *fedF*, fimbrial adhesin AC precursor; *ltcA*, heat-labile enterotoxin A subunit; *sepA*, shigella extracellular protein A; *sta1*, Heat-stabile enterotoxin ST-Ia; *stx2A*, shiga toxin 2, subunit A; *stx2B*, shiga toxin 2, subunit B; *stx2*, O139 S1191, variant e; *cea*, colicin E1. **(C)** Plasmid replicon type in *cfr*-positive *E. coli*. Orange represents the plasmid replicon type; white represents none of the genes predicted.

type of plasmid had not yet been determined; it was only known that the backbone of plasmid p737A1-27K-cfr was derived from pSTEC2018_607-F (CP075703.1, 24,412 bp). The 4,270 bp construct containing the IS*26*-*cfr*-IS*26*-*higA*-*higB*-*parK* was inserted into the plasmid pSTEC2018_607-F (Figure 4B).

cfr-IS*26* circular intermediate

Genome analysis found that both the upstream and downstream regions of the *cfr* gene in EC727A3 and EC737A1 had an IS*26* element in the same direction, forming an IS*26*-*cfr*-IS26 structure

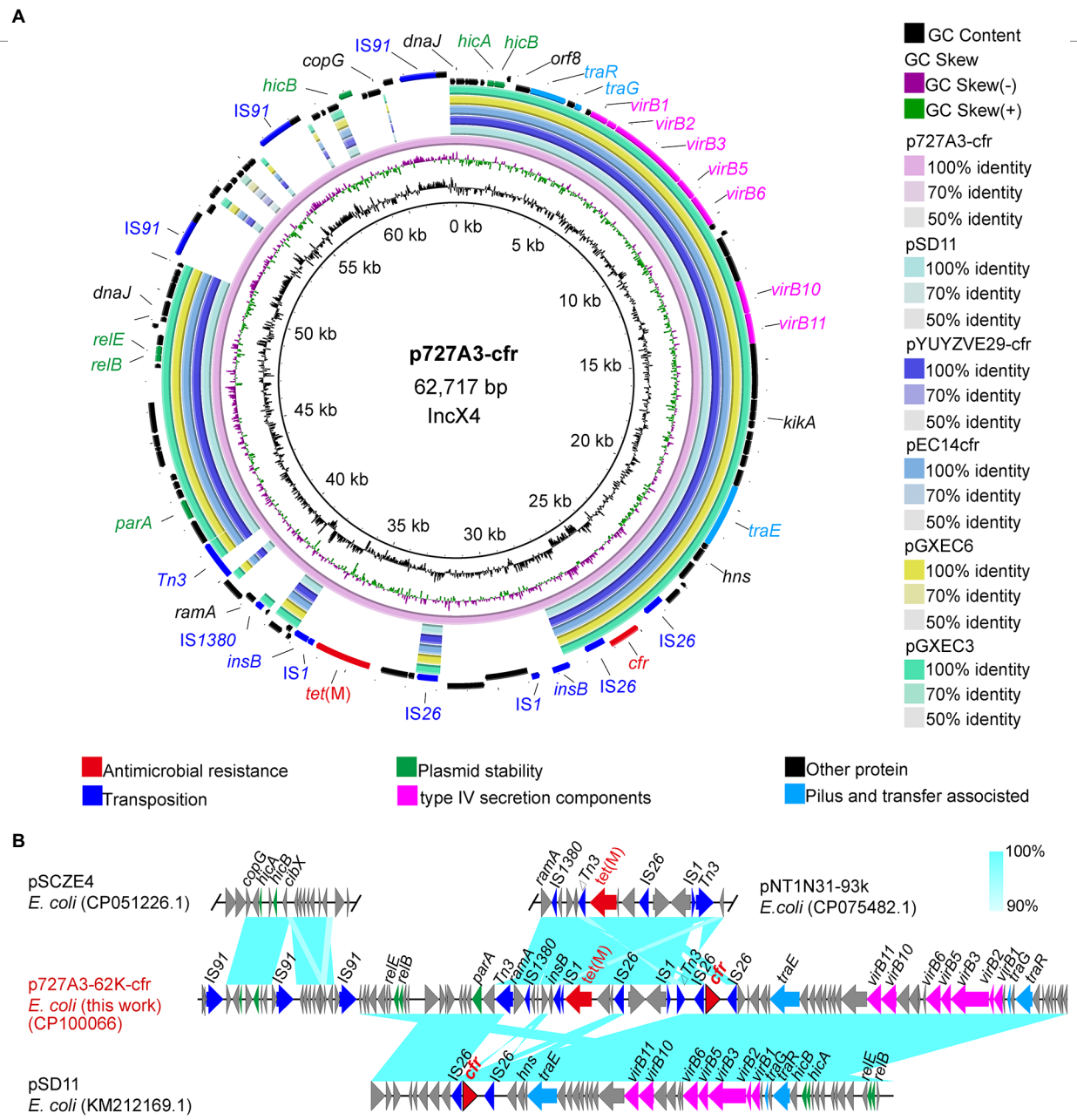

FIGURE 3
Sequence alignment of plasmid p727A3-62K-cfr and the gene–environment of *cfr*. **(A)** Comparison of the plasmid sequences between p727A3-62K-cfr, pSD11, pYUYZVE29-cfr, pEC14cfr, pGXEC6, and pGXEC3 of *E. coli* strains. **(B)** Linear comparison of the plasmid sequences of *E. coli* p727A3-62K-cfr and *E. coli* pSCZE4, pSD11, pNT1N21-93k. Open arrows indicate coding sequences (red arrows, AMR genes; green arrows, plasmid replication; blue arrows, transfer and transfer-related sequences; gray arrows, hypothetical, and unclassified) and indicate the direction of transcription.

(Figure 5A). However, there was a 343 bp size difference between the IS26-*cfr*-IS26 structures in EC727A3 and EC737A1. PCR determined that three out of eight *E. coli* strains could form *cfr*-IS*26* cyclic intermediates of two different sizes. Among them, the size of the circular intermediate formed by ECJHZ21-173 and EC727A3 was the same, at 2,365 bp (Figure 5B). The size of the *cfr*-IS*26* circular intermediate in EC737A1 was 2,022 bp (Figure 5C).

Discussion

To date, the prevalence of the *cfr* gene in *E. coli* from animals has been reported to be 0.37% in Eastern China. In previous studies, most of the *cfr* genes in *E. coli* were isolated from pigs (Deng et al., 2014; Zhang et al., 2014). As far as we know, only four *E. coli* strains of chicken origin containing the *cfr* gene have

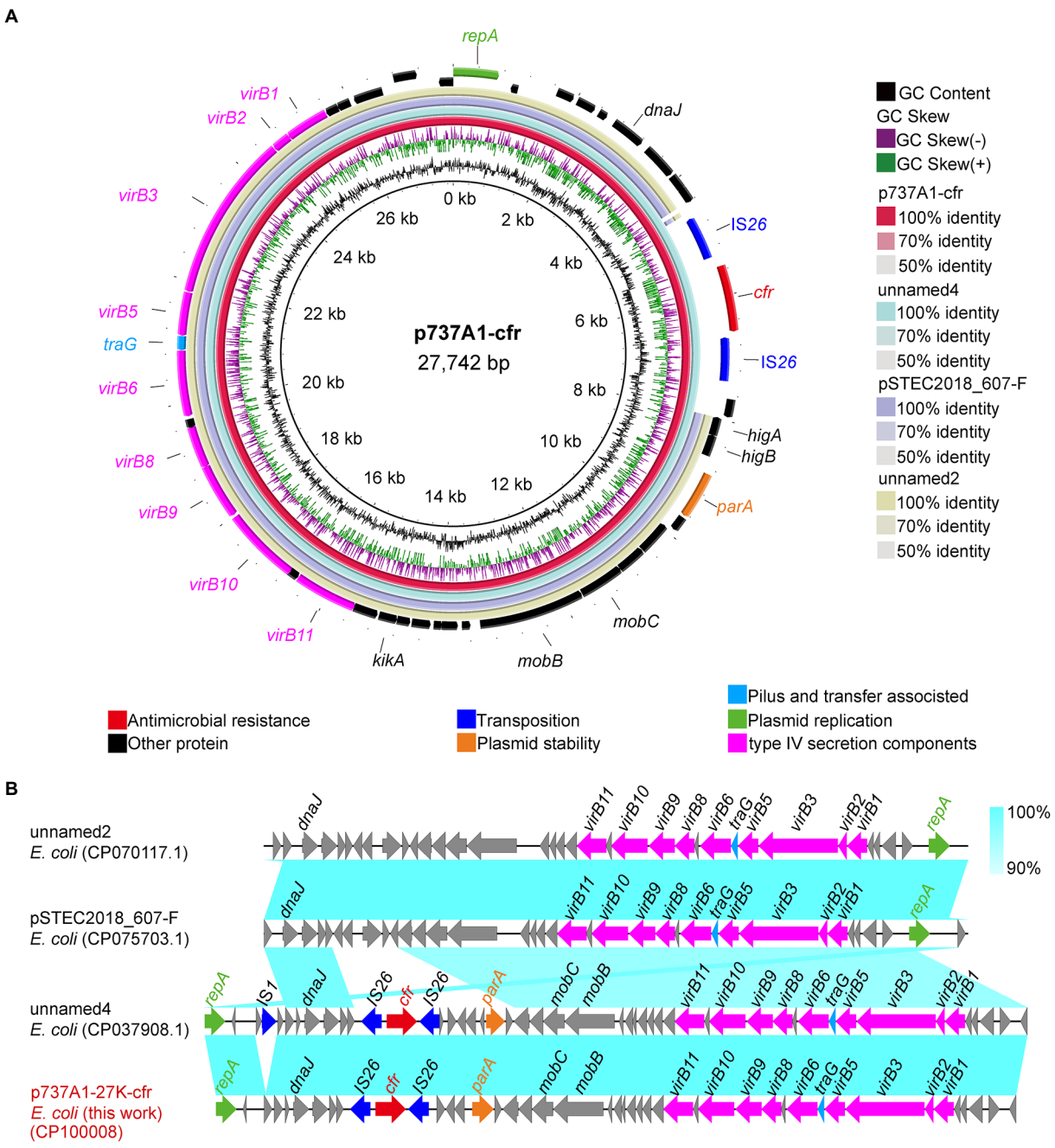

FIGURE 4
Sequence alignment of plasmid p737A1-62K-cfr and the gene–environment of *cfr*. **(A)** Comparison of the plasmid sequences between *E. coli* p737A1-27K-cfr, *E. coli* plasmid unnamed4, pSTEC2018_607-F, unnamed2, FHI99-scaffold-18_conting-14, and pEC2547-KPC-2. **(B)** Linear comparison of the plasmid sequences of *E. coli* p737A1-27K-cfr and *E. coli* plasmid unnamed4, pSTEC2018_607-F, and plasmid unnamed2. Open arrows indicate coding sequences (red arrows, AMR genes; green arrows, plasmid replication; blue arrows, transfer, and transfer-related sequences; gray arrows, hypothetical, and unclassified) and indicate the direction of transcription.

been identified in Guangdong Province, Fujian Province and Heilongjiang Province (Zhao et al., 2016; Wang et al., 2018). No *cfr* gene has been found in human clinical *E. coli* isolates. In this study, we isolated the *cfr* gene from chicken sources in addition to pigs, and the prevalence of the *cfr* gene in *E. coli* isolates was higher than the initially reported at 0.08% (1/1230) (Wang et al., 2012a). This was similar to the previously reported 0.5% (2/398) (Liu et al., 2017) but much lower than the 13.7% (85/617) recently reported in Guangdong Province, China (Ma et al., 2021). According to the official, authoritative statement, in 2018 (P.R., 2019) and 2020 (P.R., 2021), the use of phenicols was 2,123 and 3,519 tons in animal breeding in China, respectively, and

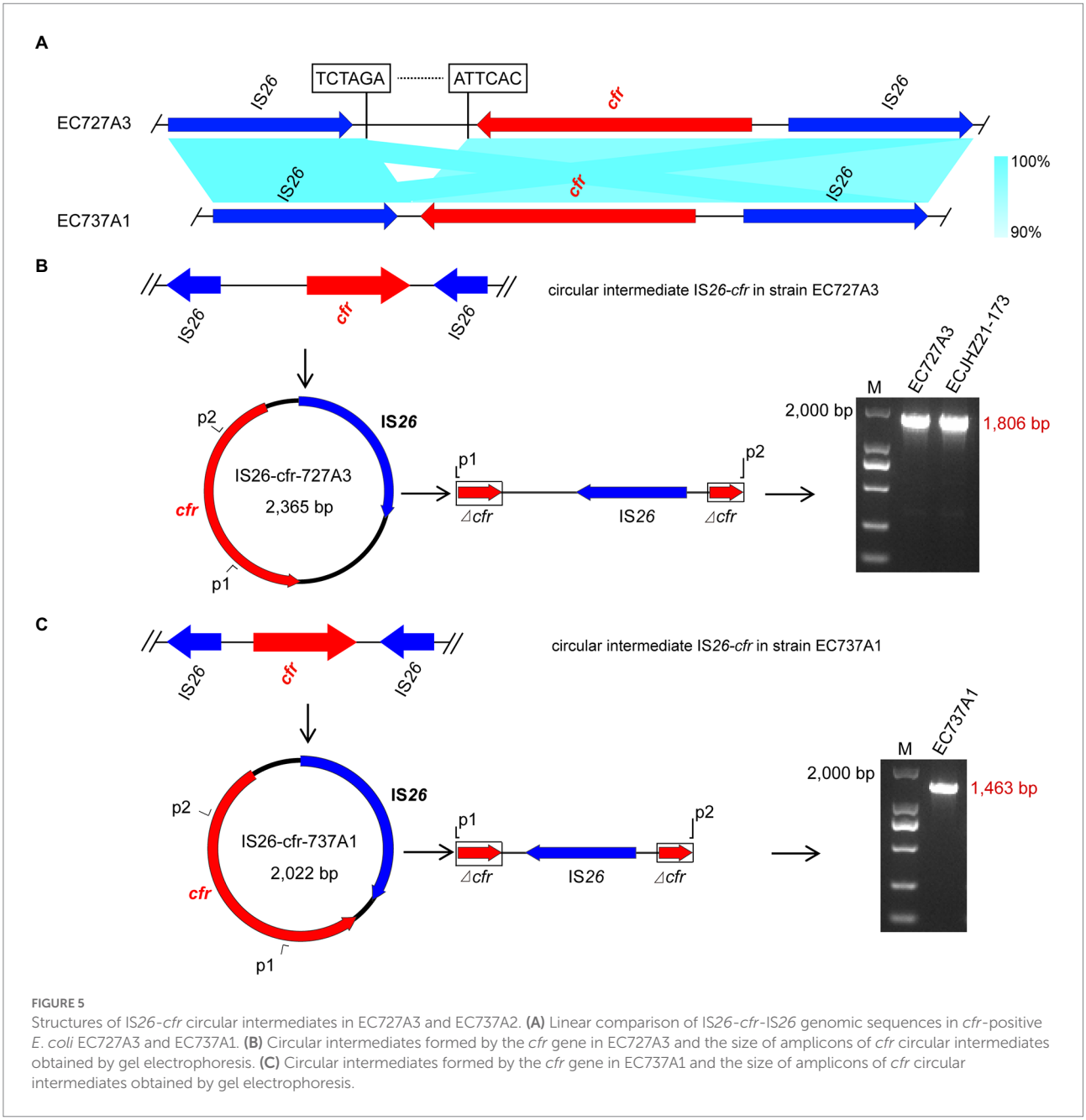

FIGURE 5
Structures of IS*26*-*cfr* circular intermediates in EC727A3 and EC737A2. **(A)** Linear comparison of IS*26*-*cfr*-IS*26* genomic sequences in *cfr*-positive *E. coli* EC727A3 and EC737A1. **(B)** Circular intermediates formed by the *cfr* gene in EC727A3 and the size of amplicons of *cfr* circular intermediates obtained by gel electrophoresis. **(C)** Circular intermediates formed by the *cfr* gene in EC737A1 and the size of amplicons of *cfr* circular intermediates obtained by gel electrophoresis.

florfenicol was the primary antimicrobials in phenicols used in livestock and poultry breeding (Van Cuong et al., 2016). Previous global or national reports show that the florfenicol resistance gene is related to the long-term use of florfenicol (Li et al., 2020). Our study indicated that the *cfr* gene dissemination was significantly different in different provinces of China, and there was a possibility of rapid spread in a small area.

IS*26* is a universal mobile element in various gram-negative bacteria, including *E. coli*, *P. multocida*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *V. diabolicus*, and *Proteus vulgaris* (Post and Hall, 2009; Harmer et al., 2014; Chen et al., 2020a; Jin et al., 2021; Zhao et al., 2021). The presence of transfer elements plays a vital role in the transfer of the *cfr* gene. Previous studies confirmed the existence of different genetic environments for the *cfr* gene in *E. coli*, with one IS*26* element on each side of the *cfr* gene being the most reported genetic environment in *E. coli* and the other two being one IS*256* element on each side of *cfr* and one IS*15* element on each side (Wang et al., 2012a, 2018; Zhang et al., 2016; Liu et al., 2017; Tang et al., 2021). In addition, IS*26* was found to form circular intermediates mediating the transmission of *cfr* genes in *V. diabolicus*. Similarly, it was also found to form circular intermediates that mediate the transmission of other AMR genes in *E. coli* (Zhao et al., 2021; Liu et al., 2022). The current study results were inconsistent with previous studies

verifying that *cfr* can form a circular intermediate of IS*26*-*cfr* during transmission and facilitate its transmission in *E. coli*.

Plasmid p727A3-62K-cfr obtained in the present study belonged to the IncX4 type. The IncX4 plasmids carrying the *cfr* gene have been found in *E. coli* isolated from Jiangsu, Guangdong, Guangxi, Liaoning, Jilin, and Heilongjiang Provinces in China (Deng et al., 2014; Mei et al., 2021). This result indicated that the IncX4-type plasmid might be a common plasmid carrying the MDR gene *cfr*. In addition, we also identified a plasmid p737A1-27K-cfr that had not yet been typed, which indicates that the types of plasmids carrying the *cfr* gene are gradually increasing, and it is necessary to pay close attention to the spread of the *cfr* gene in *E. coli*.

Conclusion

Eight strains containing the *cfr* gene were isolated from 2,165 strains of *E. coli* in 2021, seven strains were isolated from pig farms, and one strain was isolated from chicken farms, indicating that the *cfr* gene widely exists in a variety of food animals. An IncX4 type plasmid and an unknown type plasmid were found, and the IS*26*-*cfr*-IS*26* structure was verified to form a *cfr*-IS*26* circular intermediate for propagation. Since the widespread use of antibiotics, particularly florfenicol, may promote the spread of *cfr* genes among animals. It is necessary to strengthen the control of veterinary antibiotics and continuously monitor the spread of the *E. coli* multidrug resistance gene *cfr* to reduce the potential public health threat.

Data availability statement

The names of the repository/repositories and accession number(s) can be found in the article/Table 2.

Author contributions

BT and MY: conceptualization. HY: funding acquisition. BT, JN, JL, HL, and YW: investigation. JN, JL, and BT: methodology. MY and HY: supervision. JN, HL, and BT: visualization. JN and BT: writing—original draft. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.999778/full#supplementary-material

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Topically applied bacteriophage to control multi-drug resistant *Pseudomonas aeruginosa*-infected wounds in a New Zealand rabbit model

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Pseudomonas aeruginosa (*P. aeruginosa*) is a widespread, gram-negative, pathogenic bacterium that causes serious internal and external infections in humans and other animals. The increasing antibiotic resistance has complicated bacterial infection treatment, and current antibiotic therapies cannot cure all infections. Owing to this, bacteriophages (phages) have regained attention as potential therapeutics for bacterial infections. In this study, the phage "PaVOA" was isolated from hospital sewage and characterized. Next, a New Zealand rabbit skin infection model was used to determine the therapeutic efficacy of PaVOA as compared to a phage cocktail or the cephalosporin antibiotic ceftriaxone. Characterization results demonstrated that phage PaVOA belongs to the *Myoviridae* family, has a double-stranded DNA genome, is resistant to low temperatures (−20°C), is most optimal at 40°C, has good acid−base tolerance, and remains stable for 30min under 20W ultraviolet (UV) intensity. The optimal multiplicity of infection of PaVOA was 0.1, and a one-step growth curve showed a short latency period (10min), thus demonstrating its ability to rapidly kill bacteria. Furthermore, the addition of calcium (Ca) and magnesium (Mg) ions significantly increased the PaVOA titer. An *in vivo* phage kinetic curve showed that PaVOA was rapidly inactivated within the blood of New Zealand rabbits (undetectable after 12 h), and no animals died due to phage treatment. Wound healing studies showed that the phage cocktail induced a high healing rate and an acceleration of the skin remodeling process, and was more efficacious than ceftriaxone. Therefore, phage cocktail therapy represents a novel therapeutic approach in the treatment of traumatic skin infections caused by multi-drug resistant *P. aeruginosa*.

KEYWORDS

***Pseudomonas aeruginosa*, multi-drug resistant, phage therapy, phage cocktail, characterization**

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*), a gram-negative bacterium, is a common opportunistic pathogen in hospitals that can cause serious infections such as pneumonia, urinary tract infections, burn infections, and bacteremia (Moradali et al., 2017). Importantly, *P. aeruginosa* accounts for 10%–20% of all bacterial cases of ventilator-associated pneumonia (Weiner et al., 2016) and is one of the leading causes of urinary tract infections (Reynolds and Kollef, 2021). Patients with burns are more likely to be infected by *P. aeruginosa* and suffer from complications in a humid environment (Norbury et al., 2016), whilst the mortality rate of *P. aeruginosa* bacteremia is as high as 43.2%–58.8% (Thaden et al., 2017).

Antibiotic treatment for infections caused by *P. aeruginosa* and infection control is hampered by its ubiquity in the environment and its intrinsic drug resistance (Breidenstein et al., 2011; Poole, 2011). Additionally, the prevalence of multi-drug resistant (MDR) *P. aeruginosa* is increasing. In recent years, bacterial infections in Spain and the United States have had a prevalence rate of 15%–30% (Pena et al., 2015; Sader et al., 2018). Drug-resistant bacterial infections are becoming an urgent issue, with the WHO reporting that 10 million people will die per year by 2050 from drug-resistant bacterial infections (Taati Moghadam et al., 2020). Therefore, novel approaches are urgently required to treat drug-resistant bacterial infections.

Bacteriophages (phages) are viruses that are small in size, do not have a complete cellular structure, and contain only a single nucleic acid. Phages have an estimated population of over 10^{31} on Earth (Dion et al., 2020) and can be isolated from soil, seawater, sewage, and waste products. Moreover, the mechanism whereby phages kill bacteria is unique and has several advantages; they have high host specificity, are non-toxic to humans and animals, and have minimal effects on nonspecific flora (Yoon Kyung Chang et al., 2022). Importantly, phages are considered a potential alternative to antibiotics for treating bacterial infections because of their high adaptability and efficacy against MDR pathogens in various environments (Alsaadi et al., 2021; Chegini et al., 2021). The application of phages for treating MDR bacterial infections is becoming more common for treating conditions such as infected skin burns, surgical wounds, and trauma wounds. A recent animal study showed that phage therapy for wounds infected with MDR *Klebsiella pneumoniae* resulted in the highest healing efficiency observed in the study (Fayez et al., 2021), thus supporting the potential therapeutic application of phage therapies.

The human skin functions to protect underlying tissues, muscles, and organs from physical and chemical damage (Bowler et al., 2001). However, direct contact with the external environment predisposes the skin to damage, resulting in a risk of sepsis due to microbial infiltration into wounds caused by burns, surgery, or trauma (Stearns-Kurosawa et al., 2011). *Pseudomonas aeruginosa* is one of the most prevalent causative pathogens of wound infections in hospitals (Chua et al., 2016). The main component of the bacterial cell wall, lipopolysaccharide, is highly immunogenic to infected patients, and the presence of MDR *P. aeruginosa* aggravates infection and hinders treatment.

For modest skin infections, the conventional methods of treatment include debridement, washing with a large amount of saline, and iodophor disinfection. In severe cases, hydrogen peroxide may be used either alone or in combination with antibiotics to enhance the therapeutic effect; however, the presence of MDR bacteria must be considered in such cases. Moreover, topical application of probiotics can reduce bacterial infections and promote wound healing (Fijan et al., 2019). However, as the structure and function of the skin microbiome are complex, the therapeutic effect of probiotics cannot be guaranteed (Krezalek and Alverdy, 2018). Due to these issues, phage therapy has received renewed attention in recent years.

As a model organism, the New Zealand rabbit has desirable characteristics of large serum volume, sensitivity to body temperature changes, and skin sensitivity to most pathogenic bacteria. Owing to these characteristics, they are commonly used experimentally for pyrogenic, immunological, and pharmacological studies. Malachowa (Malachowa et al., 2022), among others, demonstrated the successful optimization of *Staphylococcus aureus* skin infection in rabbits, which provides a reference for the establishment of other skin infection models. The present study aimed to characterize the laboratory-isolated *P. aeruginosa* phage PaVOA and subsequently evaluate its efficacy in resolving *P. aeruginosa*-mediated wound infections in New Zealand rabbits.

Materials and methods

PaVOB

PaVOB was sourced from the Department of Surgery, School of Veterinary Medicine, South China Agricultural University, China.

Ethics statement

Animal experiments were carried out according to the regulations of the ethics committee of South China Agricultural University. All animal experiments complied with the guidelines of the China Animal Welfare Commission.

Bacterial strains and culture conditions

Twenty-eight clinically isolated *P. aeruginosa* strains from the Department of Surgery, College of Veterinary Medicine, South China Agricultural University were included in this study. A standard *P. aeruginosa* strain CMCC 10104 was used as a reference strain. All strains were grown in LB broth (Qingdao high tech Industrial Park Haibo Biotechnology Co., Ltd. Qingdao, China)

and cultured on a shaking table at 37°C for 12 h. Bacterial DNA was isolated using the TIANamp Bacteria DNA Kit (Tiangen Biotech, Beijing, China). All strains were identified as *P. aeruginosa* by PCR experiments.

Antibiotic sensitivity test

We referred to the recommendations for *P. aeruginosa* drug susceptibility testing provided by the National Committee for Clinical Laboratory Standards (NCCLS) for drug selection. A total of 22 antibiotics (Hangzhou Binhe microbial Reagent Co., Ltd. Hangzhou, China), including ciprofloxacin (CIP; 5 μg), amikacin (AK; 30 μg), ceftriaxone (CRO; 30 μg), meropenem (MEM; 10 μg), gentamicin (CN; 10 μg), doxycycline (DO; 30 μg), imipenem (IPM; 10 μg), amoxicillin (AM; 20 μg), cefepime (CPM;30 μg), ofloxacin (OF;5 μg), compound sulfamethoxazole (SXT; 15 μg), enrofloxacin (ENR; 5 μg), ampicillin (AMP; 10 μg), azithromycin (AZM; 15 μg), chloramphenicol (C; 10 μg), tetracycline (TE; 30 μg), nincomycin (MY; 10 μg), norfloxacin (NOR; 10 μg), ceftazidime (CAZ; 30 μg), mezlocillin (MEZ; 30 μg), cefoperazone (CFP; 30 μg), and tobramycin (TOB; 10 μg) were used for the antibacterial sensitivity testing of 29 *P. aeruginosa* strains using the paper diffusion method. The diameter of inhibition circles was used to evaluate the sensitivity of *P. aeruginosa* to all antibiotics tested.

Phage isolation, purification, and host range determination

Two different bacteriophages were isolated from the sewage of Nanfang Hospital of the Southern Medical University of China. For water sampling, a 0.22 μM filter membrane (Qingdao high tech Industrial Park Haibo Biotechnology Co., Ltd. Qingdao, China) was used to remove residual bacteria, followed by centrifugation of the filtered filtrate at 12,000 rpm for 15 min at 25°C, with the resulting supernatant enriched with *P. aeruginosa*. The double-layer agar plate method was then used to separate phages. Individual phage plaques were chosen for purification once their size and morphology were consistent (purified single phages). One of the lytic phages with transparent plaques was selected, and *P. aeruginosa* (PA/18) was used as the host bacteria to further study the biological characteristics of the selected phages. Moreover, the host profile of the phage was assessed using the spotting method.

Transmission electron microscopy of phage particles

Purified phage particles with a titer of 10^{11} PFU/mL were loaded onto a copper grid for 1 min, followed by negative staining with uranyl acetate (2% w/v) for 1 min and subsequent aspiration of excess liquid. After drying, the Talos™ F200S TEM (Thermo Fisher Scientific, Waltham, MA, USA) apparatus was used to observe the morphology of phages and to capture images.

Phage nucleic acid type identification

Purified phage concentrates were prepared using the PEG precipitation extraction method (Rathor et al., 2022). Phage genomic DNA was extracted using the Viral DNA Kit (OmegaBiotek, Norcross US). Isolated DNA was treated with DNase I (20 U/μg; Solarbio Science & Technology Co., Ltd., Beijing, China), RNase A (5 U/μg; Solarbio Science & Technology Co., Ltd., Beijing, China), and Mung Bean Nuclease (20 U/μg; TaKaRa Biomedical Technology Co., Ltd., Beijing, China) and incubated at 37°C for enzymatic digestion of the genome. The resulting samples were separated by 0.8% (w/v) agarose gel electrophoresis.

Phage biological characteristics

Determination of optimal multiplicity of infection

Pseudomonas aeruginosa was cultured in LB broth for 16–18 h. Overnight cultures were then diluted with LB broth to a concentration of 10^8 CFU/ml. Phages were added at MOIs of 1×10^{-4}–1×10^{2} and incubated for 4 h at 37°C in a warm oven after shaking and mixing. Cocktails were centrifuged at 12,000 rpm for 10 min followed by isolation of the supernatant to determine phage titers using the double-layer agar plate method. The experiment was repeated thrice.

One-step growth curve

Pseudomonas aeruginosa was cultured to the logarithmic growth phase, at which point the PaVOA phage was added according to the optimal MOI ratio determined previously. Cultures were placed in a 37°C incubator for 15 min prior to centrifugation at 8,000 rpm for 5 min. After discarding the supernatant, sediment was resuspended with preheated sterile LB broth. The suspension cocktail was cultured on a shaking table at 37°C, 200 rpm. Samples were taken at intervals of 10 min in the first 30 min and then every 15 min. Phage titers of samples were determined using the double-layer agar plate method. The experiment was repeated thrice, and one-step growth curves were plotted using GraphPad Prism 8 (San Diego, CA, USA) software. The burst size of the phage is equal to the amount of phage at the end of lysis divided by the initial number of bacterial cells at the time of infection (Xu et al., 2021).

Optimal phage temperature, pH, and UV stability

Aliquots of PaVOA phages with determined titers were incubated in water baths at a range of temperatures (−20°C, 4°C, 25°C, 37°C, 40°C, 50°C, 60°C, 70°C, and 80°C) for 1 h. Three parallel controls were set up for each temperature, and titers were measured using the double-layer agar plate method. Additionally, the PaVOA phage was exposed to sterile Petri dishes at a distance of 40 cm from UV light (20 W) for 1 h, and samples were taken at 5 and 10 min intervals to determine phage stability. The titers of

PaVOA phages were determined using the double-layer agar plate method at a pH range from 1 to 14, with pH conditions controlled using SM buffer. Experiments were repeated thrice.

Effect of calcium and magnesium ions on phage activity

Host bacteria (PA/18) were inoculated in LB, LB-Ca, LB-Mg, and LB-Ca-Mg liquid medium (all ion concentrations were 0.1 mol/l) and then cultured in a shaking table at 37°C, 200 rpm until the OD600 was approximately 0.3. PaVOA phages were added at the optimal MOI as previously determined and cultured for 3 h. The resulting supernatants were collected by centrifugation at 12,000 rpm for 15 min at 4°C, with phage titers being determined by the double-layer agar plate method.

Phage inactivation test in New Zealand rabbits

New Zealand rabbits (3 months old) were purchased from Guangdong Provincial Medical Laboratory Animal Center (Guangzhou, China). Purified PaVOA phage samples were diluted to a concentration of 10^9 PFU/mL with normal saline. New Zealand rabbits underwent shaving and disinfection of their ears, followed by slow injections of 1 ml diluted phage preparations through the ear marginal vein. Volumes of 500 µl whole blood were collected into anticoagulation tubes at different points (5 min, 15 min, 30 min, 60 min, 3 h, 6 h, and 24 h) throughout the experiment. Whole blood samples were centrifuged at 12,000 rpm for 10 min at 4°C with the resulting supernatants being diluted in PBS. Phage titers at each time point were determined by the double-layer agar plate method to evaluate phage survival rate.

Comparison of wound healing efficiency between PaVOA phage and ceftriaxone on *Pseudomonas aeruginosa*-infected wounds in a New Zealand rabbit model

Surgical procedures for full-thickness wound models

The efficacy of phages in the treatment of traumatic skin bacterial infection was evaluated using phages and antibiotics to treat full-thickness skin excision wounds of New Zealand rabbits inoculated with *P. aeruginosa*. Twenty-five New Zealand rabbits (3 months old) weighing approximately 1.9–2.1 kg were housed in sterilized cages and provided with adequate water and rabbit food. After 7 days of adaptive feeding, New Zealand rabbits were depilated at approximately 2 cm on the left side of the spine of their back, followed by local anesthesia using 0.5% lidocaine. Surgical scissors were then used to create square allograft wounds with a side length of approximately 2 cm (Shalaby et al., 2019; Kifelew et al., 2020). Wound infections were induced by inoculation of a 10^8 CFU/ml *P. aeruginosa* bacterial solution (PA/18) by syringe, after which wounds were covered with sterile dressings and adhesive bandages. Successful replication of the previously described model should result in purulent secretion on the wound surface, mild tissue erythema and edema, and slightly elevated skin temperature.

Following the successful replication of the wound infection model, New Zealand rabbits were randomly divided into five groups: negative control (infection-free), positive control (infection), and three treatment groups (phage alone treatment, phage cocktail treatment, and ceftriaxone treatment), with five animals in each group. The administration methods for each group were as follows: negative and positive control groups were treated with 0.9% NaCl dropped onto their sterile gauzes, whilst single bacteriophage (PaVOA, 10^8 PFU/mL), bacteriophage cocktail (PaVOA and PaVOB, 10^8 PFU/mL), and ceftriaxone groups (0.002%) received 2 ml of relevant solutions onto their sterile gauzes. Sterile adhesive dressings were then used to cover and fix all gauzes, followed by 4 days of treatment. Wound healing effects were evaluated at fixed points (days 0, 3, 7, 12, 17, and 22) using a digital camera and Vernier Calipers. Wound healing (%) was calculated using the following equation:

$$\text{Wound healing}(\%) = \left[\left(\text{wound area at time point tested} - \text{wound area at day 0}\right) / \text{wound area at day 0}\right] \times 100$$

Histological examination

Tissues for histological examination were obtained from wound edges on days 0, 3, 7, and 22. The isolated tissues were fixed in a 4% formaldehyde solution and were subsequently embedded in paraffin. Fixed sections were stained with hematoxylin and eosin (H&E).

Statistical analysis

All experimental data were collected and organized using Microsoft Excel and then plotted and analyzed using GraphPad Prism 8. Data are presented as mean ± standard deviation (SD).

Results

Antibiotic sensitivity profile

The drug sensitivity results of the 22 antibiotics tested on 29 strains of *P. aeruginosa* showed that all strains were highly resistant to cotrimoxazole, amoxicillin, and lincomycin. Additionally, more than 75% of the strains were highly sensitive to amikacin, ceftriaxone, cefepime, meropenem, ofloxacin, gentamicin, imipenem, azithromycin, tetracycline, ceftazidime, mezlocillin, cefoperazone, and tobramycin. The remaining 25% showed no obvious regularity concerning antibiotic resistance. Additional data showed that the host strain PA/18 was resistant to six antibiotics and was an MDR strain.

Phage studies

Phage morphology and host range identification

TEM showed that each PaVOA virus particle had an icosahedral head with a diameter of 66 ± 1 nm and a contracted tail with a length of 120 ± 7 nm (Figure 1A), which is a typical morphological feature of the *Myoviridae* family. Additionally, the host spectrum of PaVOA was determined by the spotting method, and the cleavage rate reached 8/29, indicating that the host spectrum of PaVOA was narrow.

Phage nucleic acid type identification

The results of nuclease-mediated digestion of the phage genome revealed no bands in the DNase I treatment group, whilst the size and position of the bands in the negative control, RNase A, and the Mung Bean Nuclease treatment groups were effectively identical, indicating that the PaVOA genome is only sensitive to DNase I, thus proving that PaVOA possesses a double-stranded DNA (dsDNA) genome.

Optimal MOI and one-step growth curve

An MOI of 0.1 resulted in the largest phage lysate titer (Table 1). The results of the one-step growth curve established according to the optimal MOI of 0.1 showed an upward trend of the PaVOA titer after 10 min, which tended to plateau at 105 min (Figure 2). Therefore, the incubation period of phage PaVOA is 10 min, and the lysis period is 20 min. According to the formula shown below, the burst amount was found to be 154 PFU/cell.

Optimal phage temperature, pH, and UV stability

The results of stability tests of PaVOA showed that the phage was able to tolerate low temperatures, and the survival rate did not change significantly between −20°C and 4°C. The highest survival rate was observed at 40°C. Decreasing survival was observed at temperatures above 40°C, and most notably at 70°C (Figure 3A). The phage survival rate was greater than 60% between pH 4–11, and the survival rate was close to 100% at pH 6, indicating that PaVOA has a good acid–base tolerance (Figure 3B). Additionally, the stability of PaVOA under 20 W ultraviolet light was studied. The results showed that PaVOA was relatively stable in the first 30 min, whereas the titer decreased sharply after 30 min, but the phage did not completely lose its activity until 60 min (Figure 3C).

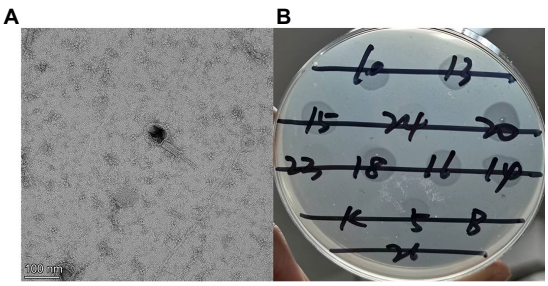

FIGURE 1
Transmission electron microscopy **(A)** and host range **(B)** of phage PaVOA.

TABLE 1 Determining the optimal multiplicity of infection of phage PaVOA.

MOI	PFU of phage PaVOA	CFU of *Pseudomonas aeruginosa* 18	Phage PaVOA titers (PFU/ml)
100	1×10^{9}	1×10^{7}	4.1×10^{8}
10	1×10^{9}	1×10^{8}	3.95×10^{8}
1	1×10^{8}	1×10^{8}	4.2×10^{8}
0.1	1×10^{7}	1×10^{8}	3.2×10^{9}
0.01	1×10^{6}	1×10^{8}	1.89×10^{9}
0.001	1×10^{5}	1×10^{8}	2.1×10^{9}
0.0001	1×10^{4}	1×10^{8}	2.23×10^{9}

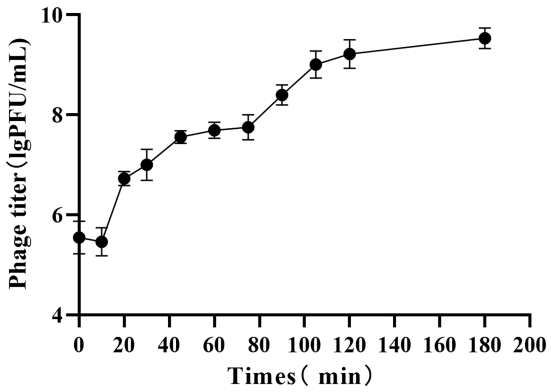

FIGURE 2
One-step growth curve of phage PaVOA.

Effect of calcium and magnesium ions on phage activity

The simultaneous addition of calcium and magnesium ions to growing PaVOA had the greatest effect on phage activity (Figure 4), which could significantly improve the titer ($p < 0.05$). The addition of magnesium ions alone could improve the titer ($p < 0.05$), whilst the addition of calcium ions alone would reduce the titer ($p < 0.05$).

Phage inactivation test in New Zealand rabbits

After the phage was injected into rabbits, phage activity decreased rapidly within 12 h. As shown in Figure 5, phage activity decreased by 25.63 ± 4.88% within 5 min, whilst 50.43 ± 9.68% of the active phage remained after 15 min. An activity of only 14.53 ± 1.38% was observed at 6 h, and no active phages in the blood were observed by 12 h. This indicated that some factors in the blood lead to the inactivation of the PaVOA.

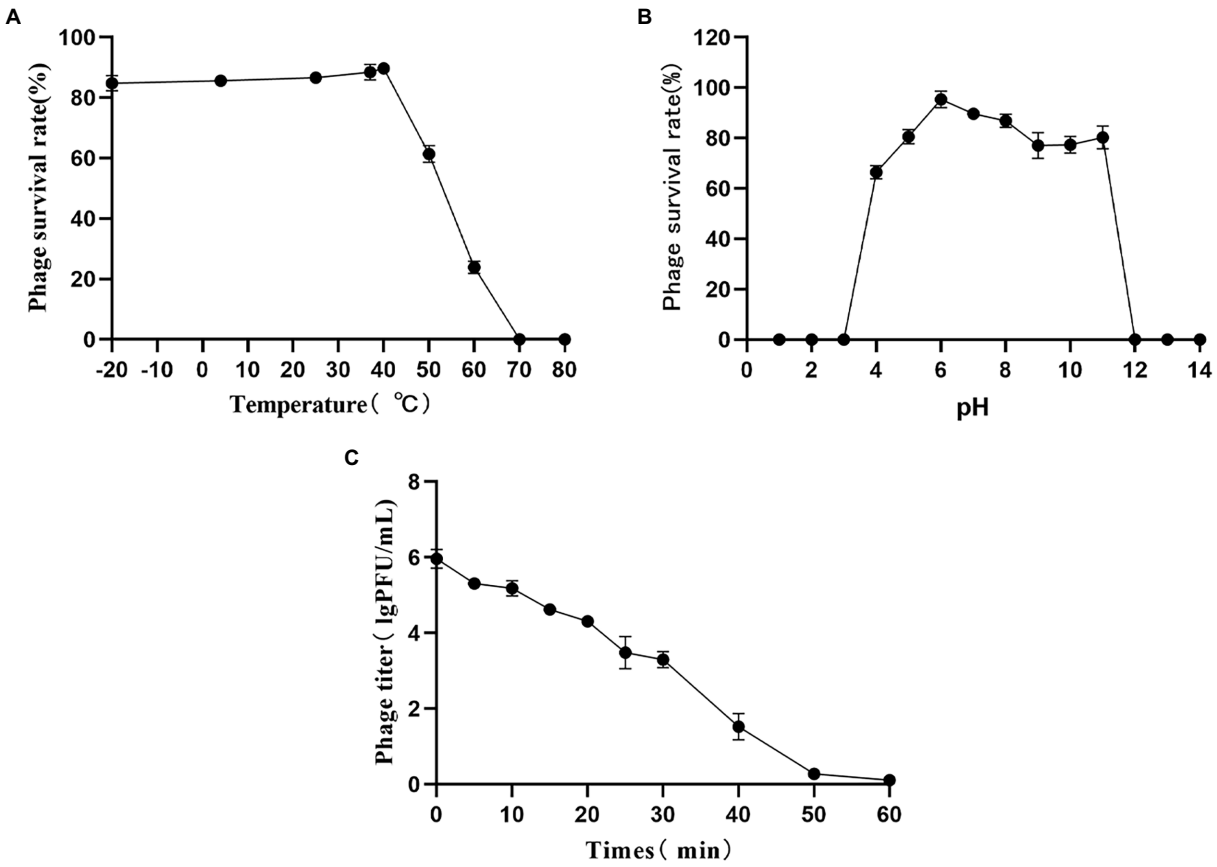

FIGURE 3
Temperature **(A)**, pH **(B)**, and UV **(C)** stability of phage PaVOA.

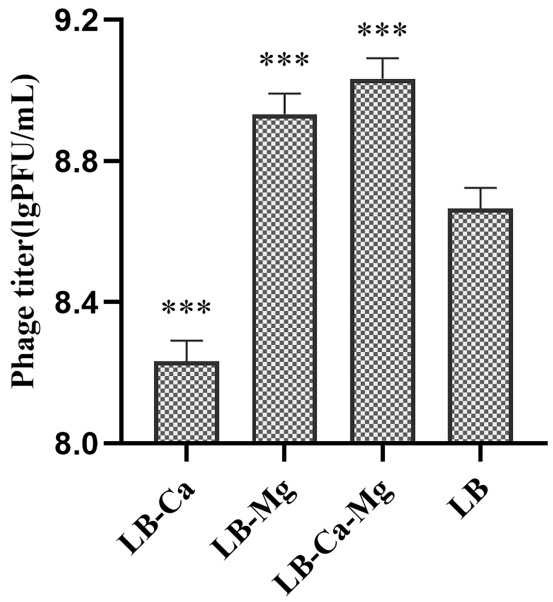

FIGURE 4
Effects of calcium and magnesium ions on the growth of PaVOA.
***$p \leq 0.001$.

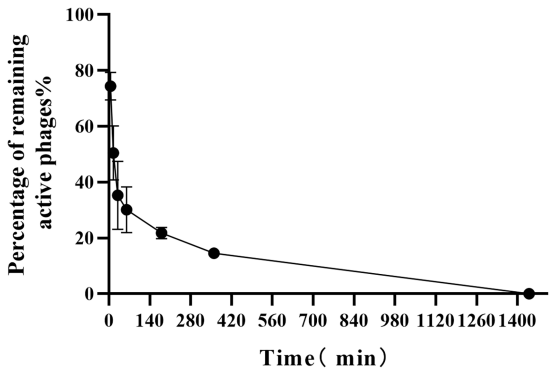

FIGURE 5
In vivo kinetics curve for PaVOA in inoculated New Zealand rabbits.

Photo documentary and wound healing analyses

The wound healing of the treatment and control groups on days 0, 3, 7, 12, 17, and 22 are shown in Figure 6A. Wound healing percentage results (Figure 6B) showed that the wounds in each group had different degrees of expansion on day 3. From

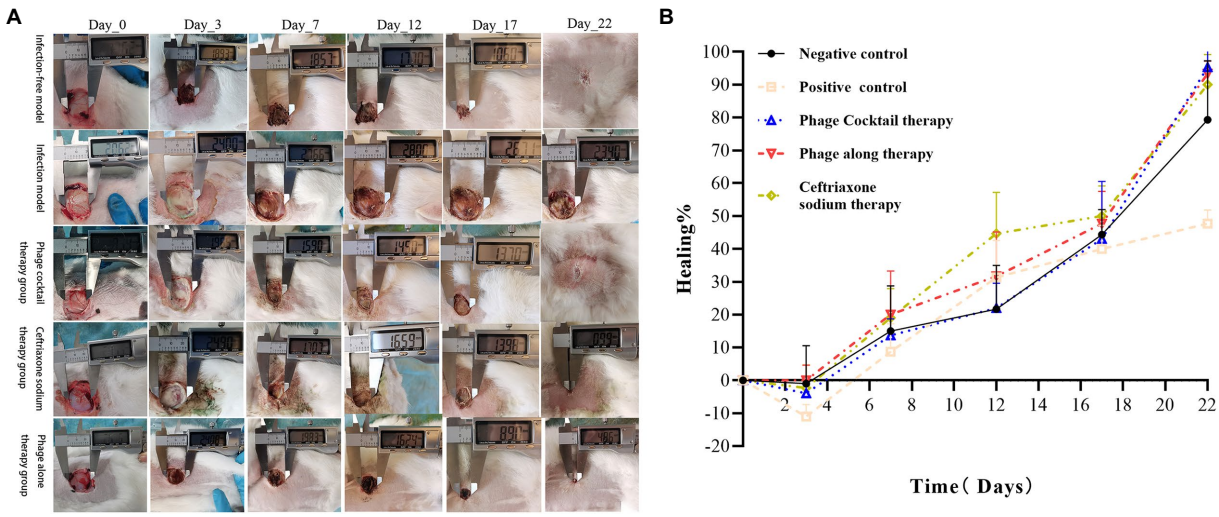

FIGURE 6
Photo documentary and wound healing. **(A)** Morphological examination of full-thickness excision wounds in New Zealand rabbits by digital photography. **(B)** Percentage of wound healing from day 0 to 22.

day 12, the wound healing rates of the three infection groups treated with phage cocktail, phage alone, and ceftriaxone were greater than that of the control, with wound healing increases of 22.0%, 31.7%, and 44.7%, respectively. On day 22, the healing rate of the phage cocktail treatment group reached 95.3%, that of the phage alone treatment group reached 93.3%, that of the ceftriaxone treatment group reached 90.0%, and that of the negative control group reached 79.3%, whilst that of the positive control group only reached 47.7%.

Histopathological analysis of wound healing

As shown in Figure 7, all five groups of New Zealand rabbits showed normal skin tissue structures on day 0, with clear epidermal, dermal, and subcutaneous tissue structures. On day 3, after damage and infection, skin sections of the five groups showed varying degrees of structural damage, with a large number of inflammatory exudates and necrotic tissue. At the end of the experiment on day 22, the skin of the negative control (no infection) group and the phage cocktail treatment group had healed completely, and the skin tissue was similar to that observed on day 0. Although the skin of the positive control group (infection without treatment) did not completely heal, skin sections showed good tissue structures. Compared with the results obtained on day 0, there were more sebaceous glands, blood vessels, and other skin appendages distributed, indicating the incomplete maturity of the healing process in the positive control group. Skin sections of the phage alone and ceftriaxone treatment groups showed obvious thickening of the epidermis, fewer skin appendages, and a large number of collagen fibers; however, these are characteristics of the remodeling stage of wound healing. Additionally, the tissue structure of skin slices was relatively clear in the phage cocktail treatment group on day 7, indicating that the wounds were in a stage of rapid healing.

Discussion

Bacterial infections are a global public health issue (Ahmed and Shimamoto, 2014; Amarillas et al., 2017), and treatment has become more complex as a result of antibiotic abuse and the emergence of MDR bacteria. Phages can accurately kill host bacteria without being limited by bacterial drug resistance and are therefore an ideal means to kill drug-resistant pathogens and pathogenic bacteria with antigenic variation. In this study, the phage PaVOA was isolated from the sewage of Nanfang Hospital in Guangzhou, China. The phage was found to have a dsDNA genome and was able to lyse 100% of the clinical isolates of *P. aeruginosa* strains and produce plaques with a diameter of 2–3 mm. According to plaque morphology and TEM image identification, it is a lytic phage belonging to the *Myoviridae* family.

Notably, phage PaVOA exhibited a narrower host spectrum as compared to phage BrSP1 (de Melo et al., 2019), the latter of which exhibited a lysis rate of 51.4% against 37 *P. aeruginosa* strains, whereas PaVOA only infected 27.6% of the tests strains. Additionally, phage PPaMa1/18 induced an 85.7% lysis of *P. aeruginosa* clinical isolates (Majdani and Shams Ghahfarokhi, 2022). When phages infect bacteria, they mainly bind to host bacterial surface receptors through interactions with the phage RBP (tail spike, tail fiber, and spike protein). As such, host ranges primarily depend on the diversity of phage RBPs. Identifying the RBP of a given phage is helpful to understand the interaction and infection mechanism between the phage and host bacteria. Furthermore, elucidating the structure of phage RBPs opens the possibility of modifying these to further expand the host range of the phage to kill more bacteria.

Studying the biological characteristics of phages will expand the implementation of phages in clinical practice. The optimal MOI of phage PaVOA in this study was 0.1, indicating that PaVOA can

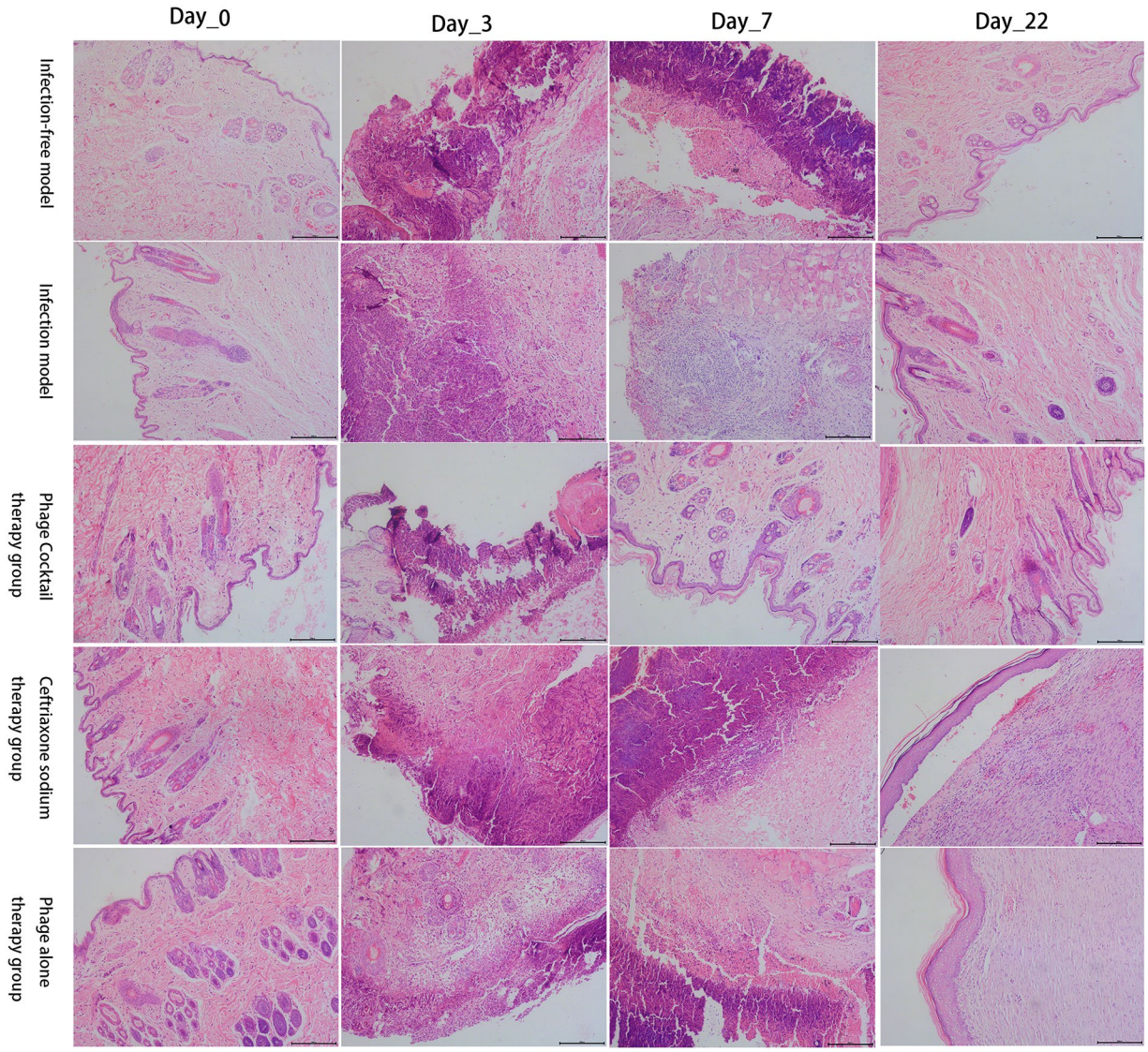

FIGURE 7
Histological analysis of the healing process of damaged skin in five groups of New Zealand rabbits. Hematoxylin and eosin (H&E) staining (×100).

infect up to 10 times more bacteria in a small order of magnitude, and can be powerful in practical antibacterial applications. The incubation and outbreak periods of phage PaVOA were 10 and 20 min, respectively. Compared to phage 2019SD1 isolated by Kumar (Kumar et al., 2021) and phage SMP isolated by Ma and Lu (Ma and Lu, 2008), the incubation and outbreak periods of phage PaVOA were shorter, indicating that the phage could infect bacteria more rapidly, thus reflecting the high efficiency of PaVOA. Moreover, during the growth phase of PaVOA, the titer can be increased by the addition of calcium and magnesium ions. It has previously been reported that metal ions play an important role in the adsorption and invasion of phages to host bacteria (Tanji et al., 2004; Moldovan et al., 2007). In this study, magnesium ions significantly promoted phage lysis of bacteria.

The adaptability of phages to their environment is crucial for their clinical application. This study identified that PaVOA activity is relatively stable within the temperature range of −20 to 60°C, with an optimal temperature of 40°C. Overall, these results are conducive to the survival of PaVOA in the environment and clinical setting. PaVOA is more than 60% active in the range of pH 4 to pH 11 and can survive in most acid–base environments. Furthermore, phage PaVOA remained stable for 30 min when exposed to a UV light of 20 W, and was not completely inactivated until after 60 min. These results are similar to results reported by Wang for the stability of phage SLPW (Wang et al., 2016). Overall, measurements of survival at a range of temperatures, pH, and ultraviolet light showed that PaVOA has a strong tolerance to low temperature, extreme pH, and high radiation conditions, which are advantageous characteristics for the clinical application of phages.

In this study, the inactivation of PaVOA in the blood was tested using a New Zealand rabbit model. Phage kinetics showed that at 15 min, the remaining percentage of active phages was

50.43 ± 9.68%. Notably, the rate of decline was fast, and the phage could not be detected in the blood at 12 h. Studies have shown that circulating B cells play an important role in phage inactivation, most likely due to B cells reacting with host immune factors to produce immunoglobulin, resulting in the loss of phage activity (Srivastava et al., 2004). Although this previous study used mice for *in vivo* experiments, we suggest that as both mice and New Zealand rabbits are mammalian species, the overall conclusions from their previous study may also apply to this study. Additionally, phage activity is related to the administration route and phage type, and previous studies reported high anti-phage activity following topical administration, whilst this was less so following oral administration (Lusiak-Szelachowska et al., 2014). In this study, phage inactivation and the absence of animal deaths during local phage treatment also suggested the safety of phage therapy in animals.

Bacterial resistance is constantly increasing, with MDR infections having already caused serious harm to humans. As such, phage therapy is being actively pursued as a substitute for antibiotic therapies in cases involving MDR bacteria. This study selected a strain of MDR *P. aeruginosa* (PA/18) that is resistant to six antibiotics to establish a New Zealand rabbit skin infection model for the comparison of phage (single or combined) and antibiotic (ceftriaxone) therapies for MDR wound infections. Overall, rabbits treated with ceftriaxone exhibited poorer wound healing than those treated with phages. Notably, treatment with a combined phage cocktail resulted in the greatest degree of wound healing. A previous study confirmed that phage cocktail treatment is effective against antibiotic-resistant *Staphylococcus aureus* diabetic foot ulcer infections (Kifelew et al., 2020). It is reported that phage cocktail therapy leads to improved efficacy, with one study suggesting that phage cocktails were able to lyse 86.7% of clinical isolates and that they had synergistic effects when used in combination with antibiotics (Malik et al., 2021). In addition, previous studies have shown that phages, which kill bacteria with minimal side effects (Chadha et al., 2016), may be more effective than antibiotic treatments in wound healing, which is consistent with the findings of this study. Topically applied bacteriophage has been reported to inhibit bacterial biofilm formation and improve wound healing (Mendes et al., 2013; Forti et al., 2018; Yang et al., 2021), which is an advantage of bacteriophage therapy. Regarding histopathological results, accelerated skin healing and remodeling were observed in the phage cocktail treatment group, which was equivalent to the wound healing in the negative control group. Skin regeneration, an indicator for assessing the efficacy of wound treatment, re-epithelization is critical to successful wound healing (Safferling et al., 2013; Rezk et al., 2022). All New Zealand rabbits treated with the phage cocktail in this study exhibited epidermal re-epithelialization and regeneration of skin appendages, similar to New Zealand rabbits before wound infection. In contrast, ceftriaxone-treated New Zealand rabbits had incomplete epidermal formation and lacked skin appendages, similar to the findings of (Rezk et al., 2022). Based on photographic records of wound healing and histopathological analysis, this study demonstrates the potential of phages to treat drug-resistant infections. Additionally, the healing rate in the phage cocktail treatment group was greater than that in the other two treatment groups, which may be attributed to the ability of the phage cocktail to reduce contamination by non-host bacteria (Hooton et al., 2011). In addition, special materials can be selected to carry phage preparations; for instance, using phages embedded in gels to treat infected wounds (Fayez et al., 2021).

Conclusion

In conclusion, the phage PaVOA discovered in this study belongs to the *Myoviridae* family, is resistant to low temperature, has good tolerance to acid–base and ultraviolet conditions, and is suitable for the environment in clinical treatment. In addition, PaVOA is quickly inactivated in the blood and does not cause animal death. A New Zealand rabbit model of MDR *P. aeruginosa* infection in skin wounds was established, and phage treatment was found to be superior to ceftriaxone treatment. Thus, the phage PaVOA has the potential to treat traumatic skin infections caused by *P. aeruginosa* and may be used as a substitute for antibiotics. With the discovery of new phages, phage cocktail therapy will enter a stage of rapid development for the treatment of MDR bacterial infections.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Ethics Committee of South China Agricultural University.

Author contributions

JinyW performed all the experiments and wrote the manuscript. WM, KZ, JingW, BL, and RW participated in the animal experiments. KJ revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1031101/full#supplementary-material

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Difference analysis of intestinal microbiota and metabolites in piglets of different breeds exposed to porcine epidemic diarrhea virus infection

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The gut microbial composition of the Luchuan (LC) piglet, one of China's native breeds, has rarely been studied, especially when compared to other breeds. This study developed a porcine epidemic diarrhea virus (PEDV) infection model in LC and Largewhite (LW) piglets, and analyzed the patterns and differences of intestinal microbial communities and metabolites in piglets of these two breeds after infection. The diarrhea score, survival time, and distribution of viral antigens in the intestine of piglets infected with PEDV differed among breeds, with the jejunal immunohistochemistry score of LW piglets being significantly higher than that of LC piglets ($P < 0.001$). The results of 16S rRNA sequencing showed differences in microbial diversity and community composition in the intestine of piglets with different breeds between PEDV infection piglets and the healthy controls. There were differences in the species and number of dominant phyla and dominant genera in the same intestinal segment. The relative abundance of *Shigella* in the jejunum of LC piglets after PEDV infection was significantly lower than that of LW piglets ($P < 0.05$). The key microorganisms differed in the microbiota were *Streptococcus alactolyticus*, *Roseburia faecis*, *Lactobacillus iners*, *Streptococcus equi,* and *Lactobacillus mucosae* ($P < 0.05$). The non-targeted metabolite analysis revealed that intestinal metabolites showed great differences among the different breeds related to infection. Spearman correlation analysis was conducted to examine any links between the microbiota and metabolites. The metabolites in the intestine of different breeds related to infection were mainly involved in arginine biosynthesis, synaptic vesicle cycle, nicotinic acid and nicotinamide metabolism and mTOR signaling pathway, with significantly positive or negative correlations ($P < 0.05$) between the various microorganisms. This

study provides a theoretical foundation for investigating the application of core microorganisms in the gut of piglets of different breeds in the digestive tracts of those infected with PEDV, and helps to tackle the antimicrobial resistance problem further.

KEYWORDS

microbiota, metabolites, porcine epidemic diarrhea, Luchuan pig, Largewhite pig

Introduction

Diarrhea has long been a worldwide issue in piglet farming. Piglet diarrhea is caused by various factors, including nutrition and infectious agents. Porcine epidemic diarrhea virus (PEDV) is a leading cause of intestinal damage in piglets, causing watery diarrhea, vomiting, dehydration, and even death (Li et al., 2012; Sun et al., 2012; Song et al., 2015). The relationships between intestinal microbial dysbiosis and diseases have recently attracted the public's interest. A growing body of research suggests that bacteria significantly influence gut barrier integrity (Mazmanian et al., 2005; Kamada et al., 2012; Manichanh et al., 2012). The intestinal microbiota of piglets is a complex system that constantly changes. Recent studies have linked intestinal barrier damage in diarrhea piglets to gut microbiota disorders (Kongsted et al., 2013; Hermann-Bank et al., 2015).

There were significant differences in the number and species of intestinal microbiota in diarrhea piglets compared to healthy piglets. In PEDV-infected piglets, the abundance of *Fusobacterium* and *Veillonella* increased in the intestinal microbiota (Koh et al., 2015; Liu et al., 2015; Huang et al., 2019). A recent study about Landrace-Yorkshire piglets infected with PEDV reported that the abundance of *Escherichia-shigella* was higher in infected piglets than in uninfected piglets, whereas the abundance of *Lactobacillus* was lower in the infected piglets (Dong et al., 2021). Another earlier study on gut microbiota in Duroc × Landrace × Large White piglets infected with PEDV showed that infection decreased the abundance of Shigella and increased the abundance of *Lactobacillus* (Wu et al., 2020). Scholars have provided a great deal of research regarding the relationship between intestinal microbiota and different diseases in recent years (Lindberg, 2014; H. Yang et al., 2018). Diet and environment also have a noticeable effect on the composition of swine intestinal microbiota (Haenen et al., 2013; Gresse et al., 2017). Additionally, differences in the types of intestinal microorganisms may exist between swine breeds or geographical locations (L. Yang et al., 2014; Cheng et al., 2018; Xiao et al., 2018).

Many studies have shown that certain microbiota members in mammal digestive tracts can help treat gastrointestinal problems. Others have used these members to treat inflammatory bowel disease, irritable bowel syndrome, and other diseases that can reduce the need for antibiotics and other medications (Amit-Romach et al., 2008; Gkouskou et al., 2014; Tomasello, 2014; Imperatore et al., 2017). Early weaning of piglets could significantly shorten the animal husbandry cycle in current livestock. Nonetheless, its stress frequently results in diarrhea, and newly introduced commercial piglets are more susceptible to intestinal stress than indigenous breeds. Increases in morbidity, mortality and economic cost can result from early weaning (Gresse et al., 2017; Wang et al., 2019).

The Luchuan (LC) pig is a Chinese domestic breed with small body size, good maternal and reproductive performance, and high-quality meat (Ran et al., 2014). Since antibiotics have been explicitly banned in the EU livestock industry, finding alternatives to antibiotic treatment was essential to address intestinal stress in piglets. Therefore, this study investigated the changes of intestinal microorganisms and metabolites in LC and Largewhite (LW) piglets related to PEDV artificial infection. Meanwhile, microorganisms and metabolites related to infection in different breeds of piglets were screened, and correlations were searched to help find suitable probiotics that would provide treatments for piglet diarrhea caused by PEDV.

Materials and methods

Experimental design

Two sows (*Sus scrofa domestica*) with the same farrowing date were chosen at a small pig farm in Guangxi province. One was a LW sow, while the other was a LC sow, a local breed unique to Guangxi province. On farrowing day, 12 piglets with an initial average body weight of 1.28 ± 0.03 kg were chosen at random, six for each group, and gender was balanced in the various groups. The umbilical cord of each piglet was trimmed and the navel disinfected with iodine. The piglets did not consume any colostrum or antibiotics and after determining good health were transferred to an experimental animal house where they were allocated into groups according to breed differences. The 12 piglets were divided into four groups ($n = 3$): LW piglets challenged with PEDV (PEDV LW group), LC piglets challenged with PEDV (PEDV LC group), LW piglets control (Control LW group), and LC piglets control (Control LC group).

TABLE 1 Primers for porcine epidemic diarrhea virus (PEDV) detection.

Primer	Sequence (5′-3′)	Primer length (bp)
PEDV-F	CGTACAGGTAAGTCAATTAC	20
PEDV-R	GATGAAGCATTGACTGAA	18
PEDV-Pro	FAM-TTCGTCACAGTCGCCAAGG-BHQ1	19

Three-day old piglets were gavaged with 2 ml of PEDV at a titer of 10^3 $TCID_{50}$. PEDV strain JS-13 used in this work was provided by Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The virulence of the PEDV strain was evaluated in the preliminary intracellular growth assay. The dose of challenge estimates for animal experiments were based on prior animal vivo assays within the laboratory for determination of the optimum infective dose. The control LW and control LC groups were left unchallenged. Clinical signs such as diarrhea score and fecal shedding were monitored daily following the challenge. Before and after the challenge, feces swabs were collected from each pig at 12 h before the challenge and 12, 24, 44, 63, 84, and 96 h after the challenge until the end of the study. All feces swabs were stored in centrifuge tubes and frozen immediately on ice until transferred to −80°C storage. After the last piglet in the PEDV group died, six piglets in the control group were euthanized.

Determination of viral loads by real-time quantitative polymerase chain reaction

Real-time polymerase chain reaction (PCR) primers and probes were designed using Primer 5 software to amplify the M gene from PEDV (Table 1). Primers were used to amplify the M fragment using synthesized cDNA extracted from PEDV positive materials as a template. The amplified products were separated using agarose gel electrophoresis, and the target fragments were cut out and purified under UV light. The purified product was ligated with pGEM-T for 2 h at 16°C before being transformed into *E. coli* DH5α competent cells. A single colony was chosen from the plate and placed in LB broth medium. After overnight incubation (37°C for 4 h), plasmids were extracted from positive clones using Thermo Scientific GeneJET Plasmid Miniprep Kit, identified by PCR, and sequenced.

A spectrophotometer was used to determine the concentration and purity of the extracted plasmid and the gene copy number. A standard plasmid was used as a template, a 10-fold gradient dilution was performed, and three repeats of each plasmid concentration were made. Simultaneously, fecal samples and a negative control were run, and the reaction was carried out on a Roche fluorescence quantitative PCR. The reaction system was 20 μL, including ddH_2O 7.6 μL, 2 × Ace qPCR Probe Master Mix10 μL, primer PEDV-F 0.4 μL, primer PEDV-R 0.4 μL, probe 0.2 μL, 50 × ROX Reference Dye1 0.4 μL, template 1 μL. The reaction conditions were as follows: 95°C, 5 min, 95°C, 10 s, 60°C, 30 s, 40 cycles. The fluorescence signal was collected during extension, and the kinetic and standard curves were obtained.

Histological observation and immunohistochemistry

Following inoculation, piglets from the control group were sacrificed on day 7, at which point all piglets in the infected groups had died of diarrhea. Their intestines were quickly removed after they died. Intestinal sections from the middle jejunum and cecum were collected and immediately fixed in 10% buffered formalin for 24 h. The tissues were removed from the formalin solution and dehydrated in graded alcohol solutions before being embedded in paraffin. The embedded-paraffin tissue samples were then cut into 4 μm sections and stained with hematoxylin and eosin (H&E). Immunohistochemistry (IHC) staining was performed with monoclonal antibody against PEDV-N protein on these samples as described previously (Curry et al., 2017a). The villus height (VH) and crypt depth (CD) of jejunum were performed using Image J software at × 100 magnification.

DNA extraction and 16S rRNA amplicon sequencing

Jejunal and cecal content samples used in microbiota analysis came from 12 piglets, as shown in Table 2. Total DNA was extracted from the jejunal and cecal contents in sterile centrifuge tubes using QIAmp DNA kit (Qiagen, Germany) according to the manufacturer's instructions. The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified using the primers 338F-ACTCCTACGGGAGGCAGCAG and 806R-GGACTACHVGGGTWTCTAAT. According to the manufacturer's protocol, the PCR products were confirmed on a 2% agarose gel, purified using an AxyPrep DNA Gel Extraction Kit AP-GX-250 (Axygen Biosciences, Union City, CA, United States), and quantified using QuantiFluorTM-ST (Promega, Madison, WI, United States). PCR-purified amplicons were pooled in equimolar amounts and paired-end sequenced (2 × 300 bp) on the Illumina NovaSeq platform, according to standard protocols from Shanghai Personal Biotechnology Co., Ltd., (Shanghai, China) (Illumina, San Diego, CA, United States). This study's 16S rRNA gene sequence data were deposited in the

TABLE 2 The information of samples collected for microbiota analysis.

The group name of the samples	Sample type	Number
Con LC J	Jejunal content	3
Con LW J	Jejunal content	3
PEDV LC J	Jejunal content	3
PEDV LW J	Jejunal content	3
Con LC C	Cecal content	3
Con LW C	Cecal content	3
PEDV LC C	Cecal content	3
PEDV LW C	Cecal content	3

TABLE 3 The information of samples collected for microbiota analysis.

The group name of the samples	Sample type	Number
Con LC J	Jejunal tissue	3
Con LW J	Jejunal tissue	3
PEDV LC J	Jejunal tissue	3
PEDV LW J	Jejunal tissue	3
Con LC C	Cecal tissue	3
Con LW C	Cecal tissue	3
PEDV LC C	Cecal tissue	3
PEDV LW C	Cecal tissue	3

GenBank Sequence Read Archive database under SRA: SRP346825.

Untargeted metabolomic analysis

After dissection, the jejunal and cecal tissues (Table 3) were quickly frozen in liquid nitrogen. The tissue was then cut on dry ice (10 mg) and placed in a microcentrifuge tube (2 mL). The tissue samples with 200 μL of sterile H_2O were homogenized using the Benchmark Beadbug homogenizer (Benchmark Scientific, United States). 800 μL of methanol/acetonitrile (1:1, v/v) was added to the homogenized solution for metabolite extraction. The mixture was centrifuged for 15 min (4°C, 14,000 × *g*). The supernatant was dried in a vacuum centrifuge. The samples were re-dissolved in 100 μL of acetonitrile/water (1:1, v/v) solution for LC-MS analysis. Analyses were performed using a UHPLC (1290 Infinity LC, Agilent Technologies) coupled to a quadrupole time-of-flight (AB Sciex TripleTOF 6600) at Shanghai Applied Protein Technology Co., Ltd.

Statistical analysis

T-tests and one-way analyses of SPSS software 19.0 were used to compare between-group differences and groups (SPSS, Inc.). $^{**}P < 0.01$ or $\Delta\Delta P < 0.01$ was considered extremely significant, while $^{*}P < 0.05$ or $\Delta P < 0.05$ was considered significant.

TABLE 4 Fecal grading.

Morphology of feces	Score
Normal and shaped feces	1
Pasty feces and not shaped	2
Semi-liquid diarrhea with some solid content	3
Liquid diarrhea with no solid content	4

Results

Differences in clinical indicators after porcine epidemic diarrhea virus infection in piglets

Within 12 h of receiving PEDV orally, some piglets in the infected group had feces with a watery appearance and a fishy odor. Some even displayed symptoms, such as vomiting and difficulty breathing. The first piglet that showed symptoms, which belonged to the PEDV LW group, died within 24 h of infection. Then, within 24∼96 h, both infected piglets in the PEDV LC and PEDV LW groups died. Each piglet was scored and averaged daily by observing fecal morphology, color, and bleeding (Table 4). The results showed that the piglets in the PEDV LC group had lower diarrhea scores and survived longer than the piglets in the PEDV LW group (Figures 1A,B). The plasmid standards amplification plot of Ct vs. log concentration was used to generate a standard curve (slope = −3.2782). After fitting the data to the standard curve, the fecal viral load values measured in the infected groups were shown in Table 5. The fecal gene copy number content was lower in the PEDV LC group than in the PEDV LW group. However, the difference was insignificant.

Histological and pathological differences in piglets infected with porcine epidemic diarrhea virus

Piglets from the two infected groups had different degrees of partial swelling of the small intestine, transparency of tissue of the intestinal wall, narrowing of the intestinal lumen, and individual blood spots after their abdominal cavities were dissected for visual inspection (Figure 1C). Tissue sections stained with H&E revealed that all piglets in the infected group had intestinal histopathological changes. The small intestinal villi in the jejunal section were damaged and lysed, and the cytoplasm appeared vacuolated. The jejunum epithelial cells were necrotic and detached, showing evidence of cell

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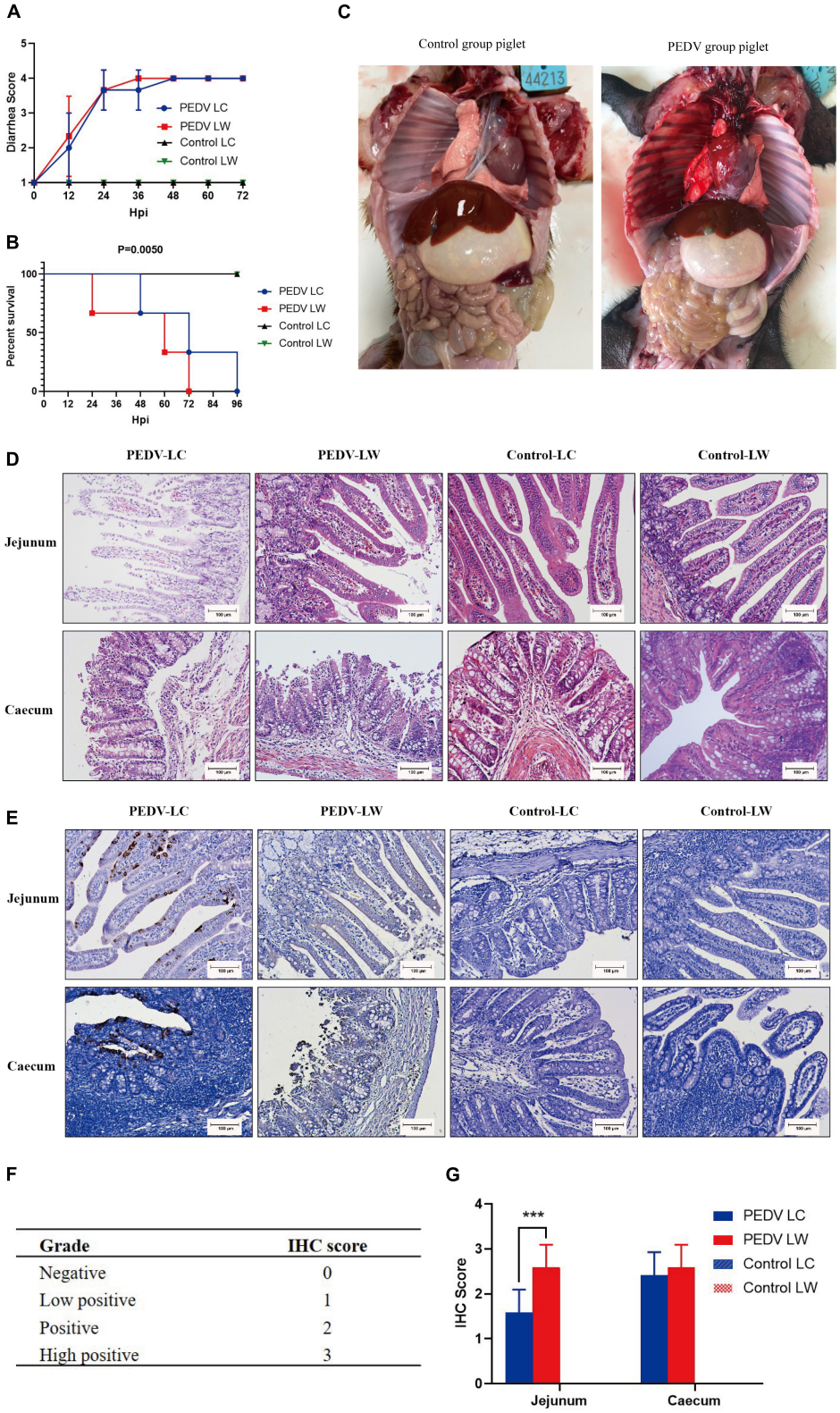

Grade	IHC score
Negative	0
Low positive	1
Positive	2
High positive	3

FIGURE 1
(A) Diarrhea score of piglets. **(B)** Survival time of piglets. **(C)** Piglet autopsy lesions. **(D)** Intestinal H&E staining (100 μm). **(E)** Immunohistochemical staining (100 μm). **(F)** Immunohistochemistry scoring criteria. **(G)** Immunohistochemistry score.

necrosis in the lamina propria. Furthermore, the cecal lesions were characterized primarily by dilated blood vessels, the indistinguishable structure of the large intestinal glands, and the disintegration and shedding of many intestinal gland cells. There were also a few lymphocytes in the cecum submucosal layer (Figure 1D). Piglets from infected groups had decreased VH to CD ratio in the jejunum ($P < 0.05$), lower VH, and tented to have decreased CD compared with the control group (Table 6).

The distribution of PEDV in the intestine was characterized by immunohistochemical staining, which showed the presence of viral antigens in both the jejunum and cecum of the experimental group (Figure 1E). Viral antigens were mainly present in the cytoplasm and lamina propria of the jejunal villous epithelium. A small number of antigens were also present in the intestinal glands of the cecum. Immunohistochemical scoring of intestinal tissue sections using Image J software according to the scoring criteria in Figure 1F showed differences in the distribution of PEDV N protein antigens in the jejunum and cecum between the PEDV LC and PEDV LW groups (Figure 1G). Also, there were significantly higher immunohistochemical scores in the jejunum of the PEDV LW group than in the PEDV LC group ($P < 0.001$). Furthermore, the PEDV LW group had more severe intestinal histopathology than the PEDV LC group and contained more antigens, as evidenced by H&E and IHC scores.

Characterization of the intestinal microbiota composition of piglets after exposure to porcine epidemic diarrhea virus

After filtering and denoising, 1,712,473 sequence reads remained out of 1,934,068 raw sequences obtained from 24 samples (including 12 jejunal and 12 cecal content samples). On average, the sequence reads were 427 bp (16–441 bp). The average number of OTUs identified after species-specific taxonomic annotations were 499 OTUs, including 232 phyla, 81 classes, 154 orders, 850 families, 2,145 genera, 3,125 species and one unclassified species.

The alpha diversity results revealed a significant difference between the PEDV LC and PEDV LW groups using the Chao1, Faith's PD, Observed species, Shannon and Simpson index in the jejunum and cecum (Figures 2A,B). Compared to the control LC group, the Chao1 and Observed species index in the cecum showed a statistically significant decrease in the control LW group (Figure 2B). The results of the flattened sparse curve show that the number of samples required for 16S rRNA analysis was achieved (Figure 2C).

According to the Venn diagram, PEDV infection decreased the number of OTUs in the jejunum and cecum of piglets from 4,724 to 3,241. Interestingly, the number of species-specific OTUs in the intestines of LC piglets was always higher than that of LW piglets whether infected with PEDV or not (Figure 2D). Furthermore, the differences in microbial community composition between the two breeds were investigated using principal coordinate analysis (PCoA) based on the Bray-Curtis metric. Overall, the LC and LW piglet groups were split into two distinct communities clustered together (Figure 2E).

Screening for species with intestinal differences in piglets of different breeds exposed to porcine epidemic diarrhea virus

Differences in species composition between groups at the phylum and genus levels were described. Firmicutes and Proteobacteria were the most abundant phyla in the jejunal and cecal communities, followed by Bacteroidetes, Actinobacteria, and Fusobacteria (Figure 3A). PEDV infection caused a decrease in the ratio of Firmicutes to Proteobacteria in the jejunum and cecum, with the percentage of Firmicutes to Proteobacteria in the jejunum of the control LC group being significantly higher than in the other groups (Figure 3B). At the genus level, the abundance of *Lactobacillus* in the jejunum and cecum showed a decreasing trend after infection with PEDV, with the abundance of *Shigella* and *Streptococcus* showed an increasing trend. Notably, the relative abundance of Lactobacillus was consistently higher in LC piglets than in LW piglets whether infected with PEDV or not. In comparison, the relative abundance of *Shigella* was consistently lower in LC piglets than in LW piglets (Figure 3C). Furthermore, Welch's *t*-test results showed that the relative abundance of *Shigella* was significantly higher in the PEDV LW group than in the PEDV LC group (Figure 3D).

Linear discriminant Effect Size (LEfSe) analysis with LDA scores > 2.0 was used to screen the bacteria further to identify microbial differences above the genus level exposed to PEDV infection (Figure 3E). *Gemellales*, *Synergistales*, and *Roseburia* were significantly different between groups (LDA > 2, $P < 0.05$). *Faecalibacterium* was significantly enriched in the cecum of the Con_LC group (LDA > 2, $P < 0.05$). Different microorganisms between subgroups were compared two-by-two and identified species levels using Metastats analysis (Figure 3F). A total of five significantly different species were obtained after performing the Welch's *t*-test, *Streptococcus alactolyticus*, *Roseburia faecis*, *Lactobacillus iners*, *Streptococcus equi,* and *Lactobacillus mucosae* ($P < 0.05$). *Lactobacillus iners* was the dominant bacterium significantly enriched in the cecum of pre-infected piglets (Con LC group). In contrast, the remaining four different species were significantly increased in the jejunum and cecum of PEDV-infected piglets ($P < 0.05$).

TABLE 5 Mortality and viral load of porcine epidemic diarrhea virus in feces.

Hours post infection (Hpi)	Number mortality		Mean log (genomics copies/μL)	
	PEDV LC	PEDV LW	PEDV LC	PEDV LW
12	0	0	4.585	5.278
24	0	1	5.000	5.045
44	0	1	5.020	5.127
63	2	2	5.015	4.841
84	2	3	5.027	–
96	2	3	4.809	–

TABLE 6 Effects of porcine epidemic diarrhea virus on jejunal morphology of piglets.

Items	Con LW	Con LC	PEDV LW	PEDV LC	*P*-value
Villus height, μm	1229.299	926.0753	455.7345	479.8395	0.0265
Crypt depth, μm	319.7322	208.9695	154.1545	117.6867	0.02
Villus height/Crypt depth	3.844778	4.590073	3.093606	4.354744	0.0012

Metabolite changes caused by exposure to porcine epidemic diarrhea virus

To better understand the metabolite changes in the intestine of piglets of different breeds exposed to PEDV infection, all metabolites in the samples were identified by the UHPLC-MS platform. The results showed that 552 metabolites were identified, with 13 main categories according to their chemical classification. The top three categories were organic acids and derivatives (17.21%), lipids and lipid-like molecules (10.688%) and organic oxygen compounds (9.058%) (**Figure 4A**).

Supervised multivariate analyses were performed to show differences between groups by using principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). PCA plots showed significant separation between sample groups and overlap of QC samples (**Supplementary Figure 1A**). OPLS-DA score plots showed substantial differences in intestinal metabolic profiles of piglets exposed to PEDV infection, and significant separation of intestinal metabolites occurred between LC and LW piglets with or without PEDV infection (**Supplementary Figure 1B**). The evaluation parameters R^2Y and Q^2 of the permutation test showed good stability in all OPLS-DA models (**Supplementary Figure 1C**).

The OPLS-DA model's Variable Importance for Projection (VIP) values were used to identify the metabolites that contributed significantly to the model. As a result, 101 different metabolites were identified in jejunal and cecal samples using the OPLS-DA VIP $>$ 1 and $P < 0.05$ criteria. The differential metabolite expression between groups was calculated using differential multiplicity analysis and visualized with histograms, and fold change (FC) values (FC $>$ 1 for upward adjustment, FC $<$ 1 for downward adjustment) that could be used as criteria for metabolite up-and down-regulation (**Figure 4B**). Of the 70 different metabolites identified in the jejunal samples, 28 were up-regulated, and 42 were down-regulated (**Supplementary Table 1**). 19 of the 31 different metabolites were significantly up-regulated, and 12 were significantly down-regulated in the cecum samples (**Supplementary Table 2**). All the different metabolites were matched against the KEGG database to obtain information on higher metabolite enrichment pathways (**Figures 5A,B**). In the jejunal content samples, certain metabolites were mainly associated with arginine and proline metabolism, arginine biosynthesis, mineral absorption, and vitamin B6 metabolism (**Table 7**, $P < 0.05$). Certain metabolites in cecum content samples were mainly enriched in the synaptic vesicle cycle, nicotinic acid and nicotinamide metabolism, and the mTOR signaling pathway (**Table 8**, $P < 0.05$).

Correlation between intestinal microbiota and metabolites that differ significantly

Spearman correlation analysis revealed a significant association between different intestinal microbiota and different metabolites in breeds of piglets infected with the PEDV or not (**Figure 5**). *S. alactolyticus* was negatively correlated with Pyridoxine, 1-Oleoyl-sn-glycero-3-phosphocholine, Creatinine, 1-Palmitoyllysophosphatidylcholine, 16-Hydroxypalmitic acid, N-Acetyl-DL-methionine, Phe-Gly and Oxyquinoline while positively correlated with DL-2-Phosphoglycerate, Taurine, gamma-L-Glutamyl-L-glutamic acid, L-Citrulline, 2-Oleoyl-1-stearoyl-sn-glycero-3-phosphoserine and Gly-Glu.

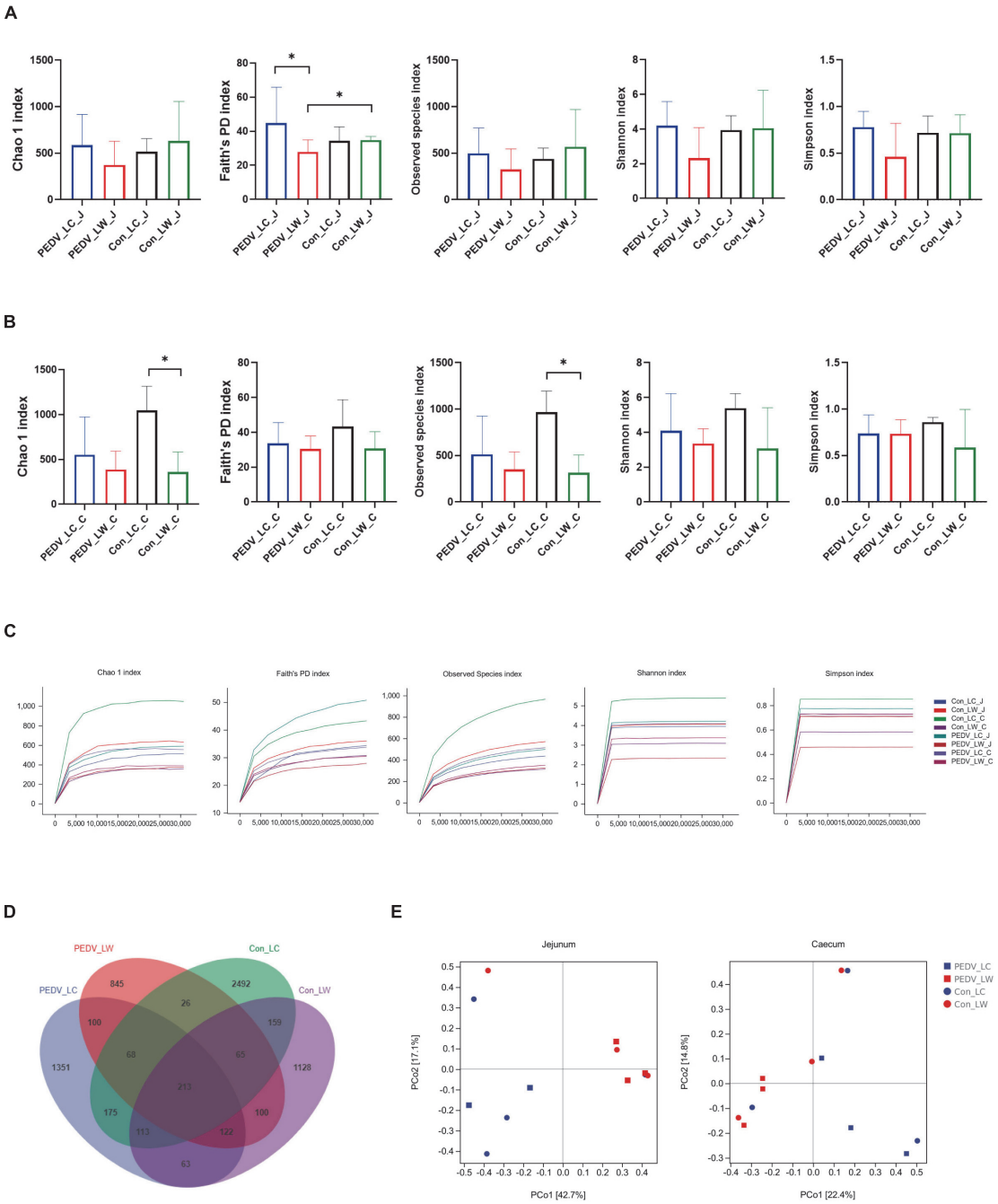

FIGURE 2
(A) Microbial alpha diversity in the jejunum. **(B)** Microbial alpha diversity in the cecum. **(C)** Rarefaction curves of alpha diversity. **(D)** OTU venn diagram. **(E)** Principal coordinate analysis (PCoA) of intestinal microorganisms in piglets exposed to porcine epidemic diarrhea virus (PEDV) infection.

R. faecis was negatively correlated with L-Pyroglutamic acid and 1-Aminocyclopropanecarboxylic acid while positively correlated with N1-Methyl-2-pyridone-5-carboxamide, Indoxyl sulfate, L-Carnosine and Urea. *S. equi* was positively correlated with L-Gulonic gamma-lactone, while *L. mucosae* was positively correlated with 1-Methylnicotinamide.

Discussion

Characteristics of intestinal microbiota in pigs of various breeds were different in terms of features and composition. In a study completed recently, scientists found that the beta diversity of gut microbes was different

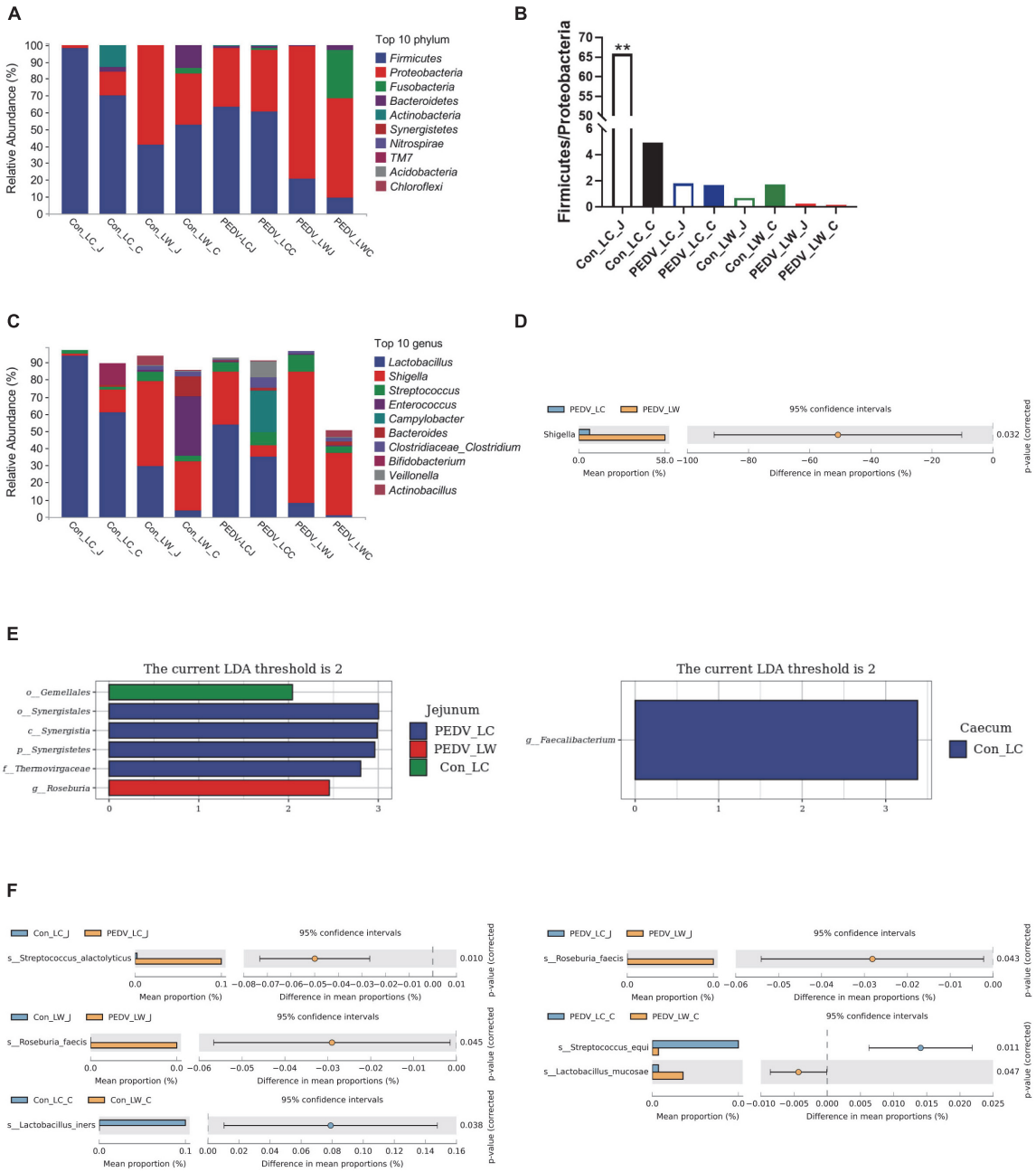

FIGURE 3
(A) Phylum-level species composition. **(B)** Ratio of firmicutes and proteobacteria. **(C)** Genus-level species composition. **(D)** Differences between groups at the genus level. **(E)** LDA values of linear discriminant effect size (LEfSe) analysis. **(F)** Intergroup differences at the species level.

between Jinhua (Chinese panda pigs) and commercial pigs (Duroc × Landrace × Yorkshire) (Curry et al., 2017b). Another recent study discovered similar differences in the intestinal microbiota composition between Duroc and Iberian pigs (López-García et al., 2021). The study of native Chinese pigs is ongoing. However, there are no reports on the characteristics of intestinal microbiota and metabolites in LC pigs. Some studies have reported that PEDV infection modified porcine gut microbial composition (Tan et al., 2019). Therefore, this study first compared the intestinal microbiota and gut metabolites between PEDV-infected and uninfected LC and LW pigs to understand their compositions and relationships further.

This study showed symptoms consistent with acute PEDV infection, such as lethargy and diarrhea, in as little as 6 h. After a while, the symptoms worsened with vomiting, dehydration and watery diarrhea. The first piglets died within 24 h,

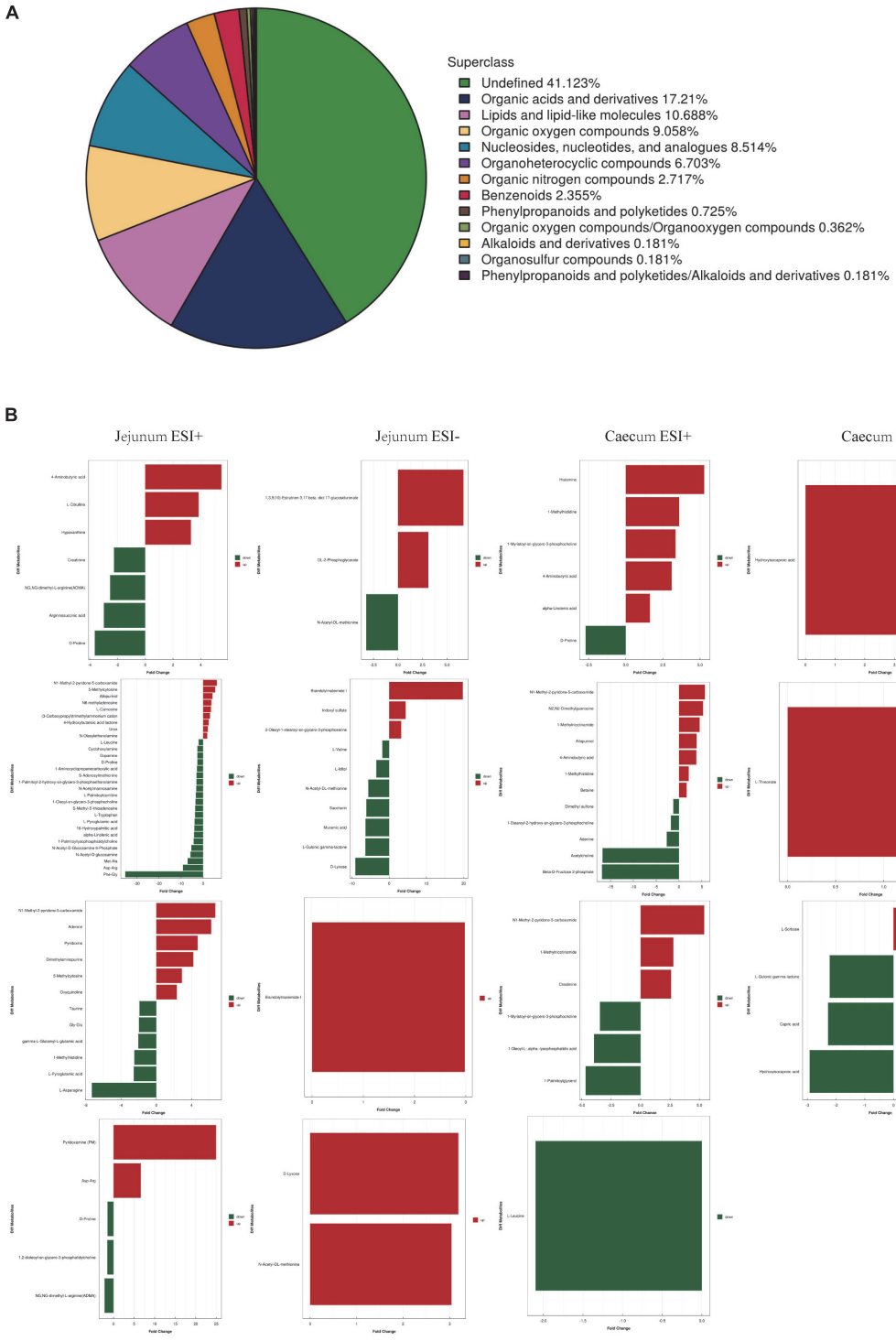

FIGURE 4
(A) Chemical classification of metabolite composition. **(B)** Fold change analysis of different metabolites.

with the earliest fecal shedding occurring within 12 to 24 h after exposure to PEDV infection at the onset of symptoms. The histopathological findings revealed intestinal villi atrophy, severe damage to the villi, and epithelial cell exfoliation. Villous epithelial cells had brown PEDV antigen signals in their cytoplasm (Jung et al., 2015; Lin et al., 2015; Xu et al., 2020). The findings of these studies agreed with the observations of disease symptoms in this study. The IHC score and survival time

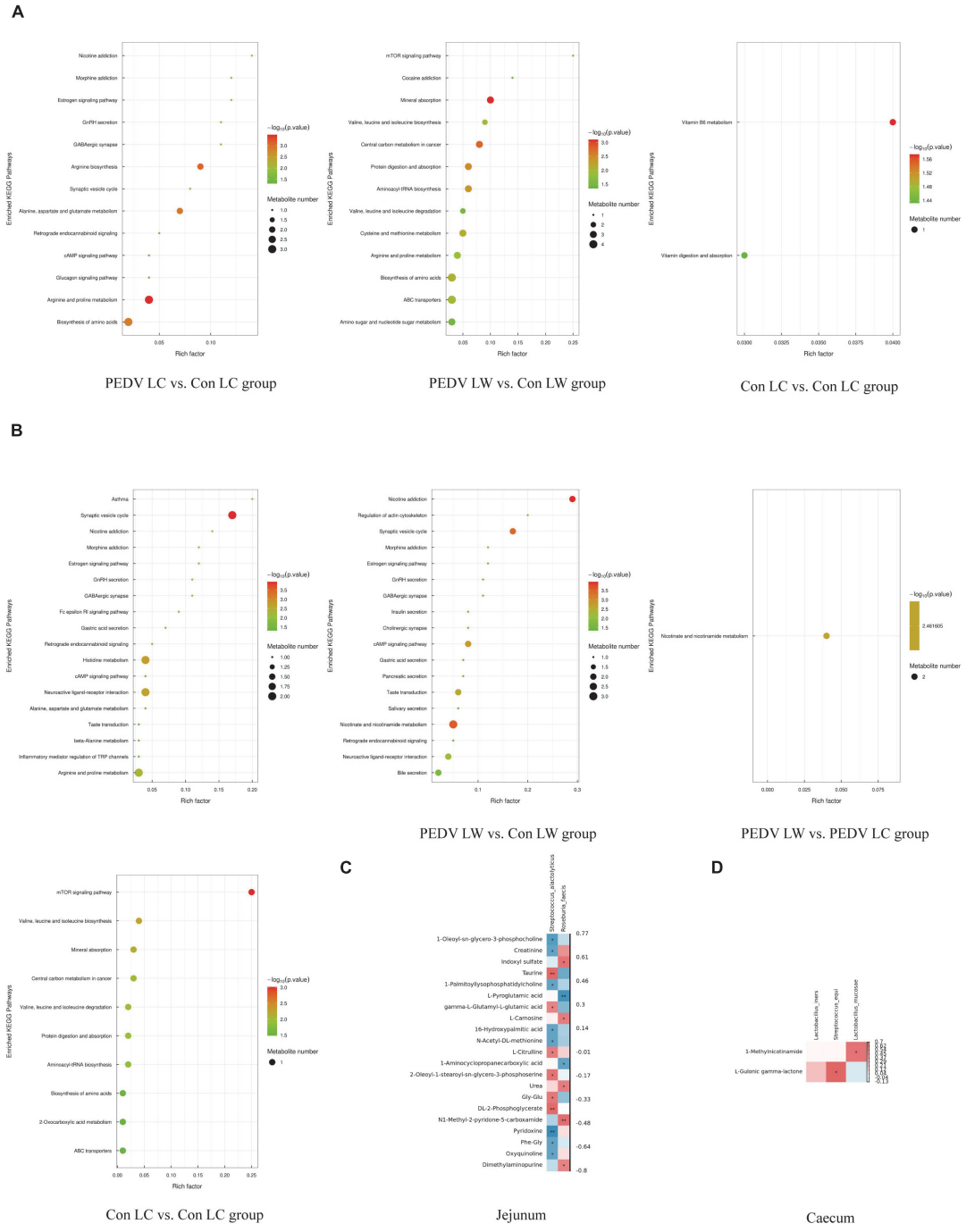

FIGURE 5
(A) KEGG enrichment pathway of different metabolites in jejunum. **(B)** KEGG enrichment pathway of different metabolites in cecum. **(C)** Spearman correlation analysis of different microorganisms and metabolites in the jejunum. **(D)** Spearman correlation analysis of different microorganisms and metabolites in the cecum.

of the LC piglet group were slightly different from those of the LW group in our results, but the differences were not significant due to the small sample size or the younger piglet age.

The present study evaluated the LC and LW piglets' intestinal microbiota displayed different bacterial community characteristics, as evaluated by alpha diversity and PCoA analysis. We found higher species richness of cecum in the LC group than in the LW group (Chao1 index and Observed species). These data revealed microbial community diversity and complexity in different piglets gut systems. Besides, differences

TABLE 7 Significant enrichment pathways for jejunal differential metabolites.

Group	Pathway name	*P*-value
PEDV LC vs. Con LC group	Arginine and proline metabolism	0.000332
	Arginine biosynthesis	0.000802
PEDV LW vs. Con LW group	Mineral absorption	0.000796
Con LW vs. Con LC group	Vitamin B6 metabolism	0.026662

TABLE 8 Significant enrichment pathways for differential metabolites in the cecum.

Group	Pathway name	*P*-value
PEDV LC vs. Con LC group	Synaptic vesicle cycle	0.000114
PEDV LW vs. Con LW group	Nicotinate and nicotinamide metabolism	0.000246
PEDV LW vs. PEDV LC group	Nicotinate and nicotinamide metabolism	0.003455
Con LW vs. Con LC group	mTOR signaling pathway	0.000962

were found in the dominant phylum, and dominant bacterial genus in the same intestine of piglets in the LC and LW groups, and the cecum of piglets of the same breed was always richer in bacterial species than the jejunum, regardless of whether they were infected with PEDV or not. Firmicutes and Proteobacteria were the dominant phyla with relative abundance greater than 1% in the jejunum of piglets whether infected with the PEDV or not, Bacteroidetes, Actinobacteria and Fusobacteria increased in the number of dominant phyla in the cecum. Three dominant genera (*Lactobacillus*, *Shigella*, *Streptococcus* and *Actinobacillus*) with relative abundance greater than 5% were identified in the jejunum. The number of dominant genera with relative abundance greater than 5% in the cecum increased to eight (*Lactobacillus*, *Shigella*, *Streptococcus*, *Shigella*, *Streptococcus*, *Campylobacter*, *Bacteroides*, and *Bifidobacterium*). Previous studies had shown high microbial diversity in the large intestinal segment, with no microorganisms in abundance above 8% at the genus level and a more complex microbial composition in the distal intestinal part than in the proximal one, which was similar to the results of this study (Martinez-Guryn et al., 2019).

The abundance of species composition showed significant differences between the control and the PEDV infected piglets. Significant changes in the microbiota composition at the genus level were found in the jejunum and cecum. The Con LC and the PEDV LC groups displayed a higher abundance of Lactobacillus and a lower abundance of Shigella than the Con LW and the PEDV LW groups in the jejunum and cecum. Many studies suggest that *Lactobacillus* sp. helps to reduce pro-inflammatory cytokine production and enhance the antiviral immune response (Waki et al., 2014; Niederwerder, 2017). *Shigella* sp. has been shown to be a common cause of bacterial deaths, and most cases present with watery diarrhea and dysentery (MacLennan and Steele, 2022). *Fusobacterium* and *Shigella* abundances increased significantly after PEDV infection, while *Psychrobacter, Prevotella,* and *Faecalibacterium* abundances decreased significantly. Others have previously made a similar observation (Afra et al., 2013; Croxen et al., 2013). Because these symbiotic bacteria were known to play an important role in maintaining homeostasis, their reduction in the control group may negatively impact them (Koh et al., 2015). PEDV infection reduced probiotics and increased pathogenic bacteria, disrupting the intestinal microbiota structure (Song et al., 2017; Huang et al., 2019). These results suggest that PEDV infection caused profound alterations in piglets' gut microbiota composition. However, the gut microbiome composition of LC pigs was significantly different from that of LW piglets, especially in the trend of changes in the microbiome composition of PEDV LC groups.

In this study, the critical microorganisms screened for differences in microbiota structure before and after infection in different breeds of piglets were *Streptococcus alactolyticus*, *Roseburia faecis*, *Lactobacillus iners*, *Streptococcus equi* and *Lactobacillus mucosae*. The significantly different microorganism *R. faecis*, belonging to the genus *Roseburia*, a butyric acid-producing bacterium, was identified in this experiment (Duncan et al., 2006), The increased content of acetic and volatile fatty acids in the cecum was beneficial for improving intestinal function and health (Duncan et al., 2006). *L. mucosae* was first isolated from the small intestine of pigs and was an anaerobic bacterium that ferments whey to metabolize L-lactate (Guerra et al., 2001). It is not clear whether the microorganisms screened for differences between groups are related to the process of PEDV infection in the host. To help clarify this, their mechanisms of action need to be further explored and elucidated. In this experiment, the metabolite 1-Methylnicotinamide that positively correlated with *L. mucosae* was upregulated in the cecum of the PEDV_LW group, indicating that the expression of this metabolite may be associated with the increased abundance of *L. mucosae*. *L. mucosae* belong to the family *Lactobacillaceae* and play an important role in gastrointestinal health (Walter, 2008; Delgado et al., 2015), was first identified by Roos et al. (2000), with probiotic properties, high viability, acid, and bile tolerance (de Palencia et al., 2011). *L. mucosae* LM1 had affinities for mucin glycan receptor analogs against pathogens such as *Escherichia coli* K88 and *Salmonella Typhimurium* KCCM 40253. This activity was demonstrated *in vitro* (Valeriano et al., 2014, 2016). 1-Methylnicotinamide was the active endogenous metabolite of nicotinamide (Alston and Abeles, 1988), involved in the nicotinic acid and nicotinamide metabolic pathways, possessed with anti-inflammatory characteristics (Gebicki et al., 2003; Bryniarski et al., 2008), and protective characteristics benefitting the gastrointestinal tract (Brzozowski et al., 2008). The specific relationship between 1-Methylnicotinamide and *L. mucosae* and whether both played a key role in the anti-PEDV infection process needs further experimental validation.

The differences in gut microbes and metabolites between the two piglet breeds observed in our study may be related to differences in the disease symptoms following PEDV infection. However, it is important to determine if there is a connection between the specific core microbiota found in LC piglets (microbiota that stays in the body with infection) and the PEDV infection process. One limitation of this study is the relatively small sample size. Future studies with a large sample size are desired to validate the discoveries in this study.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary material**.

Ethics statement

This animal study was reviewed and approved by Animal Protection and Ethics Committee and Use Committee of Foshan University.

Author contributions

WeZ, YZ, WaZ, and ZL designed the study. ZH, LM, and LS performed the research and analyzed the data. WaZ wrote the initial draft of the manuscript. JG, FW, KM, SE-A, SH, and ZL revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author WeZ was employed by Guangxi Yangxiang Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.990642/full#supplementary-material

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Genetic characteristics, antimicrobial resistance, and prevalence of *Arcobacter* spp. isolated from various sources in Shenzhen, China

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Arcobacter spp. is a globally emerging zoonotic and foodborne pathogen. However, little is known about its prevalence and antimicrobial resistance in China. To investigate the prevalence of *Arcobacter* spp. isolated from various sources, 396 samples were collected from human feces, chicken cecum, and food specimens including chicken meat, beef, pork, lettuce, and seafood. *Arcobacter* spp. was isolated by the membrane filtration method. For 92 strains, the agar dilution method and next-generation sequencing were used to investigate their antimicrobial resistance and to obtain whole genome data, respectively. The virulence factor database (VFDB) was queried to identify virulence genes. ResFinder and the Comprehensive Antibiotic Resistance Database (CARD) were used to predict resistance genes. A phylogenetic tree was constructed using the maximum likelihood (ML) method with core single-nucleotide polymorphisms (SNPs). We found that 27.5% of the samples (n = 109) were positive for *Arcobacter* spp., comprising *Arcobacter butzleri* (53.0%), *Arcobacter cryaerophilus* (39.6%), and *Arcobacter skirrowii* (7.4%). Chicken meat had the highest prevalence (81.2%), followed by seafood (51.9%), pork (43.3%), beef (36.7%), lettuce (35.5%), chicken cecum (8%), and human fecal samples (0%, 0/159). Antimicrobial susceptibility tests revealed that 51 *A. butzleri* and 40 *A. cryaerophilus* strains were resistant to streptomycin (98.1, 70%), clindamycin (94.1, 90%), tetracycline (64.7, 52.5%), azithromycin (43.1%, 15%), nalidixic acid (33.4, 35%), and ciprofloxacin (31.3, 35%) but were susceptible to erythromycin, gentamicin, chloramphenicol, telithromycin, and clindamycin (≤10%). *A. skirrowii* was sensitive to all experimental antibiotics. The virulence factors *tly*A, *mviN*, *cj1349*, *ciaB*, and *pldA* were carried by all *Arcobacter* spp. strains at 100%, and the following percentages were *cadF* (95.7%), *iroE* (23.9%), *hecB* (2.2%), *hecA,* and *irgA* (1.1%). Only one *A. butzleri* strain (F061-2G) carried a macrolide resistance gene (*ereA*). One *A. butzleri* and one *A. cryaerophilus* harbored resistance island gene clusters, which were isolated from pork and chicken. Phylogenetic tree analysis revealed that *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* were separated from each other. To our knowledge, this is the first

report of the isolation of *Arcobacter* spp. from vegetables and seafood in China. The resistance island gene cluster found in pork and chicken meat and the presence of virulence factors could be a potential risk to human health.

KEYWORDS

***Arcobacter*, whole genome sequencing, antibiotic resistance, phylogenomic analysis, zoonotic pathogen**

Introduction

Arcobacter is a globally emerging foodborne pathogen causing diarrhea, enteritis, and bacteremia in humans and diarrhea, mastitis, and abortion in animals (Ramees et al., 2017; Zambri et al., 2019; Chieffi et al., 2020; Khodamoradi and Abiri, 2020). Humans are mainly infected with *Arcobacter via* the consumption of contaminated food and water (Collado and Figueras, 2011; Šilha et al., 2019). The main strains causing diseases in humans are *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* (Van den Abeele et al., 2014; Ferreira et al., 2019).

Arcobacter-infected poultry is considered the major source of infection (Jribi et al., 2020; Khodamoradi and Abiri, 2020). The prevalence of *Arcobacter* in broiler chickens was reported as 26.0% (26/100) in Iran (Khodamoradi and Abiri, 2020), 55.7% (54/97) in the south of Chile (Vidal-Veuthey et al., 2021), and 73.33% (44/60) in Beijing, China (Wang et al., 2016). Moreover, *Arcobacter* is also found in beef, pork, vegetables, and seafood (Mottola et al., 2016, 2021; Córdoba-Calderón et al., 2017; Kim et al., 2019; Zhang et al., 2019; Jasim et al., 2021), which represent possible transmission sources to humans. However, there have been a few reports regarding *Arcobacter* isolated from various sources in China.

Although most *Arcobacter* infections are self-limited, antibiotic treatment is required for severe clinical infections (Ferreira et al., 2019). This treatment usually includes quinolones, tetracyclines, macrolides, and β-lactamase (Figueras et al., 2014). However, high resistance rates of *Arcobacter* isolates to quinolones and macrolides have been reported (Ferreira et al., 2016; Dekker et al., 2019; Jribi et al., 2020).

Currently, our knowledge is limited concerning the pathogenic mechanisms and virulence features of *Arcobacter* strains (Oliveira et al., 2018; Parisi et al., 2019; Šilha et al., 2019). It was found that 10 potential virulence genes (*iroE, irgA, tlyA, pldA, mviN, hecB, hecA, ciaB, cj1349, and cadF*) were considered important for the virulence of this pathogen (Miller et al., 2007; Rathlavath et al., 2017; Kietsiri et al., 2021). Different virulence genes have different effects on disease (Kietsiri et al., 2021). The existence of virulence factors in *Arcobacter* spp. isolated from food could threaten human health.

This study aimed to determine the prevalence of *Arcobacter* spp. in various sources in Shenzhen, China, and to identify the virulence and antibiotic resistance profiles of *Arcobacter* spp. using whole genome sequencing (WGS). Furthermore, we aimed to determine the minimum inhibitory concentrations (MICs) of 11 common antibiotics to identify the most appropriate and effective treatment for *Arcobacter* infections.

Methods

Sample collection

Between June and September 2019, 159 fecal samples were collected from adult patients with diarrhea at the top three local hospitals. In this study, informed consent was obtained from each adult patient with diarrhea. Patients >16 years of age and who experienced acute diarrhea three times or more in the previous 24 h were included. Meanwhile, a collection of 237 samples from chicken meat ($n = 69$), beef ($n = 30$), pork ($n = 30$), lettuce ($n = 31$), and seafood ($n = 27$) were purchased from two retail markets in the Nanshan center; chicken cecum samples ($n = 50$) were collected from a poultry wholesale market.

Fecal samples (~0.5 g each) were collected into the Cary-Blair medium, and food samples (~250 g each) were placed in a sterile plastic bag, and all samples were transported to the laboratory at 4°C within 4 h for bacterial isolation.

Bacterial culture, isolation, and identification

Arcobacter was isolated by an *Arcobacter* isolation kit using the enrichment and membrane filter method (ZC-ARCO-001, Qingdao Sinova Biotechnology Co., Ltd., Qingdao, China) for stool samples and a direct filtration method for food samples. Briefly, stool specimens were transferred into a 4-ml enrichment buffer, which was provided in the kit. The principal component of the enrichment buffer was the modified Preston broth containing vancomycin, trimethoprim, and amphotericin B, as described in the manual book. The enriched suspension from stool samples was incubated for 24–48 h at 37°C in a microaerophilic atmosphere consisting of 5% O_2, 10% CO_2, and 85% N2. Subsequently, 300 μl of cultured enrichment

suspension was spotted on Karmali and Columbia agar with a 0.45-μm cellulose membrane filter. At the same time, food samples were fully washed with buffered peptone water (100 ml per 250-g sample), which was concentrated by centrifugation at a low speed of 1,500 g for 15 min. Then, 300–500 ml of concentrated suspension was spotted on Karmali and Columbia agar with a 0.45-μm cellulose membrane filter. After air-drying for 40 min in a biological safety cabinet, the filter membrane was removed and the plates were incubated in an aerobic atmosphere at 30°C for 48 h.

After incubation, small, round, and whitish colonies 2 mm in diameter were plated and confirmed by Gram staining, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and real-time polymerase chain reaction (PCR). Mass spectrometry was performed using Flexcontrol software, and the results were interpreted with IVD MALDI Biotyper 2.3 software (Bruker Daltonik GmbH, Bremen, Germany). The criteria for determining the genus and species of bacteria were as follows: 2.300–3.000 points indicated reliable identification to the species level and 2.000–2.299 points indicated reliable identification to the genus level and possible identification to the species level. In this study, scores≥2.000 were considered credible. For PCR identification, a loop was used to collect suspected pure culture colonies, which were resuspended in 200 μl of ultrapure water, boiled for 10 min, and centrifuged for 10 min at 8,000 ×g. Subsequently, the supernatant was removed for PCR species identification by a realtime PCR kit (MABSKY BIO-TECH CO., LTD, Shenzhen, China). PCR amplification conditions were as follows: initial denaturation at 94°C for 5 min, followed by 45 cycles of 94°C for 15 s and 60°C for 1 min.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using an agar dilution method-based kit (ZC-CAMPY-013, Zhongchuang Biotechnology Ltd. Corp., Qingdao, China). The test was performed two times in parallel. Mueller-Hinton agar containing 11 different antibiotics was coated onto wells in a 96-well-plate to obtain the MIC of 92 *Arcobacter* strains. The cutoff criteria for each antibiotic were based on the National Antimicrobial Resistance Monitoring System (NARMS-2015: https://www.cdc.gov/narms/pdf/2015-NARMS-Annual-Report-cleared_508.pdf) for *Campylobacter jejuni* and included clindamycin (≥ 1 μg ml^{-1}), telithromycin (≥ 8 μg ml^{-1}), tetracycline (≥ 2 μg ml^{-1}), florfenicol (≥ 8 μg ml^{-1}), gentamycin (≥ 4 μg ml^{-1}), ciprofloxacin (≥ 1 μg ml^{-1}), nalidixic acid (≥ 32 μg ml^{-1}), azithromycin (≥ 0.5 μg ml^{-1}), and erythromycin (≥ 8 μg ml^{-1}), combined with MIC EUCAST (https://mic.eucast.org/search/?search%5Bmethod%5D=mic&search%5Bantibiotic%5D=-1&search%5Bspecies%5D=100&search%5Bdisk_content%5D=-1&search%5Blimit%5D=50:) streptomycin (≥ 4 μg ml^{-1}) and chloramphenicol (≥ 32 μg ml^{-1}). The quality control bacterial strain was *C. jejuni* ATCC 33560. In addition, we defined multidrug resistance (MDR) as resistance to ≥ 3 classes of antibiotics.

Extraction of deoxyribonucleic acid and WGS

Deoxyribonucleic acid (DNA) was extracted from Arcobacter isolates. One or two Arcobacter plates from blood agar plates (Huaikai biology, Guangzhou, China) were needed to obtain sufficient material for DNA preparation. Colonies were harvested using fiber swabs and resuspended in 1 ml of phosphate-buffered saline (PBS). The tubes were centrifuged at 16,000 × *g* for 6 min, and the supernatant was discarded. The resulting pellet was further processed for DNA recovery using the bacterial genomic DNA Extraction kit (T132, Tianlong, Shanxi, China) according to the manufacturer's instructions. The concentration of the double-stranded DNA (dsDNA) was examined using a Microplate spectrophotometer (Epoch, Berten Instruments Co., Ltd., Montigny-le-Bretonneux, France).

Next-generation sequencing was performed using the Illumina NovaSeq PE150 (Illumina, San Diego, CA, USA) by Novo Source Technology Co., Ltd., (Beijing, China) and BGI Genomics Co., Ltd., (Beijing, China). To sequence the genomes, a 270-bp paired-end library was constructed and then 150-bp reads were generated. FastQC v0.11.8 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and fastp v0.20.0 (https://github.com/OpenGene/fastp) software tools were applied to evaluate and improve the quality of the raw sequence data, respectively. Low-quality reads were removed in case of the quality scores of ≥3 consecutive bases ≤ Q30. The clean reads were assembled using SOAPdenovo v2.04 (http://soap.genomics.org.cn/soapdenovo.html) and spades v3.13.1 software (Prjibelski et al., 2020). Finally, the assembled sequences were subjected to gene prediction and functional annotation using the Prokka pipeline (Seemann, 2014) and glimmer software (http://ccb.jhu.edu/software/glimmer/index.shtml). The Kyoto Encyclopedia of Genes and Genomes (KEGG) and Clusters of Orthologous Groups (COG) databases were used for functional classification.

Bioinformatic analyses

The presence of virulence genes was assessed by submitting the assembled genomes to the virulence factor database (VFDB) (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi?func=VFanalyzer). ResFinder (http://cge.cbs.dtu.dk/services/ResFinder/), and Abricate software (https://github.com/tseemann/abricate), and the Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca/?q=CARD/ontology/35506) were used to predict resistance genes,

with a cutoff comprising an *E*-value of at least 1e−10. Cutoffs for identification and query coverage values were >80 and >60%, respectively. Individual missense mutations in *gyrA* conferring ciprofloxacin resistance were detected using BLASTn. Databases including KEGG, COG, SwissProt, and PHI, were used to functionally annotate and classify protein sequences. We extracted the nucleotide sequences of all annotated resistance genes from the genomes, and the genoPlotR package was used to visualize related gene clusters. Based on a previous study (Parisi et al., 2019), 10 common virulence factors (*iroE, irgA, tlyA, pldA, mviN, hecB, hecA, ciaB, cj1349, ciaB, and cadF*) were analyzed using the BLASTn method.

Phylogenetic tree analysis was performed on 177 *Arcobacter* strains, including 85 *Arcobacter* strain sequences (45 *A. butzleri*, 26 *A. cryaerophilus,* and *14 A. skirrowii*), which were downloaded from the GenBank database and 92 strains obtained in this study. Core single-nucleotide polymorphisms (SNPs) were called using Snippy 4.3.6 software (https://github.com/tseemann/snippy) with default parameters and ICDCAB83 as the reference genome. Gubbins software (Croucher et al., 2015) was used as a recombination-removal tool to gain pure SNPs without recombination. Phylogeny reconstruction was performed using the maximum likelihood (ML) method in MEGA 7 software (Kumar et al., 2016) with 1,000 bootstraps.

Data analysis

SPSS 26.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis, and the chi-squared test (χ^2) was used to compare count data between groups. A statistical probability of <0.05 ($p < 0.05$) indicated a statistically significant difference.

Results

The prevalence of *Arcobacter* spp. isolated from patients with diarrhea, poultry, beef, pork, vegetables, and seafood

Out of 159 human fecal samples, no *Arcobacter* strains were isolated. In total, 109 *Arcobacter* strains were separated from chicken meat, chicken cecum, beef, pork, lettuce, and seafood (Figure 1). *A. butzleri* was the most prevalent species (33.3%, 79/237), followed by *A. cryaerophilus* (24.9%, 59/237) and *A. skirrowii* (4.6%, 11/237). In 37 of the samples, two or three species of Arcobacter were isolated. Moreover, the prevalence percentage of *Arcobacters* spp. in chicken meat, seafood, pork, beef, lettuce, and chicken cecum was 81.2% (56/69), 51.9% (14/27), 43.3% (13/30), 36.7% (11/30), 35.5% (11/31), and 8% (4/50), respectively (Figure 1). Significantly, *Arcobacter* spp. isolated from chicken cecum (8%, 4/50) had a lower prevalence than those isolated from poultry meat (81.2%, 56/69) ($\chi^2 = 10.632$, $p = 0.001$).

Whole genome sequencing

A total of 92 strains (51 *A. butzleri* isolated from 26 chicken, eight beef, seven pork, seven lettuce, and three seafood samples; 40 *A. cryaerophilus* isolated from 29 chicken, three beef, two pork, two lettuce, and four seafood samples; and one *A. skirrowii* isolated from seafood) were characterized by WGS. WGS of Arcobacter isolates were registered in GenBank with accession numbers SAMN30871879 to SAMN30871970. Assemblies consisted of 10–132 contigs. The sequence length was predicted to be between 1,827,334 and 2,453,640 concordant bases. The estimated sizes of the genomes of the 92 strains varied from 1.82 to 2.34 Mb. The guanine-cytosine (GC) content was determined to be 27.78% for *A. skirrowii*, 27.15–27.47% for *A. cryaerophilus*, and 26.65–27.02% for *A. butzleri*.

Virulence genes and secretion systems

Genome sequencing in VFDB showed that all *A. butzleri* and *A. cryaerophilus* isolates had the following virulence factor genes: *tlyA*, *mviN*, *cj1349*, *ciaB*, and *pldA*, followed by *cadF* (95.7%), *iroE* (23.9%), *hecB (2.2%)*, *hecA*, and *irgA* (1.1%) (Figure 2). Moreover, there was no difference in the distribution between *A. butzleri* and *A. cryaerophilus*. Almost no *A. cryaerophilus* strains carried the *IrgA*, *hecA*, and *hecB* genes. None of the strains had a secretion system. *A. skirrowii* contained the virulence factor genes *tlyA*, *mviN*, *cj1349*, *ciaB*, and *pldA*.

Genetic prediction of antibiotic resistance and concordance with resistance phenotypes

All 51 strains of *A. butzleri* contained β-lactam antibiotic resistance genes (bla_{OXA464}or bla_{OXA491}), among which 27 strains contained complete genes and the remaining 24 strains contained incomplete genes. Five strains (F034-1G, F050-4G, F061-2G, F101-1G, and F114.2G) contained three tetracycline resistance genes (*tet (L)*, *tet (H)*, and tet *(M)*). F061-2G contained a macrolide resistance gene (*ere (A)*). F050-4G, which was isolated from pork, contained aminoglycoside resistance genes (*APH (3 ') -IIIa* and *ant (6) -Ia*), streptomycin resistance genes (*SAT-4*), and tetracycline resistance genes (*tet (M)*). These genes might exist as gene clusters in bacteria. The gene cluster size was 6,072 bp, with a GC content of 37.17%, and comprised five genes, including four resistance genes (Figure 3).

Seventeen *A. cryaerophilus* strains contained β-lactam resistance genes, and three of them (F015-3G, F035-7G, and

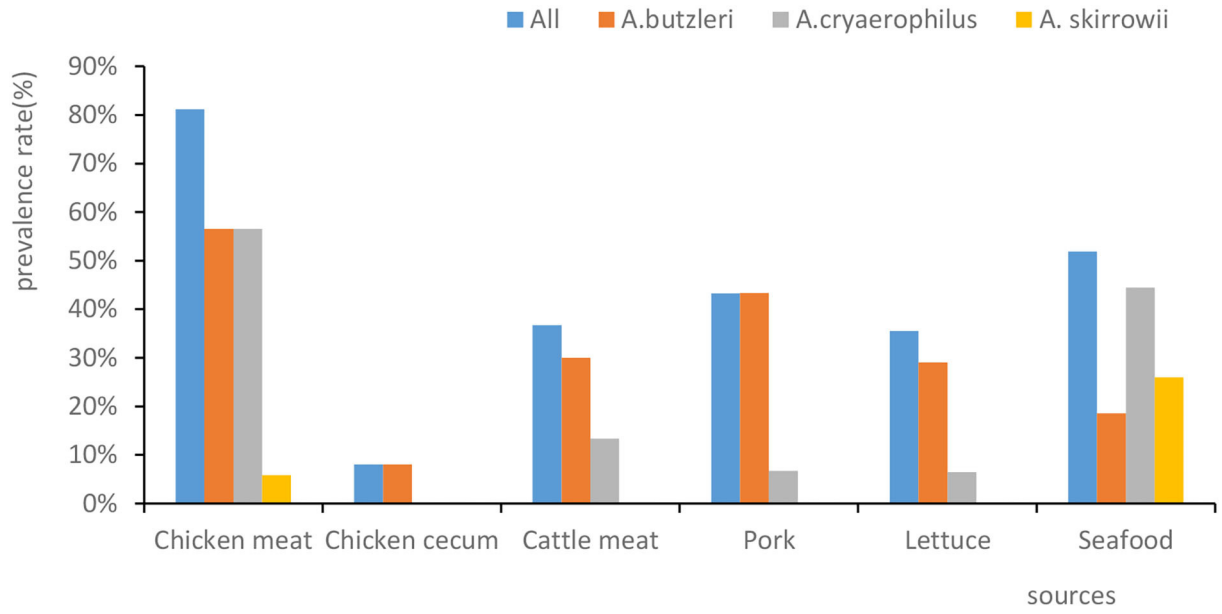

FIGURE 1
The prevalence rates of *Arcobacter* spp. isolated from different sources. The *x*-axis represents the sources. The *y*-axis represents the prevalence percentage. The color of the columns corresponds to the organisms.

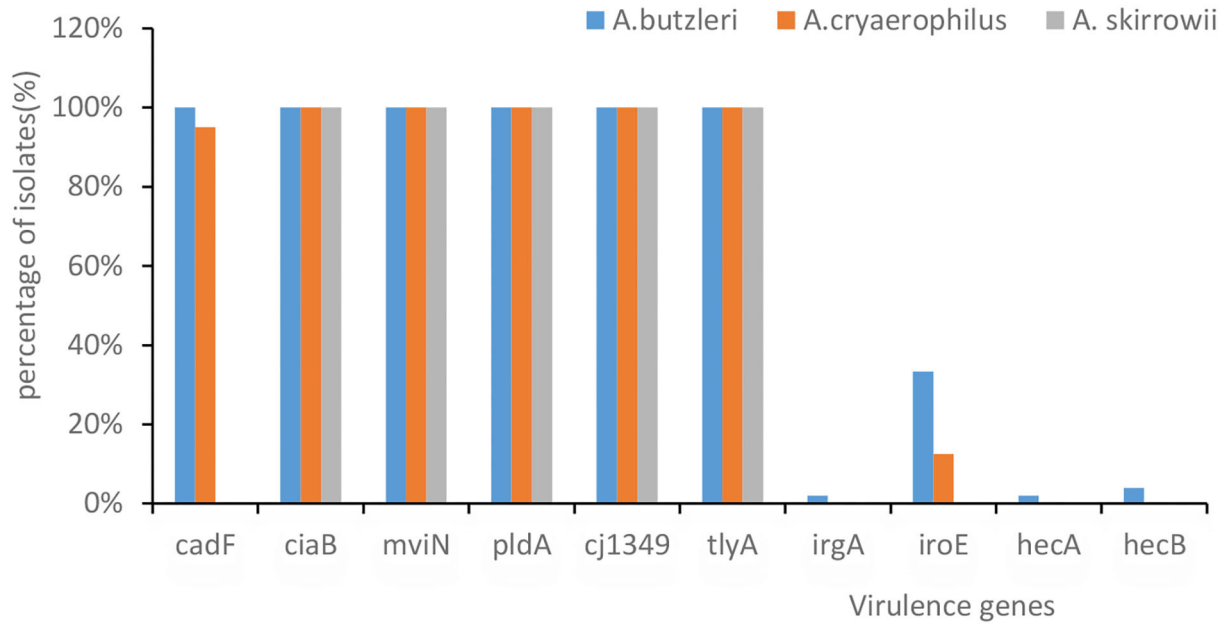

FIGURE 2
The presence of virulence-associated genes in *Arcobacter* spp. The *x*-axis represents the virulence genes. The *y*-axis represents their percentage of isolates. The color of the columns corresponds to the organisms.

F132-4G) contained tetracycline resistance genes, *tet* (Y), *tet* (H), and *tet* (M). Strain F015-3G isolated from chicken contained one MDR gene island flanked by the insertion sequence IS4 (Figure 3). The size of this MDR gene island was 9,409 bp in length and had a GC content of 33.45%. Seven resistance genes, including three aminoglycosides resistance genes, one

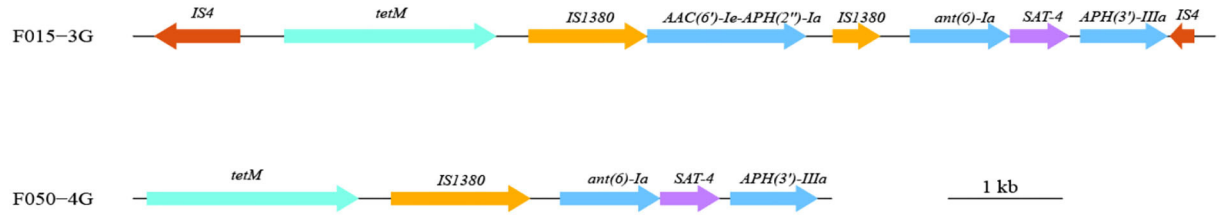

FIGURE 3
Gene cluster composition in one *A. butzleri* (F050-4G) and one *A. cryaerophilus* (F015-3G) isolate. Different colors represent different genes or categories of the resistance gene. The length and direction of the arrows indicate the size and direction of genes.

tetracycline resistance gene, one streptomycin resistance gene, and two insertion sequences (IS1380), were harbored on this island (Figure 3). This resistance island of F015-3G was similar to that carried by *Campylobacter coli* SH96 (Sequence ID: **MT107516.1**).

One strain of *A. skirrowii* (F198-3G) did not contain resistance genes. Among 51 *A. butzleri* isolates from five sources, streptomycin had the highest resistance rate (98.1%), followed by clindamycin (94.1%), tetracycline (64.7%), azithromycin (43.1%), nalidixic acid (33.4%), and ciprofloxacin (31.3%); others were <10% (Table 1). Among 40 *A. cryaerophilus* isolates from five sources, clindamycin resistance was the highest (90%), followed by streptomycin (70%), tetracycline (52.5%), nalidixic acid, and ciprofloxacin (35%); others were <8% (Table 2). In terms of MDR, 33 *A. butzleri* strains and 23 *A. cryaerophilus* strains were resistant to three or more classes of antibiotics.

Five *A. butzleri* isolates, including tetracycline resistance genes (*tet (L)*, *tet (H)*, and *tet (M)*), were phenotypically resistant to tetracycline. Thirteen *A. butzleri* isolates with *gyrA* (C254T) mutation were phenotypically resistant to ciprofloxacin. One of the *A. butzleri* isolates carried the *ere(A)* gene, which is associated with erythromycin resistance (MIC value ≥ 64 μg/ml). One *A. butzleri* isolate (F050-4G) isolated from pork had *gyrA* (C254T) mutation, contained a streptomycin resistance gene (*Sat-4*), a tetracycline resistance gene (*tet (M)*) and aminoglycoside resistance genes (*APH (3 ') -IIIa* and *ant (6) -Ia*), and was phenotypically resistant to ciprofloxacin, streptomycin, and tetracycline.

Three *A. cryaerophilus* isolates containing tetracycline resistance genes (*tet (L)*, *tet (H)*, and *tet (M)*) were phenotypically resistant to tetracycline. Fourteen *A. cryaerophilus* isolates with a *gyrA* (C254T) mutation were phenotypically resistant to ciprofloxacin. One *A. cryaerophilus* isolate (F015-3G) isolated from chicken had a *gyrA* (C254T) mutation, contained a streptomycin resistance gene (*Sat-4*), a tetracycline resistance gene (*tet (M)*), and aminoglycoside resistance genes (APH (3 ') -IIIa and ant (6) -Ia), and showed resistance to ciprofloxacin, streptomycin, and tetracycline.

Phylogenetic reconstruction

Phylogenetic tree analysis revealed that the 177 strains were mainly divided into three groups (*A. butzleri*, *A. cryaerophilus*, *and A. skirrowii*). There was no obvious aggregation phenomenon in each group according to the source of the host or the sampling site, indicating that *Arcobacter* strains showed high genetic diversity (Figure 4).

Discussion

Arcobacter spp. is globally recognized as one of the causes of acute gastroenteritis (Zhang et al., 2019; Brückner et al., 2020). The main transmission source of *Arcobacter* spp. in humans is *via* the consumption of contaminated water and food (Collado and Figueras, 2011; Zambri et al., 2019). This study provides updated information on the incidence, genetic diversity, and antimicrobial susceptibility of *Arcobacter* spp. Samples were collected from various sources in Shenzhen, China.

Our results showed that the prevalence of *Arcobacter* spp. ranged from high to low in chicken meat (81.2%, 56/69), seafood (51.9%, 14/27), pork (43.3%, 13/30), beef (36.7%, 11/30), lettuce (35.5%,11/31), chicken cecum (8.0%, 4/50), and human feces (0.0%,0/159). The prevalence of *Arcobacter* spp. in human feces ranged from 0.2 to 3.6% in studies conducted in Germany, Chile, Portugal, India, and Turkey (Patyal et al., 2011; Kayman et al., 2012; Ferreira et al., 2014; Fernandez et al., 2015; Brückner et al., 2020), which was somewhat consistent with our result that *Arcobacter* spp. was not isolated from any of the 159 fecal samples. In contrast to our findings, the prevalence of *Arcobacter* isolated from chicken meat was higher than the prevalence found in Iran (26%) (Khodamoradi and Abiri, 2020), Germany (26.8%) (Lehmann et al., 2015), and Tunisia (13.42%) (Jribi et al., 2020), but similar to that found in Beijing (73.33%) (Wang et al., 2016). Jasim et al. (2021) reported that (120/1,293) the prevalence of positive beef samples in Iraq was 9.25%, which was lower than that in the present study. Mottola et al. (2021) reported that its prevalence in ready-to-eat vegetables was 14.5% (16/110), which was lower than that in the present

TABLE 1 Minimum inhibitory concentrations (MICs) of antimicrobial agents toward 51 *A. butzleri* isolates.

CLSI antimicrobial class†	Antimicrobial agent	Percentage of all isolates with MIC(μg/ml)									
		<0.25	<0.5	0.5	1	2	4	8	16	≥ 32	≥ 64
Aminoglycosides	Gentamicin		11.8		58.8	27.5	2.0				
	Streptomycin					2.0	54.9	39.2		2.0	2.0
Ketolide	Telithromycin	7.8		17.6	21.6	37.3	7.8	5.9		2.0	
Macrolides	Azithromycin		56.9		5.9	7.8	9.8	13.7	3.9		2.0
	Erythromycin		23.5		27.5	35.3	7.8	3.9			2.0
Quinolones	Ciprofloxacin		68.6		2.0		3.9	9.8	7.8	3.9	3.9
	Nalidixic acid						23.5	33.3	9.8	5.9	27.5
Lincosamides	Clindamycin			5.9	49.0	35.3	7.8		2.0		
Phenicols	Florfenicol				3.9	47.1	39.2	7.8	2.0		
	Chloramphenicol		2.0			17.6	58.8	17.6	3.9		
Tetracyclines	Tetracycline				35.3	27.5	23.5	9.8	3.9		

† Clinical and laboratory standards institute.
Gray shadings represent the percentage of resistance isolates.

TABLE 2 MICs of antimicrobial agents to 40 *A. cryaerophilus* isolates.

CLSI antimicrobial class†	Antimicrobial agent	Percentage of all isolates with MIC(μg/ml)									
		<0.25	<0.5	0.50	1	2	4	8	16	≥ 32	> 64
Aminoglycosides	Gentamicin		40.0		32.5	25.0					2.5
	Streptomycin		2.5			27.5	35.0	27.5	2.5		5.0
Ketolide	Telithromycin	25.0	2.5	10.0	30.0	27.5	5.0				
Macrolides	Azithromycin		85.0	2.5	5.0	5.0	2.5				
	Erythromycin		42.5		32.5	22.5	2.5				
Quinolones	Ciprofloxacin		65.0		2.5			5.0	17.5	10.0	
	Nalidixic acid						15.0	40.0	10.0	2.5	32.5
Lincosamides	Clindamycin	5.0		5.0	30.0	50.0	7.5		2.5		
Phenicols	Florfenicol				7.5	45.0	40.0	7.5			
	Chloramphenicol				2.5	30.0	60.0	5.0	2.5		
Tetracyclines	Tetracycline		12.5		35.0	25.0	22.5		5.0		

† Clinical and laboratory standards institute.
Gray shadings represent the percentage of resistance isolates.

study. Zhang et al. (2019) reported that the contamination rate for seafood was 17.6% (56/318), which was lower than that reported here.

Furthermore, our results showed a lower prevalence of *Arcobacter* spp. in the chicken cecum (8%, 4/50) than in chicken meat (81.2%, 56/69) ($\chi^2 = 62.073$, $p = 0.000$), which was possibly due to cross-contamination of meat in both the slaughter and retail market environments.

Barboza et al. (2017) reported that the prevalence of chicken cecal content was 5.26% (8/152). Schönknecht et al. (2020) reported that the prevalence of *Arcobacter* spp. in the chicken cecum was 3% (1/29), which was lower than other intestinal contents. Cecal contents might not be the main *Arcobacter* reservoir inside chickens. The prevalence of *Arcobacter* spp. isolated from various sources in this study might change with seasons and climate. We will continue to conduct relevant pathogen monitoring research in the future.

In the current study, almost all strains of *Arcobacter spp.* have β-lactam antibiotic resistance genes. It has been speculated that *Arcobacter* spp. may be resistant to β-lactamase. Several studies reported that β-lactam resistance may be caused by the presence of three putative β-lactamases (AB0578, AB1306, and AB1486) identified in the RM4018 genome, which are enhanced by the occurrence of the lrgAB operon (ab0179 and ab0180) and may regulate tolerance to penicillin in *Staphylococcus* (Bayles, 2000; Groicher et al., 2000; Miller et al., 2007). However, we did not perform an experiment showing the phenotypic resistance

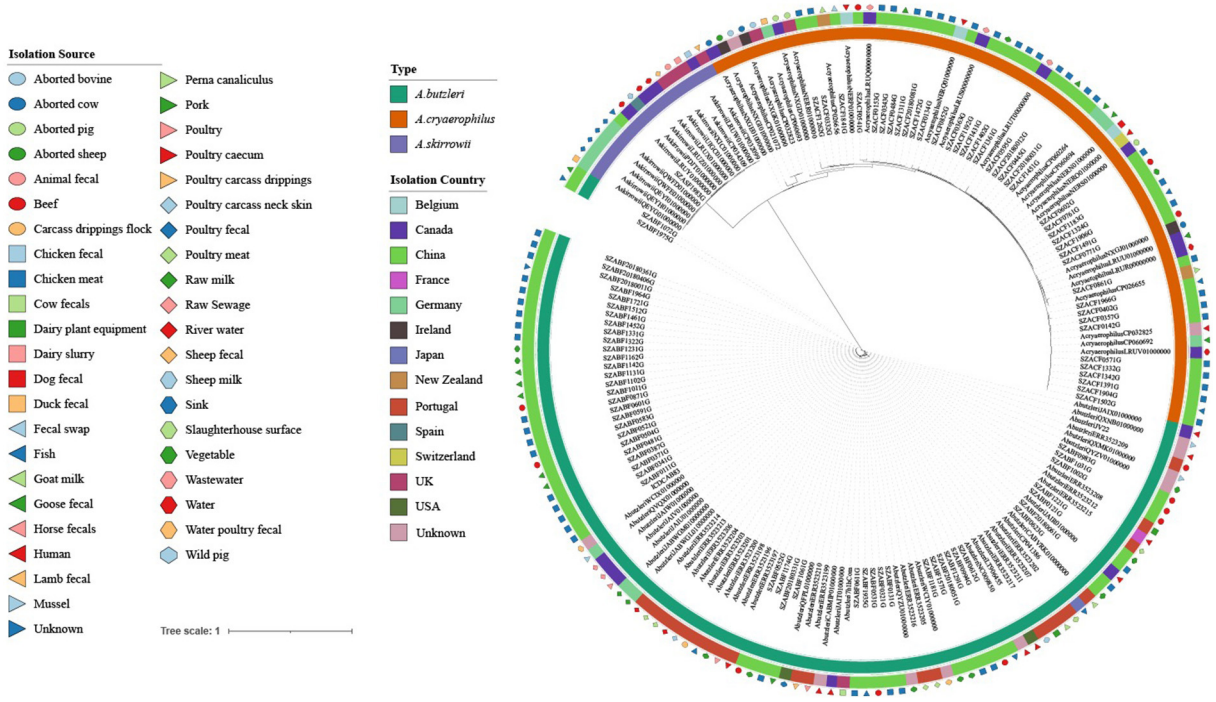

FIGURE 4
Maximum likelihood (ML) tree of the core genome alignments of strains isolated from various sources and countries with 1,000 bootstraps. Different colors and shapes indicate various sources. From the inner circle to the outer circle, strains, Type, Isolation Country, and Isolation Source are listed.

of *Arcobacter* spp. to β-lactam antibiotics. Therefore, further investigation on the resistance phenotype and mechanism of β-lactam antibiotics is needed. For severe clinical diseases caused by *Arcobacter* spp., fluoroquinolones, tetracyclines, macrolides, and aminoglycosides are recommended for treatment (Ferreira et al., 2016). A meta-analysis of *Arcobacter* spp. antibiotic resistance in 2019 (Ferreira et al., 2019) reported that fluoroquinolone resistance ranged from 4.3 to 14.0%, whereas it was 0.8–7.1% for tetracyclines, 10.7–39.8% for macrolides, and 1.8–12.9% for aminoglycosides. In this study, the resistance rates of A. *butzleri* and A. *cryaerophilus* to nalidixic acid (33.4%, 35%), ciprofloxacin (31.3%, 35%), and tetracycline (64.7%, 52.5%) were much higher than previously reported (Ferreira et al., 2019). However, Jribi et al. (2020) reported a high level of resistance to tetracycline (100%) in *Arcobacter* spp. isolated from poultry in Tunisia.

The resistance rates of 51 *A. butzleri* isolates to erythromycin, azithromycin, and telithromycin were 5.9, 43.1, and 7.9%, respectively. All *A. cryaerophilus* isolates were completely sensitive to erythromycin. The resistance rate of *Arcobacter* spp. to erythromycin is consistent with a previous study (3.6%, 3/84) (Kietsiri et al., 2021). Moreover, streptomycin resistance rates for *A. butzleri* and *A. cryaerophilus* were 41.2 and 32.5%, respectively, which were higher than those reported in a previous study (Ferreira et al., 2016, 2019). Therefore, further attention should be paid to streptomycin resistance.

One strain each of *A. butzleri* (F050-4G) and *A. cryaerophilus* (F015-3G) contained resistance island gene clusters, which contained multiple antibiotic resistance genes and were located near the transposon. The resistance island in *A. cryaerophilus* (F015-3G) was inserted into IS4. The GC content of the two resistance islands was significantly higher than that of the genome of *Arcobacter* spp. We suspected that the resistance island might have been obtained by the horizontal gene transfer. Several studies reported that the use of antibiotics in animals might cause MDR and then transfer to humans (Chang et al., 2015; Dekker et al., 2019).

Several studies (Webb et al., 2018; Hodges et al., 2021) showed that the base mutation in the *gyrA* gene was associated with a higher level of resistance to ciprofloxacin. In our study, 13 *A. butzleri* and 14 *A. cryaerophilus* isolates with *gyrA* (C254T) mutation were 100% phenotypically resistant to ciprofloxacin. Moreover, the genes *aph (3 ') -IIIa* and *ant (6) –Ia* were reported to correlate with resistance to kanamycin and streptomycin, respectively (Ntilde et al., 2018; Cho et al., 2020), and in our study, two isolates carried the ant (6) –Ia gene and were phenotypically resistant to streptomycin. In addition, isolates that carried tetracycline resistance genes were phenotypically resistant to tetracycline, whereas the occurrence of *ere(A)* gene was associated with erythromycin resistance, which was consistent with previous studies (Gao et al., 2015; Zhao et al., 2016; Webb et al., 2018).

The number of antibiotic resistance genes varied greatly between the isolates. Some isolates harbored multiple antibiotic resistance genes, and specific resistance genes were detected in the corresponding antibiotic resistance isolates. Strains containing resistance genes were resistant to the corresponding antibiotics or had higher MIC values. A C254T mutation was found in some strains, resulting in a Thr to Ile substitution at position 85 of the deduced protein sequence. This substitution in *A. butzleri* and *A. cryaerophilus* isolates could be responsible for the observed fluoroquinolone resistance. A C254T mutation in *gyrA*, which resulted in a Thr to Ile substitution in *gyrA* were found in all ciprofloxacin resistance strains. This substitution in *A. butzleri* and *A. cryaerophilus* isolates could be responsible for the observed fluoroquinolone resistance.

Arcobacter skirrowii, A. cryaerophilus, and *A. butzleri* isolated from food and originating from animals commonly carry *tlyA*, *pldA*, *mviN*, *ciaB*, *cj1349*, and *cadF* (Douidah et al., 2012; Khoshbakht et al., 2014; Parisi et al., 2019; Khodamoradi and Abiri, 2020). Rathlavath et al. (2017) reported that the majority of *A. butzleri* isolated from seafood and the coastal environment contained six common virulence genes [cadF (89.7%), cj1349 (97.2%), ciaB (95.9%), mviN (100%), pldA (91.1%), and tlyA (91.8%)] but relatively lower amounts of *hecA* (10.8%), *hecB* (19%), *iroE* (12.9%), and *irgA* (17.6%). Similarly, our study found that more than 90% *Arcobacter* contained these six common virulence factors. It was found that different virulence genes had different functions, e.g., *tlyA* encoding hemolysin and *pldA* encoding the outer membrane phospholipase A are involved in erythrocyte lysis; *mviN* is required for the biosynthesis of peptidoglycan; *ciaB* is required for the biosynthesis of peptidoglycan; and both *cadF* and *cj1349* encode fibronectin-binding protein (Parisi et al., 2019). *A. butzleri* and *A. cryaerophilus* showed no differences in the distribution of these virulence factors, which was in contrast to the results of a previous study (Sekhar et al., 2017) in which *A. butzleri* was observed to carry more of these virulence factors than *A. cryaerophilus*.

In addition to poultry, vegetables and seafood are also important transmission routes for *Arcobacter* infection in humans. The resistance island gene cluster found in pork and chicken meat and the carriage of virulence factors could be a potential health risk to human health.

Author's note

The first author, YM MD, female, was born in 1991, whose specialty is pathogenic microbiology; CJ, female, was born in 1982, majoring in analysis of genetic characteristics of *Campylobacter* and *Arcobacter*.

Data availability statement

The data presented in the study are deposited in the GenBank with accession numbers SAMN30871879 to SAMN30871970.

Author contributions

YD and MZ designed the experiments. YM, CJ, MY, HC, and JH participated in the sample collection and performed the experiments. GZ performed the genome bioinformatic analysis. YM and CJ wrote this paper. All authors read and approved the submitted manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Characteristics, pathogenic mechanism, zoonotic potential, drug resistance, and prevention of avian pathogenic *Escherichia coli* (APEC)

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Although most *Escherichia coli* (*E. coli*) strains are commensal and abundant, certain pathogenic strains cause severe diseases from gastroenteritis to extraintestinal infections. Extraintestinal pathogenic *E. coli* (ExPEC) contains newborn meningitis *E. coli* (NMEC), uropathogenic *E. coli* (UPEC), avian pathogenic *E. coli* (APEC), and septicemic *E. coli* (SEPEC) based on their original host and clinical symptom. APEC is a heterogeneous group derived from human ExPEC. APEC causes severe respiratory and systemic diseases in a variety of avians, threatening the poultry industries, food security, and avian welfare worldwide. APEC has many serotypes, and it is a widespread pathogenic bacterium in poultry. In addition, ExPEC strains share significant genetic similarities and similar pathogenic mechanisms, indicating that APEC potentially serves as a reservoir of virulence and resistance genes for human ExPEC, and the virulence and resistance genes can be transferred to humans through food animals. Due to economic losses, drug resistance, and zoonotic potential, APEC has attracted heightened awareness. Various virulence factors and resistance genes involved in APEC pathogenesis and drug resistance have been identified. Here, we review the characteristics, epidemiology, pathogenic mechanism zoonotic potential, and drug resistance of APEC, and summarize the current status of diagnosis, alternative control measures, and vaccine development, which may help to have a better understanding of the pathogenesis and resistance of APEC, thereby reducing economic losses and preventing the spread of multidrug-resistant APEC to humans.

KEYWORDS

APEC, epidemiology, virulence, zoonotic, antibiotic resistance, control strategies

Introduction

Escherichia coli (*E. coli*) colonizes the gastrointestinal tract and other mucosal surfaces of a variety of animals (Hill and Drasar, 1975). Although most *E. coli* strains are commensal and abundant, certain pathogenic strains can cause severe diseases from gastroenteritis to extraintestinal infections that affect health worldwide (Russo and Johnson, 2003). According to the anamnestic clinical reports and virulence features, pathogenic *E. coli* have been classified as either intestinal pathogenic *E. coli* (IPEC) or extraintestinal pathogenic *E. coli* (ExPEC) (Russo and Johnson, 2003). Six diarrhoeagenic pathovars of IPEC have been extensively studied. ExPEC contains newborn meningitis *E. coli* (NMEC), uropathogenic *E. coli* (UPEC), avian pathogenic *E. coli* (APEC), or septicemic *E. coli* (SEPEC) based on their original host and clinical symptoms. Among these typical infections caused by ExPEC in humans are urinary tract infections (UTIs) and neonatal meningitis (Russo and Johnson, 2000). Similarly, APEC is mostly associated with respiratory tract or systemic infections and results in a variety of diseases in chickens, ducks, and other avian species worldwide, which are economically devastating to poultry industries. There is an increasing risk of ExPEC due to its abundance and multidrug resistance (Antão et al., 2008).

More and more shreds of evidence indicated that ExPEC strains involved in animal and human infections have a highly similar range of phylogenetic and pathogenic mechanisms (Moulin-Schouleur et al., 2007; Reid et al., 2019). Moreover, the whole-genome sequencing of *E. coli* strains indicated that the genomic level of human ExPEC strains is clustered with avian isolates. Some *E. coli* strains could acquire a combination of mobile genetic elements via a horizontal exchange, to become a highly adapted pathogen capable of survival and causing a range of diseases in humans and animals (Shames et al., 2009). Thus, the APEC might be virulence genes and antibiotic-resistant genes reservoir for human ExPEC strains. It is necessary to consider the zoonotic potential of APEC (Manges and Johnson, 2012). Although the pathogenic mechanisms of APEC have not yet been completely elucidated, insights into virulence factors of APEC are increasing, which helps to develop novel strategies for controlling APEC infections. In this review, we highlight the recent advances in zoonoses' potential characteristics, antibiotic resistance, and control strategies of APEC to provide guidance for the prevention and control of avian colibacillosis.

Avian colibacillosis associated with avian pathogenic *Escherichia coli*

Avian colibacillosis is an assembly of many extraintestinal infections in chickens and other birds with APEC as etiological agents (Barbieri et al., 2015). APEC is susceptible to inducing localized and systemic types of colibacillosis with two important infection stages. The primary stage of colibacillosis was identified as infections of the reproductive tract, omphalitis, and yolk sac. Salpingitis-peritonitis-salpingoperitonitis syndrome (SPS) causes reproductive tract infections with multiple and specific symptoms (Johnson et al., 2008). Omphalitis and yolk sac infection, which are caused by fecal contamination of eggs or egg formation, affect chicks with high mortality in poultry (Matthijs et al., 2009). APEC, in the secondary colibacillosis infections stage, assures an important role in bone and joint infections affecting poultry flocks (Nolan et al., 2003). Among the several types of colibacillosis, colisepticemia was identified as the most important systemic form. Colisepticemia occurs in birds under stress and weak immunosystems through the degradation of certain biotic and abiotic factors mostly high humidity, excess temperature, high dust, viral infections, and vaccines or virulent infectious bronchitis virus in the poultry (Matthijs et al., 2009).

Epidemiology of avian pathogenic *Escherichia coli*

Avian pathogenic *E. coli* has been known and reported as a principal etiologic agent of avian colibacillosis, responsible for significant morbidity and mortality, with resultant serious economic losses to the poultry industry in the world (Ronco et al., 2017). According to its impact worldwide, serotyping was the most approved method used frequently to estimate the pathogenic potential of APEC strains. *E. coli* strains belonging to somatic (O), capsular (K), and flagellar (H) antigens. The specific O-serotypes have close correlations with pathogenic *E. coli* strains (Kauffmann, 1947). Previous studies indicated that O78, O1, and O2 were the predominant serotypes of APEC, whereas, there are different prevalent serotypes in diverse countries according to geographic distribution (Ewers et al., 2004). A recent report that O145 may be emerging as a predominant serogroup of APEC in China (Wang et al., 2022). In addition, the *E. coli* strain could be assigned to one of the main phylo-groups (A, B1, B2, and D) (Clermont et al., 2000). Significantly, strains responsible for extraintestinal infection were far more likely to be members of phylo-groups B2 or D than A or B1 (Johnson and Stell, 2000). The ExPEC, including APEC strains, mainly belong to the phylogroup B2 and a lesser extent to group D (Smith et al., 2007).

Pathogenic mechanism and virulence factors of avian pathogenic *Escherichia coli*

Pathogenic mechanism

Colibacillosis is an important part of a respiratory infection that evolves to generalize infection resulting in fibrinopurulent lesions of internal organs (Kathayat et al., 2021). Pathogenic bacteria use many strategies to sustain themselves and overcome host barriers with the adhesion of the microorganism to host cells (Mellata, 2013). Colonization is a common step in the pathogenesis of pathogenic bacteria through the ability to adhere to the host surfaces and the successful replication in the respiratory tract (Mellata, 2013). APEC enters through the respiratory tract and uses adhesins to attach to the epithelial cells, followed by survival, invasion, and replication via the presence of the invasins and complementary defense mechanisms (**Figure 1**). Then, APEC enters the bloodstream, disseminates through the vital organs, such as the lung, heart, liver, and brain, and causes significant damage and lesions (Pourbakhsh et al., 1997). Finally, APEC leads the host to death or induces illness. The resistance to phagocytosis may be an important mechanism in the development of colisepticemia when a strong correlation was observed between pathogenicity for chickens *in vivo* and the ability to resist the bactericidal effects of chicken macrophages *in vitro* (Dho-Moulin and Morris Fairbrother, 1999).

Virulence factors of avian pathogenic *Escherichia coli*

During the process of APEC infection, various virulence factors, including adhesins, iron acquisition systems, protectins, toxins, invasins, metabolism, and secretion systems (**Table 1**), play important roles in the colonization and survival of APEC (Li et al., 2010).

Adhesins

Adhesins are a cell-surface system of bacteria that adhere to the epithelial cells during the initial stages of APEC infections (Kalita et al., 2014). Initial bacterial attachment or adhesion to host cells is vital to bacterial pathogenesis and is determined by various adhesins. There are many adhesins in APEC, including type 1 fimbriae, P fimbriae, S fimbriae, flagella, curli, outer membrane protein, non-fimbrial and atypical adhesins, and temperature-sensitive hemagglutinin (Aleksandrowicz et al., 2021). Type 1 fimbriae facilitate the adherence to epithelial cells of the respiratory tract during the primary stage (Ewers et al., 2007), whereas the expression of P fimbriae and S fimbriae contribute to the late infection. The application of normal anti-type 1 fimbriae serum and D-mannose, which are the cellular receptors of the adhesin of type 1 fimbriae, could block the specific adherence of APEC strains to chicken tracheal sections (Gyimah and Panigrahy, 1988). Curli induces resistance of APEC in the host's cecum and facilitates the bacterial invasion of the whole cells (Gophna et al., 2001). Temperature-sensitive hemagglutinin (Tsh) intervenes in the colonization in the first stage of respiratory tract infection (Kostakioti and Stathopoulos, 2004).

Iron acquisition systems

Iron acquisitions have a large operating system and contribute to the growth and proliferation of APEC in the host (Rodriguez-Siek et al., 2005b). Multiple siderophores, including aerobactin, salmochelin, yersiniabactin, and transporters to sequester iron from the body fluids, were reported in the APEC. In APEC, the proportion of these operons is increased and located on large plasmids (Johnson et al., 2006). APEC strains have an important frequency of aerobactin iron acquisition system compare to non-pathogenic strains (Dozois et al., 1994).

Protectins

Protectins are structural factors, including the K1 capsule, lipopolysaccharide (LPS), and certain outer membrane proteins (OMPs), which protect bacteria from the host immune system under stress conditions (Mellata et al., 2003). The capsule is an important virulence factor for the spread of APEC in the bloodstream and septicemia (Biran and Ron, 2018). Furthermore, the *iss* gene present in the ColV plasmid plays an important role in the bacteria serum survival. In addition, protectins also mediate APEC adhesion, invasion, intracellular survival, colonization, and proliferation in the host (Huja et al., 2015).

Toxins

Toxins are biologically harmful substances that intervene in the bacterial ability to invade and cause damage to the tissues. APEC produces a low quantity of toxins, including vacuolating autotransporter toxin (Vat), cytotoxic necrotizing factor 1 (CNF1), and various hemolysins (Rodriguez-Siek et al., 2005a). These toxins play role in colonization, motility, biofilm formation, agglutination, and induction of vacuolization. Vat causes cytotoxic effects in cultured cells and attenuates virulence when its deleted (Parreira and Gyles, 2003).

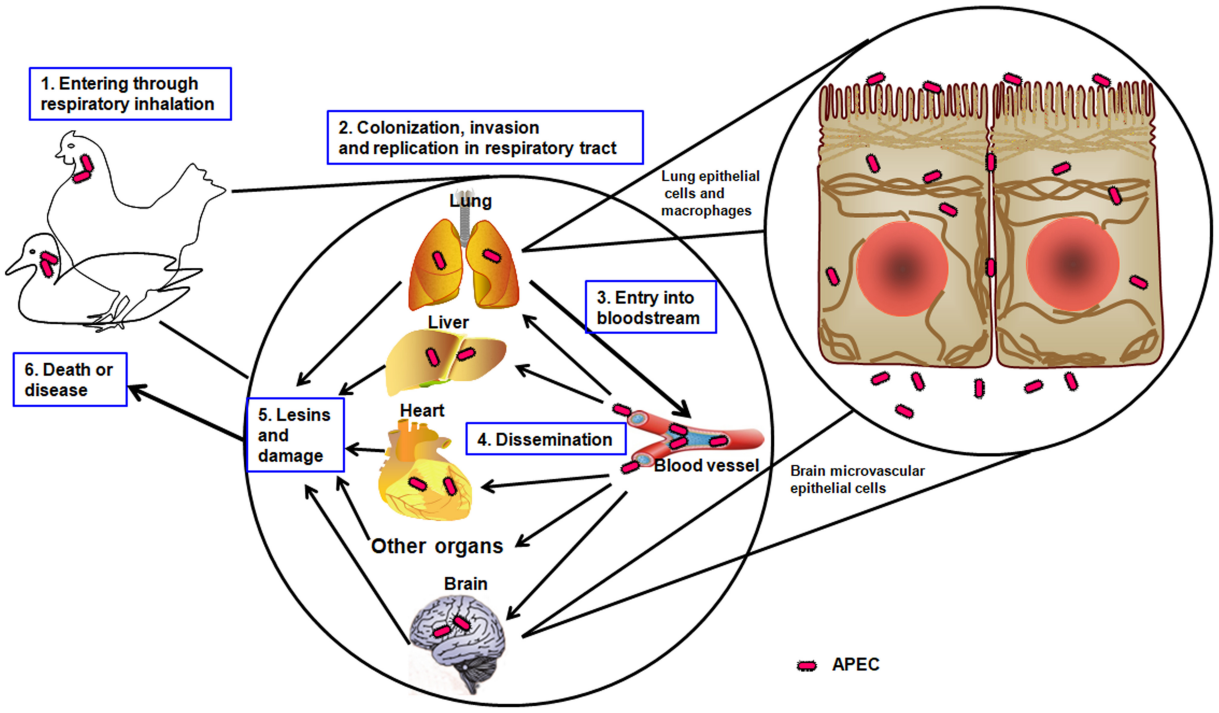

FIGURE 1
The pathogenic mechanism of APEC. The schematic illustrates the APEC infection progress in avians, such as chickens or ducks.

Invasins

Invasins facilitate the entrance of APEC into the host cells during the infection. Several genes encoding invasins, *ibeA*, *ibeB*, and *gimB* have been identified in NMEC, which contributed to the invasion of the brain microvascular endothelial cells (BMECs) (Germon et al., 2005). These invasins were present in APEC and contributed to resistance to oxidative stress, biofilm formation, colonization, and proliferation in the host (Germon et al., 2005; Wang et al., 2012).

Secretion systems

Secretion systems are needle-like structures used to secrete effector proteins, which contribute to bacterial survival and virulence (Wang et al., 2016a). Among the different bacterial secretion systems, two important secretion systems (types III and VI) were identified in APEC (Ma et al., 2014). Type III secretion system 2 (ETT2) is found frequently in pathogenic *E. coli* strains. The O1, O2, and O78 serotypes of APEC were identified as possessing important elements of ETT2 (Wang et al., 2016a). The intact and degenerate forms are identified in the O1, O2, and O78 serotypes (Wang et al., 2016b). However, the degenerative form of ETT2 may contribute to reducing the virulence and serum survival activity in bacteria (Wang et al., 2016a). Multiple components of ETT2 are involved in the pathogenicity of APEC (Wang et al., 2016a; Fu et al., 2021; Tu et al., 2021; Li et al., 2022; Xue et al., 2022; Yin et al., 2022). Our research shows that the transcriptional regulator DctR can regulate the expression of ETT2 and affect the virulence and pathogenicity of APEC (Zhang et al., 2021). In all, ETT2 has been discovered in APEC isolates and plays significant roles in bacterial virulence, adhesion, colonization, intracellular survival, serum bactericidal activity, and the downregulation of pro-inflammatory cytokine responses. The type VI secretion system (T6SS) is one of the recent nanomachine secretion systems present in Gram-negative pathogens (Yi et al., 2019). Two different forms such as multipurpose T6SS1 and conservative T6SS2 were discovered in APEC isolates. The T6SS1 intervened in the proliferation of APEC during infection, whereas T6SS2 played a role only for cerebral infection (Ma et al., 2014). Overall, the type VI secretion systems are specialized in interbacterial competition, stress sensing, biofilm formation, and virulence (Hachani et al., 2016). In addition, APEC and NMEC have similar T6SS which contributes to binding, and competition by using it to kill neighboring non-immune bacteria and pathogenesis of APEC and NMEC (Ma J. et al., 2018).

Two-component regulatory systems

Two-component systems (TCSs) are signaling proteins that play important roles in modulating bacterial fitness in different niches. Different TCSs such as CpxA/CpxR, BarA/UvrY, RstA/RstB, and PhoB/PhoR have been identified in APEC isolates (Tu et al., 2016). The CpxA/CpxR regulates surface structure assembly and stress response system implicated in APEC. In addition, CpxA/CpxR positively controls the expression of the APEC type VI secretion system 2 (Yi et al., 2019). BarA/UvrY regulates virulence properties in APEC through the adhesion, invasion, persistence, intracellular survival, resistance to serum bactericidal activity and oxidative stress, and regulation of exopolysaccharide production and expression of type 1 and P fimbriae (Palaniyandi et al., 2012). The RstA/RstB is a nitrogen metabolism TCS that contributes to iron acquisition, acid resistance, intracellular survival, and colonization (Gao et al., 2015b). The PhoB/PhoR is present in many bacterial species that respond to external phosphate concentrations and intervene in biofilm formation, motility, adhesion, invasion, and systemic dissemination (Bertrand et al., 2010).

The zoonotic potential of avian pathogenic *Escherichia coli* increases the risk of resistance

Avian pathogenic *Escherichia coli* is a potential reservoir for the contamination of human ExPEC

Several studies have shown the phylogenic similarity between APEC and human ExPEC isolates. According to phylogenetic classification, APEC isolates share significant genetic similarities with human ExPEC (Johnson et al., 2007). ExPEC (APEC, UPEC, and NMEC) share the same virulence factors and similar pathogenic mechanisms, and it may be spread between animals and humans (Johnson et al., 2008). The report demonstrated that ExPEC strains including APEC derived from specific STs may have a high zoonotic impact on humans (Johnson et al., 2008). Several specific virulence genes of the APEC strain, detected in UPEC plasmids, were shown susceptible to increasing the bacteria process to get iron in deficiency conditions (Ewers et al., 2007). Virulence genes that operate in ColV plasmids have similar functions in APEC and UPEC strains (Johnson et al., 2008). Moreover, APEC may induce high urinary infections in mice similar to UPEC and meningitis in rats similar to NMEC (Johnson et al., 2010). In addition, the ExPEC was recognized as a potential causal agent in women's health, newborns, elderly, and immunocompromised individuals in fact of an important number of urinary tract infections (UTIs), newborn meningitis, abdominal sepsis, and septicemia (Mellata, 2013).

As a foodborne pathogen, the emergence and transfer of antibiotic-resistance genes

Poultry is one of the most widely consumed meats in the world. APEC are causative agents of colibacillosis, one of the principal causes of morbidity and mortality in poultry worldwide (Nhung et al., 2017). Since poultry is usually raised under intensive conditions, infection transmission is favored, and the animals are more susceptible to diseases. So using large quantities of antimicrobials to prevent and treat disease, if overused or misused, lead to the evolution of bacteria and the rise of drug-resistant pathogens in the long term (Rahman et al., 2022). It is precisely due to the extensive use of antibiotics that APEC is severely resistant. Antibiotic-resistant APEC can not only lead to treatment failure, resulting in economic losses, but also be a source of resistant bacteria/genes that may represent a risk to human health (Nhung et al., 2017; Kim et al., 2021).

Avian pathogenic *E. coli* already carries many resistance genes and resistance to a lot of important antibiotics around the world. Colistin resistance of 2.2% was detected in isolates in Senegal, and colistin resistance of 8.7% was detected in isolates in Vietnam (Vounba et al., 2019). Particularly, there was a higher prevalence of *mcr-1* in isolates from chicken in Vietnam (53.2%), and the *mcr-1* gene was detected in 85% of 13 phenotypically colistin-resistant isolates (Vounba et al., 2019; Le et al., 2021). In addition, all colistin-resistant isolates exhibited multidrug-resistant phenotypes (Vounba et al., 2019). In Jordan, APEC resistance rates of sulfamethoxazole–trimethoprim, florfenicol, amoxicillin, doxycycline, and spectinomycin were 95.5, 93.7 93.3, 92.2, and 92.2%, respectively. At least five antibiotic-resistance genes were found in 68% of APEC isolates. The most important genes were *int1* 97%, *tetA* 78.4%, *bla*TEM 72.9%, *Sul1* 72.4%, and *Sul2* 70.2%; these resistance genes are detected in human pathogens (Ibrahim et al., 2019). Under commercial conditions in Portugal, the overall 10-year antibiotic resistance of APEC strains is amoxicillin 78%, ampicillin 73.5%, tetracycline 63.3%, doxycycline 56.4%, apramycin 34.5%, neomycin 68.2%, flumequine 39.4%, cotrimoxazole 47.7%, florfenicol 46.6%, and lincospectin 66.3% (Oliveira et al., 2022). In China, the prevalence of extended-spectrum cephalosporin-resistant strains in *E. coli* from chicken colibacillosis and raw meat separately accounted for 66.1% and 71.2% (Wang et al., 2021).

TABLE 1 Validated virulence factors in APEC.

Name/Description	Functions	Present in ExPEC	References
Adhesins			
Type I fimbriae	Colonization, biofilm formation	APEC, NMEC, SEPEC UPEC,	Ewers et al., 2007
P fimbriae	Colonization, stimulate of cytokines production,	APEC, UPEC, SEPEC,	Kariyawasam and Nolan, 2009
Flagella (FliC)	Colonization, biofilm formation	APEC, UPEC	Dziva et al., 2013
Curli	Colonization, biofilm formation	APEC, UPEC, SEPEC	La Ragione et al., 2000
Temperature sensitive hemagglutinin (Tsh)	Adherence	APEC, UPEC, NMEC	Kostakioti and Stathopoulos, 2004
Iron acquisition			
Aerobactin	Siderophore, acquisition of iron	APEC, UPEC	Gao et al., 2015a
Salmochelin	Siderophore receptor, use of Fe irons	APEC, NMEC, SEPEC, UPEC	Caza et al., 2008
SitABC	Transportation of Fe, Mn	APEC, UPEC	Sabri et al., 2008
Antiphagocytic activity/serum resistance			
Transfer protein	Inhibition of the classical pathway of complement activity	APEC, NMEC, SEPEC	Sarowska et al., 2019
Capsule	Resistance to human alpha-defensin 5	APEC, NMEC, UPEC	Thomassin et al., 2013
LPS	Reduce the environmental acidity	APEC	Yu et al., 2015
Increased serum survival (Iss)	Protect against phagocytosis	APEC, NMEC, SEPEC	Nolan et al., 2003
Toxins			
Vacuolating autotransporter toxin	Induce vacuolization of host cell	APEC, UPEC	Ewers et al., 2004
Serin protease autotransporter	Epithelium colonization, mucins degradation	APEC, UPEC	Pokharel et al., 2019
Invasins			
IbeA	Invasion, resistance to oxidative stress	APEC, NMEC, SEPEC	Cortes et al., 2008
IbeB	Invasion	APEC, NMEC	Wang et al., 2012
GimB	Adherence and invasion	APEC, NMEC, UPEC	Matter et al., 2015
Two component regulatory systems			
CpxA/CpxR	Fitness, virulence	APEC, UPEC	Yamamoto and Ishihama, 2006
BarA/UvrY	Biofilm formation, persistence	APEC, UPEC	Herren et al., 2006
RstA/RstB	Acid resistance, intracellular survival	APEC	Gao et al., 2015b
PhoB/PhoR	Intracellular survival	APEC	Bertrand et al., 2010
Secretion systems			
E. coli type III secretion system 2 (ETT2)	Virulence, intracellular survival	APEC, NMEC, UPEC	Wang et al., 2016a; Fu et al., 2021; Li et al., 2022; Xue et al., 2022; Yin et al., 2022
Type VI secretion system	Interbacterial competition, stress sensing, virulence	APEC, NMEC	Hachani et al., 2016

Previous studies indicated that APEC strains found in poultry are shown to be important reservoirs for antibiotic resistance genes (Nandi et al., 2004). Antibiotic resistance occurs with high complexity in presence of resistance-encoding genes that are found inside plasmids or chromosomal genetic material (Ibrahim et al., 2019). The antibiotic resistance genes are identified on mobile genetics elements enabling their rapid transfer among the ExPEC strains. Furthermore, animal reservoirs may be responsible for human contamination or transfer of APEC antibiotic-resistant and other commensal bacteria through the contaminated food in poultry (Hannah et al., 2009). Investigations from the poultry farm revealed the use of multiple antibiotics may present significant resistance among *E. coli* (Johar et al., 2021). The *mcr-1* gene, found in APEC, exhibited colistin resistance, demonstrating its role in colistin resistance (Eltai et al., 2018). In addition, APEC carrying the *mcr-1* gene was isolated from septicemic chickens, which may increase the difficulty of prevention and control of poultry septicemia (Ewers et al., 2016). The research has shown that β-lactamase *CTX-M*, *OXA*, *CMY,* and *TEM* genes were widespread in chicken-source *E. coli*, and *bla*CTX-M was the most predominant ESBL gene (Wang et al., 2021). The studies identify resistance genes such as *floR, cmlA, cat1, cat2, tetA, tetB, tetC, tetD, tetE, tetG, sul1, sul2, addA1*, and *addA2* among APEC isolates (Li et al., 2007; Shin et al., 2015; Ibrahim et al., 2019). The presence of the resistance gene in isolates from poultry and marketed retail meats further complicates the APEC antibiotic resistance situation and is a possible health risk for humans.

Diagnosis, prevention, and control of avian pathogenic *Escherichia coli* infection

Diagnosis

Colibacillosis infections are suspected to focus on the clinical signs and the presence of typical macroscopic lesions (Gomis et al., 1997). The laboratory diagnosis must be confirmed in the presence of such clinical signs and lesions, including traditional bacterial isolation, virulence gene detection, and serotyping. The diagnosis occurs in different regions of an infected animal such as cardiac blood and affected tissues, liver, spleen, and pericardium. Samples of the lesion were collected and prepared. Selective media like McConkey, eosinmethylene blue, or drigalki agar are used for isolation. The antigenic identification and virulence genes of isolated strains were detected by PCR; specific antigen and virulence gene detection is beneficial to identify APEC (Antão et al., 2009). Traditional biochemical reactions and ELISA methods for APEC identification are costly and time-consuming. Therefore, the PCR method has been found to be a fast and effective common technique for APEC detection (Wang et al., 2014; Lucas et al., 2022).

Prevention and control

While some families of antibiotics used as a treatment in poultry such as tetracyclines, penicillins, and aminoglycosides are also commonly administered to humans to treat bacterial infections. Therefore, the drug resistance of APEC will threaten the choice of drugs when human beings are infected with bacteria. Other multitude methods such as biosecurity measures and vaccination are also necessary for the prevention and control of infections (Wang et al., 2017).

Management and biosecurity measures

Effective prevention and control of APEC infections depend on the identification and elimination of predisposing causes of the disease. Maintaining flock biosecurity is difficult to control and prevent (Dziva and Stevens, 2008). The main objective is to reduce the level of APEC exposure by improving biosecurity, good litter, and ventilation conditions in poultry (Dziva and Stevens, 2008). The sanitation of the environmental system should be improved. Furthermore, the reducing of fecal contamination of eggs, cleaning nest boxes, and decreasing the number of floor eggs contribute to reducing the incidence risk of colibacillosis infections (Dziva and Stevens, 2008). The research shows that vitamin E has been able to interfere with bacterial biofilm and prevent *in vitro* biofilm formation (Vergalito et al., 2018). It is possible to increase the level of vitamin E in the nutrition system to prevent APEC infection.

Antibiotics for treatment

Generally, antibiotics are wildly used to prevent and treat APEC infections. The application of these antibiotics was reported to accelerate the emergence of multidrug-resistant bacteria (Rahman et al., 2022). Seriously, APEC's high levels of resistance to important antibiotics may pose a high risk to humans, because antibiotic-resistant bacteria and genes can be transmitted through the food chain to humans. Previous studies demonstrated that APEC isolates were resistant to multiple antibiotics. Thus, it is crucial and helpful to perform antibiotic susceptibility testing for the appropriate antibiotic in the treatment of avian colibacillosis (Bass et al., 1999).

Vaccines

Avian pathogenic *E. coli* infections of poultry result in significant morbidity and mortality with important economic losses. Therefore, many efforts have been made to develop effective vaccines, including inactivated vaccines, subunit vaccines, and live attenuated vaccines against APEC infections (Nesta and Pizza, 2018). **Table 2** shows the of vaccines development against APEC infection with their main findings.

Inactivated vaccines

Inactivated vaccines were developed earlier to provide the effectiveness of vaccines against homologous and heterologous challenges (Gross, 1957). These vaccines were made from inactivated predominant APEC serotype strains to control colibacillosis. The inactivated vaccines were observed to provide efficacy protection against only homologous challenges (Deb and Harry, 1978). The efficacy of the inactivated vaccines is determined by diverse parameters such as the serotypes of *E. coli* include in the vaccine, the administration methods, age of the birds, and the dose of vaccine administered to the birds (Russo et al., 2003).

TABLE 2 Summary of vaccines development against APEC infection.

Antigens[a]	Immunity route[b]	Challenge route[b]	Outcome by homologous challenge[c]	Outcome by heterologous challenge[d]	References
Inactivated vaccine					
O78	SC, IM, IP	IM or IV	Protective	Not protective	Deb and Harry, 1976
O2	SC, IM	SC	Protective	Not protective	Deb and Harry, 1978
O1	SC	Air sac	Protective	Not protective	Gyimah and Panigrahy, 1985
O1, O2, and O78	IM	N/A	Protective	N/T	Panigraphy et al., 1984
O2; O78	SC, SC	SC; SC	Protective	Protective; not protective	Melamed et al., 1991
O2, O78, and O35	SC	IT	Protective	Not protective	Rosenberger et al., 1985
Subunit vaccines					
Aerobactin	IM	Aerosol	Protective	Not protective	Le Roy et al., 1995
IROMPs	IV	Air sac	Protective	N/T	Bolin and Jensen, 1987
SRP	SC	IV	Protective	Protective	Russo et al., 2003
Pilus	SC	Air sac	Protective	N/T	Gyimah and Panigrahy, 1985
FimA	IM	Air sac	Protective	Protective	van den Bosch et al., 1993
FimH	IM	Air sac or aerosol	Not protective	Not protective	Kariyawasam et al., 2002
PapG	IM	Air sac	Protective	Protective	Kariyawasam et al., 2002
IutA	IM	Air sac	Protective	Protective	Kariyawasam et al., 2002
Iss	IM	Air sac	Protective	Protective	Lynne et al., 2012
rOmpA and rFliC, recombinant GroEL	IM	Sterile water	Protective	Not protective	Bao et al., 2013
Live attenuated vaccines					
Non-pathogenic *E. coli* O78	Aerosol	Aerosol	Not protective	N/T	Azeem et al., 2017
Non-pathogenic piliated *E. coli* (BT-7)	Aerosol, drinking water	IT	N/A	Protective	Ghunaim et al., 2014
ΔcarAB	IM	IM	N/A	Protective	Frommer et al., 1994
Δcya Δcrp	Oral	IT	Protective	N/T	Kwaga et al., 1994
Δcya Δcrp	Spray, Oral	IT	Protective	N/T	Roland et al., 1999
ΔgalE	Spray	Aerosol	Protective	N/T	Peighambari et al., 2002
DE17ΔaroAΔluxS	Spray	Aerosol	Protective	Not protective	Kariyawasam et al., 2004
ΔpurA	IM	Aerosol	Protective	Protective	Holden et al., 2014
ΔaroA	Spray	Aerosol	Not protective	Not protective	Kariyawasam et al., 2004
O78:K80	Spray	Aerosol	Protective	Not protective	Kariyawasam et al., 2004
Other vaccines					
Bacterial ghost	Spray, IM	Air sacs, IM	Protective	Not protective	Ebrahimi-Nik et al., 2018; Hu et al., 2019
Salmonella delivery FimA, OmpC, O78	Oral gavage	IM or air sac	Protective	Protective	Han et al., 2018
Lactobacillus strains expressing PapA, PapG, IutA, CS31A	Intragastric	Oral	Protective	Not protective	Ma S. T. et al., 2018
Outer membrane vesicles	IM	Air sac	Protective	Not protective	Wang et al., 2019

[a]SRP, siderophore receptor protein; IROMPs, iron-regulated outer membrane proteins; Iss, increased serum survival protein.
[b]IV, intravenous; IM, intramuscular; SC, subcutaneous; IP, intraperitoneal; IT, intratracheal; N/A, not applicable or not available.
[c]N/A, not applicable or not available.
[d]N/T, not tested; N/A, not applicable or not available.

Subunit vaccines

Subunit vaccines were produced to overcome the limit of inactivated vaccines, which were unable to protect chickens against the heterologous challenge (Bolin and Jensen, 1987). Several recombinant subunit antigens intervene to produce subunit vaccines for successful protection against heterologous challenges (Bolin and Jensen, 1987). The recombinant subunit vaccines generate strong antibody responses in recipient

birds when administered parenterally with adjuvant-containing formulations (Kariyawasam et al., 2002). Subunit vaccines may provide broader protection to more serotypes of APEC. The understanding of APEC genome sequences and pathogenic virulence genes may contribute to the development of more new subunit vaccines (Ghunaim et al., 2014).

Live-attenuated vaccines

Live vaccines are available for numerous viral, bacterial, and coccidial organisms. Live vaccines are effective and relatively economical (Frommer et al., 1994). The most successful APEC live attenuated vaccine is the *aroA* gene mutant vaccine. The live Poulvac® *E. coli* (Zoetis) vaccine includes an *aroA* mutant of a strain of serotype O78:K80 and ST23, and the *aroA* mutation attenuates the virulence of the strain and results in a requirement for aromatic amino acids, which results in reduced survival of the strain in the chicken and the environment (La Ragione et al., 2013; Han et al., 2015). The live vaccines are generally short-lived after first or initial exposure to the immunity system. These vaccines generally reduced the systemic lesions by mass administered drinking water, spray, and oral (Barbieri et al., 2012). Even though the live attenuated vaccine provides clinical protection against the challenge, it is not able to prevent completely pathological lesions (El-Mawgoud et al., 2020). Live vaccines may induce a high risk of reversion to natural virulence via back-mutations of the attenuated organism and susceptible to causing symptomatic affection (Ghunaim et al., 2014).

Other vaccines

After evaluating the efficacy of inactivated vaccines, subunit vaccines, and live attenuated vaccines, various recombinant vaccines have been investigated to protect chickens against APEC infections (Ghunaim et al., 2014). Within the tested vaccines, multiple vaccines such as outer membrane vesicles (OMVs), bacterial ghost (BG) vaccines, *Salmonella*-delivered vaccines containing APEC antigens, such as FimA and OmpC, were able to reduce the mortality and morbidity, APEC lesions as well as stimulate the antibody (immunoglobulins; IgG and IgA) responses in immunized chickens (Ebrahimi-Nik et al., 2018). In our previous study, the BG vaccine was able to achieve over 90% immune protection against virulent challenges using the same serotype O2 strain, while it showed poor cross-protection against serotypes O1 and O78 (Hu et al., 2019). Further research is needed to provide cross-protection rates between serotypes.

Conclusion and outlook

Avian pathogenic *E. coli* is considered responsible for multi-factorial illness and causes significant economic losses in the poultry industry over the world. APEC antibiotic resistance is serious, which increases the opportunity to transmit antibiotic resistance genes from APEC to human pathogens. Investigations are necessary to provide concrete evidence for the zoonotic transmission of APEC to humans. However, we highlight the crucial roles played by the different virulence factors; further investigations and studies are suggested to understand the contribution of virulence factors in APEC virulence. We should continue the efforts to identify more potential virulence factors and reveal the pathogenic mechanism, thus helping to develop a novel diagnosis method and vaccines to control avian colibacillosis. The virulence factors and drug resistance genes of APEC can be prevented from being transmitted to humans through food animals and endangering human health.

Author contributions

JH, DA, and SW: conceptualization and original draft writing—review and editing. BZ, HZ, LY, WG, XW, ZW, DW, HP, MT, and JQ: helped in revising. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Whole-genome sequencing reveals genomic characterization of *Listeria monocytogenes* from food in China

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Introduction: *Listeria monocytogenes* is a foodborne bacterium that could persist in food and food processing environments for a long time. Understanding the population structure and genomic characterization of foodborne *L. monocytogenes* is essential for the prevention and control of listeriosis.

Methods: A total of 322 foodborne *L. monocytogenes* isolates from 13 geographical locations and four food sources in China between 2000 and 2018 were selected for whole-genome sequencing.

Results: *In silico* subtyping divided the 322 isolates into five serogroups, 35 sequence types (STs), 26 clonal complexes (CCs) and four lineages. Serogroup IIa was the most prevalent serogroup and ST9 was the most prevalent ST of foodborne *L. monocytogenes* strains isolated in China. The in-depth phylogenetic analysis on CC9 revealed that ST122 clone might be original from ST9 clone. Furthermore, 23 potentially relevant clusters were identified by pair-wised whole-genome single nucleotide polymorphism analysis, indicating that persistent- and/or cross-contamination had occurred in markets in China. ST8 and ST121 were the second and third top STs of *L. monocytogenes* in China, which had heterogeneity with that of *L. monocytogenes* isolates from other countries. The antibiotic resistance genes *aacA4*, *tetM*, *tetS*, *dfrG* carried by different mobile elements were found in *L. monocytogenes* strains. One lineage II strain carrying Listeria Pathogenicity Island 3 was first reported. In addition, a novel type of premature stop codon in *inlA* gene was identified in this study.

Discussion: These findings revealed the genomic characteristics and evolutionary relationship of foodborne *L. monocytogenes* in China on a scale larger than previous studies, which further confirmed that whole-genome sequencing analysis would be a helpful tool for routine surveillance and source-tracing investigation.

KEYWORDS

***Listeria monocytogenes*, foodborne, whole-genome sequencing, genomic characterization, inlA PMSCs**

Introduction

Listeria monocytogenes, as an important foodborne pathogen, is the causative agent of human listeriosis with clinical symptoms ranging from self-limiting gastroenteritis to severe invasive infections (Swaminathan and Gerner-Smidt, 2007). It has a significant impact on public health around the world due to high mortality rate in hospitalized patients (Schlech III, 2019). Immunocompromised individuals, pregnant women, neonates and the elderly are often at high risk for the aggressive forms of listeriosis (Wilking et al., 2021). *L. monocytogenes* is widely distributed in nature and more commonly found in a variety of food products and food-associated environments (Wang et al., 2019). This bacterium can contaminate food at any time from production to consumption. It can also shed into the environment, colonize and multiply, and then cause persistent contamination and/or cross-contamination. A number of research have shown that epidemic and sporadic listeriosis were often associated with consumption of *L. monocytogenes* contaminated foods (McLauchlin et al., 2021a,b). Food contamination caused by *L. monocytogenes* poses a serious threat to food safety and public health worldwide.

L. monocytogenes has evolved into four lineages, lineage I to lineage IV. Lineage I, including serotypes 1/2b, 3b, 4b, 4d, 4e, and 7 occurs frequently among human cases of listeriosis. While lineage II including serotypes 1/2a, 3a, 1/2c, and 3c is overrepresented in food products and food-associated environments. Lineage III and IV, corresponding serotypes 4a, 4c, and 4ab, are rare and commonly isolated from animal sources (Leclercq et al., 2011). Doumith et al. developed a multiplex PCR method to classify *L. monocytogenes* strains into five serogroups by five marker genes (*lmo0737*, *lmo1118*, *ORF2819*, *ORF2100* and *prs*), namely serogroups IIa (serotypes 1/2a and 3a), IIb (serotypes 1/2b, 3b, and 7), IIc (serotypes 1/2c and 3c), IVb (serotypes 4b, 4d, and 4e) and L (4a, 4ab, and 4c; Doumith et al., 2004, 2005). Multi-locus sequence typing (MLST) remains a practical and portable method to meet the data exchange demands of different laboratories around the world (Salcedo et al., 2003). MLST can divide *L. monocytogenes* isolates into large number of sequence types (STs), which can further be grouped into serval clonal complexes (CCs). A CC of *L. monocytogenes* is defined as a group of STs, where each ST shares six of seven identical alleles with one other ST in the group (Salcedo et al., 2003). CC1, CC2, CC4, and CC6 were hypervirulent clones and strongly associated with listeriosis outbreaks. In contrast, CC9 and CC121 were hypovirulent clones and overrepresented in food sources (Maury et al., 2019).

However, traditional molecular subtyping methods, such as MLST and pulsed-field gel electrophoresis (PFGE), have insufficient discriminative power for source-trace investigations of *L. monocytogenes* contamination (Moura et al., 2016). The main reason for this is the conserved genomic backbone and the limited resolution of the technologies used (Moura et al., 2016). Whole-genome sequencing (WGS) has been widely used in the investigation of listeriosis outbreaks for a decade, for example, it facilitated to identify ST6 *L. monocytogenes* as the causative agent of the listeriosis outbreak in South Africa and guided the traceback investigations (Thomas et al., 2020). Furthermore, WGS analysis is of great significance for both biological and epidemiological studies on *L. monocytogenes*, through which we can obtain more details of molecular characteristics and refine the genetic relationship among them. *L. monocytogenes* genome-based typing and population biology study at the national level is lacking in China, although related projects have been carried out in part of local regions. A 10-year surveillance of *L. monocytogenes* in Shanghai found CC87 isolates persist in different food groups (Zhang et al., 2020). Another study found *L. monocytogenes* isolated from pork in Wuhan differed by few SNPs indicated a common supply source contamination (Wang et al., 2021). However, there are no reports analyzed isolates from the whole country or multiple provinces in China. Here, we sequenced 322 *L. monocytogenes* isolates from 13 different provinces/cities across China to determine (i) the population structure and diversity of *L. monocytogenes* from food in China based on whole-genome sequencing, (ii) the distribution of the predominant clones in the global context of corresponding clones, and (iii) the genetic characteristics, including virulence factors, stress survival genes and antibiotic resistance genes, of food-source *L. monocytogenes* in China.

Materials and methods

Selection of *Listeria monocytogenes* isolates in this study and public genomes for comparison

A total of 322 foodborne *L. monocytogenes* isolates from 12 provinces and one municipality in China between 2000 and 2018 were chosen in this study. These strains were isolated from four types of food including raw meat ($n = 107$), raw poultry ($n = 81$), ready-to-eat (RTE) food ($n = 44$), and aquatic products ($n = 31$). There were also 59 isolates recorded as unknown food sources. Details of each isolate were listed in Supplementary Table S1.

To examine the relationship between the isolates of the prevalent subtypes (ST9, ST8, ST121, and ST87) in China and the isolates from other countries, a total of 310 genomes available from GenBank were selected for comparative analysis. These genomes included 79 isolates from China and 231 isolates from 17 other countries, and the detailed information was listed in Supplementary Table S2.

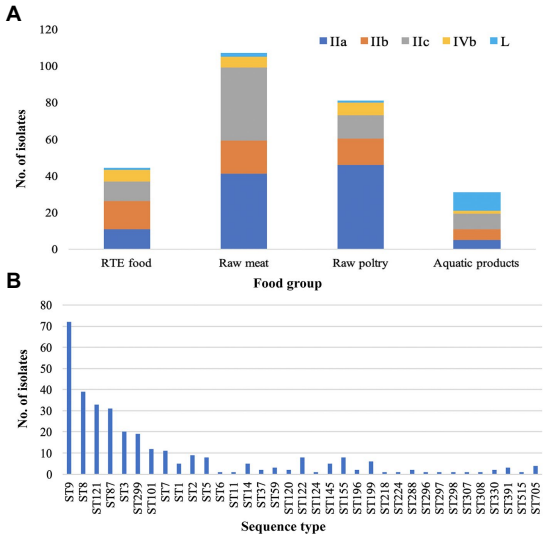

FIGURE 1
Distribution of *Listeria monocytogenes*. **(A)** Distribution of five serogroups (IIa, IIb, IIc, IVb and L) in four main food groups. **(B)** Distribution of sequence types (STs) in the 322 *L. monocytogenes* isolates.

DNA extraction and whole-genome sequencing

All *L. monocytogenes* isolates were streaked on Brain Heart Infusion agar plates and incubated at 37°C for 18 h. Genomic DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega, United States) according to the instructions of the manufacturer for Gram-positive bacteria. DNA quality and concentration were determined using NanoDrop Spectrophotometer (Thermo Scientific, United States). WGS was performed on Illumina HiSeq X PE150 platform by Novegene (Beijing, China), with a coverage rate of more than 100-fold. Low-quality reads, ambiguous sequences and adapter sequences were filtered using FastQC. Then, SOAP *de novo* v2.04 was used to assemble genome sequence. Whole genome annotation was performed using Prokka pipeline v1.14.6.

In silico subtyping and phylogenetic analysis

Serogroup identification and MLST analysis were performed *in silico* on BIGSdb-Lm platform.[1] Snippy pipeline v4.6.0 was used to generate core genome SNPs by mapping genome sequences with the reference genome sequence EGD-e (NZ_CP023861.1). The recombination was removed by Gubbins pipeline, whole-genome phylogeny of strains was inferred by FastTree. Then the core-SNPs phylogenetic tree was visualized and edited by online software iTol[2] and Phandango[3] (Hadfield et al., 2018; Letunic and Bork, 2021). Roary v3.13.0 was used to define the pan genome of strains using the outputs of Prokka annotation. Pan-genome were classified according to the principles of previously reported (Page et al., 2015). The phylogenetic tree for pan-genome was visualized using online software Phandango.

Identification of genes for stress resistance, virulence factors and antibiotic resistance

According to BIGSdb-Lm database, BLASTN algorithm was used to detect genes encoding stress resistance, virulence factors and antibiotic resistance, with the minimum of 80% coverage and 80% identity (Camargo et al., 2019).

Results

Characteristics of whole genome sequencing

The high-quality reads of each isolate with an average coverage above 100X were assembled to draft genome sequences. The genome assemblies contained seven to 48 scaffolds with the sizes ranging from 2.8 Mbp to 3.2 Mbp and the GC content about 37.87%. There were 2,748 to 3,243 protein-coding sequences predicted and annotated in the genomes in this study (Supplementary Table S3).

Serogroup identification and MLST *in silico*

Five serogroups were identified among 322 *L. monocytogenes* isolates based on five genes (*lmo0737*, *lmo1118*, *ORF2110*, *ORF2819*, and *prs*), including serogroup IIa ($n = 131$, 40.68%), IIb ($n = 68$, 21.12%), IIc ($n = 80$, 24.84%), IVb ($n = 24$, 7.45%), and L ($n = 19$, 5.90%). Serogroup distributions of *L. monocytogenes* isolates were different in four food types (Figure 1A). Serogroups IIa, IIb, IIc and IVb accounted for a similar proportion of isolates from RTE food. Serogroup IIa and IIc isolates were predominant in raw meat with the frequency of 38.31 and 37.38%, respectively. Serogroup IIa isolates were overrepresented in raw poultry with a frequency of 56.78%. For aquatic products, serogroup L was more common with a frequency of 32.26% than other four serogroups.

Based on seven-genes MLST (Salcedo et al., 2003), all the studied isolates were classified into 35 STs which were further grouped into 26 CCs. ST9 was the predominant ST ($n = 72$,

1 https://bigsdb.pasteur.fr/listeria

2 https://itol.embl.de/

3 http://jameshadfield.github.io/phandango/

22.36%), followed by ST8 ($n = 39$, 12.11%), ST121 ($n = 33$, 10.25%), ST87 ($n = 31$, 9.63%), ST3 ($n = 20$, 6.21%), ST299 ($n = 19$, 5.90%), ST101 ($n = 12$, 3.73%), and ST7 ($n = 11$, 3.42%). The remaining 27 STs contained less than 10 isolates for each (Figure 1B). Overall, 28.57, 65.53 and 5.90% of isolates were assigned to lineage I, lineage II and lineage III, respectively.

Core-SNP phylogenetic analysis

A maximum-likelihood phylogenetic tree was constructed based on the alignment of 16,383 core-SNPs that were identified by Snippy (Figure 2). Three clusters corresponding three lineages were displayed. In addition, the phylogenetic tree also showed that the genomes clustered based on CCs. Notably, all ST122 isolates belong to a sub-clade located within the clade of ST9, not next to the clade of ST9 (Supplementary Figure S1A). To clarify the refine relationship of ST9 and ST122, the phylogenetic analysis was performed based on the SNPs among genomes of the two STs isolates in this study and four ST122 genomes from public database (Accession numbers: GCA_002114825.1, GCA_002114845.1, GCA_002250305.1, and GCA_009664775.1; Supplementary Figure S1B). Another tree was constructed based on the binary presence and absence of accessory genes when the analysis of pan-genome was performed using Roary (Supplementary Figure S1C). Both the trees showed that a subset of ST9 isolates were phylogenetically closer to ST122 isolates than the other ST9 isolates, in other words, these ST122 isolates had a recent common ancestor with a subset of ST9 isolates.

Pair-wised wgSNP analysis of closely related isolates

Based on the above phylogenetic analysis, there were 87 core-SNP profiles represented by more than one isolates. To zoom in the differences among isolates within each profile, pair-wised SNP analysis was performed by Snippy separately. The numbers of SNP differences among isolates were minimal suggesting potential epidemiological links (Blanc et al., 2020). Twenty-three potentially relevant clusters were ≤ 12 pair-wised SNPs (Zhang et al., 2021; Table 1). These potential related isolates assigned into 10 STs with the top three STs of ST9, ST8, and ST299. Among these possible contamination events, eight clusters (Cluster 1, 3, 4, 7, 9, 10, 21, and 22) were caused by the isolates from different sources (market, farm or restaurant) in the same province in the same year, six clusters (Cluster 5, 6, 13, 17,19, and 23) were caused by the isolates from different food products from the same market

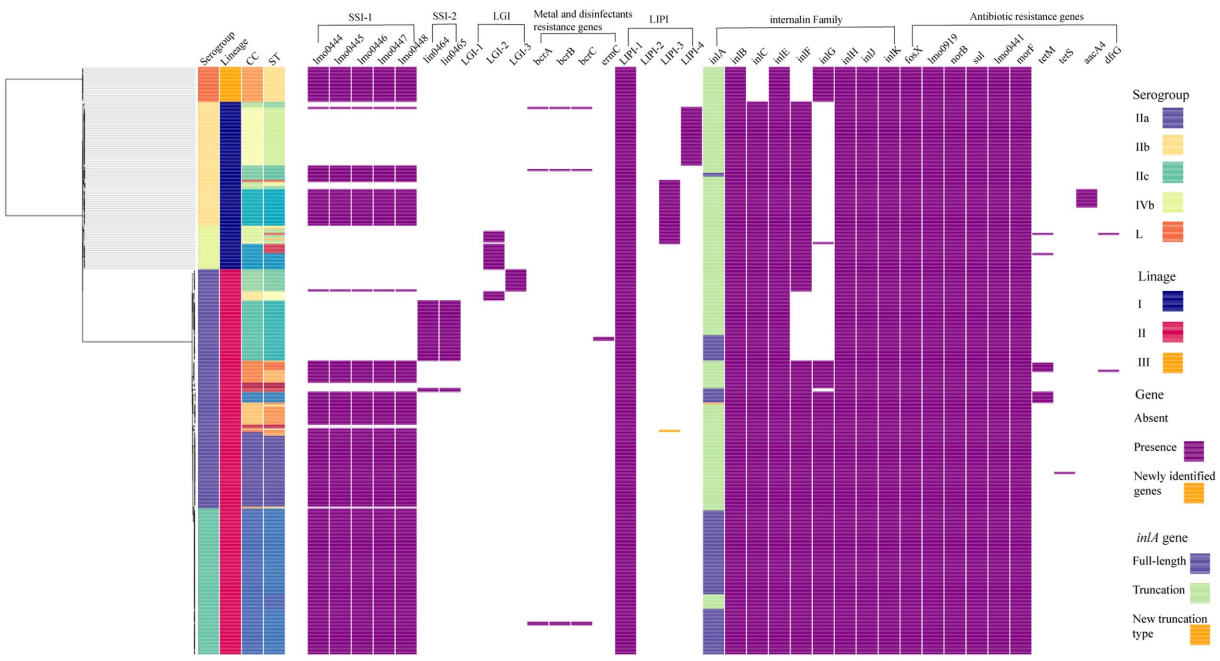

FIGURE 2
The core-SNP phylogenetic tree and genomic characterization of 322 foodborne *L. monocytogenes* isolates from China. Maximum likelihood phylogeny of 322 *L. monocytogenes genomes based* on 16,383 core-SNPs with EGD-e as the reference. Information of serogroup (dark purple boxes represent serogroup IIa, light orange boxes represent serogroup IIb, blue-green boxes represent serogroup IIc, light green boxes represent serogroup IVb, dark orange boxes represent serogroup L), lineage (dark blue boxes represent lineage I, rose red boxes represent lineage II, orange boxes represent lineage III), clonal complex and sequence type (they were clustered by different colors) are provided on the right. The distribution of different genes in *L. monocytogenes* was visualized using Phandango. The presence of a gene was marked with a purple box. The newly identified genes were marked with an orange box. For *inlA* gene, light green boxes represent full-length *inlA* gene, dark blue boxes represent truncated *inlA* gene.

TABLE 1 Strains used for pair-wised wgSNP analysis.

Cluster	ST	Isolate ID	Time	Province[a]	Market[b]	Source	Food group	Number of SNP[c]
1	ST9	ICDC_LM3007	2017	YN	M1	Minced pork	Raw meat	5
	ST9	ICDC_LM3008	2017	YN	M2	Cooked meat products	RTE Food	R
2	ST9	ICDC_LM172	2008	AH	M3	Beef	Raw meat	7
	ST9	ICDC_LM176	2010	AH	M4	Fish	Aquatic products	R
3	ST9	ICDC_LM265	2010	AH	M5	Duck	Raw poultry	3
	ST9	ICDC_LM266	2010	AH	M6	Chicken	Raw poultry	R
4	ST9	ICDC_LM340	2011	AH	M5	Pork	Raw meat	5
	ST9	ICDC_LM426	2011	AH	R1	Cooked meat products	RTE Food	R
5	ST9	ICDC_LM316	2009	ZJ	M7	Pork	Raw meat	1
	ST9	ICDC_LM318	2009	ZJ	M7	Beef	Raw meat	R
6	ST9	ICDC_LM321	2009	ZJ	M8	Chicken	Raw poultry	4
	ST9	ICDC_LM328	2009	ZJ	M8	Fish	Aquatic products	R
7	ST9	ICDC_LM180	2008	AH	M9	Fish	Aquatic products	4
	ST9	ICDC_LM184	2008	AH	M10	Fish	Aquatic products	R
8	ST8	ICDC_LM187	2008	AH	M5	Rabbit	Raw meat	11
	ST8	ICDC_LM267	2010	AH	M11	Chicken	Raw poultry	R
9	ST8	ICDC_LM480	2012	AH	M12	Beef	Raw meat	0
	ST8	ICDC_LM481	2012	AH	M13	Beef	Raw meat	R
10	ST8	ICDC_LM288	2007	ZJ	M14	Pork	Raw meat	8
	ST8	ICDC_LM300	2007	ZJ	M15	Chicken	Raw poultry	R
11	ST8	ICDC_LM2659	2009	AH	M9	Chicken	Raw poultry	0
	ST8	ICDC_LM2661	2013	AH	F1	Fish	Aquatic products	R
12	ST8	ICDC_LM186	2008	AH	M13	Chicken	Raw poultry	R
	ST8	ICDC_LM2640	2009	AH	R2	Cooked meat products	RTE Food	0
	ST8	ICDC_LM2641	2009	AH	R2	Cooked meat products	RTE Food	0
13	ST8	ICDC_LM320	2009	ZJ	M8	Chicken	Raw poultry	4
	ST8	ICDC_LM327	2009	ZJ	M8	Fish	Aquatic products	R
14	ST155	ICDC_LM292	2007	ZJ	M14	Pork	Raw meat	6
	ST155	ICDC_LM304	2008	ZJ	M16	Chicken	Raw poultry	R
15	ST705	ICDC_LM291	2007	ZJ	M14	Pork	Raw meat	2
	ST705	ICDC_LM303	2008	ZJ	M14	Pork	Raw meat	R
16	ST101	ICDC_LM114	2005	ZJ	M17	Chicken	Raw meat	1
	ST101	ICDC_LM179	2008	AH	F2	Crayfish	Aquatic products	R

(Continued)

TABLE 1 (Continued)

Cluster	ST	Isolate ID	Time	Province[a]	Market[b]	Source	Food group	Number of SNP[c]
17	ST121	ICDC_LM286	2007	ZJ	M14	Pork	Raw meat	1
	ST121	ICDC_LM297	2007	ZJ	M14	Beef	Raw meat	R
18	ST299	ICDC_LM561	2006	NX	M18	Fish	Aquatic products	5
	ST299	ICDC_LM2644	2009	AH	M6	Fish	Aquatic products	R
19	ST299	ICDC_LM190	2008	AH	M3	Beef	Raw meat	9
	ST299	ICDC_LM191	2008	AH	M3	Fish	Aquatic products	R
20	ST299	ICDC_LM192	2008	AH	M3	Chicken	Raw poultry	2
	ST299	ICDC_LM197	2009	AH	M3	Egg	Eggshell	R
21	ST3	ICDC_LM272	2010	AH	M19	Pork	Raw meat	R
	ST3	ICDC_LM274	2010	AH	M5	Fish	Aquatic products	7
	ST3	ICDC_LM275	2010	AH	M6	Chicken	Raw poultry	7
	ST3	ICDC_LM278	2010	AH	M20	Jellyfish	Aquatic products	4
22	ST145	ICDC_LM594	2014	AH	F3	Chicken	Raw poultry	R
	ST145	ICDC_LM595	2014	AH	F4	Fish	Aquatic products	1
	ST145	ICDC_LM596	2014	AH	F5	Chicken	Raw poultry	0
	ST145	ICDC_LM597	2014	AH	F6	Pork	Raw meat	0
	ST145	ICDC_LM2660	2013	AH	M13	Cooked meat products	RTE Food	2
23	ST87	ICDC_LM3033	2018	YN	M1	Chicken	Raw poultry	R
	ST87	ICDC_LM3034	2018	YN	M1	Cooked meat products	RTE Food	6
	ST87	ICDC_LM3035	2018	YN	M1	Cooked meat products	RTE Food	3

Province[a]: YN, YunNan; ZJ, ZheJiang; AH, AnHui; NX, NingXia. Market[b]: M, Market; R, restaurant; F, Farm. RTE food: read-to-eat food. Number of SNP[c]: R, reference strain for each cluster of pair-wised SNP analysis

in the same year, five clusters (Cluster 2, 8, 11, 12, and 14) were caused by the isolates from different sources in the same province in different years, two clusters (Cluster 15 and 20) were caused by the isolates from the same market in different years and two clusters (Cluster 16 and 18) were caused by the isolates from different provinces in different years.

The distribution of the prevalent subtypes of *Listeria monocytogenes* isolates from food in China in the context of global isolates

To determine the relationship between the top four prevalent STs from China (ST9, ST8, ST121, and ST87) and those from other countries, a total of 310 publically available genomes of *L. monocytogenes* were selected for comparative analysis. Here we also selected some isolates of each four ST from human cases in China to diversify the sources of isolates. For each ST, phylogenetic trees were separately constructed based on the alignment of the core-SNPs of isolates (Figure 3). The ST9 *L. monocytogenes* from China containing two isolates from clinical cases formed different clusters and were distributed across the phylogenetic tree of the global isolates (Figure 3A). The ST9 isolates in China were isolated from different regions and isolation years were clustered on the same branches, no epidemiological specific clustering was observed. For ST8, with exception of three outliers (one food-original and two clinical isolates), the ST8 isolates in China were grouped into two clusters (Figure 3B), and no evident temporal and spatial specificity were observed for the

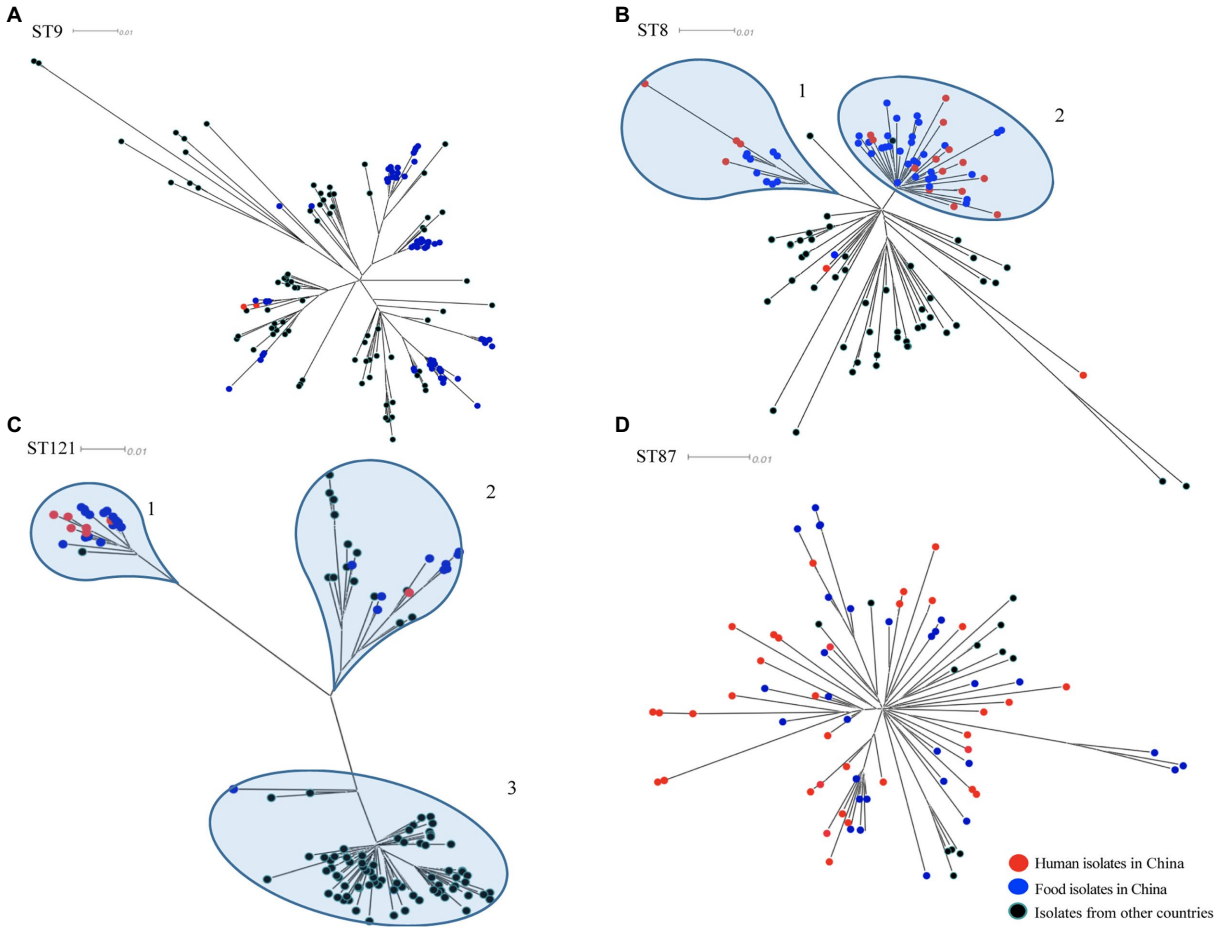

FIGURE 3
The core-SNP phylogenetic tree of prevalent subtypes of *L. monocytogenes* in China in the context of global isolates. The trees were visualized using SplitsTree 5. **(A)** Maximum likelihood phylogeny of 156 *L. monocytogenes* ST9 genomes based on 2,269 core-SNPs, annotation of SLCC2479 was used as reference. **(B)** Maximum likelihood phylogeny of 115 *L. monocytogenes* ST8 genomes based on 1,327 core-SNPs, annotation of ICDC_LM2 was used as reference. **(C)** Maximum likelihood phylogeny of 132 *L. monocytogenes* ST121 genomes based on 2,236 core-SNPs, annotation of ICDC_LM135 was used as reference. **(D)** Maximum likelihood phylogeny of 82 *L. monocytogenes* ST87 genomes based on 1,058 core-SNPs, annotation of ICDC_LM188 was used as reference. Human isolates in China are represented by red circles, food isolates in China are represented by blue circles, isolates from other countries are represented by black circles. Light blue circles represent clusters.

isolates of each cluster. For ST121, almost all isolates in China were distributed in the two out of three clusters, with exception of one food-original isolate that belonged to an international cluster. While 83.33% isolates of ST121 from other countries were grouped into the remaining cluster that was distinct from the clusters of strains isolated in China (Figure 3C). For ST87, isolates of ST87 from China and other countries were mixed with no epidemiological specific clusters observed (Figure 3D).

Stress resistance genes in the studied *Listeria monocytogenes* isolates

In this study, all 322 *L. monocytogenes* isolates were screened for the presence of stress adaptation associated genes or gene clusters, including stress survival islet 1 (SSI-1), stress survival islet 2 (SSI-2), three *Listeria* genomic islands (LGI1, LGI2, and LGI3), two benzalkonium chloride resistance determinants *bcrABC* gene cassette and the *ermC* gene. The SSI-1 was detected in 203 isolates (63.04%), including 32.61% (30/92) of lineage I isolates, 72.64% (154/212) of lineage II isolates and all the lineage III isolates. The SSI-2 was observed only in ST121 and ST196 isolates of lineage II (Figure 2). None of the studied isolates harbored LGI-1. There were 25 isolates harbored LGI-2 that includes genes putatively involved in arsenic and cadmium resistance. All CC2 and ST14 isolates carried LGI-2 consisting of 34 genes, which is consistent with the firstly reported strain Scott A (Kuenne et al., 2013). In addition, all the six CC1 isolates harbored a variant of LGI-2 previously reported by Gray et al. (2021), which carried an

additional *metC* homology gene. A variant of LGI-3 was observed in all ST101 isolates in this study, lacking four genes compared to the first reported LGI-3 in ST101 isolates by Palma et al. (2020). These four genes encode the transposase (*Tn3*), recombinase (*hin*), and Cd-resistance (*cadC* and *cadA1*), the locus-tag in strain A37-02-LmUB3PA: LmUB3PA_1699 - LmUB3PA_1702. *The bcrABC* gene cassette was observed in two isolates (each for ST5 and ST296) of lineage I, and two ST9 isolates of lineage II. Two ST121 isolates harbored the *ermC* gene (Figure 2).

Virulence factors in the studied *Listeria monocytogenes* isolates

Three *L. monocytogenes*-specific pathogenicity islands (LIPI), LIPI-1, LIPI-3, and LIPI-4, were screened among the 322 foodborne isolates. LIPI-1 was highly conserved in all isolates. LIPI-3 was identified in the isolates belonging to a subset of lineage I isolates, including CC1, CC3, CC6, CC11, CC218, CC224, and CC288. Notably, one CC11/ST11 isolate ICDC_LM277 of lineage II carried the intact LIPI-3, a gene of it had 100% coverage and 96.937% identity with previously reported *llsH* gene in lineage I. LIPI-4 was identified in all ST87 (31/31) and ST296 (1/1) isolates belonging to lineage I.

The intact *inlA* gene encoding full-length InternalinA (InlA) was observed in 226 *L. monocytogenes* isolates, including 97.83% lineage I isolates, 55.45% lineage II isolates and 100% lineage III isolates in this study (Figure 2). The truncated *inlA* caused by premature stop codon (PMSC) mutations were observed in two ST5 isolates of lineage I, and 94 (44.55%) lineage II isolates belonging to ST7 (1/11), ST9 (71/72), ST121 (14/33), ST196 (2/2) and ST199 (6/6). Nine PMSC mutation types of *inlA* were identified, one of which was firstly reported here. The novel PMSC type existed in one ST7 isolate, a truncated InlA with a shorter peptide of 312 amino acids was caused by the deletion of cytosine in position 937 (Figure 4). For the other internalin genes, *inlB*, *inlE*, *inlH*, *inlJ* and *inlK* were found in all studied isolates. The *inlC* gene was absent in all ST299 isolates, while the *inlF* gene was absent in all ST14, ST121, and ST299 isolates. In addition, the *inlG* gene was absent in all lineage I isolates except for one ST6 isolate, and a subset of lineage II isolates which belonged to ST14, ST101, ST121, and ST196.

Antibiotic resistance genes in the studied *Listeria monocytogenes* isolates

Ten antibiotic resistance genes were identified in this study (Figure 2). Six intrinsic antibiotic resistance genes, including *fosX* (resistance to fosfomycin), *lmo0441* (cephalosporin), *lmo0919* (lincosamides), *norB* (quinolones), *mprF* (cationic antimicrobial peptides) and *sul* (sulfonamides), were present in all studied isolates. Four acquired antibiotic resistance genes were identified in a small portion of the isolates. The aminoglycosides resistance gene *aacA4* was identified in 50% (10/20) of ST3 isolates, locating in a 57-kb prophage that was inserted between the *fosX* (locus tag in EGD-e: CRH04_RS08765) and *rlmD* (locus tag in EGD-e: CRH04_RS08770) genes. The *aacA4* gene positive isolates were found in three provinces in three different years, and they were isolated from four food sources, including RTE food, raw meat, raw poultry and aquatic products. The tetracycline resistance gene *tetS* was identified in one strain ICDC_LM3005 and was carried by a plasmid with a size of about 78 kb. Another tetracycline resistance gene *tetM* was observed in 13 isolates belonging to ST199 ($n = 6$), ST705 ($n = 4$), ST2 ($n = 1$), ST155 ($n = 1$), and ST515 ($n = 1$). The *tetM* gene from the ST199 isolates were carried by a transposon that was similar to Tn5801_B15 in *Enterococcus faecalis* with 100% coverage and 99.99% identity. The transposon harboring *tetM* gene was found in all ST199 isolates in this study, which were isolated from four provinces and three food sources. While the *tetM* gene from the ST705, ST2, and ST515 isolates were carried by a transposon that was similar to Tn916 in *Bacillus subtilis* with 100% coverage and 99.83% identity. Isolates carrying the Tn916 were distributed in three provinces and were isolated from three food sources. In addition, the *tetM* gene from the ST155 isolate was carried by a transposon Tn6198 that also carried the trimethoprim resistance gene *dfrG*. The *tetM* positive ST515 isolate in this study also carried *dfrG*, which was located downstream of a *tRNA-Val* gene along with two hypothetic proteins.

Discussion

As a foodborne pathogen, *L. monocytogenes* can survive and proliferate in different kinds of food and food-associated environments, even for a long time (Fagerlund et al., 2020;

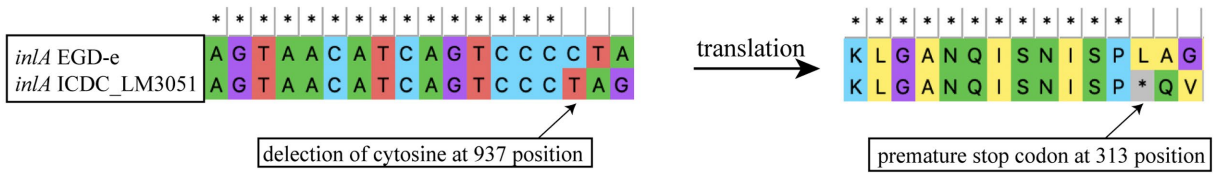

FIGURE 4
Newly identified *inlA* PMSC mutation type in *L. monocytogenes* as compared to full-length *inlA* gene from strain EGD-e.

Wieczorek et al., 2020). Since 2000, the national surveillance of *L. monocytogenes* in different food products in China has been established. Several studies have been conducted to investigate the population structure and molecular epidemiology of *L. monocytogenes* in China using molecular subtyping methods, such as PFGE and MLST (Hua et al., 2018; Wang et al., 2018). Genome-wide genotyping has been used to study the biodiversity of *L. monocytogenes* and the genetic relationships between isolates, but is limited to local areas in China. Here, we performed whole-genome sequencing and genetic characterization of 322 *L. monocytogenes* isolated from food products from 13 regions between 2000 and 2018 to provide insight into the genomic diversity, molecular characteristics, and phylogenetic relationships of *L. monocytogenes* in China.

As previously reported, serogroup IIa was the most frequently isolated serogroup in meat products and environmental surfaces (Centorotola et al., 2021). In this study, by subtyping *in silico*, serogroup IIa and ST9 were the most common subtypes of food-sourced *L. monocytogenes* in China, which was consistent with previous studies (Wang et al., 2012; Zhang et al., 2020). ST9 was a common clone of *L. monocytogenes* isolated worldwide from food especially from meat products (Moura et al., 2016). The second and the third top of STs (ST8 and ST121) in this study also belonged to serogroup IIa. All the serogroup IIc isolates belonged to CC9 including ST9 and ST122, and showed limited diversity of genomes compare to serogroup IIa, which comprised of multiple CCs. ST8 was the second prevalent ST, which was proposed as the subtype of epidemic clone 5 (ECV), and was the predominant ST responsible for the human listeriosis in Canada during 1988 and 2010 (Fagerlund et al., 2016). Previous studies have shown that ST8 *L. monocytogenes* is commonly found in both food products and human listeriosis in China (Li et al., 2018; Zhang et al., 2021). Full-length *inlA* and SSI-1 were carried by all ST8 isolates, which partially supported its full pathogenic potential and increasing tolerance of stress conditions. Therefore, more attention should be paid to ST8 *L. monocytogenes* in the surveillance in China. ST121 was identified as the third prevalent subtype of isolates from food in China. A number of genetic determinants had been identified to involved in survival and adaptation of ST121 strains in food and food associated environments, like SSI-2, the transposon Tn6188 and a high proportion of plasmids (Schmitz-Esser et al., 2021). Consistent with our previous study, ST87 had been always identified as a concerned subtype among the *L. monocytogenes* from diverse sources in China (Wang et al., 2019). And the recent study had revealed that several genetic elements, such as LIPI-4 and a conserved plasmid pLM1686, may contributed to its pathogenic potential and adaptation to harsh environment (Wang et al., 2019).

So far, no listeriosis outbreak of any scale has been reported in China, partly due to the different consumption patterns and food-handling habits. However, RTE food particularly Chinese cold dishes which were reported to increase the risk of infection by 3.43-fold (Niu et al., 2022). In this study, 15 STs isolates were detected from RTE food, some STs of them were reported to have a strong link to the outbreak and sporadic listeriosis around the world, such as ST1, ST2, ST5, ST8, and ST87 (Chen et al., 2018; Maury et al., 2019; Wang et al., 2019; Zhang et al., 2021). Therefore, it is very important to strengthen hygiene monitoring and disinfection of RTE food processing and storage environment.

Pair-wised SNPs detection was performed on the indistinguishable isolates by core-SNPs analysis based on the species of *L. monocytogenes*. Here, we found 23 potentially associated events related to *L. monocytogenes* contamination. Combining with the meta-data including isolation sources (market, restaurant or farm), isolation years and isolation regions, it suggested that food safety risks exist in multiple contamination scenarios, including long-term persistent contamination over time, cross-contamination in markets, and the spread of a single source to different markets. However, caution should be exercised in inferring relationships between isolates with closely related genomes from different provinces and over time, unless clear epidemiological information is available.

Core-SNPs analysis based on the species of *L. monocytogenes* here apparently did not provide sufficient resolution to differentiate between isolates within a given CC/ST. ST122 was a member of CC9 with a different allele of *ldh* gene (the allele number of *ldh* is four in ST9 and 62 in ST122). Deeper phylogenetic analysis showed that the emerging ST122 clone might diverge from the ST9 clone for its most recent ancestor sharing with a subset of ST9's. It suggested that the population of CC9 *L. monocytogenes* may be undergoing a diversification process.

In-depth phylogenetic study of the prevail STs in China, including ST9, ST8, ST121, and ST87, and each corresponding STs isolates from other countries, revealed the distribution of our studied isolates in the context of global isolates. ST9 *L. monocytogenes* in this study were grouped into several clusters throughout the phylogenetic tree of isolates from around the world, indicating that this clone from food in China was genetically diverse. ST9 was a prevalent clone isolated from foods, especially from meat products, in many countries (Martín et al., 2014; Maury et al., 2019; Wang et al., 2021). Different genetic characteristics of ST9 *L. monocytogenes* were spread and circulated globally with the frequent international trade of food. The majority of *L. monocytogenes* isolates of ST8 and ST121 from China were both grouped into two clusters, while isolates of these two STs from other countries were distributed in different clusters (Figures 3B,C). It indicated that ST8 and ST121 isolates from China were more closely related to another among each cluster, and showed heterogeneous with isolates from other countries. The possible reason is that these two STs are adapted to different food niches in China and other countries. The results of the phylogenetic analysis of ST87 *L. monocytogenes* in this study are consistent with that of our

previous comprehensive and in-depth study of this clone in China (Wang et al., 2019). The isolates of ST87 *L. monocytogenes* were divided into multiple sub-clusters that were divided from the root, and minor isolates from other countries were distributed among the tree without region-clustering except for four clustered United States isolates that were from environments in the same year (2017). Overall, ST-specific core-SNP analysis well revealed the genome-level diversity of clonal groups which were prevalent in China, providing unprecedented discrimination to distinguish isolates within certain clonal groups.

Due to the use of several stress-adaptive mechanisms to withstand a wide variety of stressful conditions, *L. monocytogenes* isolates are able to colonize in different ecological niches. SSI-1 contributes to *L. monocytogenes* adapting to low pH and high salt concentration, while SSI-2 helps *L. monocytogenes* survival in alkaline and oxidative stress conditions (Ryan et al., 2010). In this study, 72.99% of lineage II isolates carried SSI-1, which included all ST8 and ST9 isolates. While all the ST121 isolates belonging to lineage II carried SSI-2. These findings gave further support for lineage II isolates being more prevalent in foods and food-associated environments than lineage I isolates. Interestingly, ST299 isolates that belonged to the rare lineage III and serogroup L were identified to overrepresent in aquatic products, which is similar to the results reported by Chen et al. (2018). It has been proposed that the ST299 have a specific ecological niche associated with aquatic products and the environment, while our study found that all ST299 isolates carried SSI-1. However, the contribution of SSI-1 in adapting to aquatic ecological niches in ST299 *L. monocytogenes* requires further functional confirmation. In addition, LGI-2 carried cadmium resistance cassette and arsenic resistance cassette which were associated with adaptation to environment, and was found a propensity with hypervirulent serogroup IVb clones, suggesting possible associations with *L. monocytogenes* virulence (Parsons et al., 2020). A variant of genomic island LGI3 lacking *cadA1C* cassette was found in all ST101/CC101 isolates in this study, which was the same with the observation of the study of Gray et al. (2021). However, Palma et al. (2020) found that the remaining 25 genes of LGI3 were significantly enriched in the persistent CC101 clone from the smoked-fish plant, indicating that this variant of LGI3 still contributes to persistent contamination of the ST101/CC101 clone.

As an important foodborne pathogen, several virulence potentials ultimately contribute to the ability of *L. monocytogenes* to infect humans. Except for species-specific LIPI-1, both LIPI-3, and LIPI-4 were found in a subset of lineage I *L. monocytogenes* isolates that were overrepresented in human infection cases. Strikingly, one ST11 strain (ICDC-LM277) of lineage II was isolated from raw meat and harbored LIPI-3. The LIPI-3 was also found in the genomes of eight ST11 *L. monocytogenes* isolates which caused an outbreak of listeriosis manifesting febrile gastroenteritis in Italy in 2016 (BioProject accession no.: PRJNA436467; Maurella et al., 2018). It suggested that ST11 *L. monocytogenes* isolates carrying LIPI-3 might have an increased risk of causing gastrointestinal infection in humans. LIPI-4, which is associated with increased neural and placental tropisms of *L. monocytogenes*, was found in all ST87/CC87 and ST296/CC88 isolates in this study. ST87 had been recognized as the dominant ST in clinical listeriosis in China, particularly associated with maternal-neonatal and central nervous system infections (Wang et al., 2019). Intact InlA is more favorable for *L. monocytogenes* to cross the intestinal barrier and establish a systemic infection, 70.19% (226/322) isolates in this study have full length of InlA, while 98.61% (71/72) ST9 isolates have truncated InlA, the under representation of the ST9 isolates in human clinical cases may be due to the frequent occurrence of PMSC mutations.

L. monocytogenes has been present sensitive to most antibiotics. For the treatment of listeriosis, ampicillin and penicillin-based agent is effective against this bacterium (Fischer et al., 2020). In present study, aminoglycosides resistance gene *aacA4* was exclusively found in a subset of ST3 *L. monocytogenes* isolates, consistent with data obtained from Brazil and Poland (Camargo et al., 2019; Kurpas et al., 2020). The *aacA4* gene was carried by a 57-kb prophage, and it is strongly associated with ST, but not with isolation location or type of food source. The standard therapy for listeriosis usually combined with gentamicin (aminoglycoside antibiotic; Fischer et al., 2020), monitoring *aacA4* gene in *L. monocytogenes* isolates especially ST3 strains is important for listeriosis clinical treatment. Tetracycline resistance was the most common phenotype in food source *L. monocytogenes* in China (Yan et al., 2019), and we found that 4.3% of isolates carried the tetracycline resistance genes *tetM* or *tetS* in this study, and the *tetM* gene is associated with Tn916 and the *tetS* gene is carried by a plasmid, which was consistent with that of previous studies (Bertsch et al., 2013; Haubert et al., 2016). The high genetic identity of *tetM*-containing transposons between *L. monocytogenes* and *B. subtilis* or *E. faecalis* suggests that horizontal transfer of antibiotic genes can occur between different genera of bacteria in the same niche. In addition, the 18-kb Tn916 transposon could be found in both lineage I and lineage II isolates. It is a warning that transposon cannot be ignored for its role in the dissemination of the resistance genes across the lineages in *L. monocytogenes*. However, no evidence showed the harboring of resistance genes was related to isolation location, isolation year and the type of food source.

Conclusion

In this study, 322 *L. monocytogenes* isolates were selected and sequenced to characterize the population structure of food source isolates in China. Serogroup IIa and ST9 were identified as the most prevalent serogroup and ST, respectively. Core-SNP analysis classified all the isolates into different clusters that corresponded to the different clonal complexes which were further grouped into

three lineages. In-depth core-SNP analysis based on specific CC or ST reveals the potential epidemiological relationship of isolates in China: (i) ST122 clone might diverge from ST9 clone; (ii) persistent contamination, cross-contamination, and transmission from a single source to different markets were found in markets of China; (iii) the prevalent subtypes ST8 and ST121 were heterogeneous with worldwide isolates. Genomic analysis identified LIPI-3 in lineage II (ST11/CC11) isolates and a novel PMSC type in *inlA* gene, which increased the understanding of the genomic diversity of *L. monocytogenes*.

Data availability statement

The datasets presented in this study are deposited in the China National Microbiology Data Center (NMDC), with accession number NMDC60016487 to NMDC60016827 (https://nmdc.cn/resource/genomics/genome/).

Author contributions

SJ, CY, and YaW conceived and designed the research study. SJ, YiW, LLi, and PM performed the sample collection and DNA extraction. ZS and SJ analyzed the data. SJ, LLu, CY, and YaW write and revise the manuscript. All authors have read and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1049843/full#supplementary-material

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In vitro digestion of ESC-resistant *Escherichia coli* from poultry meat and evaluation of human health risk

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Introduction: The spread of antimicrobial resistance (AMR) has become a threat against human and animal health. Third and fourth generation cephalosporins have been defined as critically important antimicrobials by The World Health Organization. Exposure to Extended spectrum cephalosporin-resistant *E. coli* may result in consumers becoming carriers if these bacteria colonize the human gut or their resistance genes spread to other bacteria in the gut microbiota. In the case that these resistant bacteria at later occasions cause disease, their resistance characteristics may lead to failure of treatment and increased mortality. We hypothesized that ESC-resistant *E. coli* from poultry can survive digestion and thereby cause infections and/or spread their respective resistance traits within the gastro-intestinal tract.

Methods: In this study, a selection of 31 ESC-resistant *E. coli* isolates from retail chicken meat was exposed to a static in vitro digestion model (INFOGEST). Their survival, alteration of colonizing characteristics in addition to conjugational abilities were investigated before and after digestion. Whole genome data from all isolates were screened through a custom-made virulence database of over 1100 genes for virulence- and colonizing factors.

Results and discussion: All isolates were able to survive digestion. Most of the isolates (24/31) were able to transfer their bla_{CMY2}-containing plasmid to *E. coli* DH5-á, with a general decline in conjugation frequency of digested isolates compared to non-digested. Overall, the isolates showed a higher degree of cell adhesion than cell invasion, with a slight increase after digestion compared non-digested, except for three isolates that displayed a major increase of invasion. These isolates also harbored genes facilitating invasion. In the virulence-associated gene analysis two isolates were categorized as UPEC, and one isolate was considered a hybrid pathogen. Altogether the pathogenic potential of these isolates is highly dependent on the individual isolate and its characteristics. Poultry meat may represent a reservoir and be a vehicle for dissemination of potential human pathogens and resistance determinants, and the ESC-resistance may complicate treatment in the case of an infection.

KEYWORDS

virulence, ExPEC, conjugation, INFOGEST, hybrid pathogen

Introduction

The diverse family of *Enterobacteriaceae* includes one of the most studied microbes, *Escherichia coli*. Within *E. coli*, we find commensals, opportunistic bacteria, and pathogens. Some of the pathogenic variants cause infections in the intestinal tract (intestinal pathogenic *E. coli*, IPEC), while extraintestinal pathogenic *E. coli* (ExPEC) can survive in other tissues of the host and are associated with neonatal meningitis (NMEC) and sepsis and urinary tract infections (UPEC) among others (Riley, 2014). Several virulence genes characterizing ExPEC have been described (Pitout and Laupland, 2008; Pitout, 2012; Mellata, 2013), which include

genes encoding for adhesins, invasins, toxins, and siderophores and genes related to iron metabolism (Dale and Woodford, 2015). The increasing occurrence of antimicrobial-resistant ExPEC isolates has led to prolonged hospital stays and higher mortality rates (Gastmeier et al., 2012). This rise in antimicrobial resistance (AMR) has a significant impact on human and animal health (Brinkac et al., 2017; Centers for Disease Control Prevention, 2019). Recently, hybrid pathogens have been explored (Lindstedt et al., 2018), demonstrating the plasticity of the *E. coli* pangenome (Mellata, 2013) and blurring the lines between human-made bacterial classifications. An increased understanding of the dynamic flow and transmission routes of antimicrobial-resistant bacteria (ARB) and antimicrobial resistance genes (ARG) between animal and human bacterial reservoirs and host interactions is important for developing and implementing targeted measures to further prevent AMR development (VKM, 2020).

Poultry has been described as one of the main reservoirs for extended-spectrum beta-lactamase (ESBL) producing bacteria, as well as *E. coli,* which is most closely linked to human ExPEC (Carattoli, 2008). The European Food Safety Agency (EFSA) concluded in 2011 that AMR *E. coli* isolates from humans and poultry are more frequently genetically related than antibiotic-susceptible isolates and that transmission of ESBL genes, plasmids, and clones from poultry to humans is most likely to occur through the food chain (EFSA, 2011; Manges and Johnson, 2012). In 2015, the report "Assessment of AMR in the food chains in Norway" concluded that the probabilities of human exposure to ESBL-producing *Enterobacteriaceae* and their corresponding genes, from live poultry and poultry meat, were considered non-negligible (VKM, 2015). Since then, extensive measures have been taken in the Norwegian poultry industry to limit previously discovered ARB and ARGs (Mo et al., 2014, 2021; Nortura, 2016), leading to the prevalence being substantially reduced in the last few years (NORM/NORM-VET, 2017, 2019). The European Center for Disease Prevention and Control (ECDC) published in their report from 2018/2019 that the proportion of presumptive ESBL/Ampicillinase C (AmpC) producing *E. coli* was low in the animal sector, as 14 countries reported a decrease in overall prevalence, while 11 countries reported an increase of ESBL/AmpC producing bacteria (European Food Safety Authority European Centre for Disease Prevention Control, 2021).

The most common AmpC beta-lactamase encoding gene in *E. coli* is the bla_{CMY-2}, which is predominantly located on plasmids (Alfei and Schito, 2022). It has been reported to occur in bacteria from both human infections and various animal and food sources with increasing prevalence (Pires et al., 2022). Some authors have assessed that ESBL-producing *Enterobacteriaceae* from the broiler production chain is a considerable public health risk due to both their virulence and resistance characteristics (Pitout and Laupland, 2008; Liebana et al., 2013; Vounba et al., 2019). At the poultry slaughterhouse, it is unavoidable that intestinal bacteria contaminate carcasses during the slaughtering process (Rouger et al., 2017; Rasschaert et al., 2020; Boubendir et al., 2021). Consequently, as chicken filets are often sold as fresh products, compromised kitchen hygiene habits may result in consumers becoming exposed to these bacteria (Bloomfield et al., 2017; Santos-Ferreira et al., 2021). However, even though the probability of exposure of consumers to ESC-resistant bacteria may be high depending on the prevalence levels in live animals, less is known about the consequences of such exposure (Buberg et al., 2021). The potentially long timespan from exposure to the development of infection makes infection routes hard to trace, and there is a need for more comprehensive genetic analysis of poultry isolates to unravel their pathogenic potential and further evaluate their role as a possible risk to human health (Leverstein-van Hall et al., 2011; Manges and Johnson, 2012; Berg et al., 2017).

To be able to determine the consequences of exposure to Extended Spectrum Cephalosporin (ESC)-resistant *E. coli* through food, further investigations of the fate of these isolates through the digestion process are needed. Their survival in humans through intake by the oral route has not yet been quantified and questions regarding survival, horizontal spread of resistance genes in the gastrointestinal tract, their interactions with the intestinal cells, and possible alterations of characteristics during the digestion process remain unanswered. Many different protocols for digestion models have been described making a comparison of studies between researchers challenging (Kong and Singh, 2010; Mulet-Cabero et al., 2019; Li et al., 2020; Mackie et al., 2020). However, in 2014, the INFOGEST network published a static *in vitro* digestion model that aimed to harmonize human-digestion conditions by being an easy and applicable model that could be compared between studies (Minekus et al., 2014; Brodkorb et al., 2019). Despite the limited use of this model for microbiological purposes, it is appropriate for the evaluation of the growth and survival of *Listeria monocytogenes* (Pettersen et al., 2019).

This study aimed to contribute to the understanding of the human health risk represented by ESC-resistant *E. coli* from the poultry food chain. We hypothesized that these bacteria would survive the human digestive process and have the potential to interact with the host and/or to transfer their resistance traits to other gastrointestinal bacteria. We addressed this hypothesis by using the above-mentioned *in vitro* digestion model for the evaluation of selected isolates' survival, conjugation abilities, and ability to adhere to and invade human colorectal cells. Furthermore, we assessed the presence of virulence factors characteristic of ExPEC through an in-depth analysis of whole genome sequence data.

Materials and methods

Isolates and selection

A total of 141 ESC-resistant *E. coli* was isolated from domestically produced retail chicken meat in the NORM-VET programs from 2012 to 2016 (NORM/NORM-VET, 2013, 2015, 2017). All these isolates were previously whole genome sequenced and known to carry the bla_{CMY-2} gene encoding ESC resistance and have previously been included in studies by Mo et al. (2014, 2016). A selection of 31 isolates was made from this collection by including isolates from all phylogroups and the most frequent sequence types, resulting in 11 isolates from 2012, 14 isolates from 2014, and six isolates from 2016. Eighteen of the isolates were previously partly characterized by Buberg et al. (2021). An overview of the included isolates is listed in Table 1.

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TABLE 1 Isolates included in the study.

Whole ID	Year	ST	Phylogroup	Previously studied published
2012-01-3586	2012	131	B2	Mo et al., 2016; Buberg et al., 2021
2014-01-3678	2014	117	D	Mo et al., 2016; Buberg et al., 2021
2016-22-832	2016	442	B1	Buberg et al., 2021
2014-01-5656	2014	10	A	Mo et al., 2016; Buberg et al., 2021
2014-01-7037	2014	355	B2	Mo et al., 2016; Buberg et al., 2021
2016-22-220	2016	429	B2	Buberg et al., 2021
2014-01-1336	2014	1,594	A	Mo et al., 2016; Buberg et al., 2021
2012-01-1295	2012	38	D	Mo et al., 2016; Buberg et al., 2021
2012-01-707	2012	38	D	Mo et al., 2016; Buberg et al., 2021
2014-01-3680	2014	1,158	D	Mo et al., 2016; Buberg et al., 2021
2014-01-4991	2014	57	D	Mo et al., 2016; Buberg et al., 2021
2014-01-5104	2014	115	D	Mo et al., 2016; Buberg et al., 2021
2012-01-771	2012	69	D	Mo et al., 2016; Buberg et al., 2021
2014-01-7011	2014	1,944	D	Mo et al., 2016; Buberg et al., 2021
2014-01-4267	2014	191	A	Mo et al., 2016; Buberg et al., 2021
2012-01-1292	2012	38	D	Mo et al., 2016, 2017; Buberg et al., 2020, 2021
2012-01-2798	2012	3,249	A	Mo et al., 2016, 2017; Buberg et al., 2020, 2021
2016-22-1061	2016	2,040	A	Buberg et al., 2021
2012-01-1988	2012	38	D	Mo et al., 2016
2012-01-2350	2012	38	D	Mo et al., 2016
2012-01-1659	2012	10	A	Mo et al., 2016
2012-01-5334	2012	1,594	A	Mo et al., 2016
2012-01-5997	2012	10	A	Mo et al., 2016
2014-01-14	2014	38	D	Mo et al., 2016
2014-01-1676	2014	117	D	Mo et al., 2016
2014-01-2452	2014	117	D	Mo et al., 2016
2014-01-7149	2014	10	A	Mo et al., 2016
2014-01-2454	2014	38	D	Mo et al., 2016
2016-22-75	2016	1,594	A	Unpublished
2016-22-226	2016	10	A	Unpublished
2016-22-1059	2016	2,040	A	Unpublished

Isolation and species determination is described by Mo et al. (2016).
For clarification purposes and easy reading, the four last digits of the isolate ID are used when referred to in the text.

In vitro digestion model

Survival after digestion was evaluated by the static *in vitro* protocol developed by INFOGEST with minor modifications to adapt the protocol for studies of bacteria (Pettersen et al., 2019). Tests determining enzymatic activity for standardization were carried out according to the protocol before the experiment. In short, overnight cultures of all bacterial isolates in LB-broth were used in the *in vitro* digestion model. The "bacterial mixture" consisting of 0.5 ml of overnight culture in broth was added to a 5-ml Eppendorf tube (Eppendorf, Hamburg, Germany). Simulated salivary fluid (SSF) was added to obtain a 1:1 ratio. $CaCl_2(H_2O)_2$ was added to achieve a total concentration of 1.5 mM in SSF. As the bacterial mixture did not contain starch, amylase was omitted, and the mixture was incubated at 37°C for 2 min on a hematology mixer. For the gastric step, preheated simulated gastric fluid (SGF) was added to the bacterial mixture in a ratio of 1:1. The pH was adjusted to 3.0 by adding a pre-defined volume of HCl. $CaCl_2(H_2O)_2$ was added to a final concentration of 0.15 mM in SGF. Porcine pepsin (Sigma-Aldrich, batch no. SLCF7636) (2,000 U/ml), Rabbit Gastric extract (RGE15, Lipolytech) (60 U/mL), and water were mixed to achieve a 1× concentration of SGF, which was then added to the mixture before incubation at 37°C for 40 min (estimated passing time for liquid boluses). For the intestinal phase, preheated

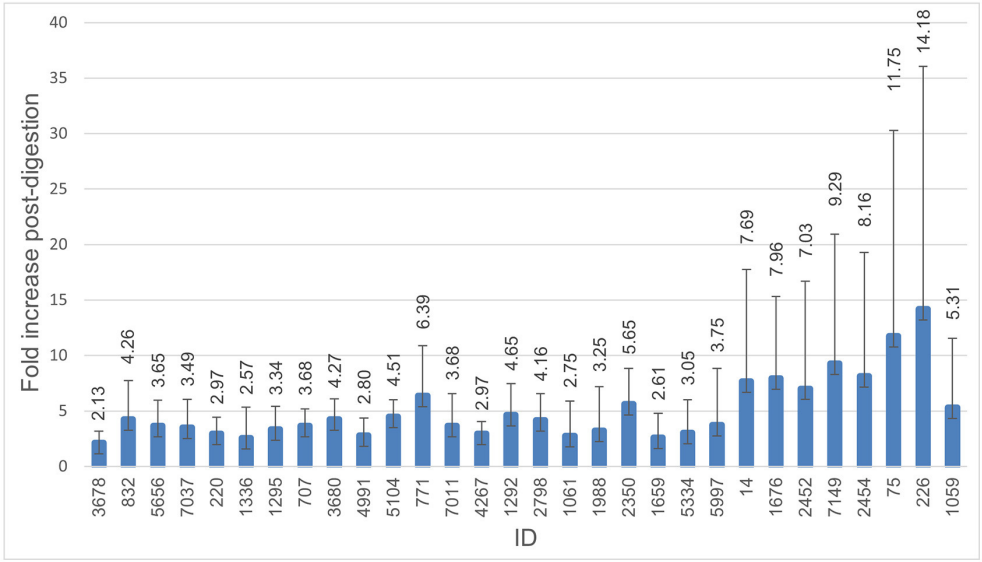

FIGURE 1
Survival through digestion. Histogram demonstrating fold-increase in CFU/ml after digestion compared to non-digested. The error bar represents the standard deviation. All isolates were able to survive digestion, and all were able to continue growth during the digestion process. Isolate 3,586 was excluded from this figure due to a failure of growth in the non-digested control.

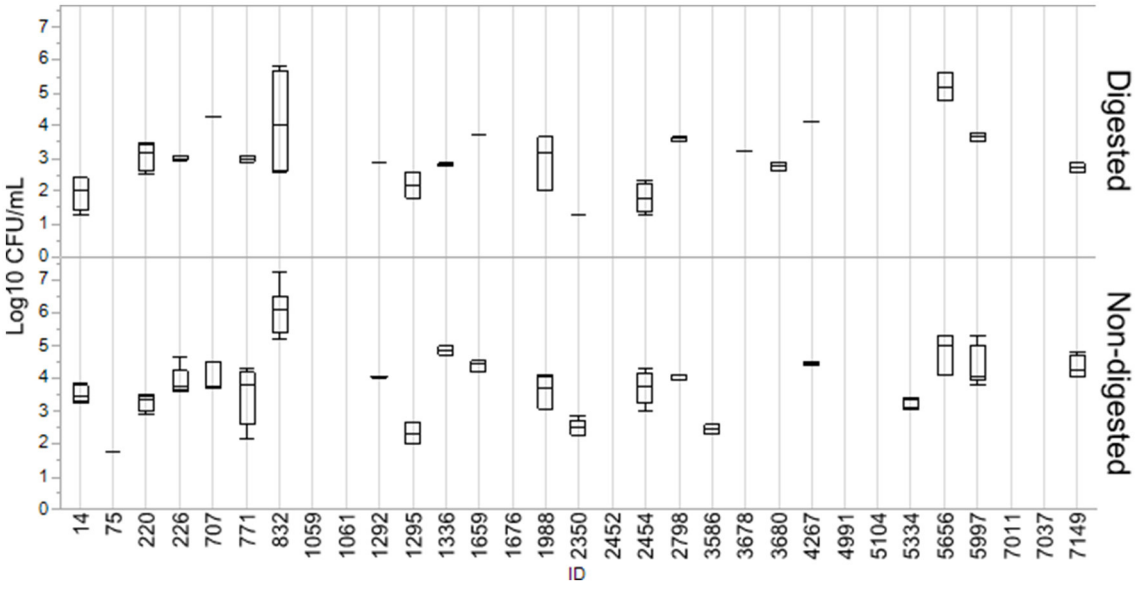

FIGURE 2
Log10 CFU/ml of transconjugants for digested and non-digested isolates. Boxes represent the quartiles and the median value, with included confidence intervals as the standard error of mean indicated by whiskers. Values are given in the number of Log10 CFU/ml. No whiskers are visible for isolates where the outer quartile is equal to the minimum or maximum value. For isolates where no transfer was detected, no box is present.

simulated intestinal fluid (SIF) was added to the bacterial mixture in a ratio of 1:1. The pH was adjusted to 7.0 by adding a pre-defined volume of NaOH. A final concentration of 10 mM bile was then added to the mixture. $CaCl_2(H_2O)_2$ was added to achieve a final concentration of 0.6 mM in SIF. Pancreatin (Sigma-Aldrich, batch no. SLCF4576) with a trypsin activity of 100 U/ml was then added to the mixture. Autoclaved ddH_2O was added to gain a 1× concentration of the SIF before samples were incubated at 37°C for 2 h on a hematology mixer. After incubation, the bacterial mixture was diluted and plated on selective agar plates (Müeller-Hinton agar containing 0.5 mg/L cefotaxime) and incubated for 24 h at 37°C. Colonies were counted manually and CFU/ml was determined after digestion. The number of CFU for the non-digested parallel was calculated by direct plating of overnight culture on selective agar plates. The experiments were carried out in triplicate.

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TABLE 2 Ability to conjugate in liquid broth.

Conjugation: digestion	Total	Conjugation after digestion	NTD after digestion
Total	100% (31)	64.51% (20/31)	35.49% (11/31)
Conjugation non-digested	67.74% (21/31)	85.71% (18/21)	14.28% (3/21)
NTD non-digested	32.25% (10/31)	20.00% (2/10)	80.00% (8/10)

NTD, no transfer detected.
Binary distribution of the number of isolates able to successfully transfer bla_{CMY-2} to *E. coli* DH5-α after digestion compared to non-digested. If no confirmable transconjugants were present on the transconjugant-selective plates, it was considered NTD.

Conjugation assay

Conjugation experiments were performed in LB-broth according to Buberg et al. (2020) with minor modifications. In short, the donors (all 31 isolates individually) and recipient strain (*E. coli* DH5-α) were grown overnight in LB-broth at 37°C reaching OD600 equivalent to a McFarland standard no. 1 (*3E+8 bacteria/ml). A volume of 500 μL of the recipient strain culture and 10 μL of the donor strain culture (donor:recipient ratio = 1:50) were mixed in 4 mL LB-broth and incubated for 4 h at 37°C. Dilutions of each mating culture were plated on Müeller-Hinton agar plates (Sigma-Aldrich, Germany) supplemented with 20 mg/L nalidixic acid and/or 0.5 mg/L cefotaxime, and incubated for 24 and 48 h at 37°C. The mating mixture was then diluted and plated on the recipient- and transconjugant-selective plates containing nalidixic acid, or both nalidixic acid and cefotaxime, respectively. The number of transconjugants was reported for quantification and comparison between the donors. Conjugation frequency was calculated by dividing the number of transconjugants by the number of recipients in CFU/mL. For control, representative colonies from each transconjugant-selective plate were plated on bromothymol lactose blue agar (Sigma-Aldrich, Germany) to distinguish transconjugants from spontaneously mutated donors (i.e., mutated to nalidixic acid resistance). In addition, PCR analysis of transconjugants was conducted to confirm that they harbored the bla_{CMY-2} gene proving conjugation.

For conjugation after digestion, the same procedure was carried out immediately after the donors had gone through the *in vitro* digestion. The experiments were carried out in triplicate.

Adhesion and invasion assay

The ability to adhere to and invade eukaryotic cells was tested in HT-29 cells (RRID: CVCL_0320) grown at 37°C (Ammerman et al., 2008) between passages 10 and 25. The cells were grown to 80% confluence, and 200 μL of cells in fresh McCoy medium (Merck, USA) with 10% Fetal Bovine Serum (GibcoTM 10270106) (referred to as McCoy+) were transferred to a microtiter plate (Corning, Costar, Fischer Scientific, USA). Plates were incubated overnight for establishing cell attachment. Cell concentration for the experiments ranged between 3 and 6 E+06 cells/ml (>90% living cells) counted with Bio-Rad TC20. Overnight culture and digested culture (digested and non-digested) of bacteria were diluted in the ratio of 1:100 in fresh LB-broth. One mL was centrifuged at 2,000 rpm for 5 min and the bacterial pellet was resuspended in 500 μL fresh McCoy+ medium without antibiotics. The bacterial suspension was diluted in the ratio of 1:100 and 50 μL of bacterial suspension was added to each well (equivalent to MOI 30:1). Plates were centrifuged at 1,000 rpm for 2 min to increase contact between bacteria and cells, and incubated for 2 h at 37°C. To assess total cell association (number of adhering and invading bacteria), the cells were washed three times with PBS (1×) to remove non-adherent bacteria and lysed with 30 μL 1% Triton X for 10 min. The lysates were serially diluted in PBS (1×) and plated on selective agar as previously described. To assess bacterial invasion, 200 μL of fresh medium with antibiotics (0.1 mg/ml gentamicin and 20 mg/mL nalidixic acid) was added to the cells before incubation at 37°C for 2 h to kill adherent bacteria. The cells were washed, lysed, and plated as described previously. Adherence was calculated by subtracting numbers from the cell invasion from the total amount of cell-associated bacteria. Digested and non-digested were compared, and experiments were carried out in triplicate.

Detection of virulence genes

An extended virulence-associated gene analysis was carried out. The sequences were scanned (i.e., BLAST search) against a custom database previously used for characterizing environmental isolates (Finton et al., 2020) now expanded to include 1,191 genes/gene variants or genetic markers. The database contains genes related to both ExPEC and IPEC as well as loci suspected to contribute to virulence, e.g., the ETT2 locus. Only matches with 95% or more nucleotide identity with 60% or more query coverage were included in the results.

Results

In vitro digestion

All isolates were able to survive digestion in the INFOGEST *in vitro* digestion model. The numbers of the colony-forming units (CFU/mL) for digested and non-digested isolates are shown in Figure 1. The number of bacteria after digestion varied between isolates and was highly increased for some isolates and replicates. Mean CFU/mL for non-digested isolates was 4.91E+08, compared to 1.82E+09 for digested isolates. All isolates showed a 1-fold increase or higher in CFU/ml, with an average of 5.31-fold increase. More than a 4-fold increase was seen in 15 of the 31 isolates, with isolate 226 displaying the highest increase in the number of CFU/ml after digestion at a 14.18-fold increase.

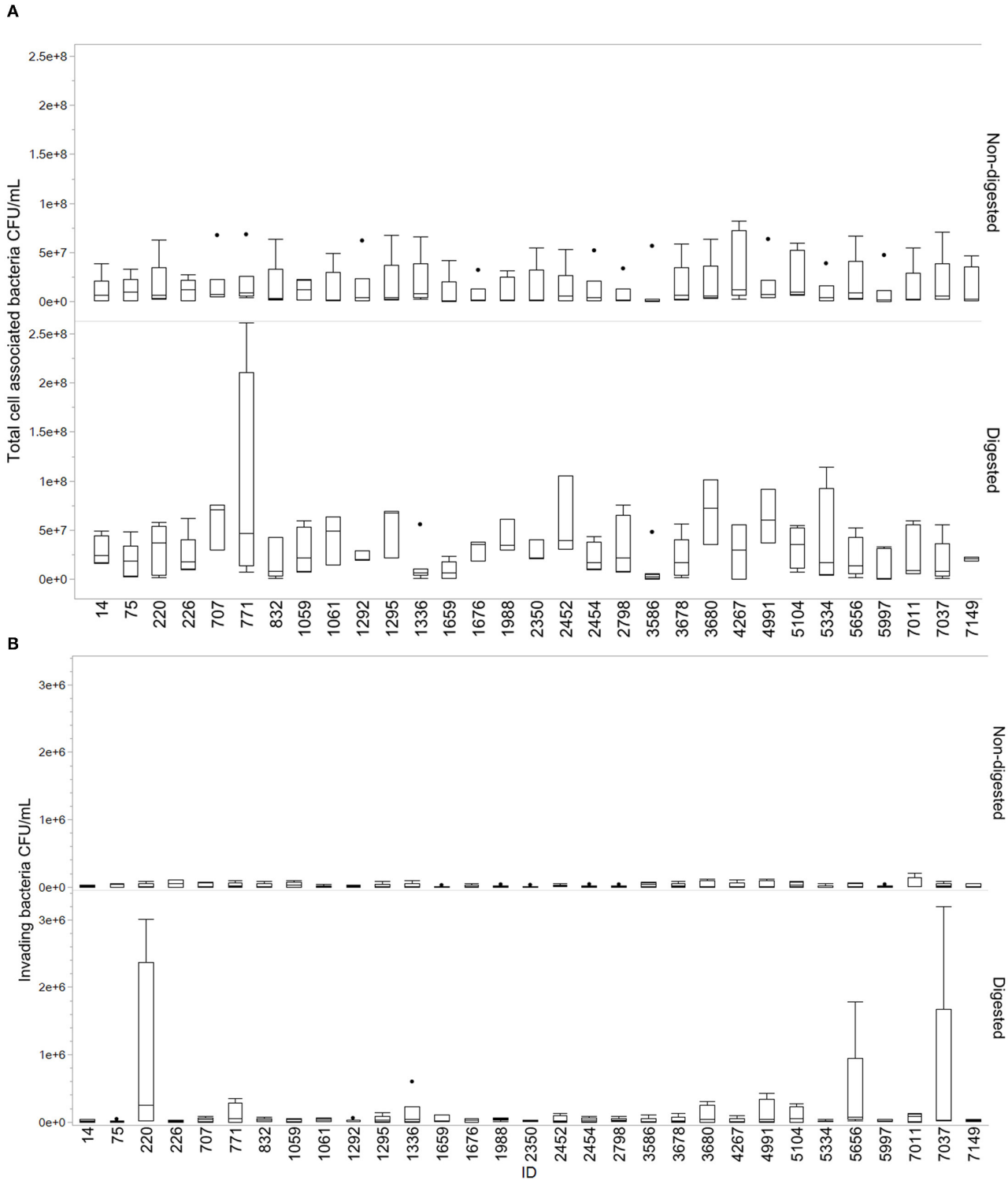

FIGURE 3
Total cell-associated bacteria and invading bacteria for digested and non-digested. Boxes represent the quartiles and the median value, with included confidence intervals indicated by whiskers. Values are given in number of CFU/mL. The dots represent outliers in the data. **(A)** Total cell-associated bacteria in Q18 CFU/mL for digested and non-digested. **(B)** Number of invading bacteria in CFU/mL for digested and non-digested.

Conjugation

Numbers of CFU/ml of transconjugants digested and non-digested for each donor are illustrated in Figure 2. Table 2 gives an overview of the sample population and highlights the variation of conjugation frequencies before and after digestion. Conjugation frequencies are available in the Supplementary material.

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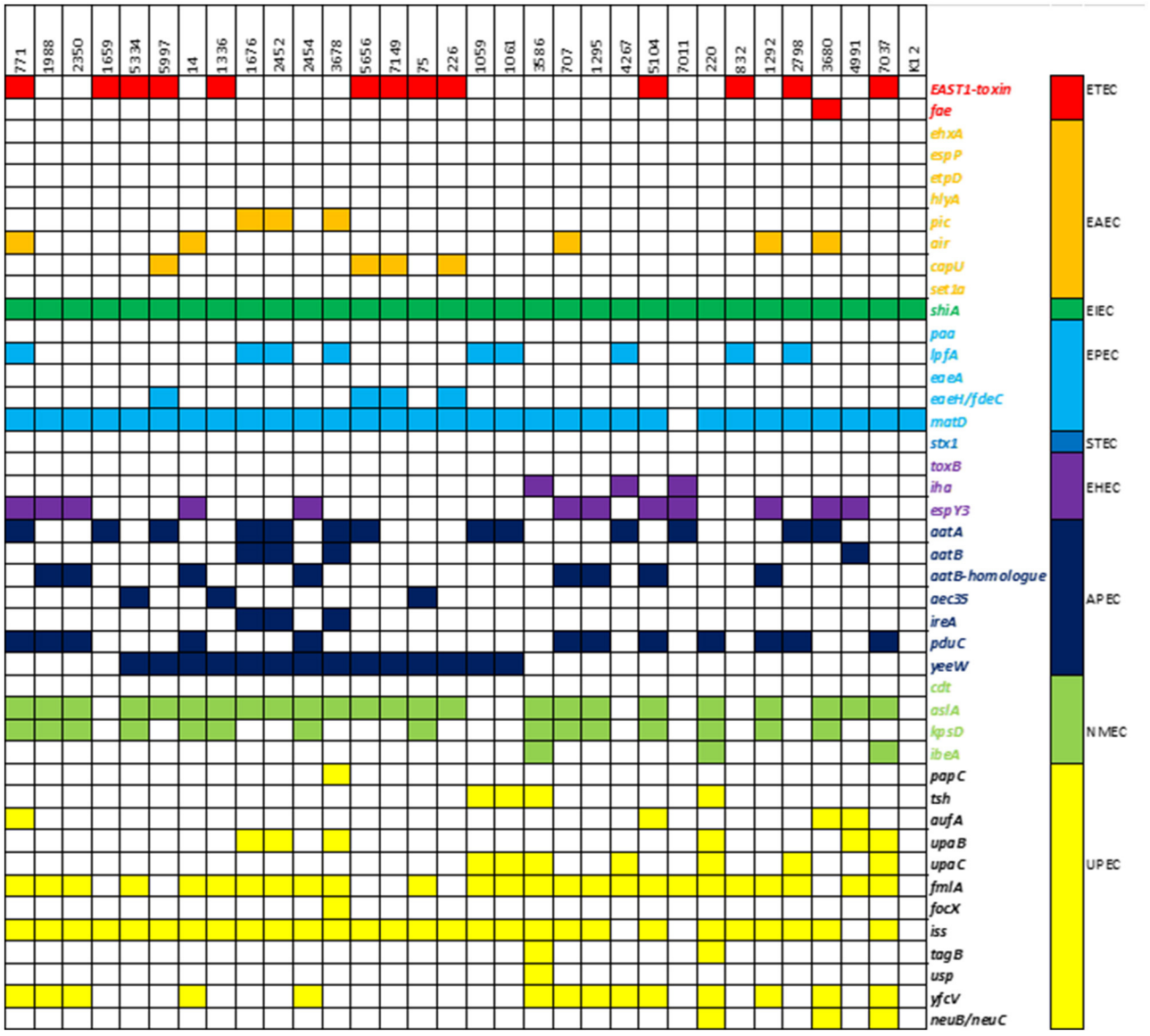

FIGURE 4
Heatmap of virulence-associated genes: presence of a selection of 41 virulence-associated genes and their respective pathogroups in the study population.

In general, most isolates that were able to transfer their bla_{CMY-2} gene displayed a decrease in conjugation frequencies after digestion. Out of the 31 isolates, 24 were able to transfer bla_{CMY-2} before or after digestion, and 19 consequently before and after. Eight isolates (7037, 4991, 5104, 2452, 1059, 1061, 1676, and 7011) were not able to conjugate at all. Conjugation frequency (the number of CFU/mL of transconjugants divided by the number of CFU/mL recipients) for each digested and non-digested isolate is reported in Supplementary Table S1. The mean conjugation frequency for non-digested was 2.04E−03, compared to digested which was 3.60E−04. Isolates 3687 and 3680 were only able to conjugate after being exposed to digestion. While isolates 0075, 3586 and 5334 were only able to conjugate before being digested, and not after. Figures are made in JMP Pro 16.0.0.

Adhesion and invasion assay

Overall, the isolates displayed a greater ability to cell adhesion compared to cell invasion. Most isolates showed an increase in total cell association after digestion compared to non-digested isolates. Results from this assay are demonstrated in Figure 3. The mean of cell adhesion for non-digested was 1.55E+07 compared to digested at 3.36E+07. For invasion, the mean for non-digested was 2.10E+04 and digested was 1.03E+05. Some variability was observed between the different replicates. Isolates 1336, 1659, 3586, and 4267 were the only isolates for which a decrease in adhesion was observed after digestion. For invasion, especially isolates 220, 5656, and 7037 stood out with highly increased invasion numbers after digestion compared to non-digested. Figures are made in JMP Pro 16.0.0.

Virulence-associated gene analysis

The presence of virulence-associated genes was compared to a reference *E. coli* K12 strain. Out of 1,191 genes in the database, our isolates had 123–193 (mean 161) different genes, compared to 107 in *E. coli* K12. Fifty-one genes were found in all isolates, including the control strain, and were considered core genes of the *E. coli* genome. An overview of 41 relevant virulence genes is presented in Figure 4, while a more detailed overview is available in the Supplementary material. According to the UPEC definition by Spurbeck et al. (2012), two of the isolates (220 and 7037) were predicted to be human UPEC isolates by containing the genes *chuA, fyuA,* and *yfcV* while being negative for *vat*. These two isolates were also both positive for other well-documented ExPEC/UPEC-associated genes, e.g., *papC, upaC, ibeA, irp1, irp2,* and *kpsMII.* In addition, isolate 7037, as the only isolate in our collection, contained the *gimB*-genomic island, which is associated with the invasion process of the host cells, particularly in NMEC strains, but is also found in APEC strains (Ewers et al., 2007). Isolate 220 held the *tagB/tagC* (Pokharel et al., 2020), and three isolates (220, 3680, and 7037) were considered K1-isolates as they held *neuB/neuC,* which is related to ExPEC virulence. The ability to acquire iron is related to pathogenicity. The majority of isolates contained iron acquisition loci in which *chuA*, *sit,* and the aerobactin loci were prominent. None of the isolates contained the ExPEC-related toxin genes *pic, sat, vat, hlyA,* or *cnf*. Genes associated with IPEC strains were detected as isolate 3680 contained *ehaA* (adhesion) and *espY3* non-LEE effector gene associated with EHEC/EPEC and *fae* (F4 fimbriae) genes associated with ETEC (Larzábal et al., 2018), and can thus be considered a hybrid pathogen.

Discussion

This study aimed to assess potential health risks represented by ESC-resistant *E. coli* from poultry meat. We showed that the selected isolates were able to survive and multiply during gastrointestinal digestion *in vitro* and that they were able to adhere to and invade human colorectal cells after digestion. In addition, we demonstrated that the stress of being digested changed the conjugation frequency of the bla_{CMY-2}-containing plasmid harbored by these bacteria. The presence of virulence-associated genes was evaluated for further determination of the pathogenic potential of the selected isolates. In general, the isolates showed a large variety of gene content despite being of the same origin and carrying the same AmpC phenotype and bla_{CMY-2} resistance gene. The impact on human health due to exposure to ESC-resistant *E. coli* from poultry meat therefore strongly depends on the individual bacterial isolate being involved.

It was an expected finding that all the investigated isolates were able to survive digestion and displayed an increase in CFU/ml after digestion (Figure 1), as the fecal-oral pathway for infection is common for *Enterobacteriaceae* (Tenaillon et al., 2010). The bacteria were not too hampered by the low pH in the gastric step and were able to replicate despite the limited nutrients available in the simulated gastrointestinal fluids throughout digestion. One study investigated the survival of acid-sensitive bacteria and showed that survival increased in the presence of solid foods (Waterman and Small, 1998). The survival of *E. coli* is therefore expected to increase further with more nutrients available in the form of a meal. If, for example, consumers get exposed to ESC-resistant *E. coli*, these bacteria might therefore reach, encounter, and subsequently interact with the resident microflora.

The current study assessed the conjugational abilities of the bla_{CMY-2} gene in a liquid broth (Table 2). The overall tendency when analyzing the conjugation data was that the frequency of spread of bla_{CMY-2} decreased after digestion (Figure 2). Several studies have suggested that stress may enhance the further spread of resistance or virulence plasmids by triggering the SOS response (Baharoglu et al., 2010; Pribis et al., 2019). As the process of digestion includes an extreme change in pH, in addition to digestive enzymes, it can be considered a stressful procedure for the bacteria. We hypothesized that this stress would increase the spread of resistance genes and increase the bacteria's ability to adhere to and invade gastrointestinal cell lines, making colonizing of the gut more likely. Some of the isolates did not transfer the bla_{CMY-2} gene to the recipient *E. coli*, as demonstrated in Table 2. The reason for this conjugation failure was not further investigated. Another interesting observation in this study is the large variation in the isolates' ability to transfer the bla_{CMY-2} gene with changing conditions, demonstrating that despite their similarities, they likely have a variable ability to adapt to the changing environment of the gastrointestinal tract. Replicon typing of the respective isolates has been previously performed by Mo et al. (2016). In brief, all isolates included in this study hold an IncK plasmid, with exception of 1061 and 2798 which hold only an IncI1 plasmid. Isolate 1336 holds both IncK and IncI1, while isolate 1295 has IncK, IncFII, and IncFIB. No correlation between conjugation and replicon type was seen in this study and thus has not been investigated further.

Adherence and invasion are important characteristics of pathogen–host interactions (Kalita et al., 2014; Desvaux et al., 2020). We observed a change in the ability of cell interaction after digestion compared to non-digested (Figure 3). The results varied between isolates, which may demonstrate that single isolates display a higher probability than others to establish themselves in the gastrointestinal tract. The act of colonizing the intestinal tract is not dependent on individual bacterial characteristics alone but is a complex dynamic involving host factors, the residing microbiota, the nutrients available, and qualities of the colonizing strain (Srikanth and McCormick, 2008; Tenaillon et al., 2010; Richter et al., 2018). Interestingly, isolate 7037 displayed an extremely high invasion rate in one of the replicates, which contributed to the increase in the mean and spread of data (Figure 3). This isolate holds the GimB operon, which is important for NMEC pathogenicity, in addition to *ibeA*, which is related to invasion (Ewers et al., 2007). Only two other isolates contained the *ibeA* gene, isolate 3586 and 220, the latter also displaying an increased cell invasion after digestion. When assessing the ability to adhere to and invade human colorectal cells, the current study found an overall increasing trend of cell association after the digestion procedure.

To our knowledge, the survival of ESC-resistant *E. coli* through an *in vitro* digestion model has not been previously studied. Our findings are in concurrence with a study using an *in situ* model that demonstrated the survival and colonizing abilities of an ESBL-resistant *E. coli* strain from poultry. However, this study only focused on the latter steps of digestion (cecum and colon) and was only performed for a single isolate (Smet et al., 2011). *In vivo* digestion

models are thought to resemble the most life-like conditions but raise ethical questions as they require living animals or human volunteers to carry them out. They are in addition time-consuming, expensive, and require specialized facilities. A large variety of *in vitro* digestion models have been established as good alternatives to *in vivo* models. The dynamic models are the ones that simulate the most accurate digestion; however, these are still expensive, and the comparison of results between different laboratories has proven difficult. Due to the recent harmonization of the INFOGEST static *in vitro* digestion model, it is now possible to standardize research regarding digestibility across laboratories. The current model has been adjusted with minor modifications to fit microbiological studies such as the survival of bacteria as used in this study. A limitation of this study is that a static *in vitro* assay does not exactly replicate the conditions in the gastrointestinal tract. However, this model has been documented to be physiologically comparable to *in vivo* porcine digestion of skim milk powder (Egger et al., 2017). In addition, dietary and genetic host factors that may affect the individual host-bacteria interactions in the gastrointestinal tract have not been considered in the current study.

Determination of different pathovars of *E. coli* based on the analysis of their virulence gene content is highly dependent on the database used, as there may be individual differences in which genes are included in the defining criteria. This study used a custom-made database containing over 1,191 genes to increase the coverage of the number of VAGs and compared the results to a common K12 *E. coli* strain. Based on the genotypic results, isolates 220 and 7037 should be classified as human UPEC. A previous analysis of a subgroup of the isolates included in this study concluded that the risk of developing UTI upon exposure to ESC-resistant *E. coli* from poultry was limited. Nevertheless, with this expanded knowledge by applying a wider VAGs search, the risk of causing disease appears to be higher than first anticipated (Buberg et al., 2021). Except for isolates 220 and 7037, there were very few virulence traits connected to known human pathogenic variants of *E. coli*. The strain 3680 contained some genes (e.g., *ehaA* and *espY3*) associated with both enterohemorrhagic/enteropathogenic *E. coli* (EHEC/EPEC) and Shigatoxin-producing *E. coli* (STEC) (Figure 4). However, the lack of specific toxins makes it questionable whether this isolate should be classified as an IPEC strain.

In summary, the current study aimed to evaluate the consequences of consumer exposure to ESC-resistant *E. coli* using a static *in vitro* digestion model and evaluated conjugation and cell adhesion and invasion for digested and non-digested isolates, in addition to performing an in-depth VAG analysis to evaluate ExPEC potential. We conclude that the pathogenic potential is highly dependent on the characteristics of the individual isolates. The isolates 7037, 220, and 3680 contained genes and characteristics classifying them as ExPEC. As they additionally encode and express ESC resistance, they may complicate treatment in the case they cause disease in a human host. This study demonstrates that poultry meat may, although to a limited extent, represent a reservoir and be a vehicle for the dissemination of potential human pathogens and plasmid-borne resistance determinants such as bla_{CMY-2}.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

MB planned, carried out the experiment, and interpretated and analyzed the results. MB wrote the manuscript with support from YW, BL, and IW. BL performed the VGA analysis. YW and IW supervised the project. All authors provided critical feedback and helped shape the research, analysis, and manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1050143/full#supplementary-material

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Mobile genetic elements drive the multidrug resistance and spread of *Salmonella* serotypes along a poultry meat production line

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The presence of mobile genetic elements in *Salmonella* isolated from a chicken farm constitutes a potential risk for the appearance of emerging bacteria present in the food industry. These elements contribute to increased pathogenicity and antimicrobial resistance through genes that are related to the formation of biofilms and resistance genes contained in plasmids, integrons, and transposons. One hundred and thirty-three *Salmonella* isolates from different stages of the production line, such as feed manufacturing, hatchery, broiler farm, poultry farm, and slaughterhouse, were identified, serotyped and sequenced. The most predominant serotype was *Salmonella* Infantis. Phylogenetic analyses demonstrated that the diversity and spread of strains in the pipeline are serotype-independent, and that isolates belonging to the same serotype are very closely related genetically. On the other hand, *Salmonella* Infantis isolates carried the pESI IncFIB plasmid harboring a wide variety of resistance genes, all linked to mobile genetic elements, and among carriers of these plasmids, the antibiograms showed differences in resistance profiles and this linked to a variety in plasmid structure, similarly observed in the diversity of *Salmonella* Heidelberg isolates carrying the IncI1-Iα plasmid. Mobile genetic elements encoding resistance and virulence genes also contributed to the differences in gene content. Antibiotic resistance genotypes were matched closely by the resistance phenotypes, with high frequency of tetracycline, aminoglycosides, and cephalosporins resistance. In conclusion, the contamination in the poultry industry is described throughout the entire production line, with mobile genetic elements leading to multi-drug resistant bacteria, thus promoting survival when challenged with various antimicrobial compounds.

KEYWORDS

***Salmonella*, genomics, poultry, antimicrobial resistance, mobile genetics elements**

1. Introduction

Food-borne diseases are taken as a main biological concern in the food industry and public health, and *Salmonella enterica* as one of the most common etiological agents (Wotzka et al., 2017). The disease caused by *Salmonella* contamination is one of the most recurrent worldwide zoonosis originated from food (Majowicz et al., 2010; EFSA, 2018; Fazza et al., 2021; Lee and Yoon, 2021). The current demand for food, as well as the production practices themselves, such as the overcrowding of cages, creates several risks of contamination, which leads to the increasing appearance and persistence of pathogens in the food industry. The main reservoir of *Salmonella* is the gastrointestinal tract of the host, yet the resulting contamination is able to spread and remain on surfaces throughout production (Golden et al., 2021).

Several serotypes of *S. enterica* have been reported in the poultry industry, with traits of concern for food safety (Oscar, 2021; O'Bryan et al., 2022), namely profiles of multi-drug resistance (MDR) and the increased prevalence of virulent serotypes in farm animals and humans (Shah et al., 2017).

Significant non-typhoid serotypes are Infantis (Mughini-Gras et al., 2021; Pardo-Esté et al., 2021), Typhimurium, Enteritidis (Karabasanavar et al., 2020), and Heidelberg (Dominguez et al., 2021), among others (Sun et al., 2021). The main cause of multi-resistant capacity is the indiscriminate use of antimicrobials, and the intensive use of cleaning and sterilization processes that are selective pressures upon the strains (Mahnert et al., 2015; Obe et al., 2021). These MDR strains are commonly found in poultry farms around the world, including Chile (Castro-Vargas et al., 2020; Pardo-Esté et al., 2021). In this context, the serotype Infantis is a worldwide-emerging serotype that is classified as one of the most prevalent non-typhoidal *Salmonella* in humans in Europe (EFSA, 2019). Furthermore, several reports indicate that the serotype Infantis is the most prevalent one in the poultry industry (EFSA, 2019; Vinueza-Burgos et al., 2019).

Diverse molecular mechanisms in non-typhoid *Salmonella* favor survival under various types of stresses (Whitehead et al., 2011; Kim et al., 2022). Such conditions, like the use of disinfection protocols or antimicrobial agents, are found in an industrial setting and can trigger a stress response. However, excessive or indiscriminate disinfection induces tolerance to these agents (Ortega Morente et al., 2013), causing the appearance of strains that are potentially resistant to antimicrobial agents along the production line, which can generate a worrying epidemiological scenario in which MDR strains can emerge.

Given this potential epidemiological risk, the rapid identification of Enterobacteriaceae strains, typing and molecular characterization of *Salmonella* using whole-genome sequencing to identify genomic profiles of interest to the poultry industry, has become a necessity (Park et al., 2014; Gymoese et al., 2019; Pardo-Esté et al., 2021). These constitute useful tools for the genomic surveillance of specific strains in outbreaks related to the industry or in epidemiological research. By typifying the whole-genome, genetic "fingerprints" that are specific for each site and time of isolation can be generated in order to further evaluate the epidemiology of an outbreak based on variances and mutations. These characterizations of *Salmonella* genomes isolated from the production line have described genetic determinants that confer resistance and virulence (de Melo et al., 2021; Mohamed et al., 2021; Zakaria et al., 2022). It is important to highlight that many of these determinants are present in mobile elements such as plasmids (Aviv et al., 2014; Kürekci et al., 2021; Tyson et al., 2021), integrons (Badouei et al., 2021), and transposons (Galetti et al., 2021). It is these genetic elements that cause a high risk of spreading antimicrobial resistance through horizontal gene transfer. Recently, an increase in the prevalence of *Salmonella* with an MDR profile has been described in the poultry industry (Gambino et al., 2022; Pławińska-Czarnak et al., 2022).

In this context, mobile genetic elements are involved in the ability of the bacteria to adapt to stress pressures (Hull et al., 2022). Despite this, the relationship of *Salmonella* and these elements within the production line environment of a chicken farm remains understudied. An understanding of these molecular factors would contribute to the comprehension and mitigation of widespread contamination. Therefore, in this study, we analyzed *Salmonella* populations isolated from a poultry farm in Chile, characterized them, and determined their genetic profiles, focusing on the presence of mobile genetic elements that contribute to pathogenicity and antimicrobial resistance.

2. Materials and methods

2.1. Study design

In this study, we characterized and compared 133 genomes of *S. enterica* isolates obtained from a production line in a poultry farm in 2018–2021, 30 isolates previously characterized (Pardo-Esté et al., 2021) and 103 isolates characterized in this work. Strains were isolated from the feed, hatchery, broiler, poultry farm, and slaughterhouse. Sampling, *Salmonella* isolation, and corroboration were performed as previously described (Pardo-Esté et al., 2021). All isolates were serotyped by Check & Trace (Check-Points BC™, Netherlands). The distribution of the isolates is detailed in Table 1 (for more details see Supplementary Table S1).

2.2. Antibiogram

All sequenced isolated were tested against a panel of 20 antibiotics using the disk diffusion method following CLSI guidelines (CLSI, 2018) for Enterobacter ales bacteria group. Antibiotic tested included: ampicillin (AMP, 10 µg); cefazolin (KZ, 30 µg); cefepime (FEP, 30 µg); ceftazidime (CAZ, 30 µg); ceftriaxone (CRO, 30 µg); ciprofloxacin (CIP, 5 µg); gentamicin (GEN, 10 µg); amikacin (AMK, 30 µg); imipenem (IPM, 10 µg); meropenem (MEM, 10 µg); ertapenem (ETP, 10 µg); tetracycline (TCY, 30 µg); ceftazidime/avibactam (CZA, 10/4 µg); piperacillin/tazobactam (TZP, 100/10 µg); trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 µg); ampicillin/sulbactam (SAM, 10/10 µg); nitrofurantoin (NIT, 200 µg); chloramphenicol (CHL, 30 µg); and aztreonam (ATM, 30 µg), all of which were supplied by OXOID (Hampshire, England). Isolates resistant to three or more antimicrobial classes were cataloged as MDR.

2.3. DNA extraction and whole-genome sequencing of 133 strains

To extract the strain genomic DNA material, we used a commercial kit (Quick-DNA Miniprep Kit, Zymo Research)

TABLE 1 Distribution of the isolated serotypes in the production line.

Serotype	Feed manufacturing	Hatchery	Broiler farm	Slaughterhouse	Poultry farm	Total
Agona	5	0	1	9	4	19
Corvallis	1	9	1	6	9	26
Heidelberg	10	2	2	15	20	16
Infantis	0	3	0	3	10	49
Senftenberg	14	4	0	3	2	23
Total	30	18	4	36	45	133

following the manufacturer's instructions. The amount and quality of the extracted DNA was evaluated by fluorometry (Qubit 3.0, Thermo Fisher Scientific), while integrity was confirmed by capillary electrophoresis (LabChip GX Touch Nucleic Acid Analyzer, PerkinElmer, Spain). The DNA samples were sent to MIGS Center (Pittburgh, PA, USA) for paired-end library construction (2 × 151 bp paired-end reads) and sequenced in the NextSeq 2000 platform (Illumina Inc., San Diego, CA, USA). We used FastQC v0.11.9 (Andrews, 2010) for quality control and Trim-Galore v0.6.6 (Krueger, 2012) for filtering and trimming (-quality 30-trim-n-retain_unparied). Moreover, we used SPAdes v3.15.2 (Bankevich et al., 2012) for genome assembly (−isolate −k 33,55,77,99,111). The quality of the contigs was evaluated using QUAST v5.0.2 (Gurevich et al., 2013) and Depth coverage was determined by assigning the reads to the assemblies using Bowtie2 v2.4.2 (Langmead and Salzberg, 2012) and Samtools v1.12 (Danecek et al., 2021). The coding sequence predictions for genes and functional annotation were carried out with Prokka v1.14.6 (Seemann, 2014) and eggNOG-mapper v2.1.01 (Huerta-Cepas et al., 2017) using the EggNOG v5.0.2 (Huerta-Cepas et al., 2019). The completeness of the assembly was evaluated by identifying the ortholog markers for specific lineages, using BUSCO v5.2.2 (Manni et al., 2021) and checkM v1.1.3 (Parks et al., 2015). The genome assemblies generated in this research have been deposited at the DDBJ/ENA/GenBank under the Bioproject: PRJNA890630.

2.4. Bioinformatics analyses

2.4.1. Genoserotyping and MLST analysis *in silico* typification and serotypification

Serotype predictions were performed by SeqSero2 v1.2.1 (Zhang et al., 2019) using the assembled genomes (−t 4 −k a). The information from the reads (−t 2 −m k) was corroborated by identifying in the data base the serotype determinants for the *Salmonella* genus (cluster *rfb*, *fliC* y *fljB*). Also, using the PubMLST data base for *S. enterica* (senterica) (Jolley et al., 2018), housekeeping genes *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA* were evaluated using mlst v1.2.1 (Page et al., 2016).

2.4.2. Core genome Single Nucleotide Polymorphism (SNP) analysis and phylogeny

To determine all the genetic markers present in all the genomes from the isolates, we use GET_PHYLOMARKER v2.3.1 (Vinuesa et al., 2018) with the default parameters together with the GET_HOMOLOGUES (Contreras-Moreira et al., 2017) data output performed with the default parameters for the 133 annotated genomes. Additionally, using SNPs-sites v2.5.1 (Page et al., 2016), we determined the SNPs in the exit alignment (−c as exit). Then, the phylogeny was generated with IQ-TREEv2.2.0-beta (Nguyen et al., 2015), using the model for substitution GTR+ ABS, with 10,000 Bootstraps and selecting the best tree every 1,000 iterations. The results were visualized using FigTree v1.4.4 including the origin and the serotype of each strain. Also, from the SNP sequences from SNPs-site, we calculated the pairwise distance matrix between the genomes using snp-dists 0.8.2., which were then visualized with the Seaborn v0.11.2 package.

2.4.3. Plasmid replicon detection and generation of draft plasmid sequences

We used PlasmidFinder v2.1 in default setting with the following parameters for threshold and coverage: 95% identity, 100% coverage, and 1×10^{-5} e-value, to identify plasmid replicons in genomes. We used the PLSDB database (Galata et al., 2019) to filter and create a specific database for each identified replicon. Then, using BLAST 2.12.0 (Altschul et al., 1990), we compared the contigs containing the identified replicon with the database created to select a reference plasmid, using the following parameters as selection criteria: >75% identity, >75% coverage and 1×10^{-5} e value. The first hit was chosen as the reference plasmid. Using BLAST, the contigs of the genome with more than 10 kbp were aligned with the reference plasmid and those that met the selection criteria were selected as part of the plasmid: >75% identity, >50% coverage and 1×10^{-5} e value. We chose an identity threshold of 75% because the replicon and the genes that make up the plasmid may be present in other bacteria and may not be conserved. On the other hand, 50% coverage was used to include discontinuous contigs, thus allowing the identification of breaks in the continuity of the plasmid due to other mobile elements, insertions or deletions. Finally, the smallest contigs (>3,000—<10,000) were aligned with the reference plasmid and evaluated by BLAST with the database created. If such hits met the criteria described above, these contigs were selected and denoted as part of the same plasmid. Selected contigs were extracted from the genome and used to generate a draft plasmid. Synteny was then ordered with the reference plasmid and saved for further analysis in fasta format. The isolates presenting the replicon of the Col plasmids were not processed in drafts due to the short sequences of the contigs and the difficulty of their assembly. However, contigs harboring the replicon and an antimicrobial resistance gene in the same sequence were analyzed as plasmids corresponding to the replicon.

2.4.4. Detection of insertion sequences, transposons and integrons

The identification of other mobile elements in the draft plasmids, such as transposases and integrases (as well as their integration sequence), was carried out by BLAST using the TnCentral (Ross et al., 2021), ISFinder (Siguier et al., 2006), and Integrall (Moura et al., 2009) databases. The cut-off thresholds were 95% identity, 100% coverage, and a 1×10^{-5} e-value.

2.4.5. Detection of virulence and resistance genes

We used the Comprehensive Antibiotic Resistance Database (CARD) to identify resistance elements (Alcock et al., 2019) to antibiotics and microbicides in the assembled genomes and the draft plasmids. Also, we used Virulence Factor Database (VFDB) to identify virulence factors. The thresholds to identify the virulence and resistance factors were 95% identity, 100% coverage, and a 1×10^{-5} *e*-value.

2.5. Statistical analyses

Antimicrobial susceptibility data were tabulated in csv format. Intermediate resistance collapsed into the susceptible category when resistance was represented as a binary variable. The presence or absence of a known resistance gene was compared with the interpretation of resistant or susceptible phenotypes when cultivated on the corresponding antimicrobial agent. Agreement measurements between phenotypic and genotypic results were performed using Cohen's Kappa statistic (κ). Where the strength of agreement Cohen's Kappa coefficient ranges from 0 to 0.2 none to slight agreement, 0.2–0.4 fair agreement, 0.4–0.6 moderate agreement, 0.6–0.8 good agreement, and 0.8–1.0 very good agreement.

3. Results

3.1. *In silico* serotypification of the *Salmonella* isolates

Overall, 133 isolates were serotyped using the microarray-based method Check&Trace (Check-Points, The Netherlands), from which the strains were classified into five serotypes: Agona, Corvallis, Heidelberg, Infantis, and Senftenberg. On the other hand, the SeqSero tool enabled us to perform analyses of genoserotypes from the WGS data for the 133 isolates coinciding with the results obtained by Check&Trace. From these analyses, we also identified 5 serotypes: 36.8% (49/133) of the isolates correspond to Infantis, 18.8% (25/133) Corvallis, 14.3% (19/133) Heidelberg, 14.3% (19/133) Agona, and 12.8% (17/133) Senftenberg. In addition, typing of the genome by MultiLocus Sequence Typing (MLST) described at least one Sequence Type (ST) strain typing for each serotype, of which the Senftenberg serotype had two types of strains.

Phylogeny of the *Salmonella* isolates.

According to the phylogeny based on the multiple alignment for the core SNPs of 546 genetic determinants present in the 133 isolates (Figure 1), we found 6 clusters that corroborated the genome typification and their grouping by serotype. In addition, it is highlighted that the serotype Infantis shows clades with isolates present in several areas of the production line, while the Corvallis and Heidelberg serotypes have few differences between the isolates of the clade, suggesting that their contamination has a clonal origin and that these two strains had then spread throughout the production line. However, the presence of the Senftenberg ST185 clade is predominant in the manufacture of the food. A similar grouping is observed with the Agona serotype, where a clade group was isolated exclusively from the feed manufacture, separating itself from the rest of the production line.

The pairwise distance matrix between the genomes resulted in a range of 0–14,993 between all the isolates (Figure 2). The maximal distance found among the isolates of Agona serotypes was 199, and was lower in the others (Infantis 123, Heidelberg 2, Corvallis 4, and Senftenberg ST185 and ST14 12 and 31, respectively). However, among the Senftenberg ST strains, their distance ranges from 11,522 to 11,544, coinciding with the result of the presence of two different strains in the Senftenberg serotype.

3.2. Replicon detection for plasmids and mobile elements

We detected plasmid replicon-associated genes in at least 104 of the 133 isolates (Table 2). Moreover, our results indicate the coexistence of multiple replicons in 20 isolates, of which Col, Col3M, IncFIB and IncI1-Iα are the most commonly identified. We reconstructed 88 draft plasmid sequences from references corresponding to the identified replicons. We determined that 56 draft plasmid sequences possessed resistance elements, including the IncFIB plasmid present in serotypes Infantis, the IncI1-Iα plasmid found in Heidelberg, Senftenberg and Corvallis, as well as the Col plasmid present in various isolates. We identified insertion sequences and transposonic elements in the plasmid drafts. The most frequent insertion sequences were IS26, IS91, IS200, IS256, IS630, IS1326, IS903, ISEch12, ISEc57, ISVsa3, and ISEcp1. Furthermore, in the IncFIB plasmid of Salmonella Infantis, we identified sequences for attl recombination corresponding to class I and class II integrons. On the other hand, the IncI1-Iα plasmid also contained the attl insertion sequence only for class I integrons.

3.3. Virulence factors and virulence profiles

From Whole Genome Secuence (WGS) analysis, we identified a total of 29,931 virulence factors among all isolates. Of these, 51.3% (15,378/29931) correspond to adherence determinants and biofilm formation, and 42.3% (12,651/29931) to secretion system effectors, such as components of the type III secretion system including the pathogenicity islands 1 and 2, along with their respective effectors. The remaining virulence factors 6.4% (1901/29931) correspond to competitive advantages, colonization, virulence regulation and stress adaptation, iron, magnesium and phosphorus metabolism, toxins, and siderophores (for more information see Supplementary Table S2).

Overall, the identified virulence-associated genetic components are homogeneous among strains. For example, in relation to adherence and biofilm formation, we found fimbrial elements commonly distributed among the isolates, such as csg, fim, lpf, and non-fimbrial elements shdA and sinH. All isolates have the type III secretion systems

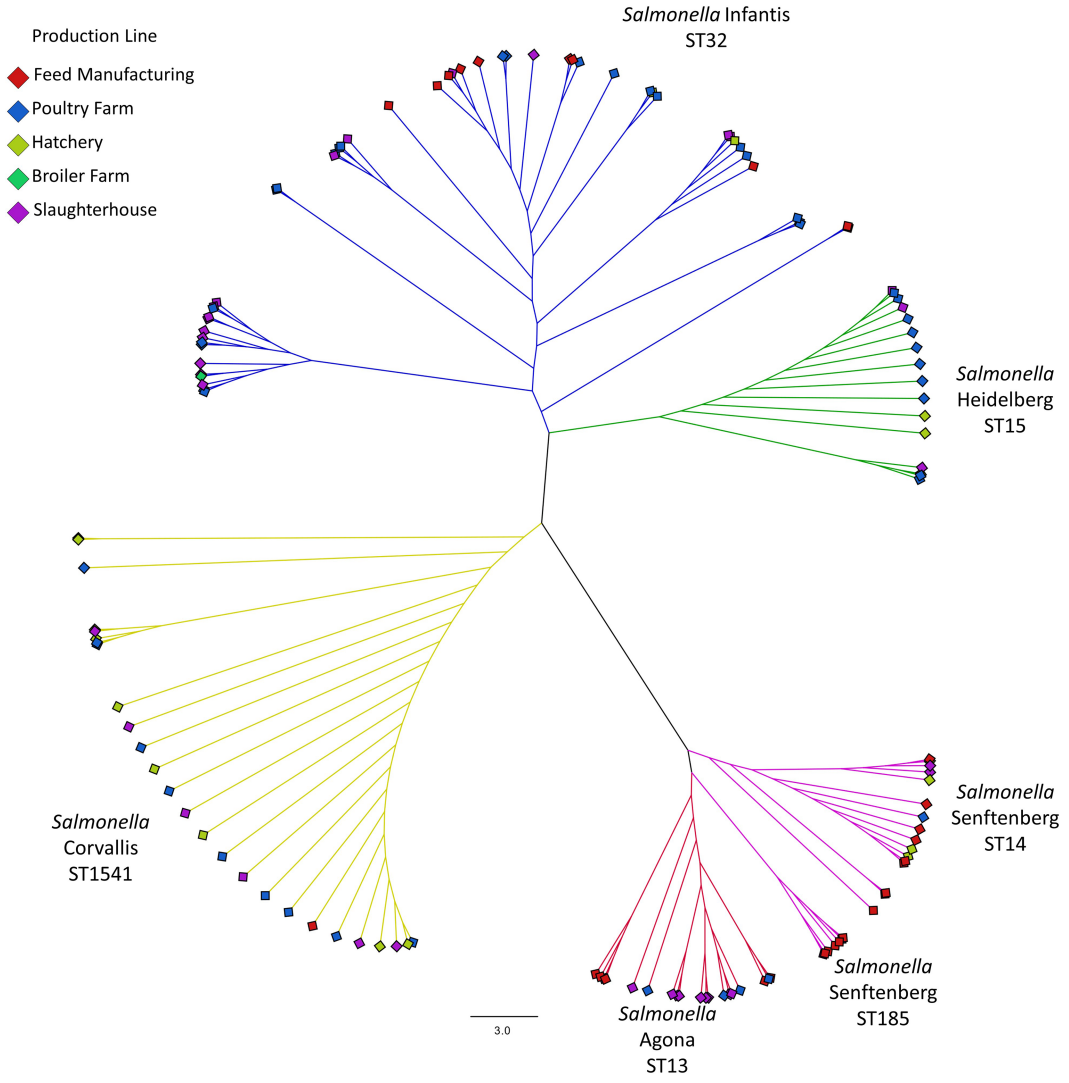

FIGURE 1
Phylogeny based on SNPs from the coregenome (546 genetic markers). The dotted lines demark the serotype group and their typification, while diamonds at the extremes of each branch correspond to the origin of the strain within the production line, coded by colors.

of SPI-1 and SPI-2. On the other hand, Salmonella Heidelberg presents the highest frequency of sodC1, which has been associated with stress response. We highlight the main differences are found between isolates possessing IncFIB and IncI1-Iα plasmids. IncFIB carriers possess ccdAB and vapBC toxin-antitoxin systems for plasmid maintenance; in addition, IncFIB possesses a fae operon related to fimbrial formation, as well as a yersiniabactin operon related to siderophore formation. In contrast, IncI1-Iα plasmid carriers exhibit the toxin-antitoxin parAB systems for maintenance. It should be noted that in isolates carrying the IncI1-Iα plasmid, they also exhibit the colicin-IB toxin.

Antimicrobial resistant genes, and their relationship with mobile elements and their agreement with the resistance phenotype.

To further characterize the isolates, we used the CARD database to determine the existence of antimicrobial resistant genes in the obtained genomes (Table 3). There are 546 antimicrobial resistant genes, mostly assigned to the plasmid sequence drafts, except *fosA7*, present on the chromosome of the Heidelberg serotypes. The serotypes with the highest amount of antibiotic resistance genes were Infantis (447/546 resistance genes) remotely followed by Heidelberg (62/546 resistance genes).

In addition, we determined that there are susceptible genetic profiles, without the presence of identified resistance genes. In this context, all Senftenberg ST185 and 11 Senftenberg ST14 isolates, the majority of Agona isolates (18 of 19 isolates identified) and 9 Heidelberg isolates are susceptible to antibiotics. All the other isolates analyzed show resistance and multi-resistant profiles, the latter reflecting the presence of possible mobile elements.

Multi-resistant profiles with the highest diversity of antibiotic resistant genes are present in strains belonging to the serotype Infantis (Table 3), including 12 genes in a single isolate. In addition, *tet(A)*, *sul1*, *ant(3′)*-Ia and *qacEΔ1* genes, which represent resistance to tetracycline, sulfonamides, aminoglycoside and quaternary ammonium disinfectant, respectively, are present in all Infantis isolates with an MDR profile.

Overall, 56 of the 133 isolates have MDR profiles, with *Salmonella* Infantis isolates accounting for 87.9% of them (47 of 56 MDR).

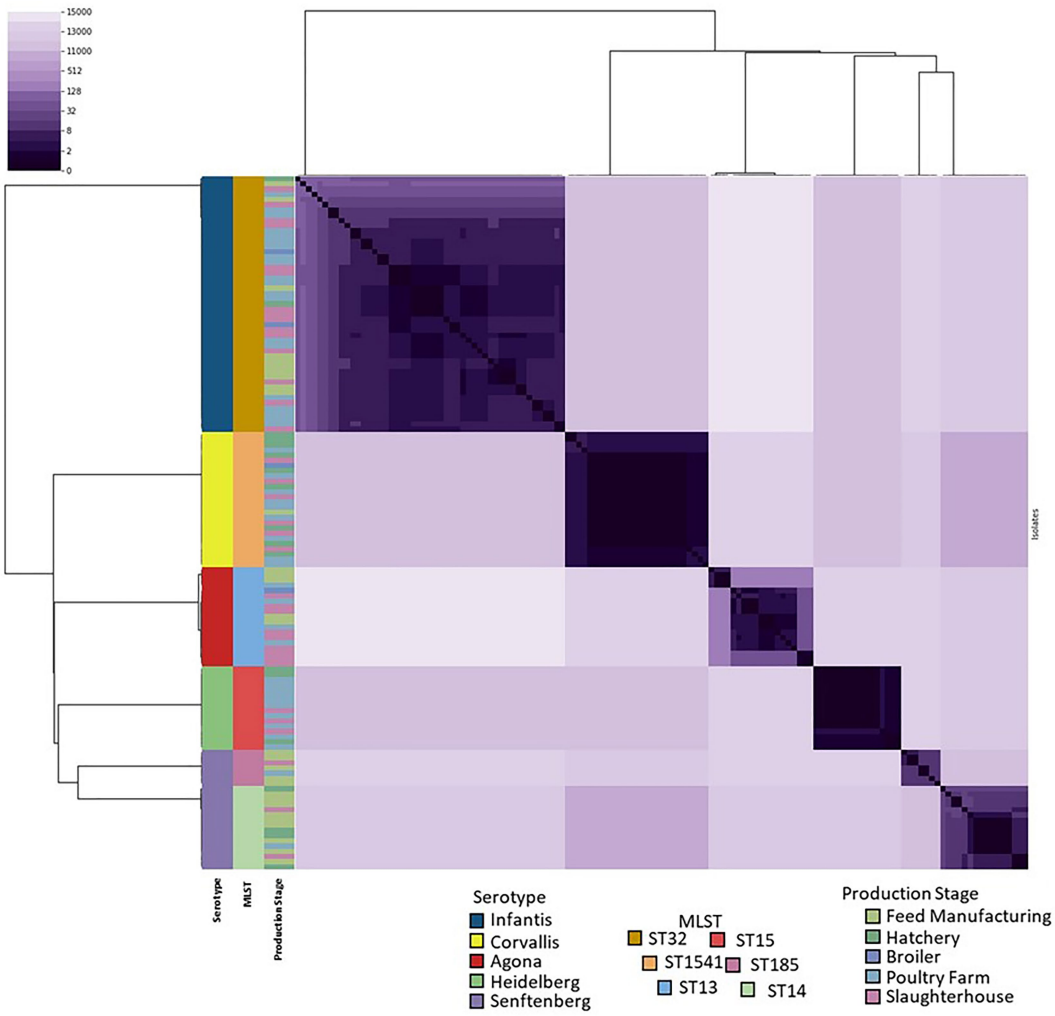

FIGURE 2
Heatmap of pairwise SNP distances of the 133 genomes. The dendrogram corresponds to the hierarchical clustering of the SNP distance matrix data. The colors on the left correspond to the serotype, genome typing and origin of the isolate within the production line.

From the results drawn from the resistance profiles of these isolates, we can infer that the poultry farm itself harbors the greatest amount of MDR profiles of all the sites sampled in this study. In contrast, the lowest frequency of isolates with an MDR profile is found in the feed manufacturing stage (Supplementary Table S1).

Furthermore, we aimed to describe the genomic context of resistance genes to relate them to mobile elements identified in the draft plasmids from the *Salmonella* isolates. For instance, in the IncFIB plasmid draft (Figure 3A), two clusters with high densities of mobile elements and resistance genes were found. Cluster A, of approximately 21 kbp (Figure 3B), has a Tn2 family transposon that contains the *tetR* and *tetA* genes that confer resistance to tetracyclines and includes a class 1 integron together with a *mer* operon for mercury resistance. The integron has insertions of the antibiotic resistance genes *ant(3′)-Ia*, *qacEΔ1* and *sul1*, which confer resistance to aminoglycosides, quaternary ammonium and sulfonamides, respectively. This cluster is suggested to be well conserved as it is present in all Infantis isolates containing the IncFIB plasmid. On the other hand, cluster B (Figure 3C), is 32 kbp in size, and is made up of various mobile elements, mainly of the IS*26* type. These mobile elements carry aminoglycoside resistance genes, such as *aph(3′)-Ia*, *acc(3)-IVa*, *aph(4)-Ia*, as well as fosfomycin resistance genes, *fosA3*. On the other hand, the presence of a transposon that contains the *floR* gene, for amphenicol resistance, is characterized by having two passenger genes that correspond to *virD2* and a gene from the *lysR* transcriptional regulator family. This cluster also includes a transposon that is truncated by the insertion of another mobile element, which contains the *fosA3* gene. This transposon includes the $bla_{\text{CTX-M-65}}$ gene, an extended-spectrum beta-lactamase, together with the *yncD-Cter/ΔiroN* gene, involved in the formation of siderophores.

In addition, all carriers of the IncI1-Iα plasmid (Figure 4) possess the $bla_{\text{TEM-1b}}$ and *dfrA1*, encoding an extended-spectrum beta-lactamase, and trimethoprim resistance, respectively. The *dfrA1* gene belongs to a class I integron that also has a pseudogenized *aac(3)-IV* gene, suggesting that the insertion of the transposon containing the $bla_{\text{TEM-1b}}$ gene disrupts the *aac(3)-IV* sequence. On the other hand, the IncI1-Iα plasmid has the *sul3* gene present in an IS*26* transposon along with two other reading frames. On the other hand, the *ant(3′)-Ia* gene is found between two transposons, IS*26* and IS*Vsa3*, and is also adjacent to *attC* insertion sequences, suggesting that this gene belonged to an integron. Other identified resistance genes, *floR* and

tetA, are found together in a transposon along with passenger genes such as *lysR*.

The drafts suggest that there are variants in the structure of the plasmid that contain the mobile elements and their resistance genes, identifying a cluster (hot spot) of approximately 17 kb where the resistance genes are concentrated.

Finally, we evaluated the consistency of the genotype and phenotype of resistance to the corresponding antibiotic (Table 4). In this context, the percentage agreement and Cohen's kappa coefficient were evaluated for each antibiotic. We observed a range of values for kappa between 0.687 and 1.0, with the lowest values recorded for beta-lactams and trimethoprim/sulfamethoxazole. Ceftriaxone resistance is due to the presence of extended spectrum beta-lactamase genes such as $bla_{\text{CTX-M-65}}$ and $bla_{\text{TEM-1b}}$, while sulfamethoxazole/trimethoprim is associated with *drfA* and *sul* genes. Therefore, the visible differences at the phenotypic level could be linked to the variability of the mobile elements within the plasmids of these isolates.

TABLE 2 Prediction of the presence of replicons from plasmids in *Salmonella* isolates.

Serotype	Replicon*	Total
Agona	Col(pHAD28)**	1
	Col3M	9
	IncH1B(pNDM-CIT)	2
	IncFIB(pHCM2)	1
Corvallis	Col(pHAD28)**	23
	Col3M	2
	IncI1-Iα**	1
Infantis	Col(pHAD28)**	3
	IncFIB(pN55391)**	47
	IncX4	2
Heidelberg	Col(pHAD28)**	15
	IncI1-Iα**	7
Senftenberg	Col(pHAD28)**	5
	IncH1B(pNDM-CIT)	5
	IncI1-Iα**	1
	IncFIB(K)	1
	IncFIB(S)	4

*Replicon detected by PlasmidFinder.
**Present in the reconstructed plasmid draft sequence (this work).

4. Discussion

This investigation aimed to study and compare the structure and genetic dynamics of the *S. enterica* isolates obtained from the production line in a poultry farm. We detected the presence of the Infantis, Heidelberg, Agona, Corvallis, and Senftenberg serotypes in several stages of production (Table 1), of which *Salmonella* Infantis was the serotype with greatest prevalence in this study. This is in accordance with the evidence that proposes that this is an emerging serotype of concern worldwide (EFSA, 2019; Lapierre et al., 2020; Li et al., 2020; Mejía et al., 2020; Pardo-Esté et al., 2021). On the other hand, typification of the genome determined the presence of six strains, where the presence of *Salmonella* Infantis ST32 is of special interest, as it has been previously-described that this is a multi-resistant, emergent strain that carries the plasmid type pESI incFIB containing several resistant elements (Alba et al., 2020; Kürekci et al.,

TABLE 3 Antibiotic classes and resistant genes identified in *Salmonella* isolates.

Antimicrobial family or agent	Antimicrobial resistant gene	Serotype (N° MDR)				
		Agona (0)*	Corvallis (1)*	Heidelberg (7)*	Infantis (47)*	Senftenberg (1)*
Quinolone	*qnrB19*	1	22	17	1	5
Aminoglycosides	*aac(3)-IV*	0	0	0	42	0
	ant(3′)-Ia	0	0	6	47	1
	aph(3′)-Ia	0	0	0	31	0
	aph(4)-Ia	0	0	0	42	0
Beta-lactams	$bla_{\text{CTX-M-65}}$	0	0	0	41	0
	$bla_{\text{TEM-1B}}$	0	1	7	0	1
Trimethoprim	*dfrA1*	0	1	7	0	1
	dfrA14	0	0	0	36	0
Phenicol	*floR*	0	1	5	40	0
Fosfomycin	*fosA3*	0	0	0	19	0
	fosA7	0	0	16	0	0
Disinfectant	*qacEΔ1*	0	0	0	47	0
Sulfisoxazole	*sul1*	0	0	0	47	0
	sul3	0	0	6	0	1
Tetracycline	*tet(A)*	0	1	5	47	1

*Number of isolates that have multi-resistant profiles (>3 genes of antibiotic resistance).

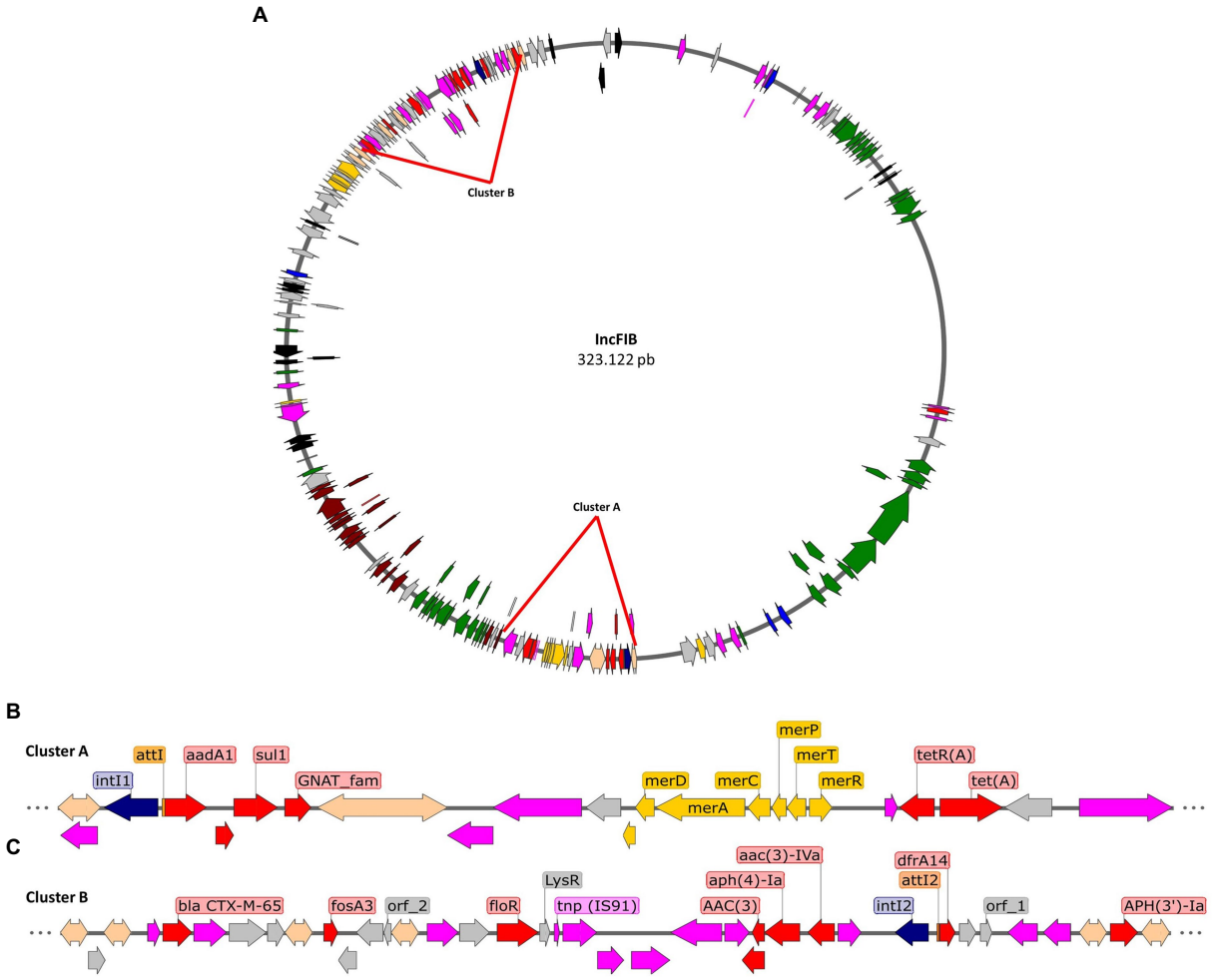

FIGURE 3
Representation of the IncFIB plasmid draft of serotype Infantis isolates. **(A)** Draft of the IncFIB plasmid, highlighting the position of clusters A and B that contain diverse mobile elements that include antibiotic resistance genes. **(B)** Genomic context of mobile and resistance elements in cluster A. **(C)** Genomic context of mobile and resistance elements in cluster B. Transposons in fuchsia arrows, integrases in blue arrows, sequence of integration in orange rectangles, antibiotic resistance genes in red arrows, metal resistance genes in yellow arrows, virulence genes in green arrows, conjugation genes in brown arrows, and other passenger genes in gray arrows.

2021; Bertani et al., 2022). In addition, we detected the presence of the serotype *Salmonella* Heidelberg ST15, which is known to be a carrier of the IncI1-α plasmid, a plasmid associated with the $bla_{\text{TEM-1b}}$ gene, an extended-spectrum beta-lactamase (Castellanos et al., 2018; van den Berg et al., 2019). The phylogeny results suggest that there is no correlation between the stage of the production line and the grouping of the strains, for all the serotypes evaluated (Figure 1), as concluded previously by Pardo-Esté et al. (2021) for *Salmonella* Infantis isolates. In addition, the phylogeny and the study of the distance by pairs of the SNPs confirmed that the diversity and propagation of the strains along the production line is independent of the serotype, denoting a high genetic relationship and a low variability in the core genome (Figure 2), suggesting that propagation is of clonal origin at all the sampled stations. This finding could be due to *Salmonella* contamination circulating and re-entering the industrial environment, as these bacteria has been linked to the process of poultry meat at various stages, including presence in incoming animals, in feed production, and even in personnel (Marin et al., 2022).

The differences between virulence factors are mainly associated with the difference between serotypes, with genomic mobile elements being the main factor contributing to the variability. Moreover, the presence of antimicrobial resistance genes also contributes to the pathogenicity of carrier bacteria. In this sense, the presence and increased expression of virulence genes linked to pathogenicity in bacteria with MDR profiles (García et al., 2011; Long et al., 2022; Salaheen et al., 2022) has been described. In this context, the presence of plasmids and other mobile elements in emerging pathogenic bacteria is very common, since the main route of acquisition of these elements is horizontal gene transfer (Vinayamohan et al., 2022).

Furthermore, in this study we found the IncFIB and Incl1-α plasmids in the strains (Figures 3 and 4), which contain a wide variety of resistance elements that have been described and linked to mobile genetic elements (Partridge et al., 2018). These plasmids have been reported in other serotypes, including IncFIB type pESI in Agona and Senftenberg, as well as in isolates that harbor both plasmids (Cohen et al., 2022; dos Santos et al., 2022), conferring a severe public health risk. However, there are some discrepancies (Table 4) between the genotype and phenotype of the strains, which could be attributed to several factors, such as promoter regions, secondary structures in the Shine-Dalgarno region, and the presence

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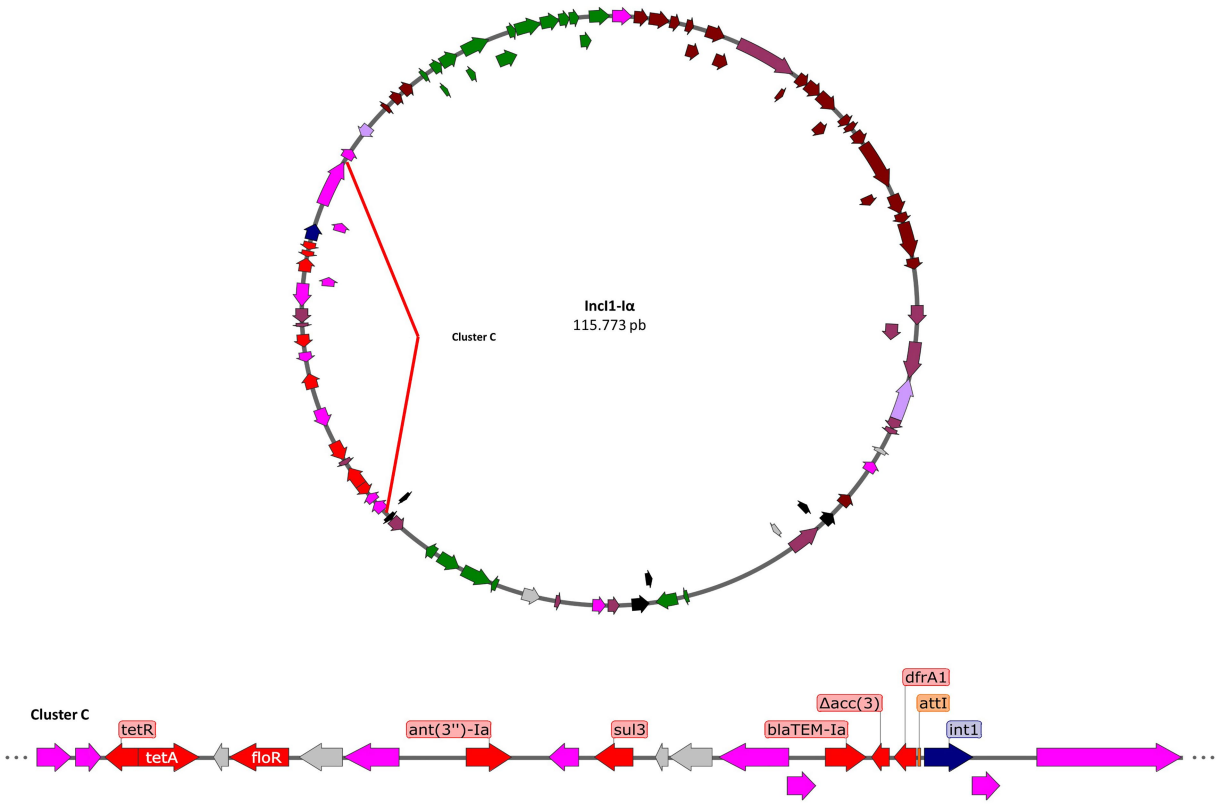

FIGURE 4
Genomic contexts of the resistant genes (red arrows) in plasmid IncI1-Iα. Transposons in fuchsia arrows, integrases in blue arrows, sequence of integration in orange rectangles, antibiotic resistance genes in red arrows, metal resistance genes in yellow arrows, virulence genes in green arrows, conjugation genes in brown arrows, and other passenger genes in gray arrows.

TABLE 4 Agreement between genotypes and phenotypes of *Salmonella* strains.

Antimicrobial family or agent	Genotype for resistant phenotype*		Genotype for susceptible phenotype		Agreement (%)	Kappa
	Resistant	Susceptible	Resistant	Susceptible		
Aminoglycosides						
Gentamicin	37	5	1	90	95.49	0.893
Kanamycin	31	0	3	82	97.41	0.936
Streptomycin	54	0	0	78	100	1.00
Beta-lactams						
Penicillin						
Ampicillin	44	5	8	76	90.23	0.793
Cephalosporins						
Cefazolin	36	13	1	83	89.47	0.762
Ceftriaxone	38	11	8	76	85.71	0.689
Fosfomycin						
Fosfomycin	34	1	0	97	99.24	0.98
Phenicol						
Chloramphenicol	43	2	7	81	93.23	0.853
Tetracyclines						
Tetracycline	48	5	4	76	93.23	0.858
Trimethoprim/ sulfamethoxazole	33	10	8	82	86.47	0.687

*Genes identified in CARD database.

of unknown or undescribed mechanisms. (Davis et al., 2011). We found the greatest presence of resistant profiles in the Poultry Farm and Slaughterhouse stages, associated with the presence of mobile genetic elements, of which the Infantis and Heidelberg serotypes were particularly multi-resistant (>=7 resistant genes per isolate; Table 2). Both serotypes have $bla_{\text{TEM-1b}}$ and $bla_{\text{CTX-M-65}}$ genes encoding extended-spectrum beta-lactamase present on plasmids, a recurrent trait in resistant bacteria in the poultry industry (Saliu et al., 2017). The presence of $bla_{\text{TEM-1b}}$ is common in plasmids; as such, Heidelberg serotypes with these characteristics have been described in chicken meat imported from the Netherlands (van den Berg et al., 2019). On the other hand, $bla_{\text{CTX-M-65}}$ in Infantis has been described in the United States as a strain of clinical importance since it is directly associated with an emerging MDR strain (Tate et al., 2017; Brown et al., 2018).

The results highlight a progressive increase in resistance in the bacteria that remain in the production line, suggesting the existence of critical points of contamination. Research has previously described that these points are related to exsanguination and evisceration, as well as to cages of contingency and transport as the main reservoir (Marin et al., 2022), resulting in the release from the host of a high density of microorganisms of different origins, contaminating equipment, and personnel.

The intensive use of antibiotics, cleaning, and disinfection protocols in the poultry industry is another aspect that must be considered when analyzing the persistence of bacterial contamination along the production line. The constant and indiscriminate use of such compounds can select for tolerance and resistance in emerging bacterial strains (Mahnert et al., 2015). On the other hand, it has also been described that the ability to form a biofilm is an important trait that is related to pathogenicity and resistance to antimicrobials (Borges et al., 2018; Sun et al., 2019; Obe et al., 2021), as bacteria contained within the biofilm are 1,000 times more tolerant to antibiotics and disinfectants. Furthermore, the cooperation between different bacteria promotes mutual survival in an industrial setting (Dieltjens et al., 2020). In this context, biofilm formation has also been reported to promote the spread of mobile genetic elements (Madsen et al., 2012).

Additionally, the current public health situation associated with the SARS-CoV-2 pandemic has led to the widespread use of disinfectants that can contribute to the appearance of bacteria with MDR profiles, with the potential to harbor mobile genetic elements (Fuga et al., 2022). Therefore, it is important to conduct genomic surveillance and understand genetic dynamics in emerging bacteria such as *Salmonella* in an industrial setting.

5. Conclusion

Mobile genetic elements produce emerging bacteria with a high capacity for resistance to antimicrobials, constituting a danger to public health and a risk to food safety. The high genetic relationship between the bacteria of the poultry industry highlights contamination of the entire production chain by emerging bacteria. This should be considered a reservoir of MDR bacteria with the potential to be transmitted to humans, either directly or through poultry-derived products. Such transmission is facilitated thanks to mobile genetic elements.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA890630.

Author contributions

GK, CP-E, JC-S, and CS: conceptualization, formal analysis, and visualization. GK: data curation. LA-T and CS: funding acquisition, resources. GK, CP-E, and CS: investigation. GK, PZ, JO-P, NG, MZ, and CS: methodology. CS: project administration. JO-P, MT, JV, and CS: supervision. GK and CS: validation. GK and CP-E: writing—original draft. GK, CP-E, JC-S, MT, and CS: writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1072793/full#supplementary-material

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Bla$_{TEM}$-positive *Salmonella enterica* serovars Agona and Derby are prevalent among food-producing animals in Chongqing, China

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Salmonella is one of the most important foodborne zoonotic pathogens, causing global morbidity and mortality in both humans and animals. Due to the extensive use of antimicrobials in food-producing animals, the antimicrobial resistance of *Salmonella* has attracted increasing attention globally. There have been many reports concerning the antimicrobial resistance of *Salmonella* from food-producing animals, meats and the environment. However, few studies on *Salmonella* from food-producing animals have been reported in Chongqing municipality, China. The aim of the present study was to determine the prevalence, serovar diversity, sequence types, and antimicrobial resistance of *Salmonella* isolated from livestock and poultry in Chongqing. Meanwhile, we also want to know the presence of β-lactamase genes, plasmid-mediated quinolone resistance (PMQR) genes and quinolone resistance-determining region (QRDR) mutations of *Salmonella* isolates. A total of 129 *Salmonella* strains were recovered from 2,500 fecal samples at 41 farms from pigs, goats, beef cattle, rabbits, chickens, and ducks. Fourteen serovars were identified, with *S.* Agona and *S.* Derby being the dominant serovars. The 129 isolates had high resistance to doxycycline (87.6%), ampicillin (80.6%), tetracycline (79.8%), trimethoprim (77.5%), florfenicol (76.7%) chloramphenicol (72.9%), and trimethoprim-sulfamethoxazole (71.3%), but were susceptible to cefepime. A total of 114 (88.4%) isolates showed multidrug resistant phenotypes. The prevalence of β-lactamase genes in *Salmonella* isolates was 89.9% (116/129), and among these isolates, 107 (82.9%) harbored *bla*$_{TEM}$, followed by *bla*$_{OXA}$ (26, 20.2%), *bla*$_{CTX-M}$ (8, 6.2%), and *bla*$_{CMY}$ (3, 2.3%). In addition, *qnrB*, *qnrD*, *qnrS*, *oqxA*, *oqxB*, and *aac(6′)-Ib-cr* were detected in 11, 2, 34, 34, 43, and 72 PMQR-producing isolates, respectively. Moreover, QRDR mutations were very common in PMQR-positive *Salmonella* isolates (97.2%, 70/72) with mutation(s) in *parC* or combinative mutations in *gyrA* and *parC*. More significantly, 32 extended spectrum beta-lactamase (ESBL)-producing isolates were identified, and 62.5% of them were found to harbor one to four PMQR genes. Furthermore, 11 sequence types were identified from the isolates, and most of ESBL-producing isolates were attributed to ST34 (15.6%) and ST40 (62.5%). The coexistence of PMQR genes with β-lactamase genes and the extensive mutations in QRDR present in *Salmonella* isolates from food-producing animals suggest a potential threat to public health. Reasonable utilization and strict control strategies for antimicrobials in animal husbandry and animal treatment are necessary to reduce the emergence and dissemination of drug-resistant *Salmonella* isolates.

KEYWORDS

serovar, multidrug resistance, ESBL, PMQR, *bla*$_{TEM}$, *parC*

1. Introduction

Salmonella is considered one of the most important foodborne pathogens causing global morbidity and mortality in both humans and animals (Nichols et al., 2021; Harrison et al., 2022; Li et al., 2022). According to data from the World Health Organization, nontyphoidal *Salmonella* infection causes 155,000 deaths worldwide, and approximately 93.8 million gastroenteritis cases are closely related to nontyphoidal *Salmonella* infection per year (Majowicz et al., 2010). According to unpublished data from Chinese Center for Disease Control and Prevention surveillance system, 549 out of 100,000 people carried *Salmonella* in 2013. As one of the most important carriers, food-producing animals can transfer *Salmonella* to humans through the food chain (Foley and Lynne, 2008; Marus et al., 2019).

Adding antimicrobials to feed and drinking water has long been an important means for farms to prevent and treat bacterial diseases, including salmonellosis. However, under long-term exposure to antimicrobials, bacteria have developed resistance to antimicrobials and even multidrug resistance (MDR), which has become a serious challenge to public health (Nichols et al., 2022). Extended-spectrum cephalosporins and quinolones are two classes of antimicrobials often used at farms, where resistance to *Salmonella* is emerging due to the misuse and overuse of antimicrobials. Resistance to cephalosporins is primarily due to the acquisition of β-lactamases that are mainly carried by transferable plasmids and transposons (Ghafourian et al., 2015). The major mechanisms of quinolone resistance have been elucidated to be chromosomal mutations in the quinolone resistance-determining regions (QRDRs) and the presence of plasmid-mediated quinolone resistance (PMQR) genes, which might aid the selection and facilitate the mutation of fluoroquinolone resistance genes (Kuang et al., 2018a). There have been numerous reports of antimicrobial resistance (AMR), a major global public health concern that has the potential to destroy the effectiveness of antimicrobials, and resistance genes about *Salmonella* around the world (Hetman et al., 2022; Canning et al., 2023; Igbinosa et al., 2023). In China, there are also many reports of AMR in *Salmonella* from food-producing animals and their products, such as pigs, poultry and cattle, involving many provinces (Tang et al., 2022; Wang et al., 2023). Moreover, many of these *Salmonella* isolates were resistant to β-lactamases and quinolone, and harbor β-lactam resistance genes and PMQR genes, as well as the chromosomal mutations in the QRDRs. However, there are few reports of AMR and resistance genes of *Salmonella* from food-producing animals in Chongqing, a province-level municipality.

In this study, we investigated *Salmonella* strains isolated from food-producing animal feces and analyzed their serovar diversity, sequence types, and AMR. Furthermore, we compared the relationship between phenotypes and genotypes regarding the resistance towards β-lactams and quinolones.

2. Materials and methods

2.1. Isolation and identification of *Salmonella*

A total of 2,500 fecal samples were collected from 1,600 pigs, 400 goats, 100 beef cattle, 50 rabbits, 300 chickens, and 50 ducks between September 2016 and May 2019 (Supplementary Table S1). Since numbers of rabbits and ducks were far lower than those of pigs and chickens in Chongqing, to avoid repetitive clones, we collected 50 fecal samples each from rabbits and ducks. Approximately 1 g of each sample was pre-enriched in 10 mL of sterile buffered peptone water (BPW) at 37°C for 8 h. Then, 0.1 mL of the suspension was added to 5 mL of tetrathionate broth (TTB) and incubated at 42°C for 18–24 h. After selective enrichment, the suspensions were streaked onto xylose lysine tergitol 4 (XLT-4) plates and incubated at 37°C for 18–48 h. Five suspected colonies were picked from each plate and confirmed by amplifying the *invA* gene, which is specific for *Salmonella*. Primers designed by our group were invA-F (5'-GAAATTATCGCCACGTTCGGGCA-3′) and invA-R (5'-TCATCGCACCGTCAAAGGA-3′). Finally, one confirmed clone was selected randomly, even if more than one clone was identified as *Salmonella* at the same plate. All identified isolates were aliquoted and stored at −80°C in Luria-Bertani (LB) broth containing 50% glycerol (Cody et al., 2008).

2.2. *Salmonella* serotyping

Salmonella isolates were serotyped using slide agglutination with hyperimmune sera (Tianrun, Ningbo, China), and the results were interpreted according to the Kauffmann-White scheme.

2.3. Multilocus sequence typing

Seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) were selected to carry out MLST. Primers (Supplementary Table S2) were synthetized according to the published data (General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China, 2017) and the sequence type (ST) was assigned according to the MLST database.[1]

2.4. Antimicrobial susceptibility testing

The antimicrobial susceptibility of *Salmonella* isolates was determined using the disk diffusion method on Mueller-Hinton agar plates and broth dilution method with cation-adjusted

1 https://pubmlst.org/bigsdb?db=pubmlst_salmonella_seqdef

TABLE 1 Prevalence and serotypes of *Salmonella* isolates from animals in Chongqing, China.

Source of sample	Number of isolates	Serovars	Number of serovars
Pigs	104/1600 (6.5%[a])	*S.* Agona (24), *S.* Anatum (2), *S.* Bredeney (1), *S.* Derby (32), *S.* Kottbus (4), *S.* London (7), *S.* Manhattan (1), *S.* Mbandaka (1), *S.* Newlands (3), *S. paratyphi* B (4), *S.* Regent (7), *S.* Rissen (8), *S.* Stanley (2), *S. typhimurium* (8)	14
Goats	11/400 (2.8%[b])	*S.* Derby (10), *S.* Stanley (1)	2
Beef cattle	3/100 (3.0%)	*S.* Derby (3)	1
Rabbits	2/50 (4.0%)	*S.* Derby (2)	1
Chickens	7/300 (2.3%[b])	*S.* Derby (7)	1
Ducks	2/50 (4.0%)	*S.* Derby (2)	1
Total	129/2500 (5.2%)		14

[a,b]Values with different letter superscripts indicate significant differences ($p < 0.05$).

Mueller-Hinton broth according to the guidelines of Clinical and Laboratory Standards Institute standards CLSI M100-S32 (CLSI, 2022) and VET08Ed4E (CLSI, 2019). Twenty-seven antimicrobials were tested by the disk diffusion method: ampicillin (AMP), cephalexin (LEX), cefazolin (CFZ), cefoxitin (FOX), cefotaxime (CTX), ceftriaxone (CRO), ceftazidime (CAZ), cefepime (FEP), imipenem (IPM), aztreonam (ATM), streptomycin (STR), kanamycin (KAN), gentamicin (GEN), amikacin (AMK), tetracycline (TET), doxycycline (DOX), chloramphenicol (CHL), florfenicol (FFC), nalidixic acid (NAL), norfloxacin (NOR), ciprofloxacin (CIP), enrofloxacin (ENO), ofloxacin (OFX), enoxacin (ENX), gatifloxacin (GAT), trimethoprim-sulfamethoxazole (SXT), and trimethoprim (TMP). However, the broth dilution method tested 26 antimicrobials, except for enrofloxacin, for there was no interpretive criterion of this drug by using broth dilution method. The results were interpreted according to the standards described by CLSI M100-S32 (CLSI, 2022) and VET08Ed4E (CLSI, 2019). *Escherichia coli* ATCC®* 25922 was used as the quality control.

2.5. Detection of β-lactamase genes, PMQR genes and mutations within the QRDR

To analyze the resistance mechanisms, we detected nine β-lactamase genes, 10 PMQR genes and identified mutations in the QRDRs of DNA gyrase and topoisomerase IV by means of PCR and sequencing. The β-lactamase genes were bla_{TEM}, bla_{CTX-M}, bla_{SHV} (Chen et al., 2004), bla_{OXA}, bla_{CMY}, bla_{PSE} (Qiao et al., 2017), bla_{PER} (Qiao et al., 2017), bla_{VEB} (Dallenne et al., 2010), and bla_{GES} (Dallenne et al., 2010), while the PMQR genes included *qnrA* (Robicsek et al., 2006), *qnrB*, *qnrC* (Cattoir et al., 2007), *qnrD*, qnr*VC*, *qnrS*, *aac(6′)-Ib-cr*, *oqxA*, *oqxB*, and *qepA*. Two DNA gyrase genes were *gyrA* (Kim et al., 2016) and *gyrB* (Hansen and Heisig, 2003), and two topoisomerase IV genes comprised *parC* (Kim et al., 2016) and *parE* (Kim et al., 2016). The primers and related parameters were listed in Supplementary Table S2. DNA was extracted using a DNA Extraction Kit (Tiangen Biotech, Beijing, China). Sequences of PCR products were aligned and analyzed using BLAST.[2] The resulting DNA sequences of all PCR products by amplifying QRDR genes were compared with the *S. typhimurium* LT2 genome as a reference.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism (Version 8.0). Differences between proportions were calculated using the Chi-square test. $p \leq 0.05$ were considered to be statistically significant.

3. Results

3.1. *Salmonella* prevalence and serovars

In this study, 129 (5.2%) isolates of *Salmonella* were obtained from 2,500 fecal samples, among which 104 (6.5%) were recovered from pigs, 11 (2.8%) from goats, three (3.0%) from beef cattle, two (4.0%) from rabbits, seven (2.3%) from chickens, and two (4.0%) from ducks (Table 1; Supplementary Table S1). Overall, the isolation rate among pigs was higher than that for other sources ($p < 0.05$). Moreover, a significantly higher prevalence of *Salmonella* was detected in pigs than in goats and chickens ($p < 0.05$). However, there was no difference in the isolation rate of strains in different years, even from different sources. Fourteen different serovars were identified from the isolates, and *S.* Derby (56, 43.4%) and *S.* Agona (24, 18.6%) were the two most common serovars (Table 1). The distribution of serovars varied among different sources. *S.* Derby (32, 30.8%) and *S.* Agona (24, 23.1%) were more prevalent than other serovars in pigs. However, most isolates recovered from goats (90.9%, 10/11) and all strains acquired from beef cattle, rabbits, and poultry were *S.* Derby, although the number of these isolates from these animals was much less than that of isolates from pigs.

3.2. Antimicrobial susceptibility testing of 129 *Salmonella* isolates

The AMR phenotypes of 129 *Salmonella* isolates exhibited high rates of resistance to doxycycline (87.6%, 113/129), ampicillin (80.6%, 104/129), tetracycline (79.8%, 103/129), trimethoprim (77.5%, 100/129), florfenicol (76.7%, 99/129), chloramphenicol (72.9%,

2 http://www.ncbi.nlm.nih.gov/BLAST/

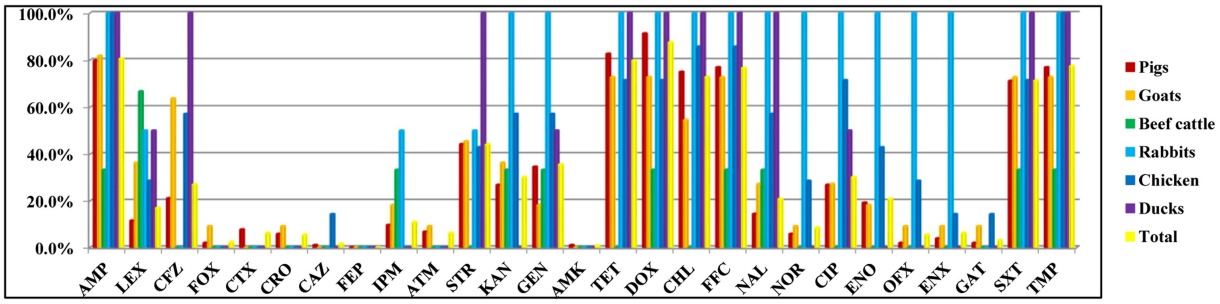

FIGURE 1
Antimicrobial resistance of *Salmonella* isolated from food-producing animals was determined by disk diffusion method. AMP (ampicillin), LEX (cephalexin), CFZ (cefazolin), FOX (cefoxitin), CTX (cefotaxime), CRO (ceftriaxone), CAZ (ceftazidime), FEP (cefepime), IPM (imipenem), ATM (aztreonam), STR (streptomycin), KAN (kanamycin), GEN (gentamicin), AMK (amikacin), TET (tetracycline), DOX (doxycycline), CHL (chloramphenicol), FFC (florfenicol), NAL (nalidixic acid), NOR (norfloxacin), CIP (ciprofloxacin), ENO (enrofloxacin), OFX (ofloxacin), ENX (enoxacin), GAT (gatifloxacin), SXT (trimethoprim-sulfamethoxazole), and TMP (trimethoprim).

94/129), and trimethoprim-sulfamethoxazole (71.3%, 92/129). However, all isolates were susceptible to cefepime and less resistant to amikacin (0.8%, 1/129), ceftazidime (1.6%, 2/129), cefoxitin (2.3%, 3/129), gatifloxacin (3.1%, 4/129), ceftriaxone (5.4%, 7/129), ofloxacin (5.4%, 7/129), aztreonam (6.2%, 8/129), and enoxacin (6.2%, 8/129; Figure 1; Table 2; Supplementary Tables S2, S3). Moreover, three (2.3%) isolates were susceptible to all antimicrobial agents, while 126 (97.7%) isolates showed resistance to at least one drug.

A total of 114 (88.4%) isolates exhibited MDR, which was defined as resistant to three or more classes of antimicrobials (Table 3). All strains belonging to *S.* Derby obtained from rabbits and poultry showed MDR profiles. A higher prevalence of MDR isolates from pigs was found among *S.* Derby (93.8%, 30/32), compared with *S.* Agona (75.0%, 18/24), although there was no significant difference. Serovars *S.* Anatum (2), *S.* Bredeney (1), *S.* Kottbus (4), *S.* London (6), *S.* Manhattan (1), *S.* Mbandaka (1), *S.* Newlands (3), *S. paratyphi* B (4), *S.* Regent (7), *S.* Rissen (7), *S.* Stanley (1), and *S. typhimurium* (8) from pigs were MDR (Table 4).

3.3. Prevalence of β-lactamase genes in *Salmonella* isolates

The prevalence of β-lactamase genes in *Salmonella* isolates was 89.9% (116/129), and of these, all isolates collected from rabbits and poultry, and 90.4% (94/104) of pig-associated strains harbored at least one β-lactamase gene. Among the isolates, 107 (82.9%) harbored bla_{TEM}, followed by bla_{OXA} (26, 20.2%), bla_{CTX-M} (8, 6.2%), and bla_{CMY} (3, 2.3%; Table 5). Strains from goats, beef cattle, rabbits and poultry carried bla_{TEM} genes and were resistant to at least one β-lactam drug except for 1 strain isolated from beef cattle. The allele of 104 bla_{TEM} was TEM-1 (44.2%, 57/129), TEM-1a (12.4%, 16/129), TEM-1b (7.8%, 10/129), TEM-116 (0.8%, 1/129), and TEM-171 (17.8%, 23/129). bla_{OXA} was found in pigs, goats, rabbits and chickens, and its genetic genotypes were bla_{OXA-1} (76.9%, 20/26) and bla_{OXA-10} (23.1%, 6/26). Three $bla_{CMY-116}$-harboring strains were detected, and two were isolated from pigs and one from duck. bla_{CTX-M} was amplified from eight pig-associated strains, and the genotypes were $bla_{CTX-M-65}$. As the predominant serovars, 79.2% (19/24) *S.* Agona and 87.5% (49/56) *S.* Derby harbored bla_{TEM}, and the proportions of bla_{TEM}-positive isolates in both serovars were lower than the proportion of bla_{TEM}-positive isolates in the total number of *Salmonella* isolates. Beta-lactamase genes in isolates carried one to three *bla* genes (Supplementary Table S4). The most prevalent genotype form was TEM-1 (31.0%, 40/129), followed by TEM-1a (11.6%, 15/129), TEM-171 (10.9%, 14/129), TEM-1b (7.8%, 10/129), and TEM-171 + OXA-1 (7.0%, 9.129). Extended spectrum β-lactamases (ESBLs) are defined as enzymes produced by certain bacteria that are able to hydrolyze β-lactam ring of broad-spectrum β-lactams such as oxyimino-cephalosporins including cefotaxime, ceftriaxone, and ceftazidime (Kawamura et al., 2017). The detection result showed that six isolates carrying $bla_{CTX-M-65}$ gene were resistant to the third-generation cephalosporins cefotaxime and ceftriaxone.

3.4. Distribution of PMQR and QRDR mutations among strains

Fifty-seven strains were resistant to at least one tested quinolone, and most of these strains harbored PMQR gene(s) (82.5%, 47/57) and/or exhibited mutation(s) in QRDR gene(s) (98.2%, 56/57). However, PMQR gene(s) and QRDR point mutation(s) were detected in 82 (63.6%) and 121 (93.8%) *Salmonella* isolates, regardless of whether they were resistant to the quinolones tested. Only *qnrB*, *qnrD*, *qnrS*, *oqxA*, *oqxB*, and *aac(6′)-Ib-cr* were detected in 13.4% (11), 2.4% (2), 41.5% (34), 41.5% (34), 52.4% (43), and 50.0% (41) of the PMQR-producing isolates, respectively (Table 6). *qnrA*, *qnrC*, *qnrVC*, and *qepA* were not present in any isolate. The genotypes of *qnrB*, *qnrD*, *oqxA*, and *oqxB* were *qnrB6*, *qnrD1*, *oqxA1*, and *oqxB5*. Nevertheless, *qnrS* represented *qnrS1* (6.1%, 5/82), *qnrS2* (13.4%, 11/82) and *qnrS10* (22.0%, 18/82). Similar to β-lactamase gene-containing isolates, there were 20 genotype forms of PMQR genes in 82 PMQR-positive strains (Supplementary Table S5). There were 14 and six isolates carrying a single PMQR gene, *qnrS10* and *oqxB5*, respectively. Moreover, 10 isolates carried the *oqxA1* and *oqxB5* genes, and eight isolates carried *qnrS2*, *oqxA1*, *oqxB5*, and *aac(6′)-Ib-cr* simultaneously. Interestingly, most *qnrS2*-positive strains (90.9%, 10/11) were positive for *aac(6′)-Ib-cr*.

QRDR mutations were very common in PMQR-positive *Salmonella* isolates (97.5%, 80/82; Table 7). A single mutation in

TABLE 2 Antimicrobial resistance profiles of *Salmonella* isolates recovered from pigs, goats, beef cattle, rabbits, chickens, and ducks as determined by disk diffusion method.

Antimicrobial	Pigs (*n*=104)	Goats (*n*=11)	Beef cattle (*n*=3)	Rabbits (*n*=2)	Chickens (*n*=7)	Ducks (*n*=2)	Total (*n*=129)
Ampicillin	83 (79.8)	9 (81.8)	1 (33.3)	2 (100.0)	7 (100.0)	2 (100.0)	104 (80.6)
Cephalexin	12 (11.5)	4 (36.4)	2 (66.7)	1 (50.0)	2 (28.6)	1 (50.0)	22 (17.1)
Cefazolin	22 (21.2)	7 (63.6)	0	0	4 (57.1)	2 (100.0)	35 (27.1)
Cefoxitin	2 (1.9)	1 (9.1)	0	0	0	0	3 (2.3)
Cefotaxime	8 (7.7)	0	0	0	0	0	8 (6.2)
Ceftriaxone	6 (5.8)	1 (9.1)	0	0	0	0	7 (5.4)
Ceftazidime	1 (1.0)	0	0	0	1 (14.3)	0	2 (1.6)
Cefepime	0	0	0	0	0	0	0
Imipenem	10 (9.6)	2 (18.2)	1 (33.3)	1 (50.0)	0	0	14 (10.9)
Aztreonam	7 (6.7)	1 (9.1)	0	0	0	0	8 (6.2)
Streptomycin	46 (44.2)	5 (45.5)	0	1 (50.0)	3 (42.9)	2 (100.0)	57 (44.2)
Kanamycin	28 (26.9)	4 (36.4)	1 (33.3)	2 (100.0)	4 (57.1)	0	39 (30.2)
Gentamicin	36 (34.6)	2 (18.2)	1 (33.3)	2 (100.0)	4 (57.1)	1 (50.0)	46 (35.7)
Amikacin	1 (1.0)	0	0	0	0	0	1 (0.8)
Tetracycline	86 (82.7)	8 (72.7)	0	2 (100.0)	5 (71.4)	2 (100.0)	103 (79.8)
Doxycycline	95 (91.3)	8 (72.7)	1 (33.3)	2 (100.0)	5 (71.4)	2 (100.0)	113 (87.6)
Chloramphenicol	78 (75.0)	6 (54.5)	0	2 (100.0)	6 (85.7)	2 (100.0)	94 (72.9)
Florfenicol	80 (76.9)	8 (72.7)	1 (33.3)	2 (100.0)	6 (85.7)	2 (100.0)	99 (76.7)
Nalidixic acid	15 (14.4)	3 (27.3)	1 (33.3)	2 (100.0)	4 (57.1)	2 (100.0)	27 (20.9)
Norfloxacin	6 (5.8)	1 (9.1)	0	2 (100.0)	2 (28.6)	0	11 (8.5)
Ciprofloxacin	28 (26.9)	3 (27.3)	0	2 (100.0)	5 (71.4)	1 (50.0)	39 (30.2)
Enrofloxacin	20 (19.2)	2 (18.2)	0	2 (100.0)	3 (42.9)	0	27 (20.9)
Ofloxacin	2 (1.9)	1 (9.1)	0	2 (100.0)	2 (28.6)	0	7 (5.4)
Enoxacin	4 (3.8)	1 (9.1)	0	2 (100.0)	1 (14.3)	0	8 (6.2)
Gatifloxacin	2 (1.9)	1 (9.1)	0	0	1 (14.3)	0	4 (3.1)
Trimethoprim-sulfamethoxazole	74 (71.2)	8 (72.7)	1 (33.3)	2 (100.0)	5 (71.4)	2 (100.0)	92 (71.3)
Trimethoprim	80 (76.9)	8 (72.7)	1 (33.3)	2 (100.0)	7 (100.0)	2 (100.0)	100 (77.5)

Unit: %.

TABLE 3 Multidrug-resistant (MDR) *Salmonella* isolated from animals in Chongqing.

Source of isolates	Number of isolates to indicated number of antimicrobial categories (%)							
	0 (%)	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)	6 (%)	Total of MDR (%)
Pigs	2 (1.9)	5 (4.8)	4 (3.8)	14 (13.5)	27 (26.0)	23 (22.1)	29 (27.9)	93 (89.4)
Goats	1 (9.1)	1 (9.1)			5 (45.5)	1 (9.1)	3 (27.3)	9 (81.8)
Beef cattle	1 (33.3)	1 (33.3)				1 (33.3)		1 (33.3)
Rabbits							2 (100.0)	2 (100.0)
Chicken					2 (28.6)	3(42.8)	2 (28.6)	7 (100.0)
Ducks							2 (100.0)	2 (100.0)
Total	4 (3.1)	7 (5.4)	4 (3.1)	14 (10.9)	34 (26.4)	28 (21.7)	38 (29.5)	114 (88.4)

parC (T57S) was detected in isolates obtained from goats (6), rabbits (2) and chickens (5), and 91.2% (62) pig-associated PMQR-positive isolates. Double mutations and triple mutations in *parC* were found in two (S57T/G72C and S57T/L131M) and one (S57T/G72C/L131M) isolates, respectively. A combination of mutations in *gyrA* (V143G) and in *parC* (S57T) was found in two isolates. However, both mutations in *gyrA* (S83L) and *parC* (S57T) were detected in one isolate.

TABLE 4 Distribution of multidrug-resistant *Salmonella* in different serotypes and animals.

Serotype	Pigs (%)	Goats (%)	Beef cattle (%)	Rabbits (%)	Chickens (%)	Ducks (%)	Total (%)
Agona	18 (75.0)						18 (75.0)
Anatum	2 (100.0)						2 (100.0)
Bredeney	1 (100.0)						1 (100.0)
Derby	30 (93.8)	8 (80.0)	1 (33.3)	2 (100.0)	7 (100.0)	2 (100.0)	50 (89.3)
Kottbus	4 (100.0)						4 (100.0)
London	6 (85.7)						6 (85.7)
Manhattan	1 (100.0)						1 (100.0)
Mbandaka	1 (100.0)						1 (100.0)
Newlands	3 (100.0)						3 (100.0)
Paratyphi B	4 (100.0)						4 (100.0)
Regent	7 (100.0)						7 (100.0)
Rissen	7 (87.5)						7 (87.5)
Stanley	1 (50.0)	1 (100.0)					2 (66.7)
Typhimurium	8 (100.0)						8 (100.0)
Total	93 (89.4)	9 (81.8)	1 (33.3)	2 (100.0)	7 (100.0)	2 (100.0)	114 (88.4)

TABLE 5 The prevalence of β-lactamase genes in *Salmonella* isolates originating from different animals.

Genotype of β-lactamase gene		Pigs (%)	Goats (%)	Beef cattle (%)	Rabbits (%)	Chickens (%)	Ducks (%)	Total (%)
TEM	TEM-1	44 (42.3)	5 (45.5)	1 (33.3)		5 (71.4)	2 (100.0)	57 (44.2)
	TEM-1a	16 (15.4)						16 (12.4)
	TEM-1b	10 (9.6)						10 (7.8)
	TEM-116	1 (1.0)						1 (0.8)
	TEM-171	17 (16.3)	3 (27.3)	1 (33.3)	1 (50.0)	1 (14.3)		23 (17.8)
OXA	OXA-1	13 (12.5)	3 (27.3)		2 (100.0)	2 (28.6)		20 (15.5)
	OXA-10	6 (5.8)						6 (4.7)
CMY	CMY-116	2 (1.9)					1 (50.0)	3 (2.3)
CTX-M	CTX-M-65	8 (7.7)						8 (6.2)

TABLE 6 The prevalence of PMQR genes in *Salmonella* isolates originating from different animals.

Genotype of PMQR genes		Pigs (%)	Goats (%)	Rabbits (%)	Chickens (%)	Ducks (%)	Total (%)
qnrB	*qnrB6*	11 (10.6)					11 (8.5)
qnrD	*qnrD1*	2 (1.9)					2 (1.6)
qnrS	*qnrS1*	3 (2.9)	2 (18.2)				5 (3.9)
	qnrS2	7 (6.7)	3 (27.3)	1 (50.0)			11 (8.5)
	qnrS10	16 (15.4)	1 (9.1)		1 (14.3)		18 (14.0)
oqxA	*oqxA1*	28 (26.9)	2 (18.2)	2 (100.0)	2 (28.6)		34 (26.4)
oqxB	*oqxB5*	39 (37.5)	2 (18.2)	1 (50.0)	1 (14.3)		43 (33.3)
aac (6′)-Ib-cr		31 (29.8)	3 (27.3)	2 (100.0)	4 (57.1)	1 (50.0)	41 (31.8)

3.5. Mechanisms of ciprofloxacin resistance among the isolates

To detect the main mechanism for the presence of 39 CIP-resistant isolates, we found that the most frequent mutation in *parC* was S57T (89.7%, 35/39; Table 8). Moreover, double mutations in *gyrA* and *parC* of three isolates mentioned above were also resistant to ciprofloxacin. Most of CIP-resistant isolates harbored at least one PMQR gene, except for three strains. Surprisingly, one isolate, resistant to ciprofloxacin, only

TABLE 7 Distribution of QRDR mutations in PMQR-positive *Salmonella* isolates.

QRDR mutation(s)						
gyrA	*parC*	Pigs	Goats	Rabbits	Chickens	Total
None	S57T	62	6	2	5	75
None	S57T, G72C	1				1
None	S57T, L131M	1				1
None	S57T, G72C, L131M	1				1
V143G	S57T	2				2

TABLE 8 Distribution of QRDR mutations and PMQR genes among the 39 ciprofloxacin-resistant *Salmonella* isolates.

QRDR mutations	PMQR genes	No. of isolates (%)
parC (S57T)		2
	qnrB6 + oqxA1 + oqxB5 + aac(6′)-Ib-cr	3
	qnrB6 + aac(6′)-Ib-cr	2
	qnrD1 + oqxA1 + oqxB5 + aac(6′)-Ib-cr	1
	qnrS1	1
	qnrS1 + oqxB5	1
	qnrS2	1
	qnrS2 + oqxA1 + oqxB5 + aac(6′)-Ib-cr	5
	qnrS2 + aac(6′)-Ib-cr	1
	qnrS10	1
	qnrS10 + oqxA1 + oqxB5	1
	oqxA1 + oqxB5	3
	oqxA1 + oqxB5 + aac(6′)-Ib-cr	4
	oqxA1 + aac(6′)-Ib-cr	2
	oqxB5	1
	oqxB5 + aac(6′)-Ib-cr	2
	aac(6′)-Ib-cr	4
gyrA (S83L) and *parC* (S57T)		1
gyrA (V143G) and *parC* (S57T)	*qnrS2 + oqxA1 + oqxB5 + aac(6′)-Ib-cr*	1
	qnrS2 + aac (6′)-Ib-cr	1
	aac(6′)-Ib-cr	1
Total		39

contained *aac(6′)-Ib-cr* and no mutation was identified at any QRDR genes.

3.6. Characterization of PMQR genes in ESBL-producing isolates

Thirty-two ESBL-producing strains were identified from the isolates, and each strain carried one of ESBL genes from three genotypes, namely $bla_{\text{TEM-116}}$, $bla_{\text{TEM-171}}$, and $bla_{\text{CTX-M-65}}$. The distribution of PMQR-encoding genes among 32 ESBL-producing *Salmonella* isolates was shown in Table 9. Twenty (62.5%) ESBL-producing *Salmonella* isolates were found to harbor one to four PMQR genes, and combinations of *qnrS2 + oqxA1 + oqxB5 + aac(6′)-Ib-cr* (30%, 6/20) was the most common combination type, followed by *oqxA1 + oqxB5* (25.0%, 5/20).

TABLE 9 Distribution of PMQR genes among ESBL-producing *Salmonella* isolates.

ESBL gene	PMQR genes	No. of isolates
$bla_{\text{TEM-116}}$	*oqxB5*	1
$bla_{\text{TEM-171}}$	*qnrS1*	1
	qnrS2 + oqxA1 + oqxB5 + aac(6′)-Ib-cr	6
	oqxA1 + oqxB5	3
	oqxA1 + oqxB5 + aac(6′)-Ib-cr	1
	oqxA1 + aac(6′)-Ib-cr	1
	oqxB5	1
	aac(6′)-Ib-cr	3
		7
$bla_{\text{CTX-M-65}}$	*oqxA1*	1
	oqxA1 + oqxB5	2
		5
Total		32

3.7. Sequence types and the relatedness with ESBL-producing isolates

In total, 11 sequence types were identified from the *Salmonella* isolates, including ST11, ST19, ST34, ST40, ST155, ST279, ST413, ST463, ST469, ST1498, and ST1499 (Table 10). ST34 (10.9%, 14/129), ST40 (36.4%, 47/129), ST463 (12.4%, 16/129), and ST469 (14.7%, 19/129) were the more popular sequence types. Most of isolates containing $bla_{\text{TEM-171}}$ (69.6%, 16/23) and the $bla_{\text{TEM-116}}$-positive isolate were identified as ST40. However, isolates harboring $bla_{\text{CTX-M-65}}$ were attributed to ST34 (62.5%, 5/8) and ST40 (37.5%, 3/8).

4. Discussion

As major reservoirs of bacteria and an important part of food chain, food-producing animals play an irreplaceable role in the

TABLE 10 Sequence types of *Salmonella* isolates, especially the ESBL-producing strains.

Sequence type	No. of isolates (%)	$bla_{TEM-116}$-positive (%)	$bla_{TEM-171}$ positive (%)	$bla_{CTX-M-65}$ positive (%)
ST11	2 (1.6)			
ST19	2 (1.6)			
ST34	14 (10.9)		2 (8.7)	5 (62.5)
ST40	47 (36.4)	1 (100.0)	16 (69.6)	3 (37.5)
ST155	13 (10.1)		1 (4.3)	
ST279	11 (8.5)		2 (8.7)	
ST413	1 (0.8)		1 (4.3)	
ST463	16 (12.4)			
ST469	19 (14.7)			
ST1498	2 (1.6)		1 (4.3)	
ST1499	2 (1.6)			
Total	129	1	23	8

transmission of *Salmonella*. It is of great significance for the prevention and control of salmonellosis in humans and animals to monitor the prevalence and dynamics of antimicrobial resistance and resistant genes of *Salmonella* originating from food-producing animals in countries or regions with extensive livestock and/or poultry breeding. In this study, we investigated the prevalence, serovar distribution, sequence types, antimicrobial susceptibility phenotypes, the emergence of β-lactamase and PMQR genes, and the occurrence of mutations in the QRDR of *Salmonella* isolates acquired from pigs, goats, beef cattle, rabbits, and poultry in Chongqing, China.

The number of samples collected from different animals was roughly consistent with the breeding density of different animals in Chongqing, and the number of samples collected from pigs (1600) and chickens (300) was much higher than those from beef cattle (100), rabbits (50) and ducks (50). Overall, 104 *Salmonella* isolates were recovered from 1,600 fecal samples of pigs, showing a prevalence of 6.5%, higher than the prevalence in Italy (3.4%; Siddi et al., 2021) but much lower than the prevalence in England (19.5%; Wales et al., 2013) and Thailand (37.54%; Phongaran et al., 2019). Moreover, the prevalence of *Salmonella* in pigs was lower than the prevalence in Shanghai (26.3%; Tian et al., 2021), Shandong (11.1%; Zhao et al., 2017) and other Chinese provinces (Li et al., 2013; Zhang et al., 2016; Kuang et al., 2018b; Zhang et al., 2019). Similarly to the prevalence of *Salmonella* from pigs, the prevalence of *Salmonella* isolated from chickens in Chongqing is lower than that of *Salmonella* from chickens in other countries and regions (Zhao et al., 2017; Caffrey et al., 2021; Sarker et al., 2021), even with a few exceptions (Li et al., 2013).

Previous studies have reported that ducks are important reservoirs of *Salmonella* (Li et al., 2013; Zhao et al., 2017; Zhang et al., 2019; Kim et al., 2021; Kang et al., 2022), and Chinese duck production exceeds 90% of all ducks globally; therefore, we investigated *Salmonella* in duck samples. The results indicate that the isolation rate of *Salmonella* in ducks is lower than in other regions of China (Li et al., 2013; Zhao et al., 2017; Zhang et al., 2019; Kim et al., 2021; Kang et al., 2022). Moreover, we isolated *Salmonella* from herbivores, including goats, beef cattle, and rabbits, with isolation rates of 2.8, 3.0 and 4.0%, respectively. Compared with other countries, the isolation rate of *Salmonella* in beef cattle and goats is low (Bosilevac et al., 2015; Thomas et al., 2020; Gutema et al., 2021). Such differences in isolation rates among different animals in our study and other reports can be interpreted based on differences in region, animal species, sample types, collection seasons, culture methods, isolation methodologies, culture media, and local environmental conditions (Kuang et al., 2015).

Among the strains isolated from pigs, 12 serovars were identified in addition to the dominant serovars Agona and Derby. These, previously reported to having been recovered in pigs, included *S.* Anatum (Li et al., 2013; Siddi et al., 2021), *S.* Bredeney (Grafanakis et al., 2001; Siddi et al., 2021), *S.* Kottbus (Toboldt et al., 2014), *S.* London (Grafanakis et al., 2001; Bonardi et al., 2016), *S.* Manhattan (Wales et al., 2013; Bonardi et al., 2016), *S.* Mbandaka (Tian et al., 2021), *S.* Newlands (Kuang et al., 2015; Li et al., 2019), *S. paratyphi* B (Phongaran et al., 2019), *S.* Rissen (Bonardi et al., 2016; Tian et al., 2021), *S.* Stanley (Kuang et al., 2015; Bonardi et al., 2016) and *S. typhimurium* (Li et al., 2013; Zhao et al., 2017; Tian et al., 2021). However, to the best of our knowledge, this is the first study reporting *S.* Regent recovered from pigs, although this serovar was first reported to be isolated from ducks in Korea (Yoon et al., 2014). This demonstrates a serovar of *Salmonella* that is not common at pig farms in other places, with clearly reported pathogenicity (Duan, 2006). Moreover, there were few reports about *S. paratyphi* B isolated from pigs (Phongaran et al., 2019), and no report about the isolation of *S. paratyphi* B from pigs in China prior to our research. Therefore, monitoring of *S.* Regent and *S. paratyphi* B in pig farms and pork products should be strengthened to prevent potential transmissions to humans through the food chain. In addition, *S.* Derby and *S.* Stanley isolated from goats have not been reported before. The main reason may be that, compared with studies on *Salmonella* obtained from pigs and poultry, there have been fewer studies on goat-associated *Salmonella*.

The resistance of isolates to different antimicrobials was positively correlated with the frequency of antimicrobial use (private communication with farmers or workers). For example, isolates have a high resistance rate to ampicillin and doxycycline, which are often

used in the breeding process. However, some antimicrobials are rarely used in farms, but the isolates have high resistance to them. For instance, the use of tetracycline is far lower than the use of doxycycline at farms in China. However, our results show that *Salmonella* isolates had almost identical resistant phenotypes to tetracycline and doxycycline. The reason may be that strains resistant to doxycycline were also resistant to tetracycline because they have the same resistance genes, such as *tet* genes (Pavelquesi et al., 2021). Moreover, the resistant phenotype of isolates to chloramphenicol, which has been banned for more than 20 years in China, was similar to that of florfenicol, which should also be related to the same resistance genes of these two antimicrobials. In general, pig, chicken and rabbit producers use antimicrobials much more frequently than producers of ruminants, reflected in the resistance to antimicrobials of isolates obtained from these two kinds of animals. The resistance rates of *Salmonella* isolated from pigs, chickens and rabbits to most tested antimicrobials were higher than those of *Salmonella* isolated from ruminants. The proportion of multidrug-resistant strains of *Salmonella* isolated from pigs, chickens and rabbits was also higher than that of *Salmonella* strains isolated from ruminants.

In this study, β-lactamase genes were amplified in 89.9% of the strains, of which 82.9% carried the bla_{TEM} gene, whose alleles were TEM-1, TEM-1a, TEM-1b, TEM-116, and TEM-171. However, we found that only eight isolates carried $bla_{\text{CTX-M}}$ gene (6.2%). Most reports on *Salmonella* isolated from food-producing animals, including those in China, and even studies in Sichuan Province adjacent to Chongqing, have shown that $bla_{\text{CTX-M}}$ was the most widespread ESBL gene. Therefore, the prevalence of bla_{TEM} was generally lower than $bla_{\text{CTX-M}}$ (Zhang et al., 2016; Luk-In et al., 2018; Kuang et al., 2018b; Zhang et al., 2019), although there are a few reports that the prevalence of bla_{TEM} was higher than that of $bla_{\text{CTX-M}}$ (Zhao et al., 2017; Al-Gallas et al., 2022). The prevalence of the β-lactamase gene, especially bla_{TEM}, is very worrisome. China's transportation network is very developed, and every village has cement concrete pavements, which makes it very convenient for people to travel but also provides convenience for pathogens to use the same developed transportation network to spread through carriers, such as animals, meat products, contaminated vehicles, and even people. *Salmonella* from food-producing animals is very easy to spread out of Chongqing through the carriers mentioned above. The bla_{TEM} gene in the *Salmonella* strains can be transferred into other bla_{TEM}-negative strains through plasmid conjugation, transformation, transduction, etc., (data not shown), resulting in the widespread prevalence of bla_{TEM}-positive *Salmonella* outside Chongqing.

The coexistence of PMQR genes in CTX-M-producing isolates has been widely reported worldwide (Li et al., 2014; Zhang et al., 2016). However, there has been no demonstrated linkage between the emergence of $bla_{\text{CTX-M}}$ and resistance to quinolones, and the emergence of $bla_{\text{CTX-M}}$ and coexistence of PMQR genes in *Salmonella* isolates. The reason was that all CTX-M-positive isolates were susceptible to seven tested quinolones and 62.5% (5/8) of isolates did not carry any PMQR gene. This indicated that $bla_{\text{CTX-M}}$ and PMQR are not necessarily cotransferred in some regions or that $bla_{\text{CTX-M}}$ may be located on chromosomes, such as CTX-M-14 (Zhang et al., 2019; Hamamoto et al., 2020).

The ESBLs phenotype mediates the resistance to third or fourth-generation cephalosporins. In the ESBL-producing isolates, 75% (6/8) of strains carrying $bla_{\text{CTX-M-65}}$ showed resistance to cefotaxime and ceftriaxone, while only one strain (4.3%, 1/23) harboring $bla_{\text{TEM-171}}$ exhibited resistance to ceftriaxone. This indicates that $bla_{\text{CTX-M-65}}$ plays a more important role in resistance to broad-spectrum β-lactams. A research from United States also supported this point (Tate et al., 2017). Therefore, although the proportion of *Salmonella* carrying the $bla_{\text{CTX-M-65}}$ gene in food-producing animals in Chongqing is low, its resistance to broad-spectrum β-lactams could not be ignored.

The mutations of QRDR genes often occurred in *gyrA*, such as S83Y, S83F, D87G, D87N, and D87Y (Zhang et al., 2016; Wang et al., 2020). The mutations of *parC* were less than the mutations found in *gyrA*, and the amino acid substitution of ParC was usually at the position of 80th (S80R; Zhang et al., 2016; Wang et al., 2020). However, in our study, mutations mainly occurred at the 57th amino acid of the ParC. Similar study has also been reported in *Salmonella* isolated from Guangdong in recent years (Chen et al., 2021), indicating an increasing trend of mutation in the 57th amino acid of the ParC.

The Ministry of Agriculture of China requested that the usage of lomefloxacin, pefloxacin, ofloxacin and norfloxacin in food-producing animals be forbidden beginning December 31, 2016. On October 21, 2021, the Ministry of Agriculture and Rural Affairs of China issued the "national action plan for reducing the use of veterinary antimicrobials (2021–2025)." These actions provided powerful measures for reducing drug-resistant bacteria from food-producing animals. Further longitudinal monitoring of antimicrobial susceptibility, serotypes and resistance genes of *Salmonella* isolates from food-producing animals in the same geographic region should be carried out to compare and evaluate the trends of above parameters.

5. Conclusion

In summary, we first analyzed the prevalence, serovar diversity, sequence types, and antimicrobial resistance and examined the β-lactamase, QRDR, and PMQR genes of *Salmonella* strains isolated from livestock and poultry at farms in Chongqing, China. Our findings demonstrated the diversity of serovars of *Salmonella* isolates. Notably, various MDR serovars of *Salmonella* are widespread, which highlights the potential risk of antimicrobial-resistant *Salmonella* foodborne infections. This study emphasizes the significant roles of bla_{TEM} genes in β-lactam-resistant isolates and *parC* mutations in quinolone-resistant isolates, especially those that carry PMQR genes.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by IACUC-20190305-01.

Author contributions

HD, HM, and YH conceived and designed the experiments. JL, HM, BZ, JH, XC, YG, MZ, MX, YZ, and YH performed the experiments. HD and HW analyzed the data. JL, JW, and YY contributed reagents/materials/analysis tools. HD and HM wrote the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1011719/full#supplementary-material

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