ANTIMICROBIAL RESISTANCE ALONG THE FOOD CHAIN: ARE WE WHAT WE EAT?

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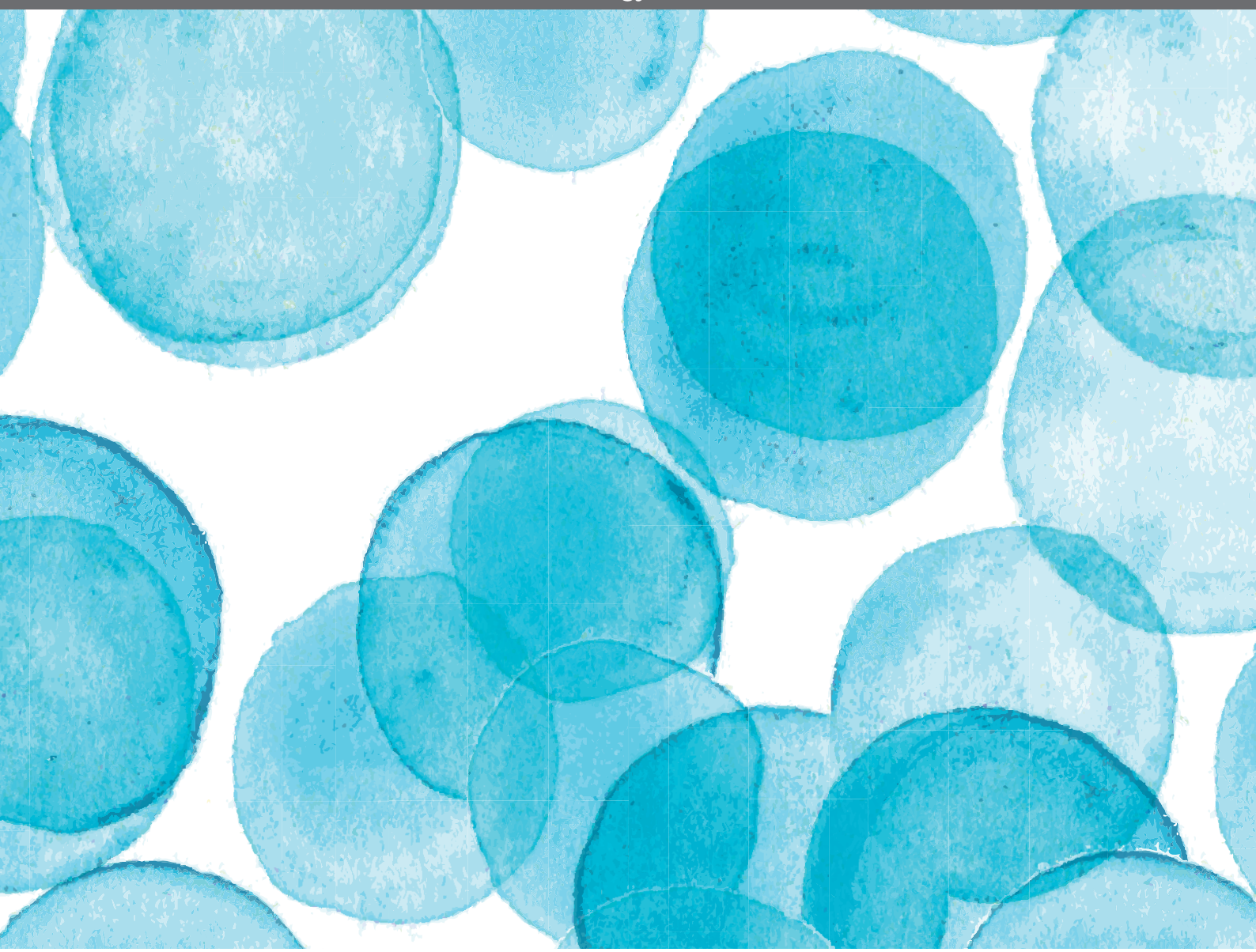

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ANTIMICROBIAL RESISTANCE ALONG THE FOOD CHAIN: ARE WE WHAT WE EAT?

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Editorial: Antimicrobial Resistance Along the Food Chain: Are We What We Eat?

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Editorial on the Research Topic

Antimicrobial Resistance Along the Food Chain: Are We What We Eat?

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Antimicrobial resistance (AR) is considered a "silent pandemic" that is responsible for more than 700,000 deaths per year; a figure that could rise to 10 million by 2050 if no action is taken (de Kraker et al., 2016; Mahoney et al., 2021). The use or rather misuse of antimicrobial drugs in hospitals has long been recognized as the main cause for the spread of antimicrobial resistant genes (ARGs) and this has become even more apparent during the SARS-CoV-2 pandemic where antimicrobial drugs were often prescribed unnecessarily to threat secondary infections (Knight et al., 2021; Russell et al., 2021; Wang et al., 2022).

In recent years, food production and agriculture among other anthropogenic activities, have exacerbated this problem (Van Boeckel et al., 2015; Wong et al., 2015; Caniça et al., 2019; Rozman et al., 2020; Schar et al., 2021). While the impact of clinical antimicrobial use and resistance has been well-documented, the contributions from other sources and how they fit into the overall prevalence of AR is less understood (Larsson and Flach, 2021). The underlying problem of AR is further complicated by the dynamic transmission of AR leading to the establishment of ARG reservoirs across various stages along the food chain (Imperial and Ibana, 2016; Hudson et al., 2017).

This Research Topic aims to offer a balanced overview of this global threat by gathering research focusing on AR along the food chain, from farm to fork. A total of 13 original research articles across China, India, Brazil, Malaysia, Spain, Iran, Egypt, and South Africa, have been published. Studies predicted that an overwhelming majority of people falling into poverty due to AR, will come from developing and underdeveloped countries, and our article collection reflects this geographical representation (Jit et al., 2020; Iskandar et al., 2021).

To demonstrate that farm animals are critical source for the dissemination of AR, Wu et al. evaluated the prevalence of *Salmonella* in a pig slaughtering house in China and found that the dehairing (66.66%) and splitting (57.14%) areas were the most contaminated with *Salmonella*. High

frequency of resistance to tetracycline, ampicillin, chloramphenicol, and nalidixic acid, was also detected in the isolates. Bioinformatics analysis predicted a high dominance of *S. Typhimurium* ST19 among the isolates while several toxin encoding virulence factors were also identified.

In another study conducted on ducks in China, Wang et al. detected many ARGs from New Delhi metallo β-lactamase (NDM)-producing *Escherichia coli* isolates. Phenotypically, they show high frequency of resistance to trimethoprim-sulfamethoxazole, gentamicin, and fosfomycin. Conjugation experiments showed that the bla_{NDM}-carrying plasmids is transferrable. Moreover, bla_{NDM} coexisted with other ARGs which suggests plausible transfer of ARGs among intestinal *E. coli* isolates of ducks.

A similar study conducted on shrimp aquaculture farms in India by Sivaraman et al. found that extended-spectrum β-lactamase (ESBL)-producing *E. coli* and *Klebsiella pneumoniae* isolates were resistant to cefotaxime, tetracycline, ciprofloxacin, and trimethoprim-sulfamethoxazole. At the molecular level, high prevalence of ARGs responsible for conferring resistance to β-lactamase (e.g., bla_{CTX-M}), tetracyclines, sulfonamide and quinolone resistance, was detected.

In another study from Brazil, Cardozo et al. also detected high prevalence of bla_{CTX-M} in ESBL-producing *E. coli* and *K. pneumoniae* isolated from chicken, chicken meat, and human feces. The authors found high frequency of $bla_{CTX-M-15}$ among the isolates although they are genetically diverse, which suggests that farm animals and their by-products, could be a source of transmission for ESBL-producing pathogens to humans.

In Malaysia, Zakaria et al. reported that *S. enteritidis* isolated from humans, poultry, and foods, was resistant to multiple drugs. ARGs responsible for resistance to aminoglycosides and tetracyclines were the most abundant, while other ARGs responsible for resistance to ampicillin, sulfonamide, and ciprofloxacin, were also detected. Similar to the findings of Cardozo et al., *S. enteritidis* isolates from the various sources share similar resistant genetic traits although they are from distinct lineages.

Zhang et al. also detected resistance to aminoglycosides in *Campylobacter* isolates from chicken and swine in China. The corresponding ARGs were determined, and conjugative experiments confirmed the transferability of aminoglycoside resistance among *C. jejuni* strains. The gene fragment responsible for the elevated resistance in recipient strains was also characterized.

Another study on aminoglycoside resistance by Lu et al. found that the prevalence rates of 16S rRNA methylation enzyme (*armA*)-harboring *Salmonella* strains were 1.1/1,000 and 8.7/1,000 in outpatient and food or environmental isolates, respectively. All *armA*-harboring *Salmonella* strains were resistant to multiple drugs. The *armA* gene was determined to be plasmid-borne and could be transferred to *E. coli* and *Acinetobacter baumannii*. Importantly, strains isolated from outpatients were genetically more identical to those from poultry than those from swine, thus inferring that poultry consumption is a credible source of infection.

In Egypt, Saber et al. examined methicillin- and vancomycin-resistant *Staphylococcus aureus* (MRSA and VRSA) isolated from ready-to-eat meat and food handlers. MRSA isolates were resistant to cefepime, penicillin, ampicillin-sulbactam, ciprofloxacin, nitrofurontoin, and gentamicin, while *VanA* and *VanB* resistant genes were detected in VRSA. Importantly, the isolates could form biofilm and they harbor several biofilm-forming genes, which suggest greater risk of colonization and dissemination.

In South Africa, Richter et al. detected multi-drug resistance in ESBL/AmpC β-lactamase(AmpC)-producing *E. coli*, *K. pneumoniae*, *Serratia fonticola*, and *S. enterica* isolates from spinach and irrigation water. Genes coffering resistance to different classes of antibiotics were detected with $bla_{CTX-M-15}$ and bla_{ACT}-types being the most dominant. *In silico* analysis predicted high similarities to human pathogens for all strains, implying contamination mediated by anthropogenic activities.

A large-scale resistome analysis of *Campylobacter spp.* genomes by Cobo-Díaz et al. found resistant determinants of β-lactams, tetracyclines, quinolones and aminoglycosides in their genomes many of which, are also frequently found together with genes conferring resistance to other antibiotics. The genomes of isolates from humans, food animals, and foods, contain higher frequency of ARGs responsible for resistance to tetracyclines and quinolones, possibly due to intense use of these drugs in veterinary and clinical settings.

Li et al. sequenced three multi-drug resistant *Listeria innocua* isolates from food. Unlike the listeriosis causing *L. monocytogenes*, *L. innocua* is not infectious but, the authors identified ARG islands in both chromosomes and plasmids. All isolates contain the pathogenicity island-4 (LIPI-4) and phylogenetic analysis revealed that they share common origins, thus suggesting transmission capability. This study advocates for surveillance on non-infectious strains to reveal the origins and concomitantly, track and contain the spread of AR in foods.

In hospitals, tigecycline and colistin are last-resort antibiotics used to threat infections. In this regard, Moghimi et al. investigated the mechanisms of tigecycline resistance and found that many non-susceptible *Klebsiella pneumoniae* isolates from human, food animals and/or laboratory selection experiments, are resistant to a combined treatment of tigecycline and colistin. All isolates from humans carried carbapenemase genes while high frequency of mutations in genes that led to increased expression of the AcrAB efflux pump, was detected. Since tigecycline is not used in animal farming, the detection of tigecycline resistance in animal isolates is thus, a clinical concern.

Environmentally sustainable approaches are being increasingly sought to eliminate ARGs from animal farming and in this regard, Peng et al. reported that treatment of animal manure with high heat effectively reduced ARGs introduced into soils. The authors showed that ARG abundance in chicken manure-treated soils was 1.41 times higher than that in mushroom residue-treated soils, but this difference was abrogated when heat-treated chicken manure-amended soils was used.

Contributions in this Research Topic incorporated a variety of approaches to collectively advance our understanding of AR across the different components beginning from farms and ending with the consumers. Through horizontal gene exchanges along the food chain, ARGs inevitably end up interacting with human microbiomes [EFSA Panel on Biological Hazards (BIOHAZ) et al., 2021]. Therefore, we hope this collection will encourage further research, and establish or expand AR surveillance in agriculture, environment, and food processing systems.

AUTHOR CONTRIBUTIONS

AW drafted the manuscript. All authors read, edited, and approved it for publication.

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Chicken Manure and Mushroom Residues Affect Soil Bacterial Community Structure but Not the Bacterial Resistome When Applied at the Same Rate of Nitrogen for 3 Years

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Animal manure is a reservoir of antibiotic resistance genes (ARGs), and direct application of the manure will lead to spread of ARGs in farmland. Here, we explored the impacts of chicken manure and heat-treated chicken manure on the patterns of soil resistome after 3 years' application, with mushroom residues set as the plant-derived organic manure treatment. A total of 262 ARG subtypes were detected in chicken manure using high-throughput qPCR, and heat treatment can effectively remove 50 types of ARGs. Although ARG subtypes and abundance were both higher in chicken manure, there was no significant difference in the ARG profiles and total ARG abundance among three manure-treated soils. Soil bacteria community compositions were significantly different among manure-treated soils, but they were not significantly correlated with soil ARG profiles. Fast expectation–maximization microbial source tracking (FEAST) was used for quantifying the contributions of the potential sources to microbial taxa and ARGs in manure-fertilized soil. Results revealed that only 0.2% of the chicken manure-derived bacterial communities survived in soil, and intrinsic ARGs were the largest contributor of soil ARGs (95.8–99.7%); ARGs from chicken manure only contributed 0.4%. The total ARG abundance in the heat-treated chicken manure-amended soils was similar to that in the mushroom residue-treated soils, while it was 1.41 times higher in chicken manure-treated soils. Thus, heat treatment of chicken manure may efficiently reduce ARGs introduced into soil and decrease the risk of dissemination of ARGs.

Keywords: high-throughput quantitative polymerase chain reaction, antibiotic resistance, chicken manure, bacterial communities, mushroom residue

INTRODUCTION

The increasing emergence and spread of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are of great concern worldwide, threatening the efficacy of modern medicines and posing risks to human health (Levy and Marshall, 2004; Su et al., 2015). ARB and ARGs have been shown to be ubiquitous in the natural environment, agricultural soil (Wang et al., 2020a),

freshwater (Xiong et al., 2015), sediments (Guo et al., 2020), human gut (McInnes et al., 2020), and organic manure (Peng et al., 2018) contain distinct ARG contents at varying abundances. Environmental microbiome is now recognized as a reservoir of ARGs observed in the clinical setting; some plasmid-mediated ARGs spread globally in recent decades have been traced to environmental and animal origins (Lee et al., 2020). Environmental research to better understand distribution and transmission of ARGs may help to reduce the anthropogenic influences on the environmental resistome.

Soil harbors a large genetic diversity at small spatial scale, favoring exchange of genetic materials by means of horizontal gene transfer that will contribute to ARG dissemination (Nesme and Simonet, 2015). The transfer of ARGs from soil bacteria to human pathogens could bring about tremendous threats to human disease control and prevention (Forsberg et al., 2012). ARGs could be transferred from soil to the plant microbiomes (Zhang et al., 2019), which is one of the ways to enter the human body. Livestock manure is the main source of ARB and ARGs in the agriculture soil environment (Peng et al., 2017; Qian et al., 2017). Previous studies have demonstrated that fertilization with pig manure or chicken manure led to an increase in soil ARGs (Peng et al., 2017; Urra et al., 2019). The composting process minimizes some chemical and biological risks (e.g., presence of pathogens) (Evanylo et al., 2008), so this option is commonly preferred before livestock manure is applied. However, compost could still lead to the increased amount of ARGs in soil (Zhu et al., 2013; Peng et al., 2015; Sun et al., 2019). ARB and ARGs in composted manure can spread and promote further development of antibiotic resistance (Kang et al., 2016; Wang et al., 2020a). Therefore, there is a vital need to further improve the effect of compost practice on ARGs carried by animal feces, monitor ARGs in manure-treated soils, and further examine manure application practices and impacts.

Organic manure originated from plants rather than animals, usually used to ameliorate soil properties, both abiotically and biotically (Feng et al., 2015). Mushroom substrate residues is rich in cellulose, lignin, vitamins, and other bioactive substances; rational utilization of mushroom residues is conducive to environmental protection and sustainable development of agriculture (Li et al., 2020). Long-term fertilization with chemical fertilizers plus mushroom residues showed a more connective and closer bacterial networks compared with chemical fertilization (Yao et al., 2020). Recent studies about ARGs mainly shed light on the dynamics of ARGs during manure treatment process (Gou et al., 2018; Awasthi et al., 2019; Wu et al., 2020) and in agricultural soils following application of animal manures (Deng et al., 2020; Liu et al., 2021). Little attention was paid to ARGs in plant-derived organic manure, which also contain a huge number of microorganisms. A previous study found that long-term (22 years) fertilization with plant-derived organic manure increased the abundance of *tetL* and *intI1* in soil (Peng et al., 2018). Mushroom residues are a kind of organic manure completely different from animal manure; their effect on soil microbial community was also quite different (Yao et al., 2020). Analyzing the effect of long-term application of mushroom residues on soil antibiotic resistome may broaden our horizons about how organic manures affect soil antibiotic resistome.

Many factors can influence the fate and migration of manure-borne ARGs in the environment. Studies showed that variations in ARGs composition were mainly structured by phylogenetic compositions of microbial communities (Forsberg et al., 2014; Su et al., 2015; Chen et al., 2016; Liu et al., 2017). Mobile genetic elements (MGEs), such as gene cassettes, integrons (capture ARGs), plasmids (conjugation and transfer ARGs), transposons, and insertion sequences (transfer ARGs) may also influence the spread of ARGs in the environment (Zhang et al., 2011). Chicken manure and mushroom residues are two completely different fertilizers: one originates from animal feces, while the other derives from plant, so they may composite completely different microbial communities and MGEs. In addition, whether sterilization of chicken manure with high-temperature can reduce the risk of ARGs contamination is still unknown. Understanding how these manures contribute to development of soil antibiotic resistome under long-term application is essential since it may direct measures ultimately aiming to diminish risks. The aim of this work was to study and contrast the impact of the 3 years' application of different manures, according to the origin (chicken manure-derived vs. mushroom residues) and sterile conditions (chicken manure vs. heat-treated chicken manure), on ARG and MGE composition in agricultural soil, as well as their relationship with bacteria communities. Studies have reported that partial replacement of chemical fertilizers with organic fertilizers could increase vegetable or grain yields and improve quality of soil and product (Wang et al., 2020b; Yi et al., 2021). In this study, different manures were used to replace 50% of chemical fertilizer, in the case of the same amount of nitrogen applied to the soil. Results from the study will be helpful in estimating the environmental loads of ARGs derived from different manures to soil, understanding how these fertilizers influence soil resistome and guiding manure application practices with the same amount of N input.

MATERIALS AND METHODS

Description of Experiment Site and Different Organic Manure

The long-term experiment field is located at the Fengqiu Agroecological Experimental Station (35°00′N, 114°24′E) in Henan Province, China. The soil is classified as Aquic Inceptisol according to United States Department of Agriculture (USDA), derived from alluvial sediments of the Yellow River, with a sandy loam texture. The long-term experiment was initiated in 2011 with different fertilization treatments rotated with winter wheat (*Triticum aestivum* L.) and summer maize (*Zea mays* L.). The treatments were arranged in a randomized block design, and each treatment had four replicated plots. These experimental plots, designed originally for a nutrient cycling study, enabled us to also examine the effect of manure application on the profiles of soil ARGs.

In the present study, we used four fertilizer treatments and one unfertilized control (CK). The fertilizer treatments included

nitrogen (N), phosphorus (P), and potassium fertilizers (F) and the combined application of NPK fertilizers with mushroom residues (MRF) or with chicken manure (CMF) or heat-treated chicken manure (KF). For the F treatment, N, P, and potassium were applied in the form of urea (200 kg/ha N), superphosphate (80 kg/ha P_2O_5), and potassium sulfate (150 kg/ha K_2O), respectively. The MRF, CMF, and KF treatments were designed to supply the same rate of total N with F: one-half of N came from urea and the other half was from mushroom residues, chicken manure, or heat-treated chicken manure. Chicken manure (CM) and mushroom residues (MR) were collected from nearby farms. Chicken manure was treated at 260°C for 4 h in an oil bath high-temperature treatment kettle to turn it into heat-treated manure (K). The organic and inorganic fertilizers were applied before sowing in June for maize and in October for wheat. The area of each plot was 30 m^2, and more information was described by Yao et al. (2020).

Soil Sampling and DNA Extraction

Soil samples were collected on September 2014, after the maize harvest. At each sampling plot, soils were collected from 0- to 20-cm layer at six random locations; the plant residues and visible roots were removed, mixed to homogenize inside a plastic bag representing the sample of the plot, and carried to lab kept on ice. The mushroom residues and chicken manure to be applied in the field during 2014 were also sampled, and three replicates were collected for analysis. Approximately 10-g soil samples or manure were stored at −80°C for DNA extraction; the rest of the soil samples were air dried and used for chemical analysis as described by Yao et al. (2020).

The microbial genomic DNA was extracted from 0.50-g soil or manure sample using the FastDNA® SPIN Kit for soil or for feces (MP Biomedicals, Santa Ana, CA, United States) according to the manufacturer's protocol. DNA concentration was determined on a ND-1000 spectrophotometer (Thermo-Scientific, Wilmington, DE, United States), and the extracted DNA was stored at −20°C until use. The DNA samples were diluted to 10× and 50× to avoid inhibitors to the PCR.

qPCR Analysis of Antibiotic Resistance Genes and Mobile Genetic Elements

High-throughput quantitative PCRs (HT-qPCR) were conducted using WaferGen SmartChip Realtime PCR system that contained a total of 370 validated primer sets as reported previously (Muurinen et al., 2017). A threshold cycle value (C_T) of 31 was used as the detection limit to differentiate between positive amplification and primer–dimers (Su et al., 2015). One negative control with no DNA template added was included in each HT-qPCR run to eliminate false-positive detections. Amplicons with multiple melting curves were removed from the analysis. Three technical replicates of each sample above the detection limit were regarded as positive detection. The $2^{-\Delta CT}$ method where ΔC_T = (C_T detected ARGs - C_T 16S rRNA gene) was used to calculate the relative abundances of ARGs and MGEs normalized to the 16S rRNA gene according to a comparative C_T method (Gou et al., 2018).

Fifteen genes of special concern ARGs including tetracycline resistance genes [*tetG*, *tetL*, tet*Z*, *tetW*, *tetM*, *tetO*, and *tetB*(*P*)], sulfonamide resistance genes (*sul1*, *sul2*, and *sul3*), macrolide resistance genes (*ermB*, *ermC*, and *ermF*), and beta-lactamase genes (bla_{CTX} and bla_{TEM}) were quantified by real-time quantitative PCR (RT-qPCR) analysis with a C1000™ Thermal Cycler equipped with the CFX96™ Real-Time system (Bio-Rad, United States). In addition, the 16S rRNA gene abundance, which has been used previously to assess the overall bacterial abundance, was quantified by using primer set 519F/907R so that the ARG abundance could be standardized against the bacterial populations. The qPCR system and primers sets as reported previously (Peng et al., 2017).

High-Throughput Sequencing of 16S rRNA Gene

The 16S rRNA gene sequencing was performed at Novegene (Beijing, China) using the Ion S5™ XL platform, and 400-bp/600-bp single-end reads were generated. The 16S V4 region was amplified using the primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACVSGGGTATCTAAT-3′).

Single-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Quality filtering on the raw reads was performed under specific filtering conditions to obtain high-quality clean reads according to the Cutadapt (Martin, 2011) (V1.9.1) quality-controlled process. The reads were compared with the reference database (Gold database) using the UCHIME algorithm (Edgar et al., 2011) to detect chimera sequences, and then the chimera sequences were removed. Then the effective tags were finally obtained. Sequences analysis was performed by Uparse software (Uparse v7.0.1001). Operational taxonomic units (OTUs) were defined at the 97% similarity level. A representative sequence of each OTU was assigned to a taxonomic level in the SILVA database. The sequences were deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (accession no., PRJNA669308).

Manure and Mushroom Residue-Associated Community and Antibiotic Resistance Gene Analyses

Fast expectation–maximization microbial source tracking (FEAST) was used to calculate the contribution of soil, mushroom residues, and chicken manure bacterial communities within treatments following the procedures as described previously (Shenhav et al., 2019) with the R package of "FEAST[1]." FEAST could unravel the origins of complex microbial communities based on the statistical model that assumes each sink is a convex combination of known and unknown sources. In this study, the chicken manure, mushroom residues, and unfertilized soil samples were defined as manure or soil "sources" and manure-treated soil samples as "sinks." OTUs

[1]https://github.com/cozygene/FEAST

present in only one sample were removed prior to the analysis, and the algorithm was run with default parameters. In order to understand the source–sink relationship in fertilized soil, FEAST was also used to predict the relative contribution of different manures to the ARGs in treated soil based on the signatures of ARGs as described previously (Chen et al., 2020).

Statistical Analysis

The effects of different manure fertilization on abundance of ARGs and soil chemical properties were compared using ANOVA performed with software SPSS 20 (IBM, Armonk, NY, United States). Tukey's honestly significant difference (HSD) test was used for comparisons among treatment means. Differences were considered significant at $p < 0.05$. The changes of ARGs and bacterial compositions across different treatments were visualized by principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) ordinations based on the Bray–Curtis dissimilarity distances. Significant difference tests in the bacterial communities and profiles of ARGs and MGEs between different treatments were conducted using ANOSIM and nonparametric permutational multivariate analysis of variance (PERMANONA, with "adonis" function) based on Bray–Curtis dissimilarities and 999 permutations. The Procrustes analysis (9,999 permutations) was used to describe the correlation between ARGs composition and bacterial communities. The above statistical analysis and redundancy analysis (RDA) were processed and plotted by R version 3.6.3 using the package vegan. Heatmap was performed with the R package pheatmap. Additionally, we tested for differential ARG abundance between manure-treated soil and chemical fertilizer treated soil (F) using likelihood ratio tests (LRTs) with the R package edgeR at a false discovery rate (FDR)-corrected value of $p < 0.05$ and exhibited with Manhattan plots based on R packages of ggplot2. Network maps based on the Spearman correlation coefficient between ARGs and MGEs were drawn using the Gephi platform based on a significant Spearman correlation (Spearman's rho > 0.9, $P_{\text{adjusted}} < 0.01$).

RESULTS

Diversity and Relative Abundance of Antibiotic Resistance Genes and Mobile Genetic Elements in Manure

The types and abundance of ARGs were analyzed by HT-qPCR in manure samples. We detected 10, 10, and 9 ARG types in manure of CM, MR, and K, respectively (**Figure 1A**). A total of 262 ARG subtypes were detected in CM, which was more than those in MR (242) and K (212) (**Figure 1A**). The total relative abundance of ARGs in CM was approximately 2.03 times and 3.92 times higher than that in K and MR (**Figure 1B**).

Compositions of ARGs in these three manures were also quite different. The main ARGs in CM belong to aminoglycoside, tetracycline, disinfectant, and sulfonamide, while MR was dominated by ARGs that belong to beta-lactam, multidrug/other, and phenicols (**Figure 1B**). MLSB resistance genes were the main residual ARGs in manure K. A different pattern was found for MGEs, with an average number of 25 MGEs detected in CM and K, while MR harbored averagely 27 MGEs. The total relative abundance of MGEs was higher in MR than that in CM and K, but the difference was not significant ($p > 0.05$) (**Figure 1B**).

Fifteen ARGs were quantified with RT-qPCR, and the result was consistent with that of HT-qPCR, while the difference among these three manures is more obvious (**Figures 1C,D**). The total relative abundance of the 15 ARGs in CM was 3.73 and 364.95 times higher than that in K and MR (**Figure 1C**). The total copy number of ARGs in MR was also lower than that of CM and K, with 16S rRNA gene copy number at the same level as K (**Figure 1D**).

Profiles of Antibiotic Resistance Genes in Soils After Different Manures Applied for 3 Years

A total of 193 ARG subtypes were detected in CMF soils, 152 detected in MRF soils, and 146 detected in KF soils. The number of ARG subtypes detected in MRF and KF was less than that in F soils (172) (**Figure 2A**). Most of these ARGs represented three resistance mechanisms: antibiotic deactivation (38%), efflux pump (36%), and cellular protection (20%) (**Supplementary Figure 1**). Phenicols and multidrug/other resistance genes were the dominant ARG types in soils (**Figure 2B**). Among three manure treatments, CMF soils had higher total relative abundance of ARGs and MGEs, but neither of the differences reached the significant level ($p > 0.05$) (**Figure 2B**).

Many ARGs were enriched in the soils fertilized with KF, CMF, or MRF (**Figure 3** and **Supplementary Figure 2**). Among them, seven ARGs, including *aadE*, *aadD*, *qacE1*, *qacH*, *lnuA*, *vatE*, and *tetL*, were significantly enriched in all three manure treatments. Aminoglycoside resistance genes *aadA1* were enriched in CMF and MRF, while MLSB resistance genes *ermB* and *ermY* were enriched in CMF and KF (**Figure 3**). There were some specific ARGs enriched in only one treatment, such as *tetV* and *tetX* in MRF soils; *aacA*, *str*, and *ermT* in CMF soils; and *tetT* in KF soils. Seven ARGs depleted in KF, including *blaOCH*, *cphA*, *erm*(*34*), *erm*(*36*), *emrD*, *cmx*(*A*), and *floR* (**Figure 3**). PCoA and ANOSIM pairwise comparisons of the overall distribution patterns of ARGs and MGEs in soils demonstrated no distinct separation among these treatments (**Supplementary Figure 3** and **Supplementary Table 1**). Network maps based on the Spearman correlation coefficient between ARGs and MGEs showed that manure fertilization reduced the network connection between different ARGs and between ARG and MGEs (**Supplementary Figure 4**). The connection edge number in KF treatment was the least among the four fertilization treatments (**Supplementary Figure 4**).

Fifteen ARGs were quantified by RT-qPCR, while *tetW* and *sul3* were not detectable in all of the soil samples. The total relative abundance of the detected 13 ARGs in CMF soils was significantly higher than that of MRF and F soils ($p < 0.05$) (**Figure 2C**), while that in KF and MRF was similar with each other ($p > 0.05$) (**Figure 2D**). In **Figures 2C,D**, CMF soils had obviously a higher abundance of *tetL*. Statistical analysis

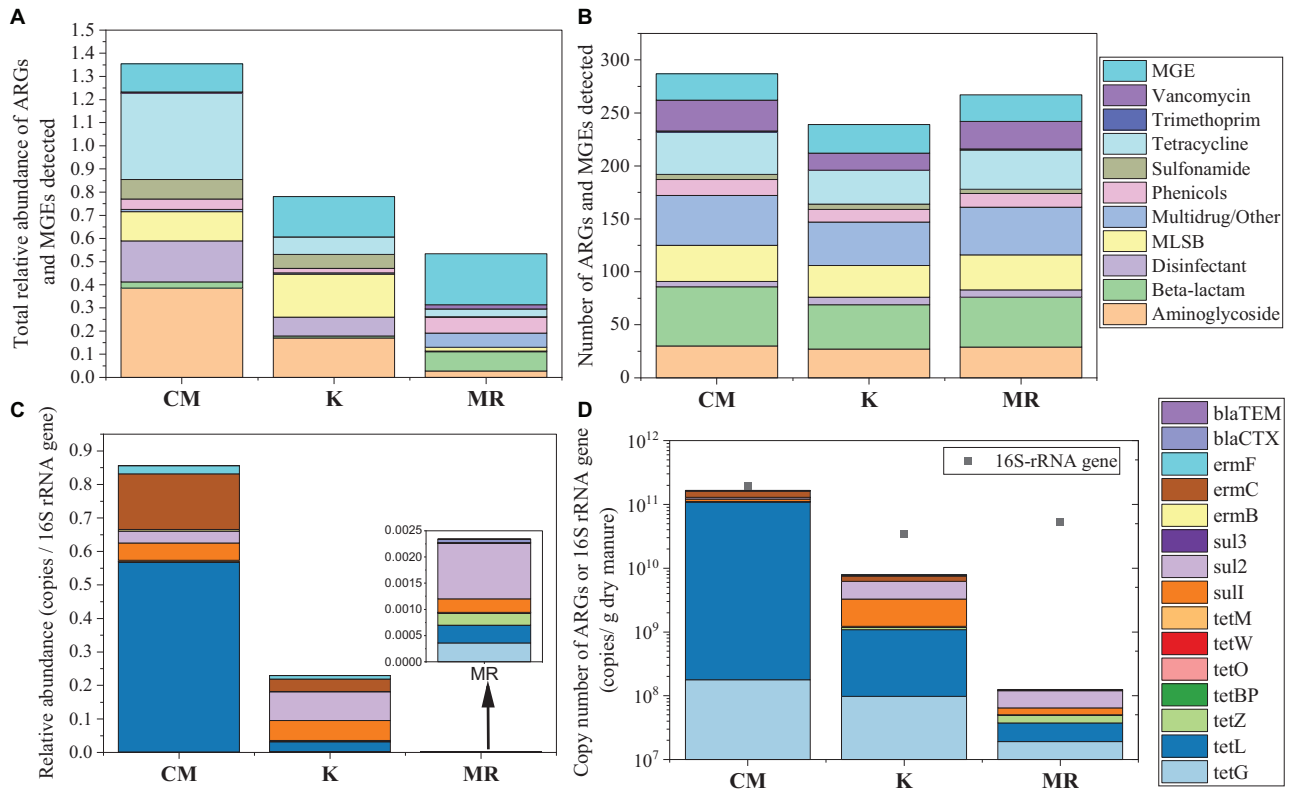

FIGURE 1 | Comparison of the average total relative abundance **(A)** and the major classes **(B)** of ARGs and MGEs in manure samples quantified by high-throughput quantitative PCR (HT-qPCR). The average relative abundances **(C)** and copy numbers **(D)** of 13 ARGs or 16S-rRNA gene in manure samples quantified by real-time quantitative PCR (RT-qPCR). CM, chicken manure; K, heat-treated chicken manure; and MR, mushroom substrate residue; ARGs, antibiotic resistance genes; MGEs, mobile genetic elements.

showed that *tetL*, *tetB*(*P*), *tetO*, and *tetM* were significantly enriched in CMF soils (**Supplementary Figure 5**). However, in results presented by HT-qPCR, *tetB*(*P*), *tetO*, and *tetM* were not significantly enriched in CMF soils (**Figure 3**).

Soil Chemical Properties and Changes in Soil Bacterial Communities

Different fertilization treatments significantly affected the soil chemical properties (**Table 1**). Compared with F, MRF treatment significantly increased the concentrations of soil organic carbon, total N, total P, available P, and available potassium ($p < 0.05$), while CMF treatment significantly increased the soil contents of total N and available P ($p < 0.05$). Except that the available P was higher in CMF-treated soil ($p < 0.05$), there was no significant difference in the soil chemical properties between KF and CMF treatments. Based on the above differences of soil chemical nutrients, MRF-treated plots obtained the highest maize yield (**Table 1**), while there was no significant difference among CMF, KF, and F treatments.

Manure bacterial communities were dominated by Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes (**Figure 4A**). The total relative abundances of these bacterial phyla in manure of K, CM, and MR, respectively, accounted for 92.7, 98.6, and 93.5% of the total bacterial 16S rRNA gene sequences (**Figure 4A**). Phylum-level relative abundance data showed that bacterial community composition in MR was obviously different with that in CM and K. MR were dominated by the phyla Bacteroidetes (47.8%), Proteobacteria (23.7%), Actinobacteria (11.7%), and Firmicutes (10.4%), while the bacterial communities in CM and K samples were dominated by Actinobacteria (43.3 vs. 32.6%), Firmicutes (38.4 vs. 39.1%), Proteobacteria (10.4 vs. 13.5%), and Bacteroidetes (6.4 vs. 7.5%). However, the soil bacteria community structures treated by different manures were similar with each other, with Proteobacteria (29.9–31.6%) tending to be the most abundant bacterial phylum followed by Acidobacteria (13.6–16.7%) and Actinobacteria (12.9–17.1%) (**Figure 4A**). Although the overall bacterial community structures in the phylum level seem similar with each other in soils (**Figure 4A**), the application of different manures still led to changes in the abundance of some bacterial families (**Figure 4B**). Many special bacterial families were enriched in manure-amended soils, such as Pseudomonadaceae, Micrococcaceae, and Planococcaceae in CMF soils (**Figure 4B**).

The NMDS ordination based on Bray–Curtis distances showed that bacterial communities were separated between manure treatments and CK (**Supplementary Figure 6**). ANOSIM

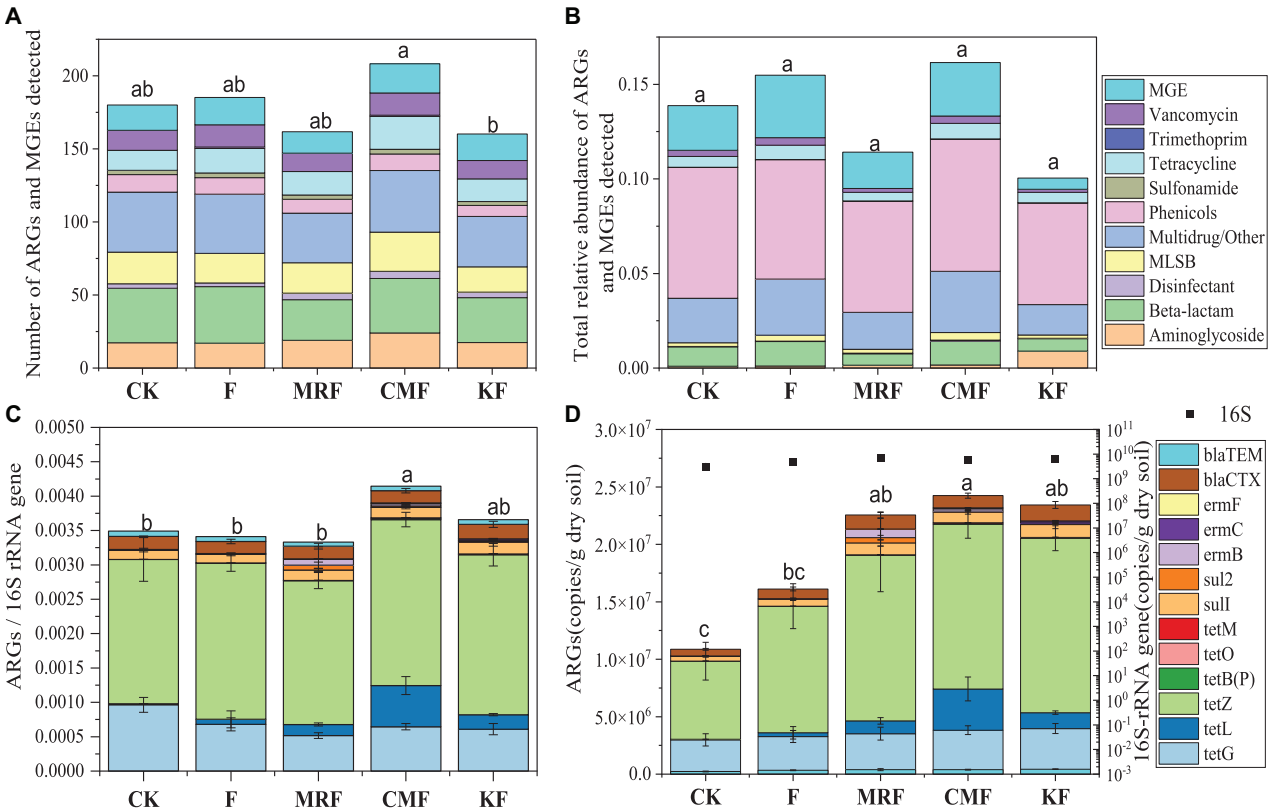

FIGURE 2 | Number of unique ARGs and MGEs detected in the soil sample of each treatment **(A)** and the average relative abundance of ARGs and MGEs in soil fertilized with each manure for 3 years **(B)** quantified by HT-qPCR. The average relative abundance **(C)** or copy numbers **(D)** of 13 ARGs in soil samples quantified by RT-qPCR. Different letters indicate significant differences based on Tukey's HSD test at $p < 0.05$. Error bars represent standard deviation ($n = 4$). CK, unfertilized control; F, NPK fertilizers; KF, the combined application of NPK fertilizers and heat-treated chicken manure; CMF, the combined application of NPK fertilizers and chicken manure; MRF, the combined application of NPK fertilizers and mushroom residues; ARGs, antibiotic resistance genes; MGEs, mobile genetic elements; HSD, honestly significant difference.

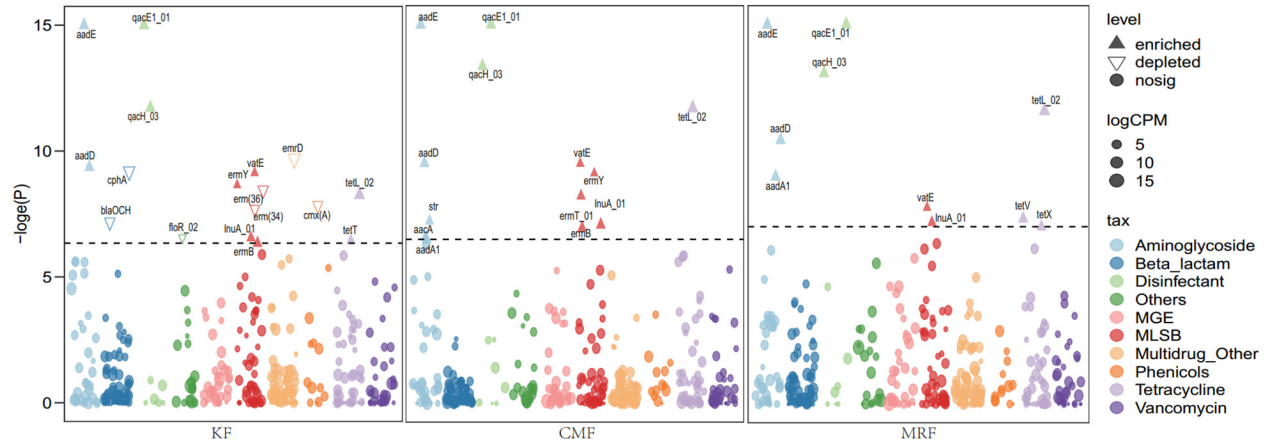

FIGURE 3 | Manhattan plots showing manure-enriched ARGs in KF, CMF, or MRF compared with F. ARGs that are significantly enriched (with respect to F) are depicted as full triangles; solid dots marked with "nosig" represent no significant difference. The color of each dot represents ARG types or MGE, and the size corresponds to their relative abundances in the respective samples. ARGs belonging to sulfonamide and trimethoprim are labeled as "Others." F, NPK fertilizers; KF, the combined application of NPK fertilizers and heat-treated chicken manure; CMF, the combined application of NPK fertilizers and chicken manure; MRF, the combined application of NPK fertilizers and mushroom residues; ARGs, antibiotic resistance genes; MGE, mobile genetic element.

TABLE 1 | The yield of maize and soil chemical properties in different treatments.

	CK	F	MRF	CMF	KF
Yield of maize (kg/plot)	12.45 ± 0.43c	31.83 ± 0.74ab	33.68 ± 0.92a	31.42 ± 1.26b	31.04 ± 1.12b
pH	8.51 ± 0.06a	8.44 ± 0.05a	8.44 ± 0.08a	8.49 ± 0.02a	8.47 ± 0.03a
Soil organic carbon (SOC) (g/kg)	6.12 ± 0.16c	6.71 ± 0.26bc	10.84 ± 1.02a	7.7 ± 0.22b	7.44 ± 0.16b
Total N (g/kg)	0.6 ± 0.01d	0.67 ± 0.02cd	0.99 ± 0.07a	0.78 ± 0.03b	0.75 ± 0.03bc
Total P (g/kg)	0.77 ± 0.03c	0.87 ± 0.02bc	0.99 ± 0.09a	0.97 ± 0.03ab	0.9 ± 0.06ab
Available P (mg/kg)	0.52 ± 0.18d	4.28 ± 0.99d	48.07 ± 8.04a	28.86 ± 5.7b	17.93 ± 4.91c
Total potassium (g/kg)	19.23 ± 0.27a	19.64 ± 0.12a	19.62 ± 0.41a	19.46 ± 0.45a	19.6 ± 0.18a
Available potassium (mg/kg)	77.12 ± 3.94c	127.73 ± 18.25b	191.6 ± 12.68a	138.58 ± 10.69b	122.91 ± 4.82b

Different lowercase letters indicate statistically significant differences at $p < 0.05$.
CK, unfertilized control; F, NPK fertilizers; MRF, the combined application of NPK fertilizers and mushroom residues; CMF, the combined application of NPK fertilizers and chicken manure; KF, the combined application of NPK fertilizers and heat-treated chicken manure.

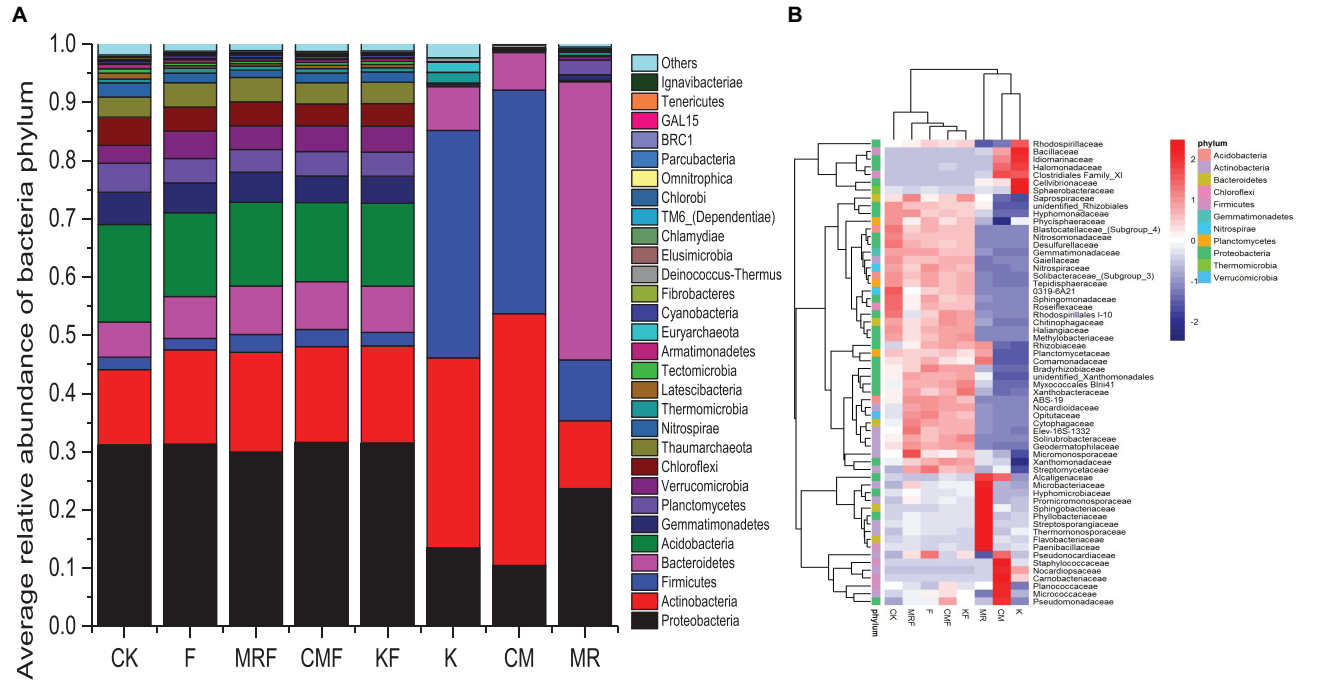

FIGURE 4 | The relative abundance of 16S rRNA gene sequences classified to each phylum **(A)** and heatmap of the top 60 bacterial families **(B)** in soil samples from different treatments and manures. CK, unfertilized control; F, NPK fertilizers; KF, the combined application of NPK fertilizers and heat-treated chicken manure; CMF, the combined application of NPK fertilizers and chicken manure; MRF, the combined application of NPK fertilizers and mushroom residues; K, heat-treated chicken manure; CM, chicken manure; MR, mushroom residues. Each color block represents the mean of four replicates.

pairwise comparisons were all significantly different except CMF vs. KF (**Supplementary Table 2**). RDA was conducted to determine the correlation of soil properties with bacterial community structures in the soils. All soil properties together explained 50.3% of the variety in soil bacterial communities. Soil organic carbon (p = 0.002), total N (p = 0.009), pH (p = 0.011), and total P (p = 0.037) were significantly correlated with bacterial structures (**Supplementary Figure 7**). However, Procrustes test analysis demonstrated that soil bacterial community compositions were not significantly correlated with ARG profiles on the basis of Bray–Curtis dissimilarity metrics (R = 0.3756, $p > 0.05$) (**Figure 5**).

Fate of Manure-Derived Bacterial Communities and Antibiotic Resistance Genes in Soil

Further, we employed FEAST to explore the fate of manure-derived bacteria in the soils. According to the analysis, manure of CM and K, respectively, exhibited 0.24 and 0.31% of the contributions for the bacterial communities in soils, while the average relative contribution of MR was 4.47% (**Figure 6**). The remaining proportions were due to "soil source" and "unknown source." The indigenous bacterial communities in the sink were defined as "soil source,"

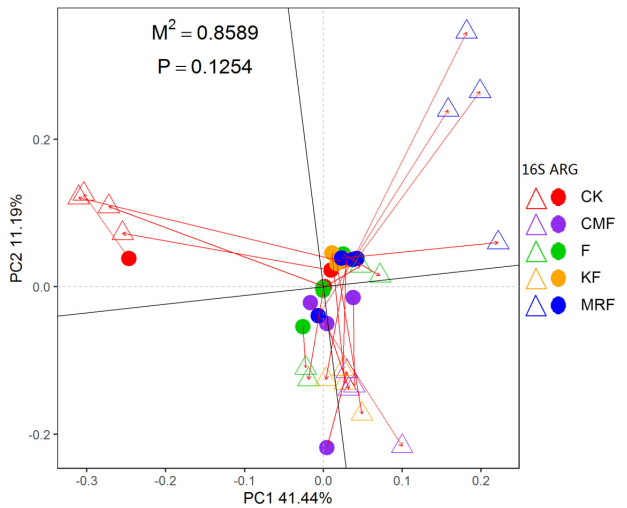

FIGURE 5 | Procrustes analysis results depicting the correlation between ARG contents (HT-qPCR data) and bacterial compositions (16S rRNA gene OTU data) on the basis of Bray-Curtis dissimilarity metrics (sum of squares $M^2 = 0.8589$, $r = 0.3756$, $p > 0.05$, 9,999 permutations). The solid dot represents ARG profiles, and the triangles indicate bacterial community. CK, unfertilized control; F, NPK fertilizers; KF, the combined application of NPK fertilizers and heat-treated chicken manure; CMF, the combined application of NPK fertilizers and chicken manure; MRF, the combined application of NPK fertilizers and mushroom residues; ARG, antibiotic resistance gene; OTU, operational taxonomic unit.

and bacterial communities in CK were used as the "soil source." Bacterial communities from MR survived more in soil than those from CM and K (**Figure 6**), which was consistent with the results of FEAST analysis for manure-derived ARGs (**Table 2**).

DISCUSSION

Chicken Manure Had Different Antibiotic Resistance Gene Profiles With Mushroom Residues and Heat-Treated Chicken Manure

In this study, 330 ARG subtypes were quantified in manure by using HT-qPCR, and the results revealed that the detected ARG subtypes and the total ARG relative abundance were both much higher in chicken manure than in mushroom residues (**Figure 1**). Evolution and enrichment of ARGs are primarily stimulated by antibiotics (Pruden et al., 2006). Veterinary antibiotics have been widely used in livestock production for the prevention of diseases or as growth promoters (Zhang et al., 2015). ARB and ARGs may accumulate during the overuse of antibiotics in intestines of livestock. Therefore, it is not difficult to understand that ARGs in

TABLE 2 | Results of FEAST analysis showing the contribution of corresponding manure to ARGs in the soils.

	Sink	Soil source	Manure source	Unknown
F as the soil source	MRF	96.4 ± 4.1%	3.6 ± 4.1%	0.00 ± 0.0%
	KF	99.6 ± 0.2%	0.3 ± 0.2%	0.04 ± 0.1%
	CMF	99.6 ± 0.2%	0.4 ± 0.2%	0.00 ± 0.0%
CK as the soil source	MRF	95.8 ± 5.6%	4.2 ± 5.6%	0.00 ± 0.0%
	KF	99.7 ± 0.1%	0.2 ± 0.1%	0.03 ± 0.1%
	CMF	99.6 ± 0.1%	0.4 ± 0.2%	0.00 ± 0.0%

FEAST, fast expectation-maximization microbial source tracking; ARGs, antibiotic resistance genes; MRF, the combined application of NPK fertilizers and mushroom residues; KF, the combined application of NPK fertilizers and heat-treated chicken manure; CMF, the combined application of NPK fertilizers and chicken manure.

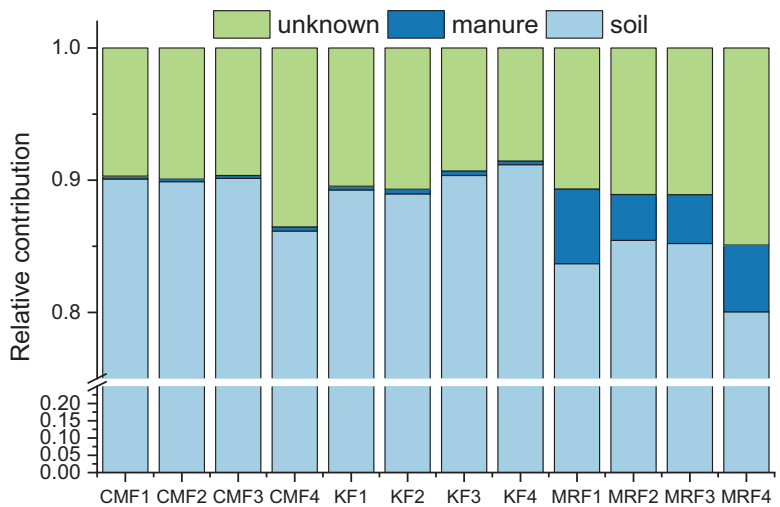

FIGURE 6 | Relative contribution of the manure-derived, soil, and unknown sources of bacterial communities in the manure-treated soils estimated using FEAST analysis. KF, the combined application of NPK fertilizers and heat-treated chicken manure; CMF, the combined application of NPK fertilizers and chicken manure; MRF, the combined application of NPK fertilizers and mushroom residues; FEAST, fast expectation-maximization microbial source tracking. Data from unfertilized control (CK) served as the soil source.

chicken manure were much more abundant than those in mushroom residue.

High temperatures (reach up to 90°C) during the fermentation process have been found to significantly enhance the removal of ARGs and MGEs from sewage sludge (Liao et al., 2018). As revealed by HT-qPCR or by RT-qPCR in this study, heat treatment (up to 260°C) can effectively remove 50 subtypes of ARGs from chicken manure and significantly reduce the total relative abundance of ARGs (**Figure 1**). Although ARGs and MGEs were still detected in the heat-treated chicken manure, most of them may exist in dead bacterial cells. Zhou et al. (2019) found that turning pig manure into biochar could noticeably reduce the level of the antibiotic resistome in soils compared with compost amendment. Although the sterilization effect of heat treatment at 260°C for 4 h may not be as thorough as that of turning it into biochar, the results of this study showed that compared with the direct application of fresh chicken manure, heat treatment of the manure also can reduce the detectable subtypes and relative abundance of ARGs in soils.

Different Manures did Not Significantly Change the Antibiotic Resistance Gene Profiles in Alkaline Soil

Antibiotic deactivation and efflux pump were found to be the two major mechanisms for antibiotic resistance in the soils applied with different manures for 3 years (**Supplementary Figure 1**). Previous studies have reported the same phenomenon in sewage sludge-applied soils (Chen et al., 2016), reclaimed water-applied soils (Wang et al., 2014), swine farms (Zhu et al., 2013), and chicken manure or cattle manure-applied soils (Wang et al., 2020a). Efflux pumps are major players in both intrinsic and acquired resistance to drugs currently in use for the treatment of infectious diseases (Hernando-Amado et al., 2016), which are either chromosomal or plasmid-encoded (Rocha-Granados et al., 2020), and deserves more attention because they are often associated with the multidrug antibiotic resistance (Wang et al., 2020a).

Common agricultural practices like fertilization and irrigation are known to affect soil resistome. Many studies have found that long-term manure application significantly increased the diversity and abundances of ARGs and MGEs (Peng et al., 2017; Han et al., 2018). However, in some studies, such significant trends of ARGs caused by long-term application of manure could not be observed (Tang et al., 2015; Wang et al., 2018). According to HT-qPCR, the distribution and total relative abundance of ARGs among manured soils had no significant difference in this study (**Figure 2**, **Supplementary Figure 3**, and **Supplementary Table 1**); soil types (Wang et al., 2020a), sources of animal manure (Zhang et al., 2017), tillage and cropping systems (Wang et al., 2018), and time (Liu et al., 2017) and application frequency (Heuer et al., 2011) may lead to the inconsistent results. In addition, a subset of ARGs analyzed in these studies may be one of the reasons; different from the result of HT-qPCR, the total relative abundance of 13 ARGs quantified by RT-qPCR was significantly higher in CMF soils (**Figure 2**). Therefore, more types of ARGs considered may provide a more comprehensive insight into the influence of manure fertilization in an agricultural environment. The different findings detected in HT-qPCR and RT-qPCR may result from the differences in primer sequences, polymerase, qPCR volume (100 nl vs. 20 μl), and number of primer sets (one pair in RT-qPCR vs. multiple primer pairs for some genes).

Antibiotic resistance genes profiles in manured soils were not significantly changed, yet among the four fertilized treatments, CMF soils had more detected ARG subtypes and higher ARG abundance level (**Figure 2**). Although differences were not significant, they still indicated that application of fresh chicken manure induced some ARG subtypes in soil. Previous studies found that application of chicken manure generally increased the abundance of some ARGs (Urra et al., 2019; Liu et al., 2021). This study further verified the result with long-term applications in the field. The soil in this study was a sandy loam (alluvial-aquic) soil with alkaline characteristics. Our previous study had found that *tetL* in this alkaline soil was enriched after long-term fertilized with plant-derived organic manure, and survival of the *Firmicutes* bacteria that carried *tetL* may be the cause (Peng et al., 2018). In this study, compared with F, the relative abundance of *tetL* quantified by RT-qPCR was also increased in different manure-amended soils (**Supplementary Figure 5**), which may be due to the same reason as our previous finding.

Small Proportion of Manure-Derived Bacteria and Antibiotic Resistance Gene Survival in Soil May Be the Main Reason for Its Minor Effects on Soil Antibiotic Resistance Gene Abundance

The succession of microbial community structure is the main factor that influenced the variation of ARGs during sewage sludge composting (Su et al., 2015), anaerobic digestion of dairy manure (Sun et al., 2016), and manure composting (Yin et al., 2017; Awasthi et al., 2019). Changes of soil resistome induced by manure application mainly resulted from the alteration of soil bacteria communities rather than the horizontal gene transfer (Liu et al., 2017). In this study, bacterial communities were significantly changed by different manure treatments (**Supplementary Figure 6** and **Supplementary Table 2**), while manure application only showed some minor effects on ARG profile (**Figure 3**, **Supplementary Figure 3**, and **Supplementary Table 1**), and bacteria community compositions were not significantly correlated with ARG profiles (**Figure 5**). These results suggested that the variation of ARG profile is not the inevitable result of bacterial community change in manure-treated soil. As mentioned above, manure application did not always lead to significant increase of ARG abundance in soil, and the reasons for this phenomenon are very complex. Finding out the reasons may be of great value for guiding us to take effective measures to reduce ARG diffusion in farmland.

Fast expectation maximization microbial source tracking and SourceTracker were commonly used machine-learning classification tools in microbial source tracking, which were used in manure-associated community analysis (Gou et al., 2018) or quantifying the contributions of potential sources to ARGs in the

river sediments (Chen et al., 2020). As a novel microbial source tracking method, FEAST has some distinct advantages on its estimation accuracy particularly if the sink community contains species/gene from an uncharacterized source (Shenhav et al., 2019; Chen et al., 2020). Here, FEAST was used to apportion the contributions of soil, mushroom residues, and chicken manure on the presence of bacteria communities and ARGs in the manure-amended soils, and results showed that MR-derived bacteria and ARGs survived more than those derived from CM and K (**Figure 6** and **Table 2**). The formation environment of mushroom residues is more similar to that of the soil environment than chicken gut microenvironment. In addition, soil microbial diversity is the key factor controlling the extent to which bacterial invaders can be established (van Elsas et al., 2012; Peng et al., 2016). Thus, the indigenous soil microbial community may also play an important role in inhibiting the CM- and K-derived bacteria survival in soil.

The total relative abundance of ARGs in CMF soils was 1.41 times higher than that of MRF and KF soils even not significantly ($p > 0.05$), and three ARGs were significantly enriched (**Figures 2**, **3**). We speculated that manure affect soil ARGs from three ways: firstly, nutrients carried by manure cause the proliferation of endogenous bacteria carrying by ARGs; secondly, ARGs from manure transfer to soil endogenous microorganisms; and finally, manure-derived ARG survival in soil. Nutrients can enhance the effect of selective pressures of ARGs (Deng et al., 2020); even sterilized manure also can result in a little increase in the relative abundance of tetracycline-resistant bacteria in soil (Peng et al., 2016). Therefore, the seven significantly enriched ARGs in three manure treatments (**Figure 3**) may result from the proliferation of endogenous resistance using nutrients carried by the manures. Bacterial abundances in manure-amended soils were not significantly increased (**Figure 2D**); this may result from the fertilization treatments in this study being loaded with equal nitrogen into the soils. From the perspective of MGEs, the total relative abundance of MGEs was not significantly different among three manure-treated soils (**Figure 2**), connections between ARGs and MGEs were not improved by manure application (**Supplementary Figure 4**). It has been demonstrated that MGEs could potentially enhance the accumulation and persistence of ARGs in manure/sludge-amended soils (Gaze et al., 2011). However, horizontal gene transfer occurrence of ARGs via MGEs may depend, at least partly, on its types; the limited distribution of some genes is possibly associated with their limited host range (Chen et al., 2016). Therefore, horizontal gene transfer via MGEs may not be the main reason leading to the minor increase of the total ARG abundance in CMF soils. Although CM-derived bacteria contributed a little proportion (0.19–0.31%) to the CMF soil bacteria community (**Figure 5**), the relative abundance of ARGs in CM was higher than that of MR and K (**Figure 1**). Thus, when the same proportion of bacteria survived in soil, CM-derived bacteria may have a higher impact on soil ARGs than that from MR and K. FEAST analysis showed that CM-derived ARGs contributed 0.4% to the ARGs in CMF soils, while K-derived ARGs contributed 0.3% (**Table 2**), and the enriched ARGs in CMF soils may be from the surplus 0.1% contribution of CM. MR carried a small amount of ARGs (**Figure 1**), so it did not significantly affect soil ARGs after being applied for 3 years, even though MR-derived ARGs contributed 3.6% to the soil ARGs (**Table 2**).

The detected ARG subtypes were significantly decreased, and the total abundance of ARGs was reduced by 29.12% in KF soils compared with that in CMF soils (**Figure 2**). This indicated that heat treatment of chicken manure may efficiently reduce the amount of ARGs introduced into soil, thus making its abundance level similar to that of MRF soils. This result further verified our early findings in mesocosm and greenhouse experiments that nutrients and tetracycline resistance genes not carried by live bacteria in sterilized manure contribute little to the accumulation of tetracycline-resistant bacteria and genes in soils (Peng et al., 2016). The total abundance of MGEs was also the lowest in KF soils (**Figure 2**), which inferred that the opportunities of ARG transfer to other species may also be reduced. In addition, after the oil bath kettle was started, the high temperature of the oil bath kettle can be maintained for a period of time after the temperature is raised, and the inner part of the equipment is closed, leaving only the steam outlet. Therefore, the energy consumption is relatively low: it needs about 48-kW electric energy to treat 1 ton of chicken manure. Whether the application of heat-treated manure can decrease the migration of ARGs in other types of soil and the removal efficiency of this oil bath high-temperature treatment kettle on ARGs from other animal manure needs further study.

CONCLUSION

In this study, 3 years' application of mushroom residue, chicken manure, and heat-treated chicken manure significantly changed the soil bacteria community compositions. However, the profiles and abundance of the ARGs in the fertilized soils were not significantly changed but only showed some minor increase in chicken manure-amended soils. In quantitative apportionment, the analysis of FEAST demonstrated that intrinsic ARGs from soil dominated 95.8–99.7% of the relative contribution of ARGs in the manure-amend soils. The contribution of chicken manure was similar to that of heat-treated manure (0.4 and 0.2%), and the contribution of both was lower than that of mushroom residues (4.2%). Although the application of the fresh chicken manure for three consecutive years had no significant effect on soil ARGs, the total abundance of ARGs had increased slightly. Heat treatment obviously reduced the total abundance of ARGs and MGEs in chicken manure, and the total ARG abundance in the heat-treated manure-amended soils was similar to that observed in the soils applied with mushroom residue. It suggests that treating animal manure with high temperature may be an environmentally sustainable technology to reduce the public health risk of ARGs derived from intensive animal farming.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI sequence read archive (SRA) repository, accession number (PRJNA669308).

AUTHOR CONTRIBUTIONS

XL and RC initiated the project. RC and YW supplied soil materials and reagents. YW contributed his scientific advice during the work. SP performed the experiments, analyzed the data, and wrote the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

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Prevalence of 16S rRNA Methylation Enzyme Gene *armA* in *Salmonella* From Outpatients and Food

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Salmonella is the primary cause of community-acquired foodborne infections, so its resistance to antimicrobials, such as aminoglycosides, is a public health issue. Of concern, aminoglycoside resistance in *Salmonella* is increasing rapidly. Here, we performed a retrospective study evaluating the prevalence of *Salmonella* harboring *armA*-mediated aminoglycoside resistance in community-acquired infections and in food or environmental sources. The prevalence rates of *armA*-harboring *Salmonella* strains were 1.1/1,000 (13/12,095) and 8.7/1,000 (32/3,687) in outpatient and food/environmental isolates, respectively. All the *armA*-harboring *Salmonella* strains were resistant to multiple drugs, including fluoroquinolone and/or extended-spectrum cephalosporins, and most (34/45) belonged to serovar Indiana. The *armA* gene of these strains were all carried on plasmids, which spanned five replicon types with IncHI2 being the dominant plasmid type. All the *armA*-carrying plasmids were transferable into *Escherichia coli* and *Acinetobacter baumannii* recipients. The conjugation experiment results revealed that the *armA*-harboring *S.* Indiana strains had a relatively higher ability to acquire *armA*-carrying plasmids. The low similarity of their pulsed field gel electrophoresis patterns indicates that the *armA*-harboring *Salmonella* strains were unlikely to have originated from a single epidemic clone, suggesting broad *armA* spread. Furthermore, the genetic backgrounds of *armA*-harboring *Salmonella* strains isolated from outpatients exhibited higher similarity to those isolated from poultry than to those isolated from swine, suggesting that poultry consumption maybe an infection source. These findings highlight an urgent need to monitor the prevalence and transmission of *armA*-harboring *Salmonella*, especially *S.* Indiana, to better understand the potential public health threat and prevent the further spread of these strains.

Keywords: *Salmonella*, *armA*, multi-drug resistant, poultry, swine

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INTRODUCTION

Aminoglycoside antibiotics are widely used in hospitals for the treatment of infections, especially those caused by Gram-negative bacteria (Doi et al., 2004), and can be used synergistically with many other antibiotics (Xia et al., 2016). Aminoglycosides were also used extensively in food-producing animals to prevent bacterial infections (Chen et al., 2007); several clinical first-line aminoglycoside agents were misused in conventional broiler chicken and swine production facilities for the prevention of infections, as well as for growth promotion (Qin et al., 2012; Xia et al., 2016).

Bacterial resistance to aminoglycosides was initially found to be due to the production of aminoglycoside-modifying enzymes, including acetyltransferases, phosphotransferases, and nucleotidyltransferases, which may modify aminoglycosides into non-functional forms (Ramirez and Tolmasky, 2010). 16S rRNA methylation enzymes were thought to be confined to environmental strains until the discovery of *armA* gene-mediated aminoglycoside resistance in *Klebsiella pneumoniae* in 2003 (Galimand et al., 2003). To date, ten different 16S rRNA methylase genes, *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *rmtF*, *rmtG*, *rmtH*, and *npmA*, have been reported in various Enterobacteriaceae strains, and the 16S rRNA methylases encoded by these genes confer high-level resistance to various aminoglycosides (Galimand et al., 2012; Wachino and Arakawa, 2012; Bueno et al., 2013; O'Hara et al., 2013). Because the 16S rRNA methylase genes are located on plasmids, they can be easily transferred to other bacteria (Wachino et al., 2007). Therefore, these genes should be monitored by surveillance as a public health precaution.

Salmonella spp., which are members of the family Enterobacteriaceae, cause a broad range of diseases in humans, mainly diarrhea and systemic infections, and are one of the greatest food safety threats (LaRock et al., 2015). These bacteria usually live in the intestinal tracts of various animals and humans and can be excreted into the environment via feces (Michael and Schwarz, 2016). *Salmonella* spp. often exhibit resistance to multiple antibiotics and are recognized as very important bacterial vectors of drug-resistance genes in the spread of antibiotic resistance (Crump et al., 2015; Kariuki et al., 2015; Wen et al., 2017). In several aminoglycoside-resistant Enterobacteriaceae (Ma et al., 2009), including *Salmonella* (Naas et al., 2011; Du et al., 2012), the most common 16S rRNA methylase gene is *armA*. Since the report of its discovery in *K. pneumoniae* strains isolated from patients in 2003 (Galimand et al., 2003), plasmids containing *armA* have been detected in diverse bacterial species (Du et al., 2012; Wang et al., 2017b; Uechi et al., 2018). The plasmids carrying *rmtB* were found in S. Typhimurium isolated from food-animal products, and found could co-spread with qepA and blaCTX-M-27 in *S.* Indiana strains from water husbandry (Fang et al., 2019). In addition, other 16S rRNA methylase genes such as *rmtC* and *rmtD* were observed in *Salmonella enterica*, *S.* Indiana and *S.* California strains from chicken samples or people, but are less common (Folster et al., 2009; Hopkins et al., 2010; Wang et al., 2017c). The other 16S rRNA methylase genes such as *rmtE*, *rmtF*, *rmtG*, and *rmtH* have never been reported in *Salmonella* strains.

However, the epidemic and transmission trends of this gene in *Salmonella* are less defined. Plasmids carrying *armA* may allow easy transmission of this gene among various bacterial species, including pathogenic strains.

The prevalence of *Salmonella* harboring plasmids containing *armA* needs to be monitored in people, food, and environmental sources. Here, we conducted a retrospective study to characterize the epidemic status and transmission of *armA* in *Salmonella* strains isolated from outpatients as well as from food and environmental sources.

MATERIALS AND METHODS

Bacterial Strains

Beginning in 2005, *Salmonella* infections in diarrheal outpatients were surveyed yearly in Shanghai. Following the expansion of the participating surveillance laboratories in June 2010, the annual number of isolated strains increased from 2011. Of the 15,782 *Salmonella* strains used in this study: 12,095 strains were collected from outpatient stools in Shanghai between 2005 and 2016; 3,206 strains were collected from various food or environmental samples, including chicken, pork, water, and seafood, in Shanghai between 2009 and 2015; and 481 strains were collected from various food or environmental samples, including chicken, pork, water, and seafood, in Guangdong between 2013 and 2015. All strains were recovered from the strain pool and isolated in CHROMagar *Salmonella* agar (CHROMagar Company, Paris, France). Suspected isolates were identified with the Vitek-II system (BioMerieux, Lyon, France).

Resistance Gene Amplification

All *Salmonella* isolates were tested for the presence of the *armA* gene by using real-time polymerase chain reaction (PCR) (**Supplementary Table 1**). All *armA*-harboring Salmonella strains were then subjected to additional PCR assays to assess the presence of aminoglycoside-resistant genes (*ant(2")-Ia*, *aph(3′)-Ia*, *aac(3)-Ia*, *aac(3)-IIa*, *aac(6′)-Ib*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *rmtF*, *rmtG*, *rmtH*, and *npmA*), extended spectrum β-lactamase (ESBL)/AmpC genes (bla_{DHA}, bla_{TEM}, bla_{CMY}, bla_{OXA}, bla_{CTX-M}, and bla_{SHV}), plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *oqxA*, *oqxB*, and *qepA*), the colistin-resistant gene *mcr-1*, and the carbapenem-resistant gene bla_{NDM}. Primers (Wu et al., 2009; Gopalakrishnan et al., 2018; Taylor et al., 2018) used in this study were showed in **Supplementary Table 1**. The variants of bla_{CTX-M} were confirmed by Sanger sequencing. The PCR amplification products were also sequenced, and the resulting DNA sequences were analyzed by using the BLAST program[1].

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed on all the *armA*-positive isolates using the reference broth

[1]http://www.ncbi.nlm.nih.gov/BLAST/

microdilution method with custom plates (PRCDCN2, Thermo) (Clinical and Laboratory Standards Institute [CLSI], 2015) for the following 20 antibiotics: ampicillin, tetracycline, erythromycin, chloramphenicol, cefazolin, ciprofloxacin, trimethoprim/sulfamethoxazole, ceftazidime, imipenem, nalidixic acid, cefoxitin, cefotaxime, gentamicin, azithromycin, ceftazidime, amikacin, tobramycin, cefepime, colistin, and tigecycline. The results were assessed with the CLSI (2015) breakpoints (Clinical and Laboratory Standards Institute [CLSI], 2015). The control strain is ATCC 25922.

Pulsed Field Gel Electrophoresis (PFGE) Analysis

All *armA*-harboring *Salmonella* strains were analyzed by pulsed field gel electrophoresis (PFGE) to determine their genetic homology. Bacterial DNA was digested with the restriction enzyme *Xba*I and then analyzed by electrophoresis for 19 h at 6 V/cm, with a pulse angle of 120°, a temperature of 14°C, and pulse times ranging from 2.16 to 63.8 s. Comparison of the resulting PFGE patterns was performed with Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium) based on the Tenover's criteria (Tenover et al., 1995). Isolates were allocated into genetic similarity clusters using a cut-off value of 80%.

Plasmid and Southern Blot Analyses

To detect the sizes of the *armA*-carrying plasmids, agarose gel plugs containing total cellular DNA were prepared and digested with S1 nuclease (TaKaRa, Dalian, China) as described previously (Barton et al., 1995). Digested plugs were subjected to PFGE using a CHEF-Mapper system (pulse times, 2.16–63.8 s; running time, 19 h; 6 V/cm). Gels were blotted onto nylon membranes (Millipore, United States). The membranes were hybridized with a digoxigenin-labeled probe directed against *armA*.

Based on the Southern hybridization results, the gel slices resulting from PFGE following digestion with S1 nuclease found to contain the *armA* fragment were excised. The DNA fragments from the gels were purified and used as templates for identifying the plasmid replicon type by PCR. A PBRT kit (Diatheva, Italy) was applied for plasmid molecular typing (Garcia-Fernandez et al., 2009).

Plasmid Elimination Test

For the SDS treatment method, 50 μl of *armA*-positive *Salmonella* suspension (OD = 0.5–0.7) was inoculated into 5 ml of LB medium with 10% SDS and then incubated in a thermostatic shaker at 37°C for 18–24 h; an SDS concentration one-half of that in which growth was first observed was chosen as the working concentration for the elimination test (Wangkheimayum et al., 2017). The products were diluted ten times with saline and then selected on agar plates containing 1000 μg/ml amikacin and on plates without antibiotics. The plasmid-eliminated strains were identified by real-time PCR.

Plasmid Conjugation

The conjugation test was performed with the broth mating method using all 45 aminoglycoside-resistant *Salmonella* strains as the donor strains and sodium azide-resistant *Escherichia coli* J53 or streptomycin-resistant *Acinetobacter baumannii* as the recipient strains. The transconjugants were selected on agar plates containing 30 μg/ml amikacin and 400 μg/ml sodium azide when using *E. coli* J53 as the recipient and on LB agar plates containing 30 μg/ml amikacin and 5,000 μg/ml streptomycin when using *A. baumannii* as the recipient. The resulting transconjugants were identified by real-time PCR targeting the *armA* gene. The transfer frequency is expressed as the number of transconjugants per total recipients.

To test the ability of the *Salmonella* strains from different serotypes to obtain an *armA*-carrying plasmid, we performed conjugation experiments using three *S.* Derby strains, five *S.* Enteritidis strains, four *S.* Typhimurium strains, four *armA*-negative *S.* Indiana strains, and three *S.* Indiana strains in which the *armA*-carrying plasmid had been eliminated as recipients and using three *E. coli* J53 strains with different *armA*-carrying plasmid lengths as donors. In total, 57 independent conjugative experiments were performed. The resulting transformants were selected on LB agar with amikacin (30 μg/ml) and streptomycin (4,000 μg/ml), and successful transconjugants were confirmed by real-time PCR. The transfer frequency is expressed as the number of transconjugants per total recipients. The transfer frequency data were analyzed with a multiway analysis of variance using SPSS 19 statistic software.

RESULTS

armA-Harboring *Salmonella* Emerged in the Diarrheal Outpatients in Shanghai District Around 2011

A total of 45 *armA*-harboring strains were detected in the 15,782 tested *Salmonella* strains. Of these, 13 (positive rate: 0.11%) were from the 12,095 outpatient strains collected in Shanghai (human source), and 32 (1.00%) were from the 3,687 food/environmental isolates.

The first *armA*-harboring strain was found in 2011 (**Supplementary Table 3**). Except in 2014, two or three *armA*-harboring *Salmonella* strains were identified each year. After the initial appearance of *armA*-harboring *Salmonella* in 2011, the prevalence rate of these strains in community-acquired diarrheal cases in Shanghai was 0.83/1,000 (13/10,234). Of the *armA*-harboring food/environmental-source strains, 22 (positive rate: 0.69%) were from the 3,206 strains collected in Shanghai and 10 (2.07%) were from the 481 strains collected in Guangdong.

To detect other concomitant aminoglycoside-resistant genes in these 45 *armA*-harboring *Salmonella* strains, PCR and qPCR were performed. Of the known aminoglycoside-modifying enzymes genes, *ant(2'')-Ia*, *aph(3')-Ia*, *aac(3)-Ia*, *aac(3)-IIa*, and *aac(6')-Ib* were detected. The positive rates of these genes were 31.1% (14/45), 11.1% (5/45), 68.9% (31/45), 8.9% (4/45), and

86.7% (39/45), respectively. The positive rates of *rmtB*, *rmtC*, and *rmtD* were 15.6% (7/45), 2.2% (1/45), and 44.4% (20/45), respectively; in contrast, the *rmtA*, *rmtE*, *rmtF*, *rmtG*, *rmtH*, and *npmA* genes were not detected in any of the isolates.

The Detected *armA*-Harboring *Salmonella* Strains Show a Highly Skewed Serotype Distribution

Among the 45 *armA*-harboring *Salmonella* strains, nine different serotypes were identified. The serotype distribution was heavily skewed toward serovar Indiana, with 34 strains (75.6%) identified as *S.* Indiana. Other identified serotypes included Thompson (three strains), Kottbus (two strains), and six different serovars with one strain each (Agona, Singapore, Schwerin, Corvallis, Goldcoast, and Infantis).

The Detected *armA*-Harboring *Salmonella* Strains Were All Multi-Antibiotic Resistant, and Most Carried Extended Spectrum β-Lactamase (ESBL)/Plasmid-Mediated Quinolone Resistance (PMQR) Genes

The results reveal that all these strains were multidrug resistant (MDR), i.e., they exhibited high levels of resistance to more than three classes of antibiotics (**Supplementary Table 3**). Resistance to amikacin, erythromycin, tobramycin, and gentamicin was observed in all 45 *armA*-harboring *Salmonella* isolates. Additionally, 40 of the strains (including 13 from outpatients and 27 from food/environmental samples) were found to be fluoroquinolone-resistant, and 35 of these fluoroquinolone-resistant strains (including 10 from outpatients and 25 from food/environmental samples) were identified as ESBL producers. In contrast, all 45 *armA*-harboring *Salmonella* strains were susceptible to both imipenem and tigecycline. The ampicillin-resistant rates of the *armA*-harboring *Salmonella* strains isolated from poultry or outpatients were much higher than that of *armA*-harboring *Salmonella* strains isolated from swine (36/37 vs. 2/4).

Among the 45 *armA*-harboring *Salmonella* isolates, the prevalence of ESBL/AmpC genes bla_{TEM-1B}, bla_{CMY}, bla_{OXA}, and bla_{SHV-12} were 46.7% (21/45), 2.2% (1/45), 68.9% (31/45), and 2.2% (1/45), respectively. The prevalence of bla_{CTX-M} was 68.9% (31/45). Notably, the extent of resistance to third generation cephalosporin mediated by different bla_{CTX-M} subtypes is distinct; we found 21 strains with $bla_{CTX-M-65}$, 6 strains with $bla_{CTX-M-55}$, 3 strains with $bla_{CTX-M-3}$, 2 strains with $bla_{CTX-M-27}$, and 1 strain with $bla_{CTX-M-14}$. The bla_{DHA} gene was not detected in these strains (**Supplementary Table 3**).

The prevalence of PMQR genes *qnrA*, *qnrD*, *qnrS*, *oqxA*, *oqxB*, and *qepA* was 2.2% (1/45), 11.1% (5/45), 15.6% (7/45), 48.9% (22/45), 48.9% (22/45), and 2.2% (1/45), respectively. The *qnrB* and *qnrC* genes were not detected. Additionally, neither the colistin-resistant *mcr-1* gene nor the carbapenem-resistant bla_{NDM} gene was found to be present in any of the 45 *armA*-harboring *Salmonella* strains.

The Detected *armA*-Harboring *Salmonella* Strains Exhibited Diverse Genomic Subtyping Patterns

All 45 *armA*-harboring *Salmonella* strains had different PFGE profiles, which generally had low similarity with one another, suggesting that these isolates are unlikely to have originated from a single clone of *Salmonella*. Only two PFGE clusters were found; one pattern group included strains SH13SF082, SH13SF466, and SH13SF540, which were isolated from food in 2013, and the other included strains SH15SF180 and SH15SF181, which were isolated from food in 2015. The strains in each cluster had indistinguishable PFGE patterns (**Figure 1**). The PFGE patterns of *S.* Indiana strains isolated from outpatients were more similar to those of the strains isolated from poultry (e.g., SH12G1005 and SH15SF559, SH13G1614 and SH15SF180/SH15SF181, SH12SF039 and SH16G1356) than to those of strains isolated from swine.

The *armA* Genes in *armA*-Positive *Salmonella* Strains Were Located on Plasmids

Pulsed field gel electrophoresis using *Xba*I and S1 nuclease digestion followed by Southern-hybridization analysis revealed that all 45 isolates, regardless of their conjugative status, carried only a single *armA*-carrying plasmid. Based on the length calculations for the hybridized bands, all the *armA*-carrying plasmids were approximately 54.7–398.4 kb in size.

We performed plasmid elimination on these 45 *armA*-harboring *Salmonella* strains via treatment with SDS and found that all the *armA*-carrying plasmids could be successfully eliminated, i.e., the *armA* gene was not detected in the plasmid-eliminated strains, which supports the conclusion that the *armA* genes in these strains were located only on plasmids. To determine the replicon type(s) of the *armA*-carrying plasmids, replicon-typing PCR, which detects the replicon sequences of plasmid types, was performed. The results show that the *armA*-carrying plasmids included IncHI2 (19 strains, 42.2%), IncFIB (12 strains, 26.7%), IncA/C (8 strains, 17.8%), IncN (5 strains, 11.1%), and IncFIA (1 strain, 2.2%). IncHI2 was the dominant type of *armA*-carrying plasmid in these *Salmonella* strains.

The *armA*-Carrying Plasmids Were Transferable, and *S.* Indiana Strains Acquired the *armA*-Carrying Plasmids More Easily

Conjugation experiments revealed that all 45 *armA*-harboring *Salmonella* isolates were able to transfer their *armA*-carrying plasmids to both *E. coli* J53 and *A. baumannii*, respectively. The transfer frequencies were low, $\sim 10^{-6}$ CFU/donor for both *E. coli* J53 and *A. baumannii* (**Supplementary Table 2** and **Figure 2**).

The transfer frequency data were analyzed by a multiway analysis of variance, and the factors used in this analysis were: five different recipients (*S.* Derby, *S.* Enteritidis, *S.* Typhimurium, *armA*-negative *S.* Indiana, and *S.* Indiana strains in which the *armA*-carrying plasmid was eliminated), and *armA*-carrying

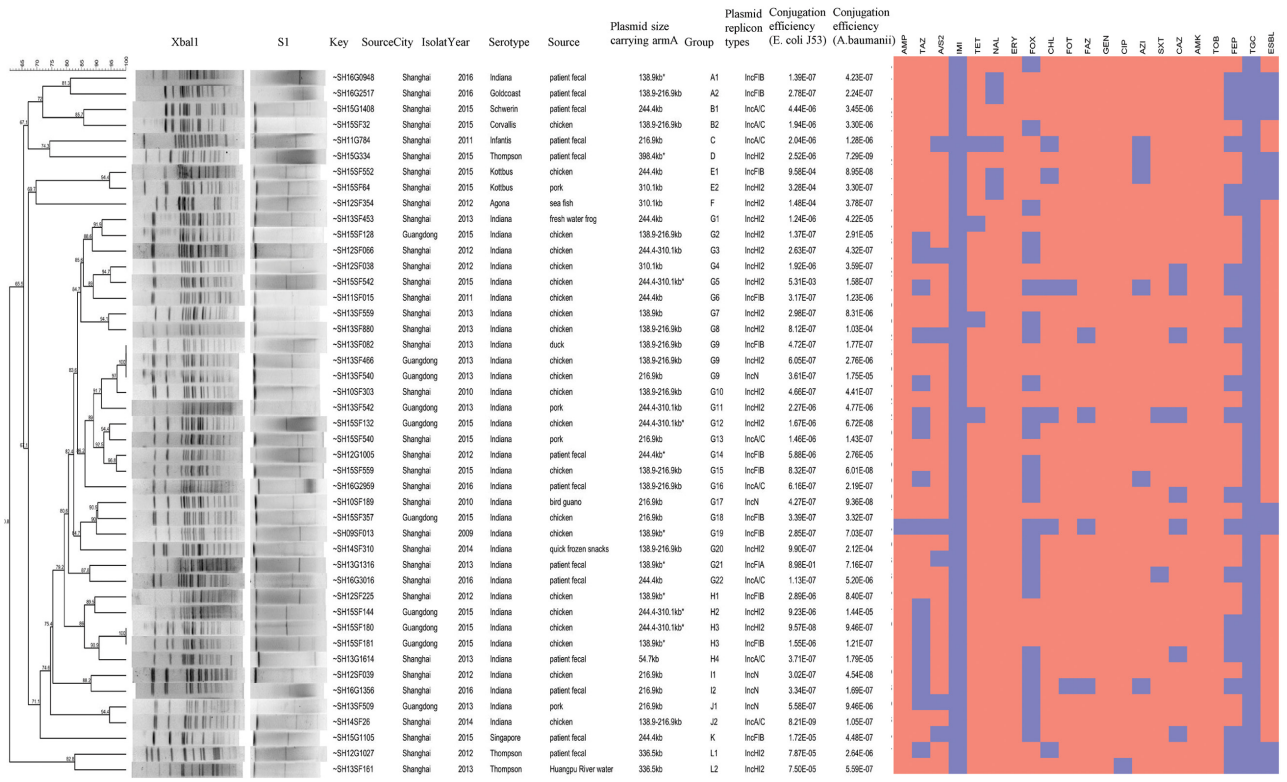

FIGURE 1 | Generated dendrograms showing the cluster analysis of *Xba*I-digested PFGE patterns of the *armA*-harboring *Salmonella* strains isolated in this study.

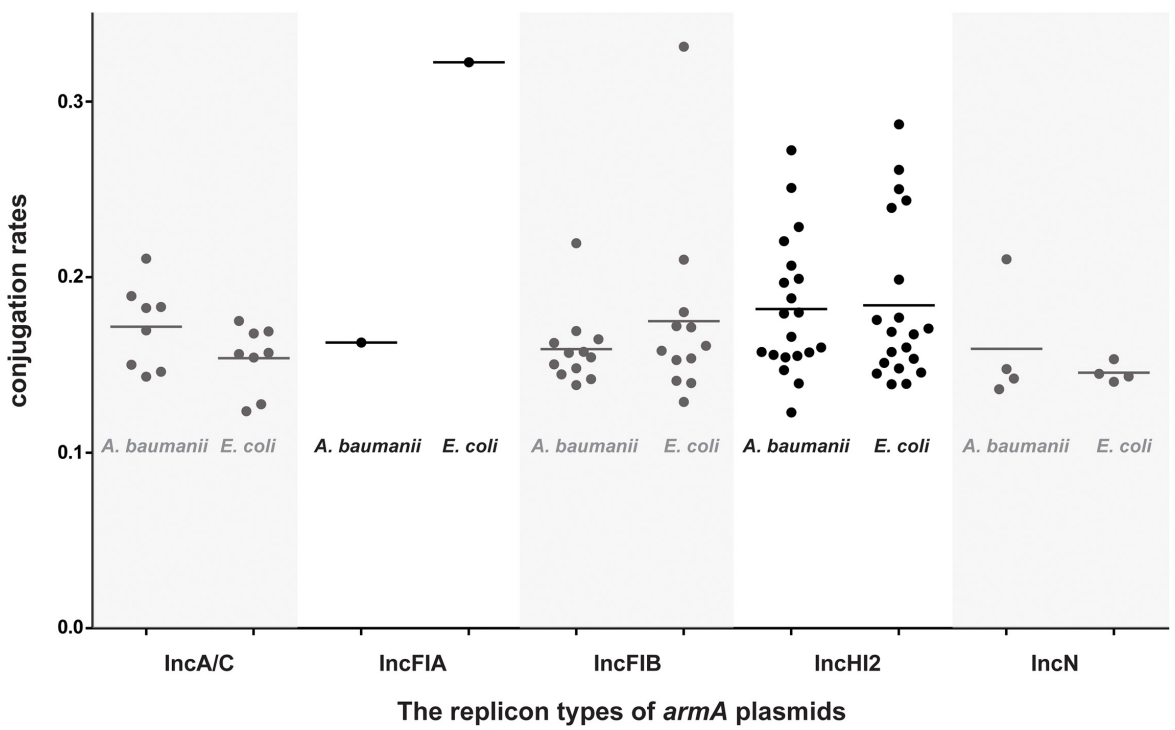

FIGURE 2 | Conjugation rates of the *armA*-harboring plasmids isolated in this study. All 45 detected *armA*-harboring *Salmonella* strains were used as the donors, and either *E. coli* J53 or *A. baumannii* was used as the recipient. Black circles show the 1/abs (log_{10} transformation of the transfer frequency) data.

plasmids of three different lengths (∼140, ∼220, and ∼300 kb) as donors. There was no significant difference among the different serotypes in their ability to acquire *armA*-carrying plasmids. In contrast, the *S.* Indiana strains in which the *armA*-carrying plasmids were eliminated were able to re-acquire *armA*-carrying plasmids with higher frequencies compared with the other recipients ($p < 0.01$, multi-way ANOVA) (**Figure 3**).

DISCUSSION

Aminoglycosides are the antibiotics used most commonly in clinical treatment and veterinary applications. Unfortunately, aminoglycoside use in economically important animals has impelled the emergence and spread of aminoglycoside-resistant bacteria, especially members of the Enterobacteriaceae family. Plasmids carrying 16S rRNA methylase genes, which are responsible for aminoglycoside resistance, have been widely recognized in Enterobacteriaceae family members; however, there are no reports of an epidemiology-based analysis of the *armA* gene prevalence in *Salmonella* spp. isolated from outpatients. Here, we examined samples from a systemic laboratory-based salmonellosis surveillance that was performed yearly from 2005 in diarrheal outpatients in Shanghai; the application of this study design should allow for a reasonable epidemiologic estimate of the prevalence of *armA*-carrying *Salmonella* strains.

The first isolation of an *armA*-harboring *Salmonella* strain occurred in 2011; fortuitously, no increasing trend for *armA* presence was found in the community-acquired infections in

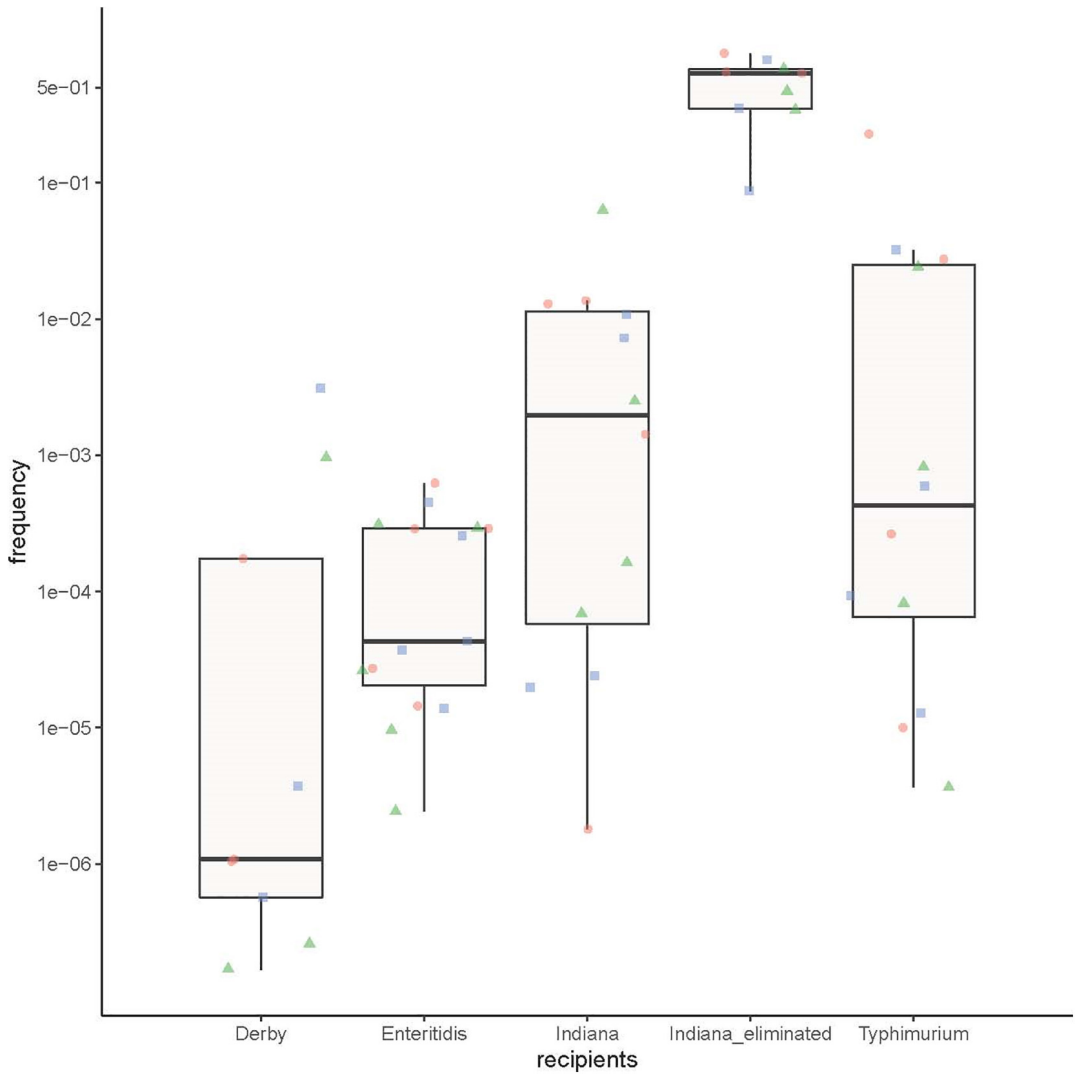

FIGURE 3 | The ability of the different *Salmonella* serotype strains to obtain an *armA*-carrying plasmid. Three *S.* Derby strains, five *S.* Enteritidis strains, four *S.* Typhimurium strains, four naturally *armA*-negative *S.* Indiana strains, and three *S.* Indiana strains in which the *armA*-carrying plasmid was artificially eliminated were used as recipients, and three *E. coli* J53 strains with different lengths of *armA*-carrying plasmid (∼140 kb: green triangles, ∼220 kb: red circles, and ∼300 kb: blue squares) were used as donors.

Shanghai. As with other clinical Enterobacteriaceae isolates (Samadi et al., 2015), the *armA* prevalence rate in clinical *Salmonella* isolates from the present work was found to remain low over the study period. However, the presence of other aminoglycoside-resistance genes was not assessed, and antimicrobial susceptibility testing was not performed on all the isolated strains from this study, only the strains with *armA*, so strains with other aminoglycoside resistance mechanisms may have remained undetected.

Salmonella strains isolated from food and food animal samples were also investigated in our study. These samples were obtained in Shanghai and Guangzhou, and the resulting isolates showed higher *armA*-positive rates compared with the outpatient isolates, suggesting that food and food animals may be a potential threat for the spread of *armA*-mediated aminoglycoside resistance to humans. Although the overall prevalence of strains harboring *armA* was low in this study, all 45 of the *armA*-harboring *Salmonella* isolates were MDR, and 35/45 strains were resistant to both cephalosporins and fluoroquinolone; notably, this indicates a high level of resistance by these strains to the antibiotics that are commonly used in the treatment of salmonellosis patients. The coexistence of the *armA* gene together with ESBL/PMQR genes in the *Salmonella* strains of animal origin may also present a potential risk to human health if the plasmids containing these genes are disseminated via contaminated food. Other studies have similarly found evidence for the coexistence of ESBL/PMQR genes with the *armA* gene in *Salmonella* strains from both food and human sources (Bouzidi et al., 2011; Fang et al., 2019). This situation may pose threats to both food safety and health management owing to two factors: (1) the active horizontal transfer of MDR genes among *Salmonella* and into other Enterobacteriaceae species, and (2) the preservation and possible spread of MDR genes through co-selection with other antibiotics that are commonly used for diarrhea treatment or for improved growth in food-producing animals. The complete sequences could provide more information about the *amrA*-bearing plasmids. Therefore, to provide more characteristic about the *amrA*-positive plasmid, we are looking forward to obtain the complete plasmid sequence base on long-reads sequencing in the future, with the cost of long-reads sequencing decline.

In the present work, we obtained *Salmonella* strains isolated from three different sources (humans, poultry, and swine). The *S.* Indiana strains isolated from outpatients had similar PFGE patterns compared with the strains isolated from poultry, suggesting that *Salmonella* with *armA*-mediated aminoglycoside resistance may be carried by poultry and transmitted from poultry products to humans. Antibiotics are used frequently and extensively in the poultry industry, which has led to the emergence of MDR *Salmonella* (Witte, 1998), and strains of MDR *Salmonella* originating from poultry have the potential to be spread to humans through the food chain (Afema et al., 2016). Poultry and poultry products are recognized as the major vehicles for *Salmonella* transmission to humans (Mezal et al., 2013). In this study, the ampicillin resistance rates of *Salmonella* strains isolated from poultry or humans were much higher than that of strains isolated from swine, which may be related to the excessive use of ampicillin antibiotics in poultry breeding and human medicine (Roth et al., 2019); this finding further supports the idea that the *armA* gene may be transmitted to humans via poultry products.

Pulsed field gel electrophoresis assessment of the 45 *armA*-harboring *Salmonella* strains obtained in this study revealed diverse genetic clones, suggesting that these strains are genetically unrelated and that clonal spread has not been the major mechanism of *armA* gene transmission in *Salmonella*. Thus, compared with the clonal spread of *armA*-harboring *Salmonella*, the transmission of *armA*-carrying plasmids may be more common. Additionally, our results indicate that the *armA* genes in all 45 *Salmonella* isolates were located on plasmids, and plasmids from these strains could be successfully transferred into *E. coli* and *A. baumannii* strains. The *armA*-harboring plasmids comprised five types; the major type, IncHI2, is an MDR plasmid that has been associated with a range of antibiotic resistance genes in *Salmonella* spp. and *E. coli* isolated from humans and food-producing animals (Zhong et al., 2017). The detection of diverse genetic clones and plasmids provides insight into the complicated and likely frequent transmission of *armA*-carrying plasmids among *Salmonella* strains and other Enterobacteriaceae species.

In the present study, 34 of the 45 *armA*-harboring *Salmonella* strains belong to serovar Indiana, raising the question of whether *S.* Indiana is more capable of obtaining these plasmids compared with *Salmonella* strains belonging to other serovars. We compared the plasmid acquisition abilities among *S.* Indiana and some other common *Salmonella* serotypes and found that the group of *armA* plasmid-carrying *S.* Indiana strains from the present study had a greater ability to obtain these plasmids. Interestingly, this level was even higher compared with the tested group of *S.* Indiana strains that lacked *armA*-carrying plasmids, suggesting that a specific genetic characteristic in these *armA*-harboring *S.* Indiana strains is required for the acquisition and maintenance of *armA*-carrying plasmids. In recent years, *S.* Indiana has been frequently isolated from chicken and broilers, as well as from poultry-farming workers, and it has gradually become one of the most common serotypes that cause animal and human salmonellosis (Xia et al., 2009; Lai et al., 2013; Gong et al., 2016). *S.* Indiana isolates that have concurrent resistance to cefotaxime, amikacin, and ciprofloxacin are widespread among chickens in China (Wang et al., 2017b). The MDR *S.* Indiana isolates in China belong to a wide variety of genotypes, suggesting that they probably evolved from many widely dispersed areas rather than from a few more local sources (Luque et al., 2009; Punia et al., 2010; Yang et al., 2010; Lai et al., 2014; Yan et al., 2014; Zhang et al., 2016). Other studies have demonstrated that *S.* Indiana isolates usually carry one or more drug resistance plasmids (Lai et al., 2013; Wang et al., 2016, 2017a), which further supports the important role of *S.* Indiana in public health.

CONCLUSION

We conducted a retrospective study using samples collected between 2005 and 2016 from diarrheal outpatients who participated in a continuous *Salmonella* infection

surveillance in Shanghai Municipality, China. Our data reveal that, since 2011, the prevalence of *Salmonella* with *armA*-containing plasmids in community-acquired diarrhea cases was low and steady but that all the *armA*-harboring *Salmonella* strains were MDR and were notably resistant to fluoroquinolone and/or extended-spectrum cephalosporins.

The use of aminoglycoside antibiotics is contributing to the rise of MDR *Salmonella*, which poses an increasing public health threat and presents a considerable challenge for the treatment of clinical infections. Future surveillance is necessary to monitor the prevalence and transmission of *armA*-harboring *Salmonella*, especially *S.* Indiana, both in humans and animals and to better understand the potential threat to public health posed by these strains.

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/s.

AUTHOR CONTRIBUTIONS

XL, MZ, and NZ wrote the manuscript. MW, BG, JL, HJ, and WX provided the technical assistance. ZL, HZa, HZo, and ZPL performed the data analysis and prepared the resources. JX, XX, and BK edited the manuscript. All authors read and approved the article.

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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.2021.663210/full#supplementary-material

Supplementary Table 1 | Primers used in this study for polymerase chain reaction.

Supplementary Table 2 | The conjugation rates of the *armA*-harboring plasmids.

Supplementary Table 3 | Basic information about the 45 armA-harboring *Salmonella* strains identified in this study and their armA-carrying plasmids.

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Occurrence and Molecular Characteristics of Extended-Spectrum Beta-Lactamase-Producing Enterobacterales Recovered From Chicken, Chicken Meat, and Human Infections in Sao Paulo State, Brazil

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This study aimed to investigate the phylogenetic diversity and epidemiology of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* from chicken, chicken meat, and human clinical isolates in Sao Paolo, Brazil, and characterize their respective ESBL-encoding plasmids. Three hundred samples from chicken cloaca, chicken meat, and clinical isolates were phenotypically and genotypically assessed for ESBL resistance. Isolates were identified by MALDI TOF-MS and further characterized by MLST analysis and phylogenetic grouping. ESBL genes were characterized and their location was determined by I-Ceu-I-PFGE and Southern blot, conjugation, transformation, and PCR-based replicon typing experiments. Thirty-seven ESBL-producing isolates (28 *E. coli* and 9 *K. pneumoniae*) that were positive for the $bla_{CTX-M-1}$ or $bla_{CTX-M-2}$ gene groups were obtained. Two isolates were negative in the transformation assay, and the chromosomal location of the genes was deduced by Southern blot. The bla_{CTX-M} genes identified were carried on plasmid replicon-types X1, HI2, N, FII-variants, I1 and R. The *E. coli* isolates belonged to nine sequence types, while the *K. pneumoniae* isolates belonged to four sequence types. The *E. coli* isolates belonged to phylotype classification groups A, B1, D, and F. This study demonstrated that isolates from cloacal swabs, chicken meat, and human feces had genetic diversity, with a high frequency of $bla_{CTX-M-15}$ among chickens, chicken meat, and human feces. Thus, this reinforces the hypothesis that chickens, as well as their by-products, could be an important source of transmission for ESBL-producing pathogens to humans in South America.

Keywords: antibiotic resistance, plasmids, extended spectrum beta lactamases (ESBLs), poultry, food chain

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INTRODUCTION

Enterobacterales carrying extended-spectrum β-lactamase (ESBLs) genes with resistance to third- and fourth-generation cephalosporins have been detected widely in livestock (Wu et al., 2018). The role of chicken meat as a potential source of multidrug-resistant bacteria that carries ESBL genes have been demonstrated in several countries including China (Wu et al., 2018), Canada (Ghosh et al., 2019), The Netherlands (Liakopoulos et al., 2016), Senegal (Vounba et al., 2019), and Brazil (Casella et al., 2017).

Brazil draws attention as the world's largest chicken meat and derivatives exporter (USDA, 2019). Therefore, European countries have demonstrated concerns regarding Brazilian imported chicken meat due to its possible role in transferring antibiotic-resistant strains from food to humans (Liakopoulos et al., 2016). Although it has been shown that human-to-human transmission in the open community has a greater impact on transmission of ESBL-producing isolates than other putative sources (Mughini-Gras et al., 2019), this concern originates from the fact that genetic determinants, such as plasmids, encoding CTX-M enzymes may be transmitted to humans *via* the food chain (Cyoia et al., 2019) and/or direct contact with animals or the environment (Mughini-Gras et al., 2019).

Several studies in Brazil have shown the presence of ESBL-encoding isolates in animals, chicken meat, and humans, but none of them has demonstrated the presence and the relationship of CTX-M-producing Enterobacteriaceae in the food chain, comprising of broilers, chicken meat, and the consumers. Therefore, we aimed to investigate the origin, phylogenetic diversity, and epidemiology of ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates from chickens and chicken meat, and their relationship with those causing clinical symptoms in humans in Sao Paulo, Brazil, as well as characterize their respective ESBL-encoding genes and plasmid replicon types.

MATERIALS AND METHODS

Bacterial Sampling, Identification, and Phenotypic Characterization

A total of 300 samples, from the northwest region of the Sao Paulo, from two farms, two clinics and two slaughterhouses within a 80 km radius, were obtained between February and October of 2014 in Sao Paulo, Brazil, consisting of: (1) 100 cloacal swabs of clinically healthy chickens originated from two different farms in the same state [50 from Farm 1 (F1) and 50 from Farm 2 (F2)], (2) 100 chicken meat samples at a local retail (1 g each) [50 from Supermarket 1 (S1), and 50 from Supermarket 2 (S2)], and (3) 100 samples of human feces collected from an equal number of patients with gastrointestinal disease without prior antibiotic treatment of two different local hospitals [50 from Hospital 1 (H1), and 50 from Hospital 2 (H2)]. After collection, samples were transferred to a selective pre-enrichment broth (Luria–Bertani broth supplemented with 1 mg/L cefotaxime) and incubated overnight at 37°C. Subsequently, they were cultured on selective MacConkey agar plates supplemented with 1 mg/L cefotaxime (Sigma-Aldrich, Germany), and incubated for 24 h at 37°C. Thereafter, five morphologically different colonies per sample were tested for ESBL production using a combination disk test as previously described (Liakopoulos et al., 2016). The species of the recovered ESBL-producing isolates was determined by MALDI-TOF mass spectrometry (MALDI Biotyper, Bruker, Germany).

ESBL-Gene Typing

The genomic DNA was extracted by DNeasy Blood and Tissue Kit (QIAGEN, Germany). The presence of ESBL genes was assessed by microarray analysis using the Check-MDR CT-101 (Check-Points, Wageningen, Netherlands) and characterized by polymerase chain reaction (PCR) and sequence analysis as previously described (Dierikx et al., 2013). Sequence data were analyzed using Sequencher version 4.2 (Gene Codes Corporation, United States), and the sequences obtained were compared to ones deposited in GenBank.

Bacterial and Plasmid Typing

All *E. coli* and *K. pneumoniae* isolates were characterized by multilocus sequence typing (MLST) according to Achtman's[1] and Pasteur's[2] schemes, respectively. *E. coli* phylotyping was performed according to Clermont et al. (2013). After transformation and/or conjugation, plasmid characterization was performed by PCR-based replicon typing (PBRT) on transformants and/or transconjugants as previously described (Carattoli et al., 2005).

Genetic Support of the bla_{CTX-M} Genes

The localization of ESBL genes on plasmids was assessed by transformation and/or conjugation experiments. For transformation experiments, the plasmids were extracted using Qiagen Plasmid Midi Kits (Qiagen, Netherlands) and electro-transformed in ElectroMaxTM H10BTM cells (Gibco Invitrogen, United States). Conjugation assays were performed in Luria–Bertani medium (LB-medium) using a rifampicin-resistant, indole-negative *E. coli* K12 strain as the recipient (Liakopoulos et al., 2016). Transformants were selected on MacConkey agar containing 1 mg/L cefotaxime, whereas transconjugants on MacConkey agar contained 1 mg/L cefotaxime and 100 mg/L rifampicin. The chromosomal location of the ESBL genes, when necessary, was confirmed by I-Ceu-I-PFGE followed by Southern blot hybridization, as previously described (Liakopoulos et al., 2016).

RESULTS AND DISCUSSION

From the MacConkey agar screening coupled with the combination disk test, we recovered 25 ESBL-producing isolates from 100 chicken cloacal samples (25%), seven isolates from 100 chicken meat samples (7%), and five isolates from 100 human fecal samples (5%). MALDI-TOF MS revealed

[1]http://mlst.ucc.ie/mlst/dbs/Ecoli

[2]http://bigsdb.pasteur.fr/klebsiella/

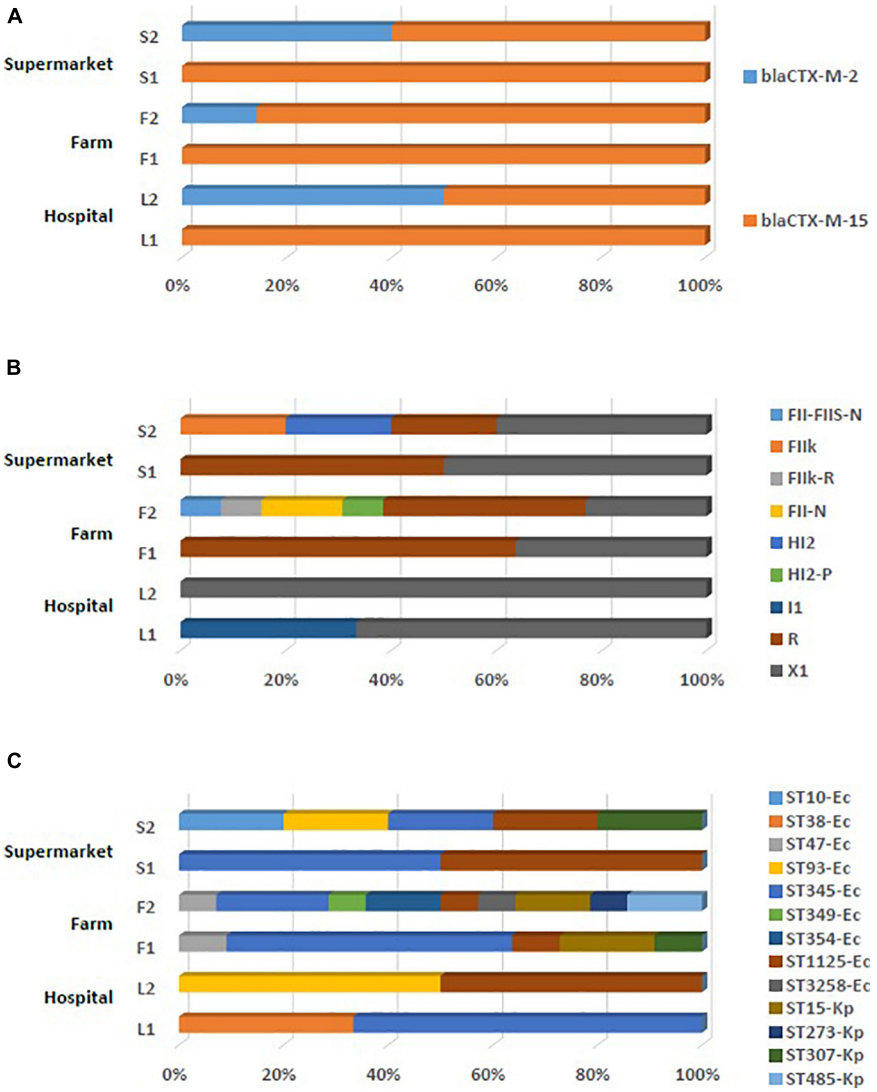

FIGURE 1 | Distribution of ESBL gene types **(A)**, plasmid replicon types **(B)**, and isolate sequence types **(C)** among the ESBL-producing isolates recovered from each of the reservoirs tested. Refer to **Table 1** for the more detailed molecular characteristics of each isolate recovered.

that these 37 isolates composed of nine *K. pneumoniae* and 28 *E. coli* strains. Interestingly, ESBL-producing *K. pneumoniae* is so far detected rarely among poultry (Zhuo et al., 2013; Daehre et al., 2018; Projahn et al., 2019). A micro-array showed that our isolates were positive for $bla_{CTX-M-1}$ or $bla_{CTX-M-2}$ group genes. Through sequencing, we have shown that all $bla_{CTX-M-1}$-group-harboring isolates encoded the $bla_{CTX-M-15}$ gene, and all $bla_{CTX-M-2}$-group encoded the $bla_{CTX-M-2}$ gene, with $bla_{CTX-M-15}$ being the most prevalent ESBL gene (86.4%; 32/37) among isolates of all sources (**Figure 1A**). Specifically, our data indicated that the $bla_{CTX-M-15}$ gene was present in 91.3% (23/25) of the chicken cloacal isolates, in 71.4% (5/7) of the chicken meat isolates, and in 83.3% (4/5) of the human feces isolates (**Table 1**). Despite the $bla_{CTX-M-2}$ and $bla_{CTX-M-8}$ genes being the most predominant ESBL genes so far in South America, the $bla_{CTX-M-15}$ gene has recently emerged in clinical isolates and is now detected as often as the $bla_{CTX-M-2}$ gene in humans, while it has only been reported sporadically from chickens in Brazil (Botelho et al., 2015; Ferreira et al., 2016; Casella et al., 2018). Either these cases reflect direct contamination through human handling or the potential emergence of the $bla_{CTX-M-15}$ gene in chickens on farms, they pose the risk for further spread of the $bla_{CTX-M-15}$ gene within the poultry production pyramid. We identified only five isolates encoding the $bla_{CTX-M-2}$ gene**,** in particularly two *E. coli* from cloaca, two from chicken meat and one from human feces, contrary to the previous studies documenting the high prevalence of the $bla_{CTX-M-2}$ gene among isolates recovered from chicken meat in Brazil (Casella et al., 2018). Overall, the detection of *E. coli* and *K. pneumoniae* isolates carrying bla_{CTX-M} genes raises concerns about the broad dissemination of these antimicrobial resistance determinants in Brazil.

TABLE 1 | Isolate origin, identification, ESBL gene, plasmid type, sequence type, and phylotype.

Isolate	Origin	Reservoir	MALDI-TOF MS	PCR-ESBL	Sequencing	Plasmid	MLST	Phylogroup
1	Cloacal swab	F1	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	IncX1	47	B1
2	Cloacal swab	F1	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	IncX1	345	B1
3	Cloacal swab	F1	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	IncX1	1125	B1
4	Cloacal swab	F1	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	R	345	B1
5	Cloacal swab	F1	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	R	345	B1
6	Cloacal swab	F1	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	R	345	B1
7	Cloacal swab	F1	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	R	345	B1
8	Cloacal swab	F1	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	R	345	B1
9	Cloacal swab	F1	*K. pneumonie*	CTX-M-1g	$bla_{CTX-M-15}$	IncX1	307	-
10	Cloacal swab	F1	*K. pneumonie*	CTX-M-1g	$bla_{CTX-M-15}$	R	15	-
11	Cloacal swab	F1	*K. pneumonie*	CTX-M-1g	$bla_{CTX-M-15}$	R	15	-
12	Cloacal swab	F2	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	IncN IncFII	354	F
13	Cloacal swab	F2	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	IncN IncFII	354	F
14	Cloacal swab	F2	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	IncN IncFII IncFIIS	349	D
15	Cloacal swab	F2	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	IncX1	345	B1
16	Cloacal swab	F2	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	IncX1	345	B1
17	Cloacal swab	F2	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	IncX1	1125	B1
18	Cloacal swab	F2	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	R	345	D
20	Cloacal swab	F2	*E. coli*	CTX-M-2g	$bla_{CTX-M-2}$	IncHI2 IncP	47	A
19	Cloacal swab	F2	*E. coli*	CTX-M-2g	$bla_{CTX-M-2}$	N/A	3258	D
21	Cloacal swab	F2	*K. pneumonie*	CTX-M-1g	$bla_{CTX-M-15}$	R	15	-
22	Cloacal swab	F2	*K. pneumonie*	CTX-M-1g	$bla_{CTX-M-15}$	R	15	-
23	Cloacal swab	F2	*K. pneumonie*	CTX-M-1g	$bla_{CTX-M-15}$	R	485	-
24	Cloacal swab	F2	*K. pneumonie*	CTX-M-1g	$bla_{CTX-M-15}$	R	485	-
25	Cloacal swab	F2	*K. pneumonie*	CTX-M-1g	$bla_{CTX-M-15}$	R IncFIIK	273	-
27	Human feces	H1	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	IncX1	345	B1
28	Human feces	H1	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	IncX1	345	B1
26	Human feces	H1	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	IncI1	38	D
29	Human feces	H2	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	IncX1	1125	A
30	Human feces	H2	*E. coli*	CTX-M-2g	$bla_{CTX-M-2}$	N/A	93	B1
31	Chicken meat	S1	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	IncX1	1125	D
32	Chicken meat	S1	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	R	345	B1
33	Chicken meat	S2	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	IncX1	345	B1
34	Chicken meat	S2	*E. coli*	CTX-M-1g	$bla_{CTX-M-15}$	IncX1	1125	B1
35	Chicken meat	S2	*E. coli*	CTX-M-2g	$bla_{CTX-M-2}$	IncHI2	10	Non-typable
36	Chicken meat	S2	*E. coli*	CTX-M-2g	$bla_{CTX-M-2}$	R	93	D
37	Chicken meat	S2	*K. pneumonie*	CTX-M-1g	$bla_{CTX-M-15}$	IncFIIK	307	-

N/A: Not applied as the ESBL gene for these isolates was confirmed to be encoded on the chromosome.

Transformation experiments revealed that the ESBL genes were plasmid-encoded in 35 of the 37 isolates with these plasmids belonging to diverse replicon-types (**Figure 1B**). In particular, plasmid replicon-types detected for the $bla_{CTX-M-15}$ gene were R, X1, FIIK, I1, N-FII(-FIIs), and R-FIIk, whereas for the $bla_{CTX-M-2}$ gene were R and HI2(-P). In the two *E. coli* isolates that were negative for transformation and the subsequent conjugation experiments, suggesting that the $bla_{CTX-M-2}$ gene was not located on a plasmid (**Table 1**), we confirmed the chromosomal location of the $bla_{CTX-M-2}$ gene by I-Ceu-I-PFGE followed by Southern blot hybridization (**Supplementary Figure 1**). Although the $bla_{CTX-M-2}$ gene has been previously documented in different genetic backgrounds as well as on plasmids highlighting multiple integration events and transmission pathways, it has been mostly reported to be chromosomally encoded (Casella et al., 2018). In our study, only a limited number of $bla_{CTX-M-2}$ genes were chromosomally encoded, suggesting that plasmids are important facilitators of their spread among the recovered isolates. Overall, our data highlight the contribution of plasmids on the epidemiology of ESBL-producing Enterobacterales of poultry and human origin in Sao Paulo, Brazil.

Clermont's classification demonstrated that our *E. coli* isolates belonged to A, B1, D, and F groups with one isolate being not classifiable. The B1 was the most prevalent with 64.7% (11/28) of cloacal isolates belonging to this group, followed by 17.6% (3/17) of D, 11.7% (2/17) of F, and 5.8% (1/17) of A. Similarly, B1 was the most prevalent group among the isolates that we recovered

from human clinical samples (60%; 3/5) and chicken meat (50%; 3/6). Isolates assigned to the B1 group have been previously associated with mostly intestinal pathogenic *E. coli* with high virulent potential in animal models (Morcatti et al., 2018).

Multilocus sequence typing classification demonstrated that the 28 *E. coli* isolates belonged to nine sequence types (ST10, ST38, ST47, ST93, ST345, ST349, ST354, ST1125, and ST3258) with the ST345 and ST1125 being the most prevalent ones (46.4 and 17.8%, respectively) (**Figure 1C**). *K. pneumoniae* isolates were assigned to four sequence types (ST15, ST273, ST307, and ST485) with ST15 being the most prevalent (44.4%) one (**Figure 1C**). Of note, we observed some known epidemic clones among the *E. coli* (i.e., ST10 and ST38) and *K. pneumoniae* (i.e., ST15 and ST307) isolates (Woodford et al., 2011; Mathers et al., 2015; Navon-Venezia et al., 2017). *K. pneumoniae* ST15 isolates harboring $bla_{CTX-M-15}$, are emerging among patients with respiratory tract infections in China (An et al., 2012; Xu et al., 2019) and have been previously isolated from companion animals in Paris (Morcatti et al., 2018). As previously described, our data reveal clonal diversity among the recovered isolates and highlight Brazilian poultry meat as a reservoir of ExPEC lineages (i.e., ST10) (Casella et al., 2018).

As observed by Casella et al. (2018), we show genetic identity in the ESBL gene, plasmid type, isolate ST, and phylogroup suggesting clonal similarity in *K. pneumoniae* isolates between F1 and F2 ($bla_{CTX-M-15}$, R, ST15) but also *E. coli* isolates among (1) F1, F2, H1, and S2 ($bla_{CTX-M-15}$, IncX1, ST345/B1); (2) F1 and S1 ($bla_{CTX-M-15}$, R, ST345/B1); and (3) F1, F2, and S2 ($bla_{CTX-M-15}$, IncX1, ST1125/B1) (**Table 1**). In addition, we observed genetic identity in ESBL gene and plasmid type suggesting plasmid spread among (1) F1, F2, H1, H2, S1, and S2 ($bla_{CTX-M-15}$, IncX1) and (2) F1, F2, and S1 ($bla_{CTX-M-15}$, R) (**Table 1**). Overall, our data highlight a complex epidemiology of ESBL-producing Enterobacterales driven by both clones and plasmids, as well as the potential transmission of these clones and plasmids along the poultry meat chain to humans and/or vice versa.

In conclusion, we demonstrated that despite their overall genetic diversity, isolates from cloacal swabs, chicken meat, and human feces present genetic similarities highlighting that Brazilian chickens, as well as their by-products, may be an important source of transmission for ESBL-producing pathogens to humans. In addition, we indicated the occurrence and high frequency of *E. coli* and *K. pneumoniae* isolates harboring the $bla_{CTX-M-15}$ gene from chicken and chicken meat products in South America for the first time.

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/s.

AUTHOR CONTRIBUTIONS

MC conceptualized and designed the study. AL and LP aided with data analysis, and manuscript preparation and revision. AK aided in data acquisition. MBo in data acquisition and manuscript preparation. All authors read, contributed to, and approved the final manuscript.

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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.2021.628738/full#supplementary-material

Supplementary Figure 1 | Chromosomal localization of the $bla_{CTX-M-2}$ gene by I-Ceu-I-PFGE and Southern blot hybridization. Columns 1 (Isolate 19) and 2 (Isolate 30) depict the hybridization results using an intragenic $bla_{CTX-M-2}$ gene probe, whereas columns 3 (*S. enterica* ser. Braenderup strain H9812—marker), 4 (Isolate 19), and 5 (Isolate 30) depict the hybridization results using intragenic 16S rDNA probes.

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ORIGINAL RESEARCH
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The Emergence and Molecular Characteristics of New Delhi Metallo β-Lactamase-Producing *Escherichia coli* From Ducks in Guangdong, China

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This study aimed to determine the prevalence and transmission characteristics of New Delhi metallo β-lactamase (NDM)-producing *Escherichia coli* from ducks in Guangdong, China. In this study, a total of 28 NDM-producing *E. coli* isolates were recovered from 88 unduplicated diseased duck samples (31.8%) from veterinary clinics in Guangzhou, Foshan, Qingyuan, and Huizhou. Two variants, bla_{NDM-1} and bla_{NDM-5}, were detected and the latter was present in 89.6% of the isolates (25/28). Multilocus sequence typing (MLST) analysis indicated that these *E. coli* isolates possessed six distinct STs, and ST156 was the most prevalent followed by ST648, ST746, ST354, ST10, and ST162. In addition, phylogenomic analysis found that two of the isolates that were recovered from a single sample possessed different genomes, and the bla_{NDM}-carrying IncX3 plasmids may be horizontal transfer between *E. coli* isolates in the intestinal tracts of ducks. Whole-genome sequencing (WGS) analysis further revealed that bla_{NDM} co-existed with other 25 types of antimicrobial resistance genes (ARGs), of which 16 ARGs were highly prevalent with detection rates >50%, and a high incidence of coproducing bla_{NDM} and *mcr-1 E. coli* isolates (22/88, 25.0%) was detected in ducks. This study underscores the importance of surveillance for bla_{NDM}-harboring microbes in ducks.

Keywords: transmission, MCR-1, *escherichia coli*, duck, bla_{NDM}

INTRODUCTION

Carbapenems are critically important for the treatment of infections caused by multidrug-resistant Gram-negative bacteria. However, carbapenemase-producing *Enterobacteriaceae* have become a major global public health threat (Nordmann et al., 2011). In particular, the New Delhi metallo β-lactamase (NDM) was initially found in *Escherichia coli* and *Klebsiella pneumoniae* isolates in India in 2018 (Yong et al., 2009). Since then, bla_{NDM}-positive isolates have been found globally (Wu et al., 2019). bla_{NDM} genes have been found in species belonging to 11 bacterial families, and the *Enterobacteriaceae* are the major hosts of bla_{NDM} (Wu et al., 2019; Zhai et al., 2020).

Carbapenem antibiotics have never been licensed for veterinary use in any country worldwide; however, there have been sporadic reports of bla_{NDM}-positive isolates from a variety of animal hosts. bla_{NDM}-positive *E. coli* isolates were frequently detected from swine in multiple geographic areas in China (Ho et al., 2019). Similar to the detected rates of bla_{NDM} gene in swine, bla_{NDM}-positive *E. coli* isolates were highly prevalent in commercial broiler farms (Wang et al., 2017). In addition, several bla_{NDM}-positive *E. coli* isolates from backyard animals shared closely related core single nucleotide polymorphisms (SNP) with human isolates (Li et al., 2019). A recent study has reported that bla_{NDM}-positive *Enterobacteriaceae* were detected from migratory birds in China (Liao et al., 2019). Therefore, continued monitoring for bla_{NDM}-positive Enterobacteriaceae in food-producing animals is urgently required.

Duck production has the potential to play a major role in the agricultural economy, and Asian countries alone contribute 84.2% of total duck meat produced in the world (Biswas et al., 2019). According to Food and Agriculture Organization (FAO) data (2019), duck meat production rose from 2.64 to 3.02 million tons from 2015 to 2019 in Asia, while in China, duck meat production rose from 2.19 to 2.50 million tons from 2015 to 2019 in China (FAO, 2019). The data show that China is the largest producer and consumer of cultivated duck in the world (Chen et al., 2020). bla_{NDM}-positive *E. coli* isolates were highly prevalent along the Chinese poultry production chain, including commercial broiler farms, slaughterhouses, and supermarkets (Wang et al., 2017). Therefore, the prevalence of bla_{NDM}-positive *E. coli* isolates from duck should be addressed through continued monitoring.

Although we previously reported that the rate of bla_{NDM}-positive *E. coli* isolates at three duck farms in Guangdong was significantly higher than four other provinces in China, the sample collected was limited in western Guangdong province (Wang et al., 2021). Thus, in this study, we furthermore examined the epidemiology and molecular characterization of bla_{NDM}-positive *E. coli* isolates recovered from ducks in representative areas for breeding ducks of Guangdong, China.

MATERIALS AND METHODS

Ethics Statement

The Institutional Review Board of South China Agricultural University (SCAU-IRB) approved the protocols. All animals were sampled under authorization from the Animal Research Committees of South China Agricultural University (SCAU-IACUC).

Sampling Information

A total of 88 unduplicated samples, including 42 liver samples and 46 caecum samples, were collected from 88 diseased ducks, these diseased ducks were sent to the veterinary clinical diagnostics laboratory in Foshan University from duck farms in Guangzhou, Huizhou, Foshan, and Qingyuan of Guangdong province (**Figure 1**). Briefly, all sample was added to 1 ml of LB Broth and incubated for 16–18 h in 37°C, followed by inoculating on MacConkey plates containing 2 mg/L meropenem

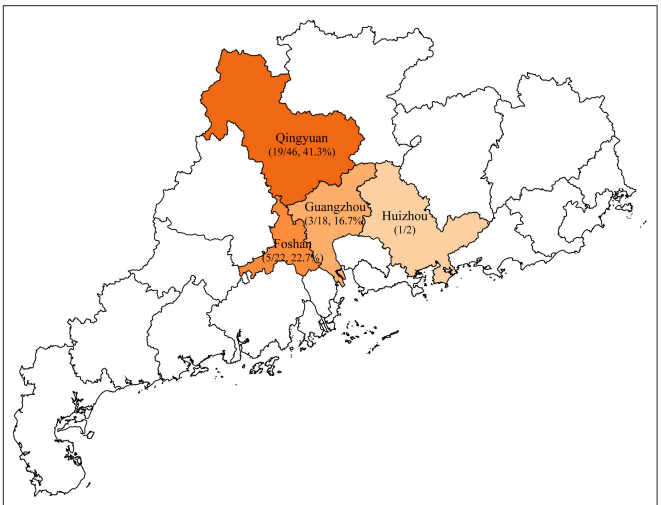

FIGURE 1 | Sample collection areas in Guangdong, China. Municipalities included in the study are shaded in orange and the rate of bla_{NDM}-positive *Escherichia coli* isolates are indicated by the depth of color.

for 12 h. Multiple red clones were selected for identification using MALDI-TOF MS Axima™ (Shimadzu-Biotech Corp., Kyoto, Japan) and 16S rRNA sequencing. For carbapenem-resistant *E. coli* isolates, five major carbapenem resistance genes, namely, bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{OXA-48}, and bla_{VIM}, were detected by PCR using previously described primers (Poirel et al., 2011). Samples were collected after obtaining consent from farms and veterinarians.

Antimicrobial Susceptibility Testing

Antibiotic susceptibility testing was performed by the agar dilution method and interpreted according to the Clinical and Laboratory Standards Institute guidelines (CLSI M100-S28) for the following antimicrobials: gentamicin, amikacin, meropenem, imipenem, aztreonam, cefotaxime, ceftazidime, cefoxitin, florfenicol, ciprofloxacin, fosfomycin, trimethoprim-sulfamethoxazole, and tetracycline (CLSI, 2018). Susceptibility to colistin and tigecycline were assessed by broth microdilution as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST Version 6.0) (EUCAST, 2016). *E. coli* ATCC 25922 was used as the quality control strain.

Conjugation Assay and Southern Blotting

To investigate the transferability of the resistance genes, a conjugation assay was performed for all bla_{NDM}-positive *E. coli* isolates with the streptomycin-resistant *E. coli* C600 as the recipient strain. Donor strains and *E. coli* C600 were mixed and applied to a 0.22 μm filter in LB agar plates for 16–18 h. The mixture culture was then diluted and spread on selective MacConkey agar plates containing both 1 mg/L of meropenem and 2,000 mg/L of streptomycin to recover transconjugants. Transconjugants were confirmed by PCR and Pulsed Field Gel Electrophoresis (PFGE) patterns. S1-PFGE and Southern blotting were performed to obtain plasmid size, and the *Salmonella enterica* serotype, Braenderup H9812, was used as the standard size marker.

Whole-Genome Sequencing (WGS)

Total DNA was extracted from *bla*$_{NDM}$-producing *E. coli* isolates using a Genomic DNA Purification Kit (TIANGEN, Beijing, China) as per the instructions of the manufacturer. WGS was performed with the Illumina Hiseq 2500 System (Novogene Guangzhou, China) using the paired-end 2 × 150-bp sequencing protocol. The draft genome was *de novo* assembled using the SPAdes version 3.9.0 (Bankevich et al., 2012). All genome assemblies of 28 *E. coli* isolates were deposited in GenBank and are registered with BioProject number PRJNA669620. Then, the sequence types, replicon types, and antibiotic resistance genes of all the isolates were identified by the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/). The sequence comparison of *bla*$_{NDM}$-carrying plasmids was performed using Mauve and Brig (Darling et al., 2004; Alikhan et al., 2011). *In silico* phylotyping of *E. coli* was carried out using the ClermonTyping method (http://clermontyping.iame-research.center/). Phylogenetic trees for the *bla*$_{NDM}$-producing *E. coli* isolates were structured using CSI Phylogeny (v1.4), and *E. coli* (18FS1-1) was used as the reference genome (Kaas et al., 2014). The corresponding characteristics of each isolate were visualized using the online tool iTOL (https://itol.embl.de/). The population structure of each phylogenetic tree was defined using rhierbaps (Tonkin-Hill et al., 2018). The genome assemblies were analyzed using a gene-by-gene approach and the allelic distance from the core genome multilocus sequence typing (MLST) (cgMLST) was visualized in a minimum-spanning tree using BacWGSTdb 2.0 (Feng et al., 2021).

RESULT

Bacterial Isolation and Detection of Carbapenemase Genes

In this study, a total of 28 (31.8%) *bla*$_{NDM}$-producing *E. coli* isolates were recovered from 88 collected samples

TABLE 1 | Bacterial information and antimicrobial resistance profiles.

Isolates	Samples	Collection data	Sources	Variants	*bla*$_{NDM-}$carrying-Plasmid	Resistance phenotype
18FS1-1	18FS1	20160320	Caecum	*bla*$_{NDM-5}$	IncX3	GEN, IMP, ERT, ATM, CTX, CAZ, FOX, CIP, FFC, TET, S/T
18FS1-2	18FS1	20160320	Caecum	*bla*$_{NDM-5}$	IncX3	GEN, AMK, IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET, S/T, CS
18FS2-1	18FS2	20160320	Liver	*bla*$_{NDM-5}$	IncX3	IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET, S/T, FOS
18FS3-1	18FS3	20160320	Liver	*bla*$_{NDM-5}$	IncX3	GEN, IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET, FOS
18FS4-1	18FS4	20160320	Caecum	*bla*$_{NDM-5}$	IncX3	GEN, IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET
18FS4-2	18FS4	20160320	Caecum	*bla*$_{NDM-5}$	IncX3	IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET
18FS5-2	18FS5	20160320	Liver	*bla*$_{NDM-5}$	IncX3	IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET
18FS7-1	18FS7	20160320	Caecum	*bla*$_{NDM-5}$	IncX3	GEN, IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET, S/T, FOS
18FS7-2	18FS7	20160320	Caecum	*bla*$_{NDM-5}$	IncX3	GEN, IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET, S/T
18FS7-3	18FS7	20160320	Caecum	*bla*$_{NDM-5}$	IncX3	GEN, IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET, S/T
18FS15-1	18FS15	20160320	Caecum	*bla*$_{NDM-5}$	IncX3	GEN, IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET, S/T
18FS16-2	18FS16	20160320	Liver	*bla*$_{NDM-5}$	IncX3	GEN, IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET, FOS
18FS16-3	18FS16	20160320	Liver	*bla*$_{NDM-5}$	IncX3	GEN, AMK, IMP, ERT, ATM, CTX, CAZ, FOX, CIP, FFC, TET, S/T
18FS17-3	18FS17	20160320	Caecum	*bla*$_{NDM-5}$	IncX3	GEN, IMP, ERT, ATM, CTX, CAZ, FOX, CIP, FFC, TET, S/T
18FS18-1	18FS18	20160320	Caecum	*bla*$_{NDM-5}$	IncX3	GEN, IMP, ERT, ATM, CTX, CAZ, FOX, CIP, FFC, TET, S/T, CS
18FS18-2	18FS18	20160320	Caecum	*bla*$_{NDM-5}$	IncX3	GEN, IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET
18FS23-1	18FS23	20160320	Caecum	*bla*$_{NDM-5}$	IncX3	GEN, AMK, IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET, S/T
18FS24-1	18FS24	20160320	Liver	*bla*$_{NDM-5}$	IncX3	GEN, IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET, S/T, FOS
20FS11-1	20FS11	20160402	Caecum	*bla*$_{NDM-5}$	IncX3	GEN, AMK, IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET, S/T
20FS11-2	20FS11	20160402	Caecum	*bla*$_{NDM-1}$	Untypable	GEN, AMK, IMP, ERT, ATM, CTX, CAZ, FOX, CIP, FFC, TET, S/T, CS, FOS
20FS12-2	20FS12	20160402	Liver	*bla*$_{NDM-1}$	Untypable	IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET, S/T, CS, FOS
20FS14	20FS14	20160402	Liver	*bla*$_{NDM-1}$	Untypable	GEN, AMK, IMP, ERT, ATM, CTX, CAZ, FOX, CIP, FFC, TET, S/T, CS
20FS19	20FS19	20160402	Caecum	*bla*$_{NDM-5}$	IncX3	GEN, IMP, ERT, ATM, CTX, CAZ, FOX, CIP, FFC, TET, S/T
20FS22	20FS22	20160402	Liver	*bla*$_{NDM-5}$	IncX3	IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET, S/T
21FS11-2	21FS11	20160409	Caecum	*bla*$_{NDM-5}$	IncX3	GEN, IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET, S/T
22FS12-2	22FS12	20160416	Liver	*bla*$_{NDM-5}$	IncX3	GEN, AMK, IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET, S/T, CS
22FS18	22FS18	20160416	Liver	*bla*$_{NDM-5}$	IncX3	GEN, AMK, IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET, S/T, CS, FOS
22FS24	22FS24	20160416	Liver	*bla*$_{NDM-5}$	IncX3	GEN, AMK, IMP, ERT, CTX, CAZ, FOX, CIP, FFC, TET, S/T, CS, FOS

Samples were collected from the caecum and liver of ducks. MEM, meropenem; CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin; FFC, florfenicol; CIP, ciprofloxacin; TET, tetracycline, S/T, trimethoprim/sulfamethoxazole; IMP, imipenem; ERT, ertapenem; GEN, gentamicin; FOS, fosfomycin; CS, colistin; AMK, amikacin; ATM, aztreonam; TIG, tigecycline.

in the veterinary clinical diagnostic laboratory in Foshan University from duck farms in Guangdong province. The isolation rates of bla_{NDM}-positive *E. coli* from different districts were 41.3% for Qingyuan (19/46), 22.7% for Foshan (5/22), and 16.7% for Guangzhou (3/18) (**Figure 1**). Within these groups, we identified only two bla_{NDM} variants: bla_{NDM-5} (25/28, 89.3%) and bla_{NDM-1} (3/28, 10.7%) (**Table 1**).

Antibiotic Susceptibility Testing

All bla_{NDM}-positive *E. coli* isolates showed reduced susceptibility to meropenem with MICs of 4– >64 mg/L (**Supplementary Table 1**) and were concurrently resistant to imipenem, ertapenem, cefotaxime, ceftazidime, cefoxitin, florfenicol, ciprofloxacin, and tetracycline (**Table 1**). Moreover, these isolates exhibited high rates of resistance to trimethoprim/sulfamethoxazole (22/28, 78.6%), gentamicin (23/28, 82.1%), and fosfomycin (12/28, 42.9%) but lower rates for amikacin (9/28, 32.1%), colistin (8/28, 28.6%), and aztreonam (7/28, 25%).

Phylogenetic Analysis of NDM-Positive *E. coli* Isolates

Whole-genome sequencing data were generated for the 28 bla_{NDM}-positive *E. coli* isolates. The results of WGS demonstrated that these isolates were divided into six distinct STs: ST156 (8/28, 28.6%), ST648 (7/28, 25.0%), ST746 (5/28, 17.9%), ST354 (3/28, 10.7%), ST10 (3/28, 10.7%), and ST162 (2/28, 7.1%) (**Figure 2**). Clonotyping revealed seven *fumC* and *fimH* (CH) types and exhibited further divergence between clones. The most prevalent clonotypes were C29:H38 (n = 7), C4:H1084 (n = 7), and C7:H54 (n = 5), which belong to ST156, ST648, and ST746, respectively. The remaining clonotypes were C11:H0 (n = 3), C65:H32 (n = 2), C88:H58 (n = 2), C7:H1353 (n = 1), and C88:H27 (n = 1) (**Figure 2**). The *E. coli* isolates from the present study were classified using Clermont Typing and the majority belonged to groups B1 (10/28, 35.7%) and F (10/28, 35.7%) from Foshan and Qingyuan, respectively. A phylogenetic tree was established using the 28 bla_{NDM}-positive *E. coli* isolates. All isolates were classified into four clades lineage, and the major Lineage IV included seven (25%) isolates belonging to ST648 and exhibited high levels of the identity of pairwise single nucleotide polymorphisms (SNP) $\leq$ 21 (**Figure 2**). Notably, in five cases, two isolates were possessing a collection of different genomic characteristics that were recovered from the same samples (18FS1, 18FS4, 18FS16, 18FS18, and 20FS11) (**Table 1**). For instance, both ST354 (18FS1-1) and ST648 (18FS1-2) were recovered from sample 18FS1 and shared 22411 SNPs. This scenario is worrying and indicates the development of diversity in the population of bla_{NDM}-positive *E. coli* isolates from ducks. To further assess the relationship between the 28 isolates, the genome assemblies were analyzed using a gene-by-gene approach, and the allelic distance from the core genome MLST (cgMLST) was visualized in a minimum-spanning tree using BacWGSTdb 2.0. Based on ST, geographic location, and position in the network, the resulting network shows that the isolates were grouped in accordance with their ST and place of isolation. The MLST type was clustered together, except ST746; 18FS4-1 were more closed to the ST156 cluster (**Supplementary Figure 1**).

Antibiotic Resistance Genes

We additionally identified the presence of 25 types of antimicrobial resistance genes (ARGs) that conferred resistance to nine classes of antibiotics including aminoglycosides, β-lactams, MLS (macrolides, lincosamides, and type B streptogramin), fluoroquinolones, sulfonamide, fosfomycin, rifampicin, colistin, and tetracyclines. Among these, 16 ARGs were highly prevalent with detection rates >50%, including *aac*, *aph*, bla_{CTX}, bla_{OXA}, bla_{TEM}, *cmlA*, *floR*, *mdf*, *mph*, *oqxA/B*, *qnrS*, *dfrA*, *sul*, *ARR*, *mcr-1*, and *tet* (**Figure 2**).

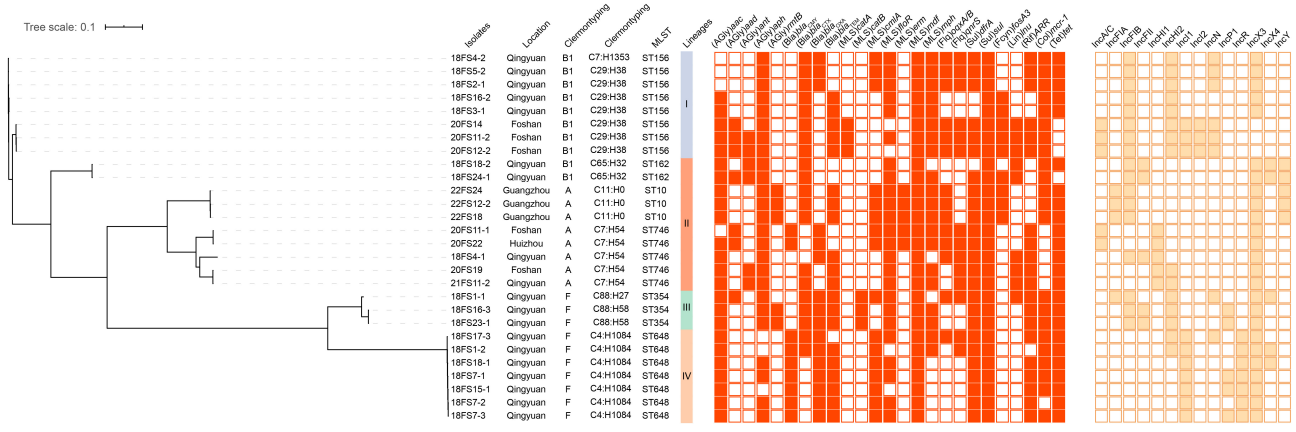

FIGURE 2 | Analysis of bla_{NDM}-positive *E. coli* isolates from ducks in Guangdong, China. Relationships among 28 bla_{NDM}-positive *E. coli* isolates are indicated using a maximum likelihood tree. Red-filled squares indicate possession of the indicated antimicrobial resistance genes (ARGs) and brown-filled squares indicated plasmid Inc type.

Plasmid Analysis

Conjugation experiments were performed using the bla_{NDM}-positive *E. coli* isolates collected from ducks, and all the bla_{NDM}-carrying plasmids were successfully transferred to the recipient strain *E. coli* C600^{str}. S1-PFGE and hybridization analyses confirmed that bla_{NDM} genes from 25 isolates were located on ∼ 50 kb plasmids, and the others genes were located on ∼ 140 kb ($n = 2$) and ∼ 200 kb ($n = 1$) plasmids (**Supplementary Figure 2**). As shown in **Supplementary Figure 3**, the ∼ 50 kb plasmids carrying bla_{NDM} were the IncX3 incompatibility group and were similar to bla_{NDM-5}-carrying IncX3 plasmid from an ST25 *K. pneumoniae* isolated from human peritoneal fluid in China (Acc. No. KU761328). In addition, we found that IncX3 plasmids were carried by two different *E. coli* isolates that were recovered from the same sample in four out of five cases (**Table 1**). This provides evidence for the horizontal transfer of bla_{NDM}-carrying IncX3 plasmids in the intestines of ducks. WGS analysis demonstrated that 14 different Inc types were present on plasmids in the 28 bla_{NDM}-positive *E. coli* isolates (**Figure 2**). Except for the IncX3 plasmid, IncFIB (17/28, 60.7%), IncHI2 (15/28, 53.6%), and IncI1 (10/28, 35.7%) plasmids were highly prevalent in these isolates (**Figure 2**).

DISCUSSION

In this study, a total of 28 (28/88, 31.8%) bla_{NDM}-producing *E. coli* isolates were recovered from ducks; this result is similar to our previous report that indicated ahigh prevalence of bla_{NDM}-positive *E. coli* isolates at duck farms in western Guangdong province. Although there was an overwhelming dominance of bla_{NDM-1} and bla_{NDM-5} in the clinical and livestock isolates (Shen et al., 2018; Zhai et al., 2020), bla_{NDM-5} was more prevalent than bla_{NDM-1} among the tested duck farms. This finding is similar to the previous report of the high prevalence of bla_{NDM-5} in the chicken production chains (Wang et al., 2017).

The 28 bla_{NDM}-positive *E. coli* isolates belonged to six distinct STs (ST156, ST648, ST746, ST354, ST10, and ST162) discussed in the current study, of which, ST156, ST648, and ST746 were the most prevalent. ST156 and ST648 have been associated with the dissemination of bla_{NDM-5} and *mcr-1*-producing *E. coli* isolates (Yang et al., 2016). The bla_{NDM}-positive ST746 and ST354 prevalent in ducks and poultry have become a primary reservoir for bla_{NDM}-positive ST746 *E. coli* isolates in China (Wang et al., 2021). Phylogenomic analysis found that two of the isolates that were recovered from a single sample possessed different genomes, which indicates the development of diversity in the population of bla_{NDM}-positive *E. coli* isolates from ducks.

Whole-genome sequencing analysis further revealed that bla_{NDM} coexisted with other 25 types of ARGs, of which 16 ARGs were highly prevalent with detection rates >50%. Of note, *mcr-1*, conferring resistance to the last-resort antibiotic colistin, was detected in 22 bla_{NDM}-positive *E. coli* isolates. Some recent studies have reported that bla_{NDM} and *mcr-1* coproducing *E. coli* isolates were recovered from chicken (37/739, 5.0%), swine (16/105, 15.2%), and duck (11/92, 12.0%) farms (Kong et al., 2017; Wang et al., 2017, 2021). There was also a high incidence of the co-harboring of *mcr-1* and bla_{NDM} in chickens (21/78, 26.9%) (Liu et al., 2017). In this study, we also discovered a high prevalence of bla_{NDM} and *mcr-1* co-carrying *E. coli* isolates from diseased ducks (22/88, 25.0%) in Guangdong. In addition, the existence of bla_{NDM-5} is associated with multiple resistances, including aminoglycosides, sulfonamide, and fluoroquinolones. These can further promote the spread and persistence of carbapenem-resistant microbes in the poultry industry (Grönthal et al., 2018; Zhai et al., 2020).

In the current study, all bla_{NDM-5} genes identified were carried by IncX3 plasmids. IncX3 plasmids may serve as one of the major platforms on which bla_{NDM} genes are evolving with the generation of new NDM variants, such as $bla_{NDM-1/4/5/6/7/13/17/19/20/21}$ (Wu et al., 2019; Zhai et al., 2020). However, a high prevalence of bla_{NDM-5}-carrying IncX3 plasmid from bacteria of animal farms in China (Zhai et al., 2020). The bla_{NDM}-carrying IncX3 plasmids have a narrow host range and have been mainly found in *Enterobacteriaceae* worldwide, which may be an association to highly conjugatable and stable and exert no fitness costs on their bacterial hosts (Johnson et al., 2012). In addition, this study found that IncX3 plasmids were carried with two different *E. coli* isolates recovered from a single sample in four out of five samples. This provides evidence for the horizontal transfer of bla_{NDM}-carrying IncX3 plasmids between *E. coli* isolates in the intestine of ducks.

CONCLUSIONS

In conclusion, we identified 28 bla_{NDM}-positive *E. coli* isolates from diseased ducks in Guangdong, China. Notably, this is the first study to report the development of diversity in the population of bla_{NDM}-positive *E. coli* isolates from ducks. WGS analysis further determined that bla_{NDM} coexisted with other ARGs, including *mcr-1*, and bla_{NDM}-carrying IncX3 plasmids were most likely horizontally transferred between *E. coli* isolates in the duck intestinal tract. This study underscores the importance of surveillance for bla_{NDM}-harboring microbes in ducks and indicates a high likelihood for the spread of carbapenem resistance from the poultry production chain to humans.

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/genbank/, PRJNA669620.

AUTHOR CONTRIBUTIONS

M-GW wrote the first draft of the manuscript. X-PL, YY, and L-XF contributed to conception and design of the study. DW, R-SY, LJ, D-TC, and S-LZ performed the statistical analysis. JS and Y-HL wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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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.2021.677633/full#supplementary-material

Supplementary Figure 1 | Minimum spanning tree analysis of the cgMLST profiles of 28 isolates. Numbers on lines correspond to the number of target genes for which allelic differences were detected. Circle colors are used to differentiate the city of origin of the isolates.

Supplementary Figure 2 | S1-PFGE and hybridization of the plasmids of bla_{NDM}-positive *Escherichia coli* isolates. Lane M: XbaI-digested genomic DNA of reference *Salmonella enterica* serotype Braenderup strain H9812, and the bands on membrane represent the plasmids where bla_{NDM} genes are located on.

Supplementary Figure 3 | The sequence comparison and map generation of bla_{NDM}-carrying plasmids with reference IncX3 plasmid (Accession Number: KU761328) from an ST25 *Klebsiella pneumoniae* isolated from human peritoneal fluid in China.

Supplementary Table 1 | Minimum inhibitory concentration of 28 bla_{NDM}-positive E. coli isolates from duck in Guangdong China.

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Whole Resistome Analysis in *Campylobacter jejuni* and *C. coli* Genomes Available in Public Repositories

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Campylobacter spp. are the most frequent agent of human gastroenteritis worldwide, and the spread of multidrug-resistant strains makes the clinical treatment difficult. The current study presents the resistome analysis of 39,798 *Campylobacter jejuni* and 11,920 *Campylobacter coli* genomes available in public repositories. Determinants of resistance to β-lactams (Be) and tetracyclines (Te) were the most frequent for both species, with resistance to quinolones (Qu) as the third most important on *C. jejuni* and to aminoglycosides (Am) on *C. coli*. Moreover, resistance to Te, Qu, and Am was frequently found in co-occurrence with resistance to other antibiotic families. Geographical differences on clonal complexes distribution were found for *C. jejuni* and on resistome genotypes for both *C. jejuni* and *C. coli* species. Attending to the resistome patterns by isolation source, three main clusters of genomes were found on *C. jejuni* genomes at antimicrobial resistance gene level. The first cluster was formed by genomes from human, food production animals (e.g., sheep, cow, and chicken), and food (e.g., dairy products) isolates. The higher incidence of *tet(O)*, associated with tetracycline resistance, and the *gyrA* (T86I) single-nucleotide polymorphism (SNP), associated with quinolone resistance, among genomes from this cluster could be due to the intense use of these antibiotics in veterinary and human clinical settings. Similarly, a high incidence of *tet(O)* genes of *C. coli* genomes from pig, cow, and turkey was found. Moreover, the cluster based on resistome patterns formed by *C. jejuni* and *C. coli* genomes of human, turkey, and chicken origin is in agreement with previous observations reporting chicken or poultry-related environments as the main source of human campylobacteriosis infections. Most clonal complexes (CCs) associated with chicken host specialization (e.g., ST-354, ST-573, ST-464, and ST-446) were the CCs with the highest prevalence of determinants of resistance to Be, Qu, and Te. Finally, a clear trend toward an increase in the occurrence of Te and Qu resistance determinants on *C. jejuni*, linked to the spread of the co-occurrence of the bla_{OXA-61} and *tet(O)-tet(O/W/O)* genes and the *gyrA* (T86I) SNP, was found from 2001 to date in Europe.

Keywords: *Campylobacter jejuni*, resistome, whole genome sequencing, host specialization, livestock and human sources, *Campylobacter coli*

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INTRODUCTION

Thermophilic *Campylobacter* species, and specially *Campylobacter jejuni* and *Campylobacter coli*, are the most frequent agents of human gastroenteritis both in industrialized countries (Kaakoush et al., 2015; European Food Safety Authority, 2018) and in low- and middle-income countries (Fischer Walker et al., 2013; Liu et al., 2015), with *C. jejuni* accounting for up to 80% of all human *Campylobacter* gastroenteritis cases (Bronnec et al., 2016), with estimations of over 800,000 cases per year between 2000 and 2008 (Scallan et al., 2011). Moreover, *C. jejuni* is the most frequent foodborne bacterial pathogen globally, with developing countries being the most affected (Yahara et al., 2017).

Campylobacter jejuni and *C. coli* are present in the digestive tract of different animals, including birds, cattle, sheep, pigs, and pets (Thépault et al., 2017), although chicken is recognized as the main source of human infection in most countries (Domingues et al., 2012; Newell et al., 2017; Cody et al., 2019). Despite consumption of contaminated raw or under-cooked poultry meat being the main source of human infection (Domingues et al., 2012), other sources of infection include contaminated water, unpasteurized/incompletely pasteurized milk, or the direct contact with animals and the environment (Lévesque et al., 2008; de Perio et al., 2013; Levallois et al., 2014; Fernandes et al., 2015; Mossong et al., 2016).

Antimicrobial-based therapy for campylobacteriosis is indicated only in patients with severe and invasive gastroenteritis. Macrolides (mainly erythromycin), fluoroquinolones (mainly ciprofloxacin), and tetracyclines are the most commonly used and effective antibiotics (Aarestrup et al., 2008; Silva et al., 2011). Unfortunately, these antimicrobial agents have analogs widely employed in veterinary settings, and their overuse or misuse have favored the emergence and spread of resistant *Campylobacter* isolates (Van Boeckel et al., 2015; Asuming-Bediako et al., 2019). Furthermore, the continuous increase in resistance to certain antimicrobial classes has been accompanied by a rise in the occurrence of multidrug-resistant (MDR) *C. jejuni* and *C. coli* from farm to fork (Mourkas et al., 2019).

Whole genome sequencing (WGS) is a powerful tool that offers high-resolution sub-typing of *Campylobacter* isolates, which is of value for outbreak investigations (Clark et al., 2016; Lahti et al., 2017). Apart from multilocus sequence typing (MLST) schemes, based on the analysis of the sequence of several house-keeping genes, WGS also allows to predict relevant phenotypes, such as antibiotic resistance, in *Campylobacter* (Zhao et al., 2016). Moreover, recent studies have found high concordance between the presence of antimicrobial resistance determinants in WGS data and phenotypic resistance among *Campylobacter* isolates from Latvia (Meistere et al., 2019), England and Wales (Painset et al., 2020), and Poland (Wysok et al., 2020), supporting the use of WGS data as a good predictor for phenotypical antimicrobial resistance.

Taking into account the increasing relevance of *C. jejuni* and *C. coli* as foodborne pathogens and the availability of a high amount of their genomes in different public repositories, with the corresponding information on the year, country, and source of isolation of the sequenced strains, the objectives of the current study are as follows: (i) to study the host association of the most abundant clonal complexes (CCs) of *C. jejuni* and the most abundant sequence types (ST) within the main CCs on *C. jejuni* and *C. coli*; (ii) to compare the prevalence of antimicrobial resistance (AR) factors within *C. jejuni* and *C. coli* genomes; (iii) to perform an exhaustive resistome genotypic analysis and link AR patterns to country/continent, source of isolation, and *C. jejuni* CCs; and (iv) to assess temporal changes in such AR patterns along the last 20 years.

MATERIALS AND METHODS

Downloading Genomes and Metadata for Each Genome

Metadata associated to each genome was obtained by downloading the table obtained after using "*Campylobacter jejuni*" and "*Campylobacter coli*" as keywords (i) on PATRIC Taxon View website[1]; (ii) through the Export dataset section on the PubMLST-*Campylobacter jejuni/coli* website[2], where all isolates were selected; and (iii) by using 02.NCBI_Download_metadata.R script[3] for the NCBI repository. This script basically downloads the information obtained from https://www.ncbi.nlm.nih.gov/biosample/BIOSAMPLE?report=full&format=text, where BIOSAMPLE is substituted by the NCBI Biosample code for each genome analyzed, with these genomes being those of the *C. jejuni* and *C. coli* strains compiled in the prokaryotes.txt file in ftp://ftp.ncbi.nlm.nih.gov/genomes/GENOME_REPORTS/.
The three metadata files obtained (for the NCBI, PATRIC, and PubMLST repositories) were manually inspected and adapted to merge them in a unique file. The collection_date, host_name, country, and continent columns were manually inspected to homogenize the collection date (to year of collection), isolation source, and location format (to country and continent).

C. jejuni and *C. coli* genomes publicly available at NCBI,[4] PATRIC,[5] and PubMLST[6] repositories were downloaded on February 2021 using the *download_genomes* pipeline made for such task (see footnote 3). Briefly, the lists of available *C. jejuni* and *C. coli* genomes were constructed from metadata files for the PATRIC and PubMLST repositories and from the *prokaryotes.txt* file[7] for the NCBI repository. Those genomes that were present on more than one repository were discarded from the NCBI or PubMLST dataset using *03.ena2ncbi.rb* and *04.remove_repetition* scripts (see footnote 3), and then, all selected genomes were downloaded using the corresponding script for each database from download_genomes github repository (see footnote 3).

[1] https://patricbrc.org/view/Taxonomy/2#view_tab=genomes
[2] https://pubmlst.org/bigsdb?db=pubmlst_Campylobacter_isolates&page=plugin&name=Export
[3] https://github.com/JoseCoboDiaz/download_genomes
[4] ftp://ftp.ncbi.nlm.nih.gov/genomes/
[5] ftp://ftp.patricbrc.org/
[6] https://pubmlst.org
[7] ftp://ftp.ncbi.nlm.nih.gov/genomes/GENOME_REPORTS/prokaryotes.txt

Genome Analyses

All genomes were analyzed through MLST by using the mlst github pipeline (Seemann), which employs data from the PubMLST.org website (Jolley et al., 2018). Antimicrobial gene detection and detection of single-nucleotide polymorphisms (SNPs) conferring antimicrobial resistance were performed using the ResFinder database version updated at March 12, 2021 (Zankari et al., 2012) and PointFinder database updated at February 1, 2021 (Zankari et al., 2017), respectively. Both analyses, together with MLST, were performed for each genome by using the staramr pipeline (phac-nml/staramr) and *08.staramr_auto.rb* script (see footnote 3) to automatize the analyses for the dozens of thousands genomes analyzed.

Statistical Analysis

The R-packages *tidyr* and *dplyr* were used to organize the data matrix, while the R-packages *ggplot2* and *pheatmap* were employed for the representation of line plots, boxplots, and heatmap plots using R version 3.6.1 (R Core Team, 2019) and *t*-test to determine statistical differences. Heatmap plots were done using the percentage of genomes carrying each AR determinant (gene or SNP) that belonged to the same isolation source, clonal complex, or isolation year. The Euclidean distance and the complete (= UPGMA) clustering method were employed for the clustering of columns and rows.

Only those isolation sources with more than 50 genomes, excluding the *unknown* group, were selected for obtaining heatmap plots of antibiotic resistance genes (ARGs) or antibiotic families versus isolation source to avoid the introduction of biases due to the low number of genomes. Similarly, European genomes were uniquely selected for temporal variation analyses in the resistome, due to the fact that the highest number of genomes belonged to Europe and North America, but an important proportion of North-American genomes had missing information in relation to the year of isolation. Moreover, only European genomes from isolates obtained in those years when at least 100 genomes were available (i.e., years between 1997 and 2018, with the exception of 1999, 2000, and 2002) were selected for this task.

RESULTS

Global Overview

A total of 39,798 *C. jejuni* and 11,920 *C. coli* genomes were analyzed. The *C. jejuni* genomes belonged to 41 CCs, with ST-21 CC being the most abundant (23.8% of the analyzed genomes) followed by ST-353 and ST-45 CCs (8.9 and 7.8% of the analyzed genomes, respectively), while *C. coli* genomes belonged to 21 CCs, with ST-828 CC as the main CC (84.8% of analyzed genomes). Remarkably, 13.7% of the *C. jejuni* and 13.43% of *C. coli* genomes were not assigned to any known CC. The main isolation source was humans (37.1% of *C. jejuni* genomes and 14.8% of *C. coli* genomes), followed by chicken, cows, and sheep for *C. jejuni* (10, 3, and 1.2% of genomes, respectively) and chicken, pigs, cows, and sheep for *C. coli* (9, 5.5, 2.3, and 1.3% of genomes, respectively). Unfortunately, 45.6 and 60.6% of *C. jejuni* and *C. coli* genomes, respectively, had missing information on the isolation source. A total of 18,812 *C. jejuni* (47.3%) and 7,959 *C. coli* (66.8%) genomes were from North-American isolates, while 18,787 *C. jejuni* (47.2%) and 3,328 *C. coli* (27.9%) genomes belonged to European isolates. The genome codes, collection year, continent, country, host and MLST information for all the analyzed *C. jejuni* and *C. coli* genomes were presented to **Supplementary Tables 1, 2**, respectively.

The most abundant AR factors were associated with resistance to β-lactams and tetracyclines for both species, although β-lactam resistance was most prevalent on *C. jejuni* (81.6 vs. 65.0%). AR factors against aminoglycosides and antibiotics of the macrolides-lincosamides-streptogramins-pleuromutilins group (MLSP) were most abundant on *C. coli* genomes (34.8 and 2.9%, respectively) than on *C. jejuni* (6.7 and 0.5%, respectively), while resistance to quinolones was much more frequent on *C. jejuni* (28.7%) then on *C. coli* (0.6%) genomes (**Figure 1**). Moreover, up to 7.8 and 23.3% of *C. jejuni* and *C. coli* genomes, respectively, did not harbor any known AR factor (**Figure 1**). Remarkably, 39.9 and 21% of *C. jejuni* and *C. coli* genomes, respectively, had only AR genes or SNPs associated with resistance to only β-lactams, while 41.7 and 44.0% of the genomes harbored determinants of resistance to β-lactams combined with determinants of resistance to other antibiotic families. Similar observations were made for resistance to tetracyclines, which were seen as unique resistance factors on 4.6 and 5.1% of *C. jejuni* and *C. coli* genomes, respectively, while 38.6 and 41.6% of genomes harbored resistance to tetracyclines combined with resistance to other antibiotic families (**Figure 1**). It is worth highlighting the high level of co-occurrence of resistance to aminoglycosides, β-lactams, and tetracyclines on *C. coli* genomes (20.2%) and to β-lactams, quinolones, and tetracyclines on *C. jejuni* genomes (15.4%) (**Figure 1**).

A total of 120 and 68 different AR genes and SNPs were detected for *C. jejuni* and *C. coli* genomes, respectively. bla_{OXA-61} and *tet(O)* genes were the most extended AR determinants on both *C. jejuni* and *C. coli* genomes (they were carried by 69.5 and 36.4% of *C. jejuni* and 35.8 and 43.6% of *C. coli* genomes, respectively). The *gyrA* (T86I) SNP was the third most prevalent AR factor on *C. jejuni* genomes (28.5%), while *aph(3′)-III*, $bla_{OXA-489}$, *aadE-Cc*, and $bla_{OXA-193}$ were present in more than 10% of the *C. coli* genomes (**Figure 1**).

Moreover, while 33.48% of *C. jejuni* genomes carried only the bla_{OXA-61} gene, 10.7% simultaneously harbored bla_{OXA-61} and *tet(O)*, and 9.8% had *gyrA* (T86I), bla_{OXA-61}, and *tet(O)* in combination, with these three ARG genotype patterns being the most abundant on *C. jejuni* genomes (**Table 1**). The presence of only the bla_{OXA-61} gene was observed on 10.4% of the *C. coli* genomes, with it being the main genotype pattern found, followed by only the $bla_{OXA-489}$ gene and bla_{OXA-61} together with *tet(O)*, with 6.9% and 6.6% of prevalence, respectively (**Table 1**).

Geographical Distribution of CCs and ARGs-SNPs

The first global analysis of *C. jejuni* CC distribution showed differences by continent. The ST-21 CC was the most abundant

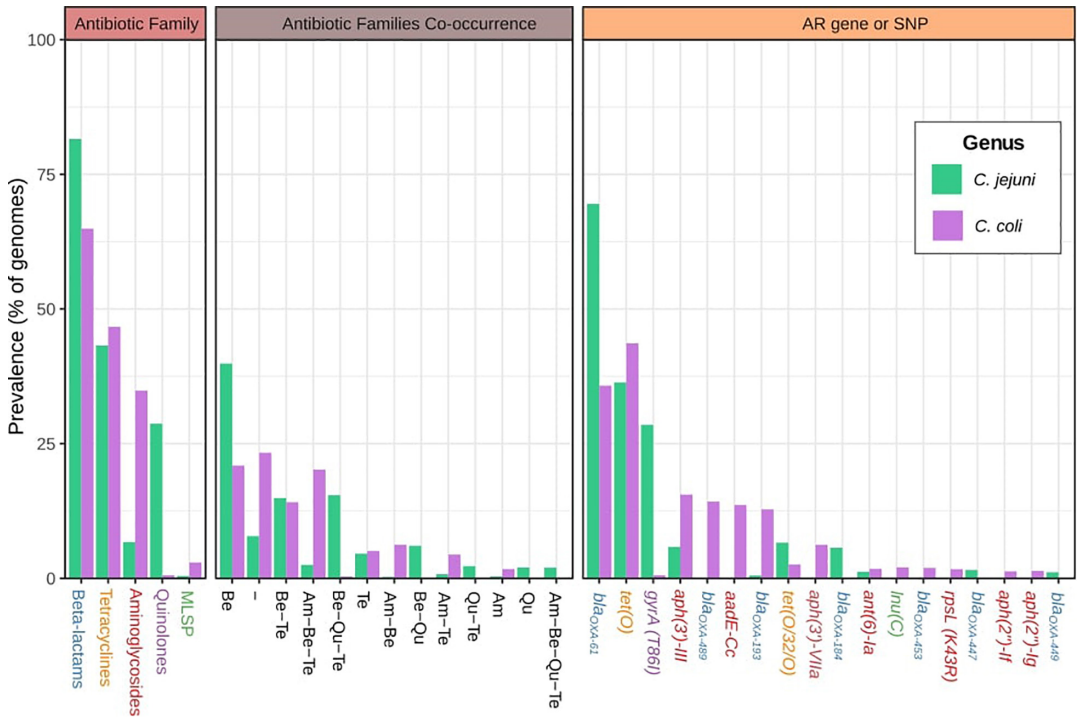

FIGURE 1 | Global antibiotic resistance gene (ARG)-single-nucleotide polymorphism (SNP) distribution on *C. jejuni* and *C. coli* genomes. Antimicrobial resistance families, family co-occurrence, and most abundant ARGs or SNPs among the *C. jejuni* and *C. coli* genomes analyzed. Am, aminoglycosides; Be, beta-lactams; Qu, quinolones; Te, tetracyclines. Gene names are colored according to the corresponding antimicrobial family [blue: beta-lactams, orange: tetracyclines, red: aminoglycosides, purple: quinolones, and green: macrolides-lincosamides-streptogramins-pleuromutilins group (MLSP)]. Hyphen (-) indicates no ARGs or SNPs detected.

in Asia, Europe, and North America (26.9, 25.0, and 23.0% of genomes, respectively), while ST-353 CC was predominant in South America and Africa (23.7 and 15.6%, respectively) and ST-354 CC was dominant in Oceania (60.3%) (**Figure 1**). These patterns are not observed in all the countries from the same continent. For example, ST-353 CC was abundant in the United States and Brazil (12.8 and 33.5% of genomes, respectively), but it was less represented on Canadian and Peruvian genomes (2.2 and 7.0%, respectively), with Canada having a high prevalence of ST-45 CC (18.8%) (**Figure 2**). The main differences among countries within the same continent were found in Europe, with 93.3% of ST-21 CC genomes in Denmark; 34.4% of ST-45CC genomes in Finland; and 26.7, 25, and 39.5% of ST-21 CC genomes in France, United Kingdom, and Israel, as the main CCs (**Figure 2**).

The bla_{OXA-61} gene had the highest prevalence among *C. jejuni* genomes in all continents (from 36.5 to 92.4% of total genomes), except for South America, which had the *gyrA* (T86I) SNP as the main AR factor (39.1%) (**Figure 3A**). Oceania had the highest prevalence of *tet(O)*, followed by North America and Asia (60, 46.9, and 42.7%, respectively), while the *gyrA T86I* SNP had the highest abundance on genomes obtained in Asia and Oceania (73.9 and 59.2%, respectively), which cluster together and separated from those from other continents (**Figure 3A** and **Supplementary Figure 1A**). There were some discrepancies at country level, where two main clusters were obtained, the second one containing genomes from Taiwan, Israel, New Zealand, Luxemburg, and China, countries with no geographical or even socio-economical relation, which harbored the highest prevalences of *tet(O)* gene and *gyrA* (T86I) SNP (**Figure 3B** and **Supplementary Figure 1B**).

The *tet(O)* and bla_{OXA-61} genes were the most abundant on *C. coli* regardless of the continent, with prevalences ranging from 16.9 to 36.1% and 13.7–28.1% of genomes, respectively. The *aadE-Cc* and *aph(3′)-III* genes showed an important presence on European genomes (22.6 and 13.6%, respectively), while *ant(6')-Ia* had an important prevalence on North and South American genomes (11.8 and 15.6%, respectively) and the *gyrA (T86I)* SNP on Asian genomes (12.2%) (**Figure 3C**). At country level, using those countries with at least 20 genomes, the cluster formed by genomes from China, Egypt, Switzerland, Peru, Canada, United States, and Vietnam had a higher prevalence of a*ph(3′)-III*, bla_{OXA-61}, and *tet(O)* genes than the cluster formed by genomes from the United Kingdom, Sweden, Australia, India, and Chile (**Figure 3D** and **Supplementary Figure 1**).

Host Specialization and ARG-SNP Distribution by Source of Isolation

The analysis of the occurrence of *C. jejuni* CCs in each of the isolation sources, excluding human, showed three clear clusters of CCs. Chicken specialist and generalist CCs formed the

TABLE 1 | Most abundant ARG genotypes on the *C. jejuni* and *C. coli* genomes analyzed, ordered by decreasing relative abundance.

# Pattern	ARG genotype – *C. jejuni*	# ARGs	# Genomes	% Genomes	% Accumulate
1	*bla*$_{oxa-61}$	1	13,326	33.48	33.48
2	*bla*$_{oxa-61}$ - *tet(O)*	2	4,259	10.70	44.19
3	*bla*$_{oxa-61}$ -*gyrA* (T86I) - *tet(O)*	3	3,915	9.84	54.02
4	–	0	3,119	7.84	61.86
5	*bla*$_{OXA-61}$ - *gyrA* (T86I)	2	1,944	4.88	66.74
6	*tet(O)*	1	1,793	4.51	71.25
7	*bla*$_{oxa-61}$ -*gyrA* (T86I) - *tet(O/32/O)*	3	1,456	3.66	74.91
8	*bla*$_{oxa-61}$ - *tet(O)* - *aph(3′)-III*	3	860	2.16	77.07
9	*bla*$_{OXA-184}$	1	836	2.10	79.17
10	*gyrA* (T86I)	1	799	2.01	81.18
11	*gyrA* (T86I) - *tet(O)*	2	711	1.79	82.96
12	*bla*$_{OXA-447}$	1	529	1.33	84.29
13	*tet(O)* - *bla*$_{OXA-184}$	2	507	1.27	85.57
14	*bla*$_{oxa-61}$ - *tet(O/32/O)*	2	498	1.25	86.82
# Pattern	**ARG genotype – *C. coli***	**# ARGs**	**# Genomes**	**% Genomes**	**% Accumulate**
1	-	0	2,780	23.32	23.32
2	*bla*$_{OXA-61}$	1	1,236	10.37	33.69
3	*bla*$_{OXA-489}$	1	826	6.93	40.62
4	*bla*$_{OXA-61}$ - *tet(O)*	2	784	6.58	47.20
5	*tet(O)*	1	478	4.01	51.21
6	*bla*$_{OXA-61}$ - *tet(O)* - *aph(3′)-III*	3	417	3.50	54.70
7	*bla*$_{OXA-193}$	1	322	2.70	57.41
8	*bla*$_{0XA-193}$ - *tet(O)* - *aph(3′)-III*	3	302	2.53	59.94
9	*bla*$_{OXA-193}$ - *tet(O)*	2	284	2.38	62.32
10	*bla*$_{OXA-61}$ - *tet(O)* - *aadE-Cc*	3	283	2.37	64.70
11	*bla*$_{OXA-489}$ - *tet(O)*	2	269	2.26	66.95
12	*bla*$_{oxa-61}$ - *aph(3′)-VIIa*	2	201	1.69	68.64
13	*tet(O)* - *aadE-Cc*	2	196	1.64	70.28
14	*bla*$_{OXA-453}$ - *tet(O)*	2	186	1.56	71.84
15	*bla*$_{OXA-193}$ - *tet(O)* - *aadE-Cc*	3	181	1.52	73.36
16	*bla*$_{OXA-61}$ - *aadE-Cc*	2	173	1.45	74.81
17	*blaOXA-193* - *tet(O)* - *aph(3′)-VIIa*	3	167	1.40	76.21
18	*bla*$_{0XA-489}$ - *tet(O)* - *aph(3 ′)-III*	3	157	1.32	77.53
19	*tet(O)* - *aph(3′)-III*	2	124	1.04	78.57
20	*tet(O/32/O)*	1	123	1.03	79.60

Only ARG genotype patterns with relative abundance > 1% are presented.

biggest group, which was clearly subdivided into two sub-clusters according to host range. Interestingly, chicken was the most frequent source found on the generalist sub-cluster (**Figure 4A**). Previous studies (French et al., 2005; Griekspoor et al., 2010; Revez et al., 2011; Sheppard et al., 2014; Dearlove et al., 2016; Mourkas et al., 2020) have classified up to eight *C. jejuni* CCs as host generalist and 13 CCs as host specialist, including three CCs specialized in cattle infection, seven in chicken infection, and other three in wild bird infection or environmental persistence (**Table 2**). According to the clustering obtained, ST-460, ST-464, ST-574, ST-607, and ST-658 CCs, previously not classified according to their host specialist/generalist behavior, can be classified as chicken specialist CCs, while ST-52 CC can be classified as a generalist CC (**Figure 4A** and **Table 2**). On the other hand, ST-283 CC, previously described as a specialist chicken CC, was not located within the chicken specialist sub-cluster (**Figure 4A**), so a change to generalist host range can be proposed according to the data here presented. Another important observed cluster was the cluster of cattle specialist CCs, which also included the ST-22 CC, previously described as generalist (**Figure 4A**). In this case, the percentage of genomes assigned to the cow source among cattle specialist CCs, which ranged from 44.1 to 66.7% (excluding genomes isolated from human), was not as high as that of genomes assigned to chicken source for chicken specialist CCs, which was in all cases above 90%. In addition, cattle specialist CCs had similar percentages of abundance in the cow source than generalist CCs in the chicken source (**Figure 4A** and **Supplementary Figure 2A**). Therefore, it is important to remark the high level of specialization of those CCs considered as chicken specialist, compared to other specialist groups. Moreover, a ST host specialization analysis performed with the most abundant CCs showed that ST-45 CC harbors STs

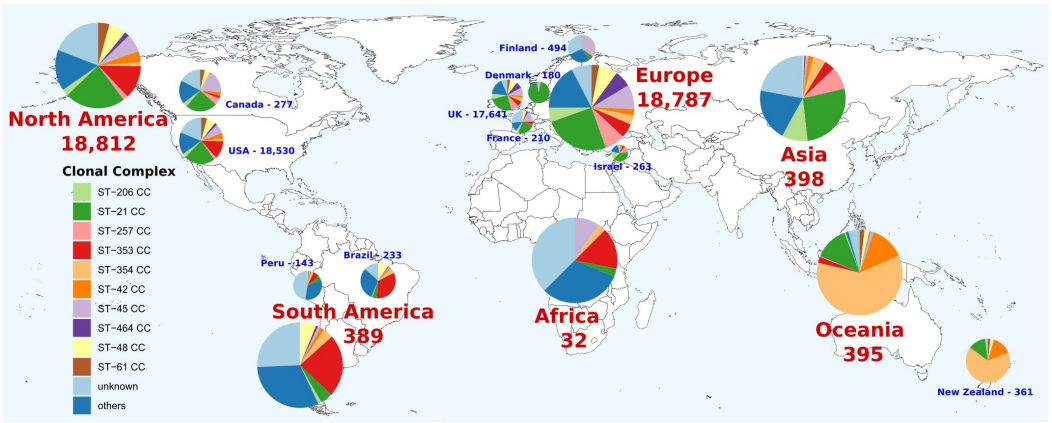

FIGURE 2 | Geographical distribution of *C. jejuni* clonal complexes (CCs). Only countries with more than 100 genomes were represented. The number indicates the genomes available on the corresponding continent or country. Colors within pie charts represent the percentage of genomes belonging to the main CCs, with low abundant CCs grouped as "others" and genomes with unknown CCs as "unknown."

with medium levels of chicken specialization, while ST-353 and ST-354 CCs contain STs highly specialized for chicken (**Figure 3B** and **Supplementary Figure 2B**). Finally, the ST-21 CC contains three STs specialized in cattle infection (ST-8, ST-806, and ST-982), two specialized in chicken infection (ST-50 and ST-53), one ST specialized on sheep infection (ST-262), and two generalist STs (ST-19 and ST-21) (**Figure 4B** and **Supplementary Figure 2B**). The ST-858 CC, which was the main one found for *C. coli* genomes, harbors up to seven STs (ST-4425, ST-830, ST-1119, ST-872, ST-1614, ST-828, and ST-829) that could be considered as chicken infection specialist, three pig specialized STs (ST-1058, ST-854, and ST-887), one cattle specialist ST (ST-1068), and five host generalist STs (ST-1016, ST-1055, ST-827, ST-825, and ST-962) (**Figure 4C** and **Supplementary Figure 2C**). The complete list of STs analyzed, from both *C. jejuni* and *C. coli* genomes, and their host specilization were indicated on **Supplementary Table 3**.

The analysis of the occurrence of AR determinants in genomes from each of the isolation sources revealed three main clusters of both *C. jejuni* and *C. coli* genomes. The main cluster of *C. jejuni* genomes related to food production sources, which showed a higher prevalence of bla_{OXA-61} (from 44.7 to 100%) and of the *gyrA* (T86I) SNP (from 1.5 to 41.1%) than genomes from the second cluster obtained [from 0 to 12.84% for bla_{OXA-61}; from 1.3 to 4.6% for the *gyrA* (T86I) SNP] (**Figure 5A** and **Supplementary Figure 3A**). This second cluster harbored genomes from isolates coming from host animals not linked to industrialized food production, such as jackdaws, crows, and other wild birds (**Figure 5B**). The lower prevalence of bla_{OXA-61} and the higher diversity of other genes associated with resistance to β-lactams among the genomes from this second cluster indicate a lower selection pressure to keep this prevalent bla_{OXA-61} gene in non-livestock animal populations. The last cluster of *C. jejuni* genomes was formed by genomes from duck, other animals, and environmental waters and presented an intermediate prevalence of bla_{OXA-61} compared to the other two clusters obtained (**Figure 5A** and **Supplementary Figure 3A**). Similar clusters were found for *C. coli* genomes, with pig, cow, and turkey forming the cluster with the highest prevalence of *tet(O)* and bla_{OXA-61}, with *tet(O)* being more prevalent than on *C. jejuni* genomes while bla_{OXA-61} was less prevalent (**Figure 5B** and **Supplementary Figure 3B**). A second cluster of *C. coli* genomes was formed by genomes from dairy products, humans, chicken, and other animals and was characterized by an intermediate prevalence of *tet(O)* and bla_{OXA-61} genes, compared to the other clusters (**Figure 5B** and **Supplementary Figure 3B**). Finally, the third cluster, with the lowest prevalence of AR determinants, was formed by genomes from environmental samples, sheep, and duck. It is important to highlight the high prevalence of *aadE-Cc* on genomes from pigs and $bla_{OXA-489}$ on genomes from sheep (**Figure 5B**).

ARG-SNP Distribution Among *C. jejuni* CCs

Up to four main clusters were obtained in the analyses performed at antibiotic family level. Cluster 1 harbored four CCs with the highest prevalence of determinants of resistance to tetracyclines and quinolones, while cluster 2, formed by 20 CCs, had the second main prevalence for tetracycline and quinolone resistance determinants (**Figure 6A** and **Supplementary Figure 4A**). Cluster 3, formed by seven CCs, had the lowest prevalence of quinolone and tetracycline resistance determinants (**Figure 6A** and **Supplementary Figure 4A**), and cluster 4, formed by six CCs, had the lowest abundance of β-lactam resistance determinants (**Figure 6A** and **Supplementary Figure 4A**). At gene level, three clusters were found: cluster 1, harboring four CCs with a high prevalence of *gyrA* (T86I) SNP, bla_{OXA-61}, and *tet(O)*; cluster 2, composed of 20 CCs with a high prevalence of bla_{OXA-61}; and cluster 3, with 14 CCs showing the lowest prevalence of *gyrA* (T86I) SNP, bla_{OXA-61}, and *tet(O)* (**Figure 6B** and **Supplementary Figure 4B**). No clear association between genotype patterns at antibiotic family

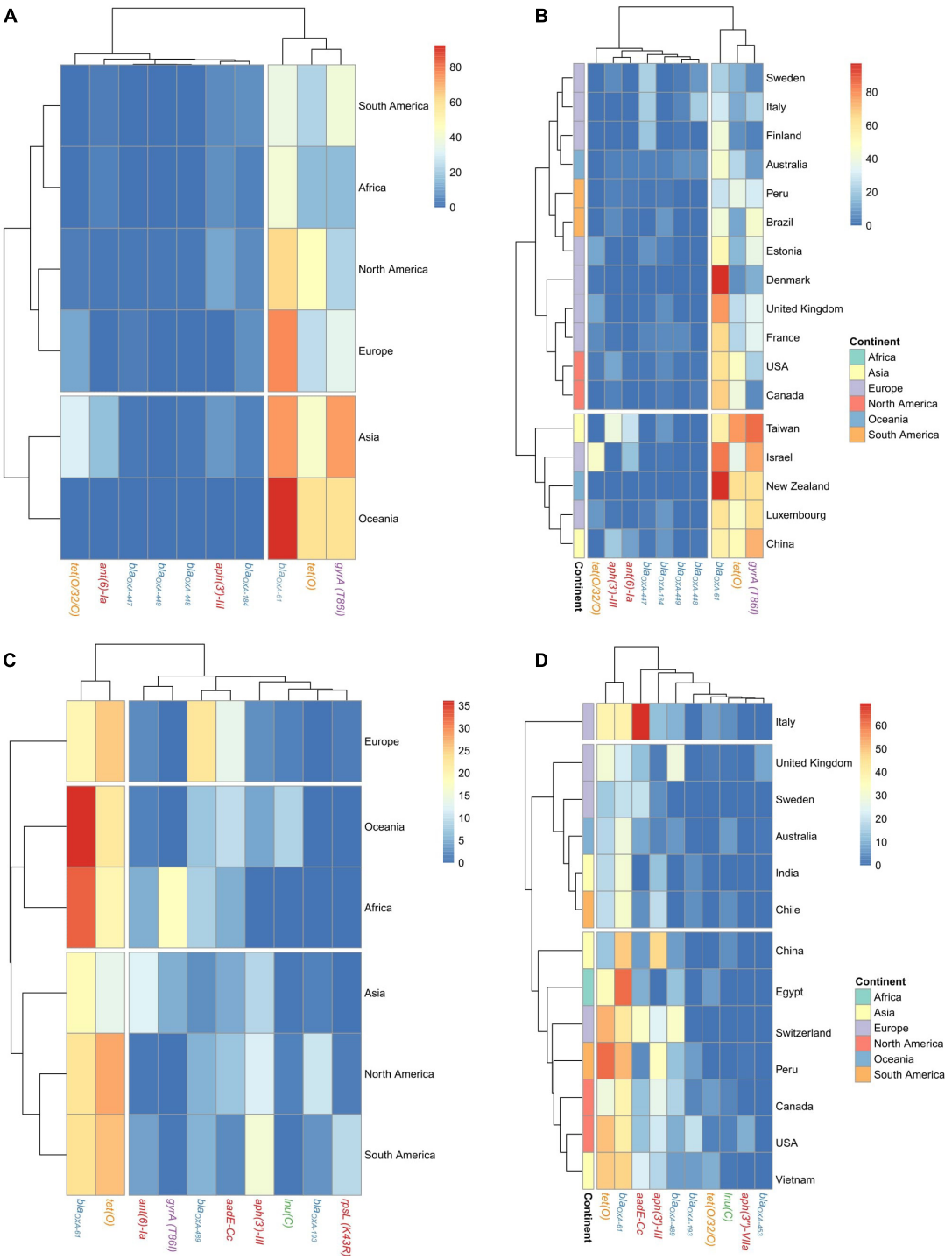

FIGURE 3 | Geographical distribution of AR genes and SNPs on *C. jejuni* and *C. coli* genomes. Heatmap plots showing the distribution of the main AR genes and SNPs on *C. jejuni* genomes among **(A)** continents and **(B)** countries and on *C. coli* genomes among **(C)** continents and **(D)** countries. Countries with less than 20 genomes were discarded for this analysis. Gene names are colored according to the corresponding antimicrobial family (blue: beta-lactams, orange: tetracyclines, red: aminoglycosides, purple: quinolones, and green: MLSP).

level and host specialist/generalist status was observed. As an example, cluster 1, characterized by a high prevalence of ARGs linked to resistance to tetracyclines and quinolones, was mainly formed by chicken specialist CCs, but other chicken specialist CCs were located across the other three remaining clusters (**Figure 6A**). A similar absence of a

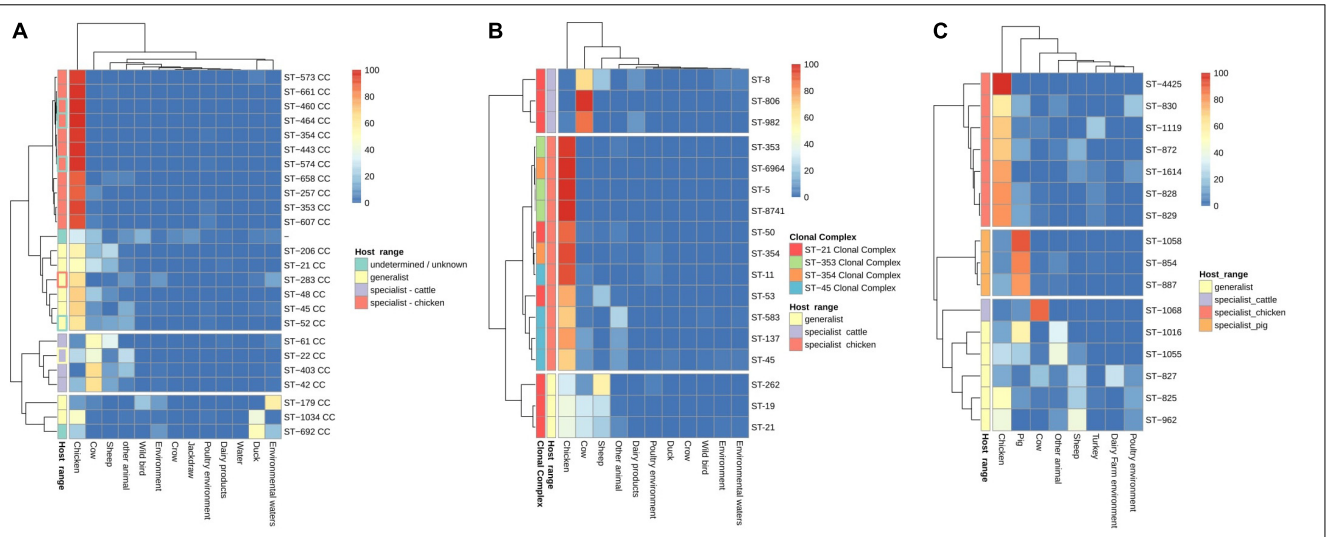

FIGURE 4 | CC and sequence type (ST) host specialization. Heatmap plot showing **(A)** percentage of *C. jejuni* genomes per CC, **(B)** *C. jejuni* genomes per ST within the four main CCs, and **(C)** *C. coli* genomes per ST within ST-828 CC belonging to each isolation source. Genomes belonging to the human isolation source were removed before the calculation of the relative abundances, and CCs/STs with 20 or less genomes were removed to avoid biases due to the low number of genomes. The *Host range* classification of *C. jejuni* CCs is according to previous publications (see **Table 1**), except for those with highlighted edges, where edge color indicates previous classification while fill color indicates the new assignment, according to the results obtained in the current study.

clear association with the host range of each CC was observed at gene level.

Temporal Changes of AR Determinants on Europe

Focusing on temporal changes in the repertoire of AR determinants among the *C. jejuni* European genomes, there was a clear stability in the occurrence of β-lactam ARGs along time (with mean prevalence values per each 5-year period ranging from of 88.8 to 93.3%), while an important increase was observed in the prevalence of determinants of resistance to tetracyclines, from a mean value of 29.8% in the 2000–2004 5-year period to 44.0% in the 2015–2018 period, and quinolones, from 21.0% in the 2000–2004 5-year period to 42.4% in the 2015–2018 period (**Figure 7A** and **Supplementary Figure 5A**).

These observations were linked to an increase in the occurrence of both the *tet(O)* and *tet(O/W/O)* genes and the *gyrA* (T86I) SNP (**Figure 7B** and **Supplementary Figure 5B**), which were the main determinants of resistance to tetracyclines and quinolones, respectively. Furthermore, the increases along time in the *tet(O/W/O)* gene and the *gyrA* (T86I) SNP were observed in both human and chicken genomes (**Figure 7C** and **Supplementary Figures 5C,D**). Finally, the enhanced occurrence over time of resistance to tetracyclines and quinolones was mainly correlated with a significant increase in bla_{OXA-61}–*tet(O)*–*gyrA* (T86I) and bla_{OXA-61}–*tet(O/W/O)*–*gyrA* (T86I) multi-drug resistance genotype patterns on *C. jejuni* genomes obtained from human and chicken, which showed mean prevalence values of 10.5 and 6.1% in the 2000–2004 5-year period and 25.6 and 29.9% in the 2015–2018 period, respectively, representing a significant increase in co-occurrence of such genes (**Figure 7D** and **Supplementary Figures 5E,F**).

Similar temporal analyses were performed on *C. coli* genomes, but no clear trends of AR determinant increase along time were detected. The observed slight increases in the prevalence of determinants of aminoglycoside and tetracycline resistance (**Figure 8A**), highly correlated with an increase of *addE-Cc* and *tet(O)* prevalence (**Figure 8B**), were associated with a differential prevalence on genomes from cows and pigs, with more *C. coli* genomes being sequenced on the last years (**Figures 8C,D**).

DISCUSSION

At a first global view, the distribution of *C. jejuni* CCs was different among continents, with ST-21 CC as the main one in North America, Europe, and Asia; ST-353 CC in South America and Africa, and ST-354 CC in Oceania. Moreover, this distribution by continent was not maintained among all the countries within a given continent, i.e., Denmark harbored a strong dominance of ST-21 CC genomes while Finland had ST-45 CC as the most abundant CC. Surprisingly, a lack of main association within developing–developed countries and AR composition on *C. jejuni* and *C. coli* genomes was clearly found, maybe due to the unequal distribution of both CC-STs and host origin of isolation among countries and even the amount of sequenced genomes for each country.

Results obtained in the current study reveal the association of certain *C. jejuni* CCs and both *C. jejuni* and *C. coli* STs to particular isolation sources. In this regard, it is worth mentioning the high abundance of CCs linked to chicken as a host. Most of these *C. jejuni* CCs had been already classified as a chicken specialist (Sheppard et al., 2014), and our study confirmed these previous associations, while ST-283 CC, previously described as a chicken specialist (Sheppard et al., 2014), can be considered as a

TABLE 2 | Clonal complex classification by their host specialization according to previous publications and to the results of the current study.

Species	Clonal complex	# Genomes	# Non-human genomes	Host specialization	References
C. jejuni	ST-21 CC	9,477	5,660	Generalist	Sheppard et al., 2014; Dearlove et al., 2016
C. jejuni	ST-22 CC	799	497	Generalist *	Revez et al., 2011
C. jejuni	ST-4I CC	28	22	–	–
C. jejuni	ST-42 CC	1,390	1,024	Specialist_cattle	Sheppard et al., 2014
C. jejuni	ST-45 CC	3,103	1,978	Generalist	Sheppard et al., 2014; Dearlove et al., 2016
C. jejuni	ST-48 CC	2,667	1,466	Generalist	Sheppard et al., 2014
C. jejuni	ST-49 CC	381	280	–	–
C. jejuni	ST-52 CC	639	390	Generalist	Present study
C. jejuni	ST-61 CC	1,422	1,110	Specialist_cattle	Sheppard et al., 2014; Mourkas et al., 2020
C. jejuni	ST-177 CC	26	23	Specialist_wild-birds/envs	Sheppard et al., 2014
C. jejuni	ST-179 CC	208	198	Specialist_wild-birds/envs	Sheppard et al., 2014
C. jejuni	ST-206 CC	1,570	677	Generalist	Sheppard et al., 2014
C. jejuni	ST-257 CC	1,857	797	Specialist_chicken	Sheppard et al., 2014
C. jejuni	ST-283 CC	290	127	Specialist_chicken *	Sheppard et al., 2014
C. jejuni	ST-353 CC	3,550	2,643	Specialist_chicken	Sheppard et al., 2014
C. jejuni	ST-354 CC	1,220	598	Specialist_chicken	Sheppard et al., 2014
C. jejuni	ST-362 CC	31	9	–	–
C. jejuni	ST-403 CC	533	362	Specialist_cattle	French et al., 2005
C. jejuni	ST-433 CC	16	7	–	–
C. jejuni	ST-443 CC	905	482	Specialist_chicken	Sheppard et al., 2014
C. jejuni	ST-446 CC	55	16	–	–
C. jejuni	ST-460 CC	256	180	Specialist_chicken	Present study
C. jejuni	ST-464 CC	1,531	679	Specialist_chicken	Present study
C. jejuni	ST-508 CC	393	299	–	–
C. jejuni	ST-573 CC	216	177	Specialist_chicken	Sheppard et al., 2014
C. jejuni	ST-574 CC	326	91	Specialist_chicken	Present study
C. jejuni	ST-607 CC	447	320	Specialist_chicken	Present study
C. jejuni	ST-658 CC	368	68	Specialist_chicken	Present study
C. jejuni	ST-661 CC	117	86	Specialist_chicken	Sheppard et al., 2014
C. jejuni	ST-677 CC	145	17	Generalist	Griekspoor et al., 2010
C. jejuni	ST-682 CC	9	9	Specialist_wild-birds/envs	Sheppard et al., 2014
C. jejuni	ST-692 CC	87	64	–	–
C. jejuni	ST–702 CC	18	16	–	–
C. jejuni	ST-828 CC	23	14	Generalist	Dearlove et al., 2016
C. jejuni	ST-952 CC	23	19	–	–
C. jejuni	ST-1034 CC	120	73	Generalist	Griekspoor et al., 2010
C. jejuni	ST-1150 CC	1	1	–	–
C. jejuni	ST-1275 CC	23	16	–	–
C. jejuni	ST-1287 CC	31	20	–	–
C. jejuni	ST-1304 CC	6	6	–	–
C. jejuni	ST-1332 CC	31	25	–	–
C. jejuni	Unknown	5,460	4,472	–	–
C. coli	ST-828 CC	10,106	8,590	–	–
C. coli	ST-1150 CC	174	161	–	–
C. coli	others	39	32	–	–
C. coli	Unknown	1,601	1,368	–	–

*Those CCs with less than 20 genomes with a known non-human source of isolation were not employed for the specialist/generalist host association analysis. The asterisk indicates CCs with new classification of host specialism/generalist behavior proposed, according to the data obtained in the current study. ST-22 CC is proposed to be cattle specialist instead of generalist and ST-283 CC to be generalist instead of chicken specialist (see **Figure 4**).*

generalist according to our findings. Moreover, among previously not assigned CCs, ST-460, ST-464, ST-574, ST-607, and ST-658 CCs can be considered as a chicken specialist, ST-52 CC as a generalist, and ST-22 CC as a cattle specialist, according to the results here reported. The high abundance of genomes from generalist ST-21 and chicken specialist ST-353 CCs agrees with previous reports that have described these CCs as highly prevalent among clinical, food, and animal samples from Israel

(Rokney et al., 2018); clinical cases, dairy cattle, and broiler products in Lithuania (Ramonaite et al., 2017); chicken in Senegal (Kinana et al., 2006); or human and broiler carcasses in Belgium (Elhadidy et al., 2018). The high abundance of ST-21, ST-48, ST-45, and ST-257 CCs on isolates obtained from human samples has been previously highlighted in the United Kingdom (Haldenby et al., 2020). Interestingly, the STs within ST-45 CC, previously considered as a generalist CC, could be considered as chicken specialist STs. Conclusions on host specialization have to be taken carefully. For example, the generalist ST-21 CC harbored STs considered as chicken specialist (ST-50 and ST-53), cow specialist (ST-8, ST-806, and ST-982), and generalist (ST-19, ST-21, and ST-262). This host specialization differences within ST-21 CC could be due to the paraphyletic phylogeny of this CC, which was found as the ancestor CC of the cattle specialist ST-61 CC (Sheppard et al., 2014; Mourkas et al., 2020). The prevalence of ST-353, ST-354, ST-50, and ST-45 on chicken hosts and ST-21 as a host generalist were previously reported by using all the profiles on PubMLST (Pascoe et al., 2020), which are twice the number of genomes available on that web repository and employed in the current study. That supports the proposed host specialization classification, despite the possible biases introduced due to the isolates selected or employed for whole genome sequencing within all the available collections of bacterial isolates.

Determinants of resistance to β-lactams and tetracyclines were the most extended among the 39,798 *C. jejuni* and 11,920 *C. coli* genomes analyzed, with bla_{OXA-61} as the main β-lactam resistance factor on *C. jejuni*; bla_{OXA-61}, $bla_{OXA-489}$, *and* $bla_{OXA-193}$ on *C. coli*; and *tet(O)* for tetracycline resistance on both species. Determinants of resistance to quinolones [due mainly to the *gyrA* (T86I) SNP] and to aminoglycosides [by *aph(3′)-III*, *aadE-Cc*, and *aph(3′)-VIIa* genes, among others) were also highly prevalent on *C. jejuni* and *C. coli* genomes, respectively. Phenotypic resistance to β-lactam antibiotics in *C. jejuni* is frequently linked to the production of β-lactamase enzymes, encoded by bla_{OXA} genes (Jonker and Picard, 2010), while tetracycline and quinolone resistance has been often associated with the *tet(O)* gene (Meistere et al., 2019) and the *gyrA* (T86I) SNP, respectively (Kinana et al., 2006; Meistere et al., 2019; Abraham et al., 2020; Joensen et al., 2020). Indeed, a high prevalence of bla_{OXA-61} and *tet(O)* has been found on *C. jejuni* isolated from human, chicken, and cattle in Spain in a study where SNPs were not screened (Mourkas et al., 2019) and on *Campylobacter* spp. in a global study analyzing 237 genomes from the RefSeq NCBI database (Rivera-Mendoza et al., 2020). Moreover, AR determinants of aminoglycoside resistance on *C. coli* and quinolone resistance on *C. jejuni* were mainly found on genomes associated with multidrug resistance, together with AR determinants for β-lactams and tetracyclines.

The increase in resistance to quinolones on *Campylobacter* isolates during the last years has been reported worldwide, including the United States (Nachamkin et al., 2002), China (Wang et al., 2016), France (Gallay et al., 2007), Vietnam (Nguyen et al., 2016), and Peru (Pollett et al., 2012), among other countries, and the usage of fluoroquinolones in poultry farms has been associated with an increase in resistance to quinolones in chicken and human *Campylobacter* isolates (Wieczorek and Osek, 2013). An increased occurrence of determinants of resistance to β-lactams, tetracyclines, and quinolones was found among those *C. jejuni* and *C. coli* genomes from human and food animal origin. The clear differences observed in the resistome between *Campylobacter* genomes from animals employed for food production and those from non-livestock animals can be likely related to the selective pressure exerted by antibiotic use in veterinary settings. Isolates from livestock species were characterized by a higher incidence of bla_{OXA-61}, associated with resistance to β-lactam antibiotics, such as amoxicillin or ampicillin, antibiotics that are frequently employed for veterinary uses. Quinolones and tetracyclines are still available for treatment of livestock all over the world (WHO, 2017), so this is likely the main cause of the resistance levels found for such antibiotics on isolates from food-related animals. Antibiotic resistance represents a great concern mainly in livestock animals due to the misuse of antibiotics. As an example, erythromycin (macrolide), ampicillin (β-lactam), tetracycline (tetracycline), nalidixic acid, and ciprofloxacin (both belonging to the quinolones family) are the drugs toward which the most resistant isolates were found in a recent review on *Campylobacter* spp. in Sub-Saharan African countries (Hlashwayo et al., 2020), which is in agreement with results found globally or also with the temporal changes among European isolates described in the current study.

Although the current study does not involve a source attribution analysis, the similar ARG genotype patterns and levels of prevalence of bla_{OXA-61}, *tet(O)*, and *gyrA* (T86I) found among human, chicken, and turkey *C. jejuni* genomes, and of bla_{OXA-61} and *tet(O)* among human and chicken *C. coli* genomes, suggest a high level of relatedness among isolates from these origins. Poultry has been reported as the main source of human infection in studies carried out in different locations including Denmark (Joensen et al., 2020), Sub-Saharan Africa (Gahamanyi et al., 2020), or Lithuania (Ramonaite et al., 2017). On the other hand, other authors have identified as important sources of human infection both chicken and ruminants in France (Berthenet et al., 2019), the United States (Kelley et al., 2020), Israel (Rokney et al., 2018), and Sub-Saharan Africa (Hlashwayo et al., 2020) or chicken and wild birds in Baltic countries (Mäesaar et al., 2020). Moreover, the detection of no major differences in the resistance profiles among isolates from different points of the chicken meat processing chain (Dramé et al., 2020) suggests that AR spread is originated on upstream steps, such as the animal feeding, where the animals are exposed to antibiotics. A clear link between human and chicken *Campylobacter* isolates has been previously demonstrated by combining pulsed-field gel electrophoresis (PFGE) to subtype strains and antibiotic resistance phenotypic testing (Zhao et al., 2015).

The spread of AR genes among *Campylobacter* isolates from humans, animals, and the environment has been reported previously (Mourkas et al., 2019), and it should explain the presence of high levels of ARGs conferring resistance to tetracyclines in human isolates despite these antibiotics are not clinically employed in human medicine. Moreover, tetracycline is broadly employed in the poultry industry worldwide (Van Boeckel et al., 2015), which could be an important reservoir for resistant strains. Furthermore, higher levels of resistant *C. jejuni*

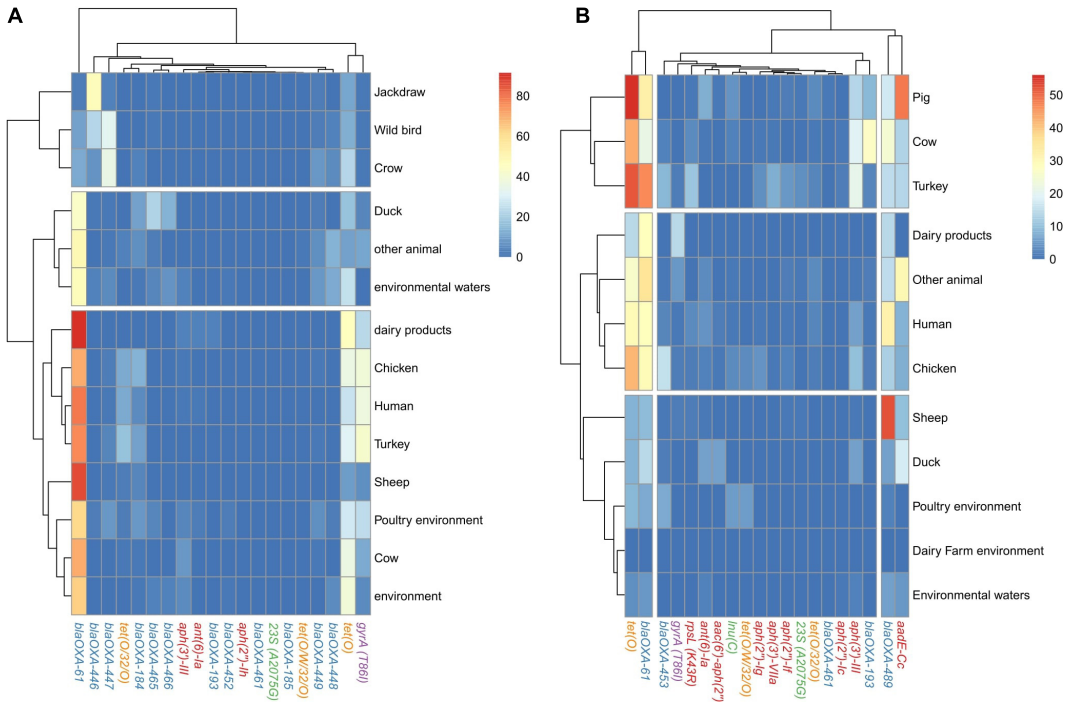

FIGURE 5 | AR genes and SNP distribution among *C. jejuni* and *C. coli* genomes by source of isolation. Heatmap plot showing the percentage of **(A)** *C. jejuni* genomes and **(B)** *C. coli* genomes per isolation source carrying ARGs or SNPs associated with AR, showed in columns. Only the main 20 ARGs or SNPs are presented, because the clustering by isolation source remained the same as when the 120 and 68 AR determinants were used for *C. jejuni* and *C. coli*, respectively (data not shown). Only isolation sources containing more than 50 genomes are presented. Gene names are colored according to the corresponding antimicrobial family (blue: beta-lactams, orange: tetracyclines, red: aminoglycosides, purple: quinolones, and green: MLSP).

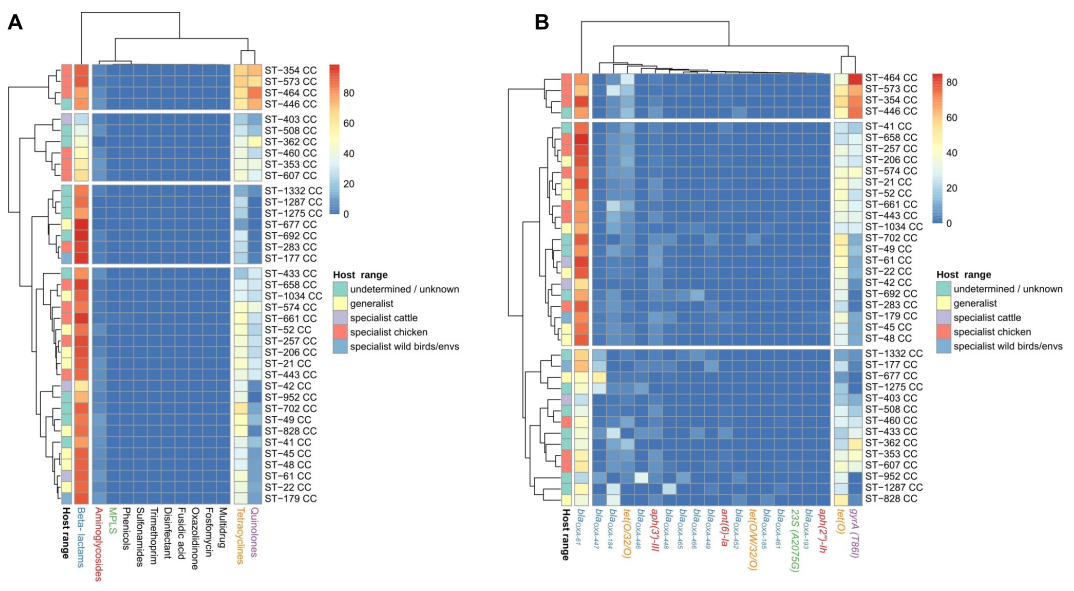

FIGURE 6 | AR genes and SNP distribution among *C. jejuni* CCs. Heatmap plot showing the percentage of genomes per CC carrying **(A)** ARGs or SNPs associated with resistance to antibiotic families and **(B)** particular ARGs or SNPs, showed in columns. Only the main 20 ARGs or SNPs are presented, because the clustering by isolation source remained the same as when the 120 AR determinants were used (data not shown). The *Host_range* classification of CCs is according to previous publications (see **Table 1**), except for those with highlighted edges, where edge color indicates previous classification while fill color indicates the new assignment, according to the results showed in **Figure 4**. Gene names are colored according to the corresponding antimicrobial family (blue: beta-lactams, orange: tetracyclines, red: aminoglycosides, purple: quinolones, and green: MLSP).

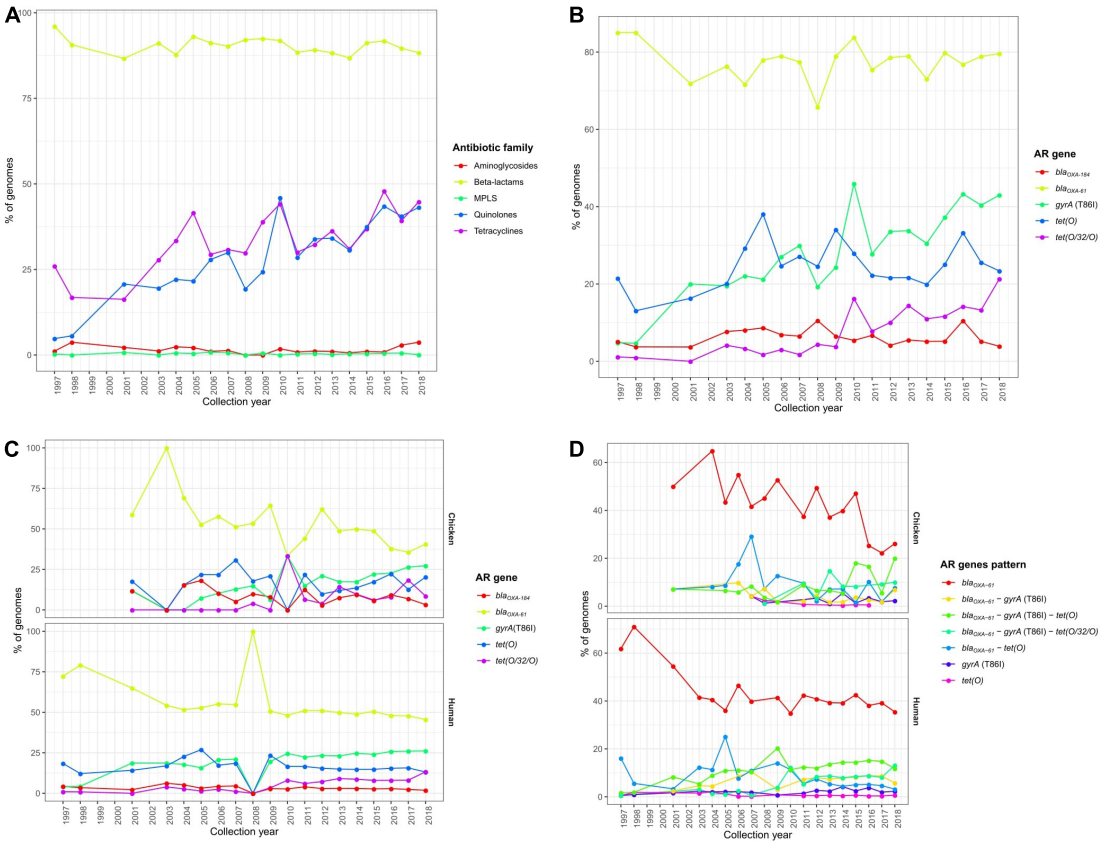

FIGURE 7 | Temporal changes in the resistome of European *C. jejuni* genomes. Prevalence of **(A)** ARGs or SNPs associated with resistance to different antibiotic families and **(B)** the main ARGs or SNPs found on all European *C. jejuni* genomes and **(C)** main ARGs or SNPs and **(D)** AR patterns on *C. jejuni* genomes from chicken and human sources along the last 20 years.

and *C. coli* isolates were observed among isolates from pigs than from wild boars in Italy (Marotta et al., 2020), where an important amount of multidrug-resistant strains were isolated from pigs, being the over-use of antibiotics the main cause of the spread of multidrug resistance among pig isolates.

The bla_{OXA-61} gene is highly prevalent among *Campylobacter* spp. isolated from poultry (Griggs et al., 2009). The high diversity and abundance of determinants of resistance to β-lactams different to bla_{OXA-61} among isolates from non-food-related animals and environments could be due to the ability of *Campylobacter* spp. for intrinsic production of β-lactamases in the absence of selective pressure (Cody et al., 2012). The bla_{OXA-61} gene, chromosomally encoded (Rivera-Mendoza et al., 2020), can generate many variants with few SNPs, differing from other bla_{oxa}-encoded proteins in only one (e.g., $bla_{OXA-193}$) or two amino acids (e.g., $bla_{OXA-489}$). The bla_{OXA-61} gene and aminoglycoside resistance genes are confined to *C. jejuni* and *C. coli* genomes within *Campylobacter* species, while *gyrA* T86I SNP was mostly presented on *C. jejuni* (Rivera-Mendoza et al., 2020). Unfortunately, ACT to ATT mutation on *gyrA* position 86, the most common on *C. coli* strains with resistance to quinolones (Zirnstein et al., 2000), was not included on PointFinder database, which includes ACA to ATA mutation, the most prevalent on *C. jejuni*. So that, the prevalence of quinolone resistance among *C. coli* genomes found on the present study can be due to this technical issue.

The clonal complexes ST-354, ST-573, ST-464, and ST-446, the first three considered chicken specialist, were the CCs with the highest prevalence of ARGs linked to β-lactam, tetracycline, and quinolone resistance, due to the presence of the bla_{OXA-61}, *tet(O)*, and *gyrA* (T86I) determinants for all of them. Some of these CCs (ST-354, ST-446, and ST-464) have been previously associated with ciprofloxacin (quinolone) resistance in human isolates (Cody et al., 2012). ARGs were more randomly distributed among CCs than among isolation sources. Hence, ARG genotype patterns were more closely related to the source of isolation (and probably the use of antibiotics) than to the lineage of the strains, as also remarked previously (Cody et al., 2012).

An increase in the occurrence of genotypes of resistance to tetracyclines and quinolones, mainly due to the spread of bla_{OXA-61}–*gyrA* (T86I)–*tet(O)* and bla_{OXA-61}–*gyrA (T86I)*–*tet(O/W/O)* multi-drug resistance genotypes, was observed along the last 20 years in European *C. jejuni* genomes. The main reason for this temporal trend could be the continuous

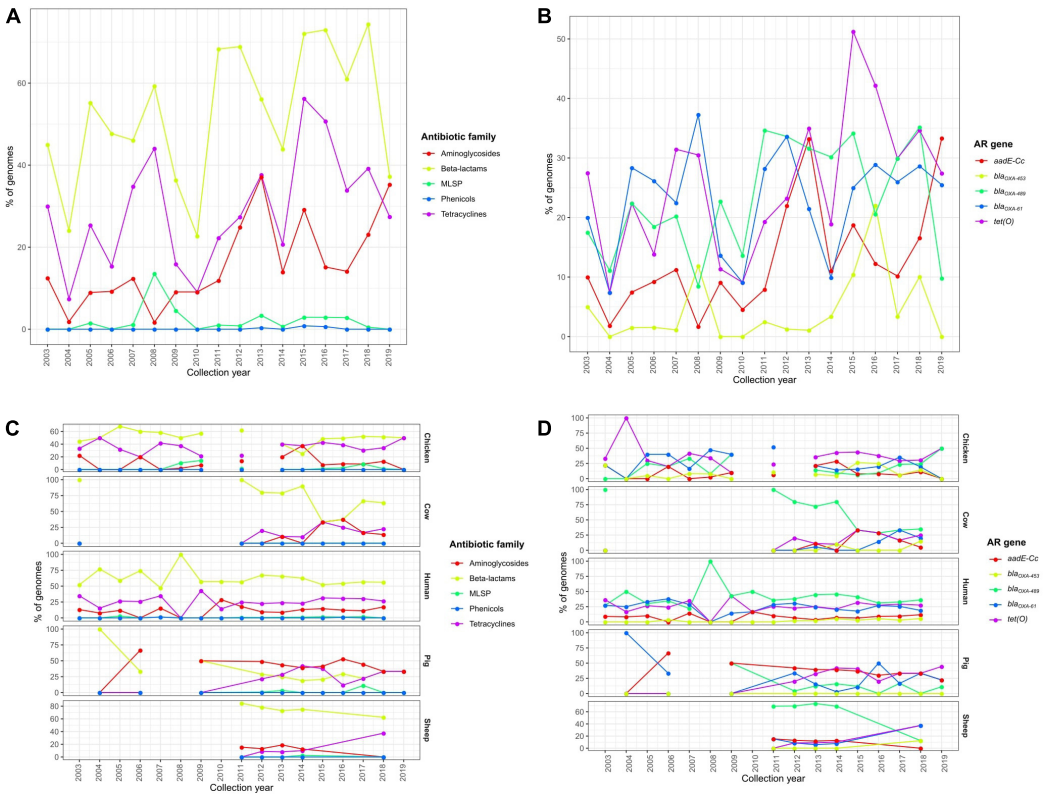

FIGURE 8 | Temporal changes in the resistome of European *C. coli* genomes. Prevalence of **(A)** ARGs or SNPs associated with resistance to different antibiotic families and **(B)** the main ARGs or SNPs found on all European *C. coli* genomes and **(C)** the main ARGs or SNPs and **(D)** AR patterns on *C. jejuni* genomes from chicken, cow, human, pig, and sheep sources along the last 20 years.

misuse of tetracyclines and quinolones, still broadly employed on livestock all over the world (WHO, 2017). Interestingly, fluoroquinolones are not utilized in Australian livestock, where *Campylobacter* isolates from chicken meat show very scarce levels of resistance to fluoroquinolones, always due to *gyrA* (T86I) SNPs (Abraham et al., 2020). On the other hand, an increase over time in the prevalence of the *gyrA* (T86I) SNP among isolates of *C. jejuni* associated with human gastrointestinal disease has been reported in the United Kingdom from 1993–1996 to 2008–2009 and even to 2016–2017 (Haldenby et al., 2020). This spread of resistance to quinolones is an important concern in some countries, such as Latvia, where resistance to quinolones was found, due to *gyrA* (T86I), in 90% of the *C. jejuni* isolates obtained from clinical and food-related sources from 2008 to 2016 (Meistere et al., 2019). Remarkably, it has been demonstrated that high levels of fluoroquinolone resistance can persist in poultry even after discontinued use of these antibiotics (Price et al., 2005) and that resistance can rapidly emerge in poultry flocks (Humphrey et al., 2005). Some fitness benefits linked to the *gyrA* (T86I) SNP could be the reason for such high resistance prevalence in settings where the use of fluoroquinolones has been reduced, as has been proposed by Haldenby et al. (2020). Another example of temporal increases in fluoroquinolone resistance among *Campylobacter* species was observed on fecal samples from cattle in France between 2002 and 2006, with the fluoroquinolone resistance rate rising from 29.7% to 70.4% (Châtre et al., 2010).

In conclusion, the current work represents the biggest study to date, as far as we know, of the resistome markers harbored in *C. jejuni/coli* genomes, with 39,978 *C. jejuni* and 11,920 *C. coli* genomes *in silico* screened to search both for antimicrobial resistance genes and SNPs conferring antimicrobial resistance. The results obtained suggest the association between antimicrobial use in veterinary settings, in particular in poultry production, and the spread of resistance to Be, Qu, and Te and evidence the rapid expansion in Europe in the last two decades of determinants of resistance to these antibiotic families, especially among poultry and human isolates. The workflow employed is available to be used in future global resistome analyses for other species of interest, or even to be adapted to other kinds of microbiome analyses.

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.

AUTHOR CONTRIBUTIONS

AÁ-O and JC-D created the study design. JC-D and PG wrote and employed the Ruby and R scripts and downloaded genomes, made data analysis, and plotted the figures. JC-D and PG wrote the first draft of the manuscript and AÁ-O revised it. All authors revised the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

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Genomic Investigation of *Salmonella* Isolates Recovered From a Pig Slaughtering Process in Hangzhou, China

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The pig industry is the principal source of meat products in China, and the presence of pathogens in pig-borne meat is a crucial threat to public health. *Salmonella* is the major pathogen associated with pig-borne diseases. However, route surveillance by genomic platforms along the food chain is still limited in China. Here, we conducted a study to evaluate the dynamic prevalence of *Salmonella* in a pig slaughtering process in Hangzhou, Zhejiang Province, China. Fifty-five of 226 (24.37%) samples were positive for *Salmonella*; from them, 78 different isolates were selected and subjected to whole genome sequencing followed by bioinformatics analyses to determine serovar distribution, MLST patterns, antimicrobial resistance genes, plasmid replicons, and virulence factors. Moreover, phenotypic antimicrobial resistance was performed using the broth dilution method against 14 antimicrobial agents belonging to 10 antimicrobial classes. Our results showed that samples collected from the dehairing area (66.66%) and the splitting area (57.14%) were the most contaminated. Phenotypic antimicrobial resistance classified 67 of 78 isolates (85.90%) as having multidrug resistance (MDR), while the highest resistance was observed in tetracycline (85.90%; 67/78) followed by ampicillin (84.62%; 66/78), chloramphenicol (71.80%; 56/78), and nalidixic acid (61.54%; 48/78). Additionally, serovar prediction showed the dominance of *Salmonella* Typhimurium ST19 (51.28%; 40/78) among the 78 studied isolates, while plasmid prediction reported the dominance of IncHI2A_1 (20.51%; 16/78), followed by IncX1_1 (17.95%; 14/78) and IncHI2_1 (11.54%; 9/78). Virulence factor prediction showed the detection of *cdtB* gene encoding typhoid toxins in two *Salmonella* Goldcoast ST358 and one *Salmonella* Typhimurium ST19, while one isolate of *Salmonella* London ST155 was positive for genes encoding for the siderophore "yersiniabactin" and the gene *senB* encoding for enterotoxin production. From this study, we conclude that pig slaughterhouses are critical points for the dissemination of virulent

and multidrug-resistant *Salmonella* isolates along the food chain which require the implementation of management systems to control the critical points. Moreover, there is an urgent need for the implementation of the whole genome sequencing platform to monitor the emergence of virulent and multidrug-resistant clones along the food chain.

Keywords: *Salmonella*, antimicrobial resistance, plasmid replicons, virulence factors, pig slaughterhouse, whole genome sequencing

INTRODUCTION

Salmonellosis is a global zoonotic disease, caused by *Salmonella* and characterized by self-limited gastroenteritis in immunocompetent adults, in which typical symptoms like diarrhea, fever, abdominal cramps, and vomiting occur between 6 and 72 h (usually 12–36 h) after ingestion of bacteria and the illness lasts from 2 to 7 days [World Health Organisation (WHO), 2018]. It might also cause severe invasive infection, particularly in immunocompromised patients (Deen et al., 2012; Xu X. et al., 2020). Recently, it was estimated that *Salmonella* was responsible for about 180 million (9%) of the diarrheal illnesses that occur globally each year, causing about 298,000 deaths (41%) of all diarrheal disease-associated deaths (Besser, 2018). In China, a study based on the literature review estimated that the incidence of nontyphoidal salmonellosis was 626.5 cases per 100,000 persons (Mao et al., 2011; Xu Y. et al., 2020). Moreover, it has been reported that *Salmonella* was responsible for approximately 70∼80% of foodborne pathogenic outbreaks in China (Jun et al., 2007).

Salmonella spp. are Gram-negative rod-shaped bacteria, facultatively anaerobic, and belong to the family Enterobacteriaceae. To date, more than 2,600 serovars have been described among *Salmonella* species; among them, only a few serovars were mostly linked to human and/or animal infections, including Typhimurium and Enteritidis (so-called majority serovars) for human infections (Xu X. et al., 2020), Gallinarum and Pullorum for poultry infections (Xu Y. et al., 2020), Dublin for cattle infections (Paudyal et al., 2019), and Choleraesuis and Typhisuis for pig infections (Boyen et al., 2008; Asai et al., 2010). Generally, animal farms are considered natural reservoirs of *Salmonella*, especially poultry and pigs (Li et al., 2013; Zhou et al., 2017; Xu Y. et al., 2020). *Salmonella* could colonize the digestive tract of animals and are excreted in feces and spread into the environment (Kagambèga et al., 2013; Bonardi, 2017; Jiang et al., 2019), then transmitted to humans *via* the food chain (Ed-Dra et al., 2018; Wang et al., 2019; Wilson et al., 2020; Liu et al., 2021). Therefore, several studies have reported the presence of *Salmonella* in foods of animal origin, especially meat products (Ed-Dra et al., 2017; Jiang et al., 2021; Liu et al., 2021).

Pork meat is considered the most frequently contaminated food and the major source of *Salmonella* infections in humans (Bonardi, 2017; Wilson et al., 2020). In fact, pig farms seem to be a suitable environment for the replication and the persistence of *Salmonella* (Lettini et al., 2016; Bonardi, 2017; Vico et al., 2020). However, the slaughtering process which is located downstream of the pig-breeding process and upstream of pork sales is a critical step in determining the contamination/decontamination of animal carcasses and thus the meat products (Zhou et al., 2018). Moreover, the application of good hygienic practices in slaughterhouses has great importance and could participate in reducing the prevalence of *Salmonella* in the final meat products (Rahkio and Korkeala, 1996; Biasino et al., 2018). During the slaughtering process, animals pass through different processing stages with complicated manipulations (Zhou et al., 2018). However, since pigs are considered reservoirs of pathogens, they could contaminate/cross-contaminate the carcasses or muscle tissues during the slaughtering process. In fact, it has been demonstrated that slaughter practices, such as splitting the head and incising tonsils, were associated with higher levels of hygiene indicator bacteria and *Salmonella* in pig carcasses (Biasino et al., 2018). Therefore, the surveillance of *Salmonella* along the slaughtering process and its environment is with a high priority to determine the key points that are responsible for the contamination of carcasses and the final meats.

Recently, whole genome sequencing followed by bioinformatics analysis was considered as a cost-effective method for the diagnosis and characterization of foodborne pathogens (Biswas et al., 2020; Liu et al., 2020, 2021; Yu et al., 2020). As proof-of-concept, we conduct a study in a pig slaughterhouse in Hangzhou (Zhejiang Province, China), to obtain *Salmonella* isolates from different sources. The recovered strains were subjected to whole genome sequencing followed by *in silico* analysis to determine serovar distribution, multilocus sequence types, plasmid replicons, antimicrobial resistance, and virulence genes. Moreover, phenotypical antimicrobial resistance was investigated by the broth dilution method and compared with genotypical resistance.

MATERIALS AND METHODS

Sample Collection and Characterization of *Salmonella*

The present study was conducted in Linpu Pig Slaughterhouse in Xiaoshan, Hangzhou (China). The capacity of the studied slaughterhouse was approximately 1,000 pigs per day. A sampling visit was organized during December 2018 allowing the collection of 226 samples from different origins (pig carcasses, swab samples, environmental samples, equipment samples, operator samples, intestinal content samples, hepatobiliary samples, and sewer samples) along the slaughtering process of pigs (**Table 1**). The sampling method was in accordance with those described in previous studies (Cai et al., 2016; Zhou et al., 2017). The isolation of *Salmonella* was performed from different samples according

TABLE 1 | Sampling design and prevalence of *Salmonella* from different sources.

Sources	No. of samples	No. of positive samples	Percentage of contamination
Slaughtering process			
Live animal area	15	2	13.33%
Bleeding area	6	1	16.66%
Washing area	4	0	0%
Scalding area	10	0	0%
Dehairing area	6	4	66.66%
Cleaning the beating area	4	2	50%
Splitting area	14	8	57.14%
Clean area after splitting	4	1	25%
Carcass trimming area	7	1	14.58%
Meat inspection area	7	1	14.58%
Disinfection and precooling area	6	2	33.33%
Others			
Sewer outlet	6	2	33.33%
Bile samples	30	7	23.33%
Intestinal samples	47	19	40.42%
Liver samples	30	4	13.33%
Mesenteric lymph node samples	30	1	3.33%
Total	226	55	24.37%

TABLE 2 | Distribution of the studied *Salmonella* isolates according to sampling sources.

Sampling sources	No. of isolates	Grouping samples	No. of isolates
Inventory area	4 (5.13%)	Carcass swabs before splitting (CSBS)	19 (24.36%)
Bloodletting area	3 (3.85%)		
Dehairing area	11 (14.10%)		
Cleaning the beating area	1 (1.28%)		
Splitting area	12 (15.38%)	Carcass swabs after splitting (CSAS)	15 (19.23%)
Carcass trimming area	1 (1.28%)		
Meat inspection area	2 (2.56%)		
Bile samples	11 (14.10%)	Hepatobiliary samples (HS)	16 (20.51%)
Liver samples	5 (6.41%)		
Stool sample	22 (28.20%)	Fecal samples (FS)	22 (28.20%)
Sewer mouth sample	6 (7.69%)	Sewer samples (SS)	6 (7.69%)
Total	78 (100%)	Total	78 (100%)

to the protocols described previously (Jiang et al., 2019; Liu et al., 2021). Then, molecular confirmation of presumptive isolates was carried out by the amplification of *invA* gene according to the protocol previously described (Zhu et al., 2015; Liu et al., 2021).

Among the 226 collected samples, 55 were positive for the presence of *Salmonella*. However, since some samples present more than one presumptive isolate, we decided to select 78 different *Salmonella* isolates that show differences in morphological and biochemical criteria for genome sequencing and analysis (**Table 2**). Serotyping of the PCR confirmed *Salmonella* isolates were performed according to White–Kauffmann–Le Minor scheme by slide agglutination method to define O and H antigens using commercial antisera (SSI Diagnostica, Hillerød, Denmark).

Genomic DNA Extraction

All the obtained *Salmonella* isolates (n = 78) were selected for genomic DNA extraction according to the protocol described previously (Liu et al., 2021). Briefly, a broth culture of each *Salmonella* isolate was prepared by inoculation of a pure colony in Luria–Bertani broth followed by incubation at 37°C under 180 rpm shaking conditions. Then, DNA extraction was conducted by using TIANamp bacteria DNA kit (Tiangen Biotech, Beijing, China) according to the instructions of the manufacturer. The quantification of the extracted DNA was performed by the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, United States), as per the instructions of the manufacturer.

Genomic Sequencing and Bioinformatic Analysis

The genomic DNA library was constructed using NovaSeq XT DNA library construction kit (Illumina, San Diego, CA, United States, No: FC-131-1024), followed by genomic sequencing using Illumina NovaSeq Platform with NovaSeq 6000 SP Reagent Kit (300 cycles). The raw sequence reads were checked for quality and assembled using SPAdes v3.12.0 (Bankevich et al., 2012). Virulence gene prediction was conducted based on the virulence factors database (VFDB) (Chen et al., 2005). Moreover, *in silico* serotyping of *Salmonella* strains was performed by the SISTR web tool, whereas sequence types, antimicrobial resistance genes (ARG), and plasmid replicons were detected using the assemblies of the samples on the in-house Galaxy platform (Afgan et al., 2016), in combination with mlst v2.16.1[1] and abricate v0.8 (Zankari et al., 2012), including the CGE ResFinder database (updated on February 19, 2021) with a similarity cutoff of 90% for ARG and PlasmidFinder database (updated on February 19, 2021) with a similarity cutoff of 95% (Carattoli et al., 2014).

Phenotypic Antimicrobial Resistance Testing

The antimicrobial resistance of the isolated *Salmonella* strains was evaluated phenotypically by the broth dilution method to determine the minimum inhibitory concentration (MIC) of a panel of 14 antimicrobial agents belonging to 10 antimicrobial classes according to the protocol described previously (Jiang et al., 2021; Liu et al., 2021). The obtained results were interpreted according to the recommendation of the Clinical Laboratory Standard Institute guidelines (CLSI, 2017). The tested antimicrobial agents were as follows: penicillins (ampicillin: AMP, 0.25–128 μg/ml), β-lactamase inhibitors (amoxicillin/clavulanic acid: AMC, 0.125/0.062–128/64 μg/ml), cephems (ceftiofur: CF, 0.125–128 μg/ml; cefoxitin: CX, 0.125–128 μg/ml), aminoglycosides (gentamicin: GEN, 0.031–64 μg/ml; kanamycin: KAN, 0.25–128 μg/ml; streptomycin: STR, 1–128 μg/ml), tetracyclines (tetracycline:

[1]https://githubcom/tseemann/mlst2016

TET, 0.062–128 μg/ml), fluoroquinolones (ciprofloxacin: CIP, 0.015–16 μg/ml; nalidixic acid: NAL, 0.5–128 μg/ml), folate pathway inhibitors (trimethoprim/sulfamethoxazole: TST, 0.25/4.75–32/608 μg/ml), polypeptides (colistin: COL, 0.031–64 μg/ml), macrolides (azithromycin: AZI, 0.25–128 μg/ml), and phenicols (chloramphenicol: CHL, 0.5–128 μg/ml). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as the quality control strains to validate the antimicrobial susceptibility testing. However, strains showing a decrease in susceptibility (intermediate) were merged with resistant strains for ease of analysis, and the multidrug-resistant (MDR) strains were defined by resistance to at least three antimicrobial classes.

Data Analysis

GraphPad Prism 8 software (San Diego, CA, United States) was used for data analysis and generation of the figures. For antimicrobial susceptibility testing, the results of intermediate susceptibility were merged with resistance. Then, each phenotypically antimicrobial susceptibility test result (resistant or susceptible) was compared with the detection (presence or absence) of the corresponding resistance gene by *in silico* analysis. The isolates that are positive for at least one antimicrobial resistance gene among an antimicrobial class were considered as resistant to the corresponding antimicrobial class. The coherent results group together the isolates that are resistant or susceptible for both phenotypical and genotypical results. However, the incoherent results correspond to the isolates that are phenotypically resistant and genotypically susceptible or phenotypically susceptible and genotypically resistant for an antimicrobial agent. The percentage of incoherence corresponds to the difference between the results obtained by phenotypical and genotypical tests for each antimicrobial agent.

RESULTS

Salmonella Prevalence, MLST Pattern, and Serovar Distribution

The results obtained in this study showed that 55 of 226 (24.37%) samples were contaminated by *Salmonella* (**Table 1**). According to the sampling points along the pig slaughtering process, our results showed that the samples collected from the dehairing area were the most contaminated (66.66%), followed by those collected from the splitting area (57.14%). However, samples collected from the washing area and scalding area were not contaminated (**Table 1**). Additionally, from the 55 samples, 78 different *Salmonella* isolates were obtained, purified, and subjected to whole genome sequencing. The genomic prediction of serovars and MLST patterns showed the distribution of five different serovars and six MLST patterns, namely, Typhimurium ST19 (n = 40), Typhimurium ST34 (n = 14), London ST155 (n = 14), Rissen ST469 (n = 7), Goldcoast ST358 (n = 2), and Derby ST40 (n = 1) (**Table 3**). Additionally, serotyping performed by *in silico* analysis and slide agglutination methods provided the same results.

Phenotypic Antimicrobial Resistance

The antimicrobial resistance of the isolated *Salmonella* strains was evaluated against 14 antimicrobial agents belonging to 10 classes or categories. The phenotypic antimicrobial profiles were classified as resistant, susceptible, and intermediate according to the criteria of the Clinical Laboratory Standard Institute guidelines and the results are presented in **Table 4** and **Supplementary Material 1**. Our findings showed that tetracycline (85.90%; 67/78) and ampicillin (84.62%; 66/78) were the most resistant antimicrobial agent, followed by chloramphenicol (71.80%; 56/78) and nalidixic acid (61.54%; 48/78). Additionally, after considering the results of intermediate resistance as resistant strains, our findings showed that 89.74% (70/78) of isolates were resistant at least to one antimicrobial class, 87.18% (68/78) were resistant to at least two antimicrobial classes, and 85.90% (67/78) were resistant to at least three antimicrobial classes and were considered as MDR (**Figure 1A**).

According to the sources, it appears that *Salmonella* isolates recovered from sewer samples (SS) and hepatobiliary samples (HS) were more resistant to the tested antimicrobial agents compared with those collected from other sources (**Figure 2B**). Moreover, among different serovars identified in this study, *Salmonella* serovars Derby and Goldcoast appear to be the most resistant to the tested antimicrobial agents (**Figure 2A**). However, it should be noted that this conclusion cannot be generalized since only one strain of *Salmonella* Derby and two strains of *Salmonella* Goldcoast have been identified in this study. Additionally, *Salmonella* Typhimurium isolates from ST34 appear to be more resistant than isolates from ST19 (**Figure 2A**).

Antimicrobial Resistance Gene Prediction

The whole genome sequences of the 78 isolated *Salmonella* strains were subjected to *in silico* detection of antimicrobial resistance genes. The results obtained showed the detection of 35 different genes encoding resistance to nine antimicrobial classes (**Figure 3** and **Supplementary Material 2**). The most detected genes were bla_{TEM-1B} encoding resistance to penicillins (74.36%; 58/78), *sul2* encoding resistance to sulfonamides (87.93%; 51/58), *tet(A)* encoding resistance to tetracyclines (64.10%; 50/78), *floR* encoding resistance to phenicols (64.10%; 50/78), and *qnrS1* encoding resistance to fluoroquinolones (60.26%; 47/78) (**Figure 1C**). Moreover, 64 of 78 isolates (82.05%) harbor the resistance genes of more than two classes (**Figure 1B**). However, regarding the serovar distribution, it appears that *Salmonella* Typhimurium ST34 harbors more diversified antimicrobial resistance genes while *Salmonella* London ST155 appears to be poor in resistance genes (only one strain that harbors the genes *cat* and *tet(J)* encoding resistance to phenicols and tetracyclines classes, respectively) (**Figure 4A**). Moreover, our results showed that *Salmonella* isolates obtained from carcass swabs after splitting (CSAS) and HS harbor more resistance genes compared with those isolated from other sources (**Figure 4B**).

The relation between phenotypical antimicrobial resistance and the presence/absence of corresponding resistance gene obtained by *in silico* analysis was evaluated and the results are

TABLE 3 | Allelic profiles, serogroups, serovars, and MLST patterns of *Salmonella* isolated from different sources.

Serogroup	Serovar	MLST pattern	Allelic type							Source[a]				
			aroC	*dnaN*	*hemD*	*hisD*	*purE*	*sucA*	*thrA*	CSBS	CSAS	HS	FS	SS
Group O:4 (B) (*n* = 55)	Typhimurium	ST19 (*n* = 40)	10	7	12	9	5	9	2	6/40 (15%)	8 (20%)	5 (12.5%)	18 (45%)	3 (7.5%)
		ST34 (*n* = 14)	10	19	12	9	5	9	2	2 (14.29%)	2 (14.29%)	10 (71.43%)	0 (0%)	0 (0%)
	Derby	ST40 (*n* = 1)	19	20	3	20	5	22	22	0 (0%)	1 (100%)	0 (0%)	0 (0%)	0 (0%)
Group O:3,10 (E1) (*n* = 14)	London	ST155 (*n* = 14)	10	60	58	66	6	65	16	11 (78.57%)	1 (7.14%)	0 (0%)	2 (14.29%)	0 (0%)
Group O:7 (C1) (*n* = 7)	Rissen	ST469 (*n* = 7)	92	107	79	156	64	151	87	0 (0%	3 (42.86%)	1 (14.29%)	0 (0%)	3 (42.86%)
Group O:8 (C2-C3) (*n* = 2)	Goldcoast	ST358 (*n* = 2)	5	110	35	122	2	19	22	0 (0%)	0 (0%)	0 (0%)	2 (100%)	0 (0%)

[a]*CSBS, carcass swabs before splitting; CSAS, carcass swabs after splitting; HS, hepatobiliary samples; FS, fecal samples; SS, sewer samples.*

presented in **Table 5**. Our results showed that ciprofloxacin has the higher incoherence percentage (55.13%; 43/78) for which several isolates were genotypically positive but phenotypically negative, while cefoxitin presents the lower incoherence percentage (1.28%; 1/78).

Virulence Gene Prediction

In this study, the presence of 117 genes that are implicated in virulence and pathogenicity mechanisms of *Salmonella* was evaluated among the genomes of the 78 *Salmonella* isolates. The results are summarized in **Supplementary Material 3**. Our results showed that the number of detected genes ranged from 88 to 113 per isolate. Among the 78 isolates, three isolates (two *Salmonella* Goldcoast ST358 and one *Salmonella* Typhimurium ST19) were positive for the gene *cdtB* encoding typhoid toxin production, and these isolates were all isolated from fecal samples (FS). Additionally, only one *Salmonella* isolate (*Salmonella* London ST155) isolated from CSBS sample was positive for the genes encoding for the siderophore "yersiniabactin" (*fyuA*, *ybtA*, *ybtE*, *ybtP*, *ybtQ*, *ybtS*, *ybtT*, *ybtU*, *ybtX*, *irp1*, and *irp2*) and for the gene *senB* encoding for enterotoxin production. However, the typical virulence factors carried on *Salmonella* Pathogenicity Island 1 and 2 (SPI-1 and SPI-2) were detected in all the studied isolates.

Plasmid Profiles

The results of the prediction of plasmid replicons in the 78 *Salmonella* isolates are presented in **Figure 5** and **Supplementary Material 4**. Our results showed that the most abundant plasmid replicon was IncHI2A_1 (20.51%; 16/78), followed by IncX1_1 (17.95%; 14/78) and IncHI2_1 (11.54%; 9/78). The number of plasmid replicons ranged from 1 to 4 per isolate, while 42 of 78 (53.85%) *Salmonella* isolates do not harbor any plasmid. Regarding serovars, our results showed that *Salmonella* Typhimurium ST19 had a large number of different plasmids replicons (five plasmids), followed by *Salmonella* Goldcoast ST358 and *Salmonella* Derby ST40 (four plasmids). However, regarding the sampling sources, our results showed that the isolates recovered from FS harbor a large number of plasmid replicons (seven types of plasmids), followed by those recovered from CSAS (five types of plasmids), while *Salmonella* isolates recovered from SS do not harbor any plasmid.

DISCUSSION

Pig slaughterhouses are critical points of the meat processing chain; they are situated downstream of the pig-breeding process and upstream of pork sales. Since reception, animals undergo different complicated manipulations and are in contact with slaughterhouse facilities, workers, etc., which favors the contamination/cross-contamination of animal carcasses and, thus, the meat products (Arguello et al., 2013; Zhou et al., 2018). However, comparison between the contamination rate of *Salmonella* in pigs in the preslaughter stage and in the postslaughter stage revealed that the prevalence in the preslaughter stage often seems to be lower (Jiang et al., 2019). In this regard, Colello and his group conducted a study along the production chain of pig farms and showed that the prevalence of *Salmonella* in farms (2.6%) and slaughterhouses (2.0%) was lower than that observed in boning rooms (8.8%) and retail markets (8.0%) (Colello et al., 2018). Additionally, Jiang et al. reported that the prevalence of *Salmonella* in pigs at the farm stage was 11.77%, lower than that observed in the slaughtered pigs (45.23%) (Jiang et al., 2019), demonstrating the criticality of the slaughtering process in determining the quality and safety of derived pig food products.

In this regard, we conducted a study to evaluate the prevalence of *Salmonella* during the pig slaughtering process. Our results showed that 55 of the 226 samples (24.37%) were contaminated by *Salmonella*. These results were lower than those reported previously in pig slaughterhouses in other Chinese regions (ranged between 29.2 and 46.6%) (Bai et al., 2015; Li et al., 2016;

TABLE 4 | Antimicrobial susceptibility interpretation of the isolated *Salmonella* strains (*n* = 78).

Antibiotic agent	Abbreviation	Breakpoint interpretive criteria (μg/ml)[a]			Results in percentage (%)		
		S	I	R	S	I	R
Penicillin:							
Ampicillin	AMP	≤ 8	16	≥ 32	15.38% (12/78)	0% (0/78)	84.62% (66/78)
β-Lactam combination:							
Amoxicillin/clavulanic acid	AMC	≤ 8/4	16/8	≥ 32/16	78.21% (61/78)	21.79% (17/78)	0% (0/78)
Cephems:							
Cefoxitin	CX	≤ 8	16	≥ 32	98.72% (77/78)	1.28% (1/78)	0% (0/78)
Ceftiofur	CF	≤ 2	4	≥ 8	96.15% (75/78)	1.28% (1/78)	2.56% (2/78)
Aminoglycosides:							
Gentamicin	GEN	≤ 4	8	≥ 16	92.31% (72/78)	1.28% (1/78)	6.41% (5/78)
Kanamycin	KAN	≤ 16	32	≥ 64	89.74% (70/78)	1.28% (1/78)	8.97% (7/78)
Streptomycin[b]	STR	≤ 8	16	≥ 32	67.95% (53/78)	10.26% (8/78)	21.79% (17/78)
Fluoroquinolones:							
Ciprofloxacin	CIP	≤ 0.06	0.12–0.5	≥ 1	73.08% (57/78)	19.23% (15/78)	7.69% (6/78)
Nalidixic acid	NAL	≤ 16	–	≥ 32	38.46% (30/78)	–	61.54% (48/78)
Tetracyclines:							
Tetracycline	TET	≤ 4	8	≥ 16	14.10% (11/78)	0% (0/78)	85.90% (67/78)
Phenicols:							
Chloramphenicol	CHL	≤ 8	16	≥ 32	25.64% (20/78)	2.56% (2/78)	71.80% (56/78)
Macrolide:							
Azithromycin	AZI	≤ 16	–	≥ 32	93.59% (73/78)	–	6.41% (5/78)
Polymyxins:							
Colistin	COL	≤ 2	–	≥ 4	78.21% (61/78)	–	21.79% (17/78)
Folate pathway inhibitors:							
Trimethoprim/sulfamethoxazole	TST	≤ 2/38	–	≥ 4/76	55.13% (43/78)	–	44.87% (35/78)

[a] *S, sensitive; I, intermediate resistance; and R, resistant.*
[b] *For streptomycin, we used the same MIC breakpoints as for netilmicin.*

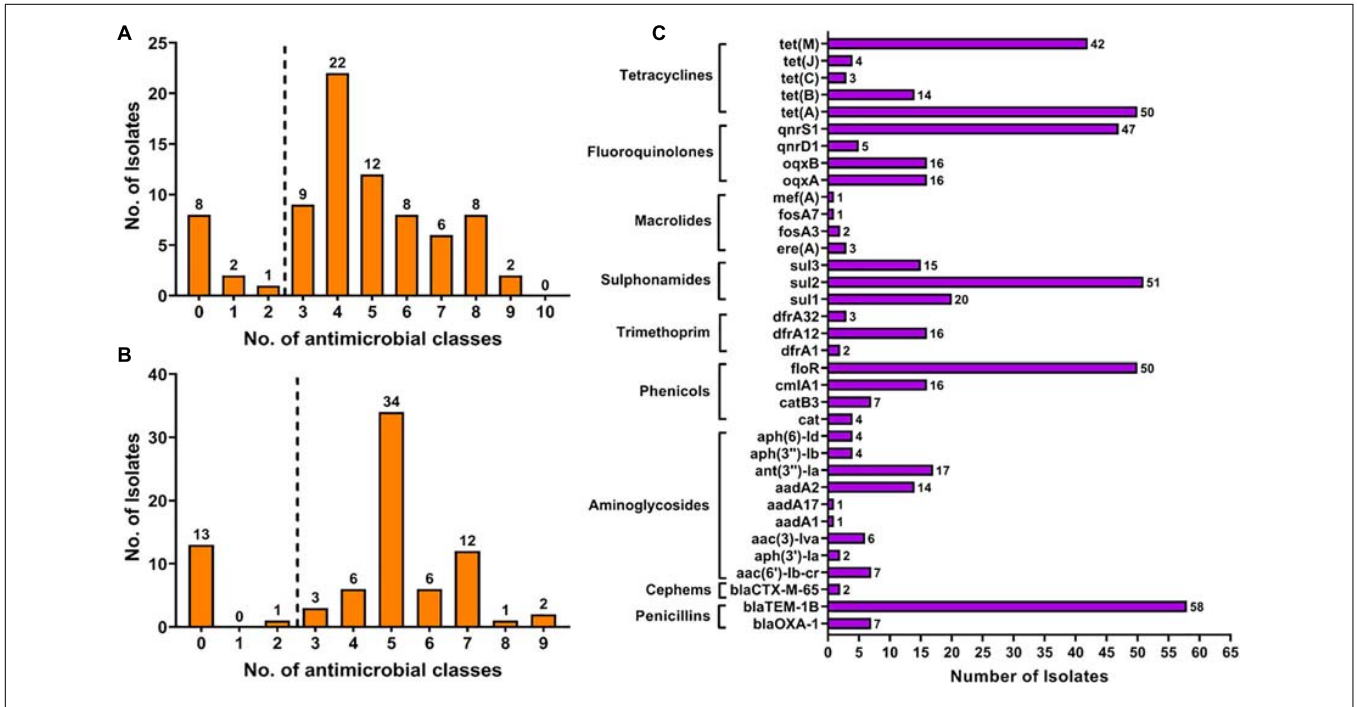

FIGURE 1 | The distribution of multiple drug resistance isolates according to the results obtained by phenotypical **(A)** and genotypical **(B)** tests. The detection of antimicrobial resistance genes **(C)** showed the high prevalence of resistance gene encoding resistance to penicillins, phenicols, sulfonamides, fluoroquinolones, and tetracyclines.

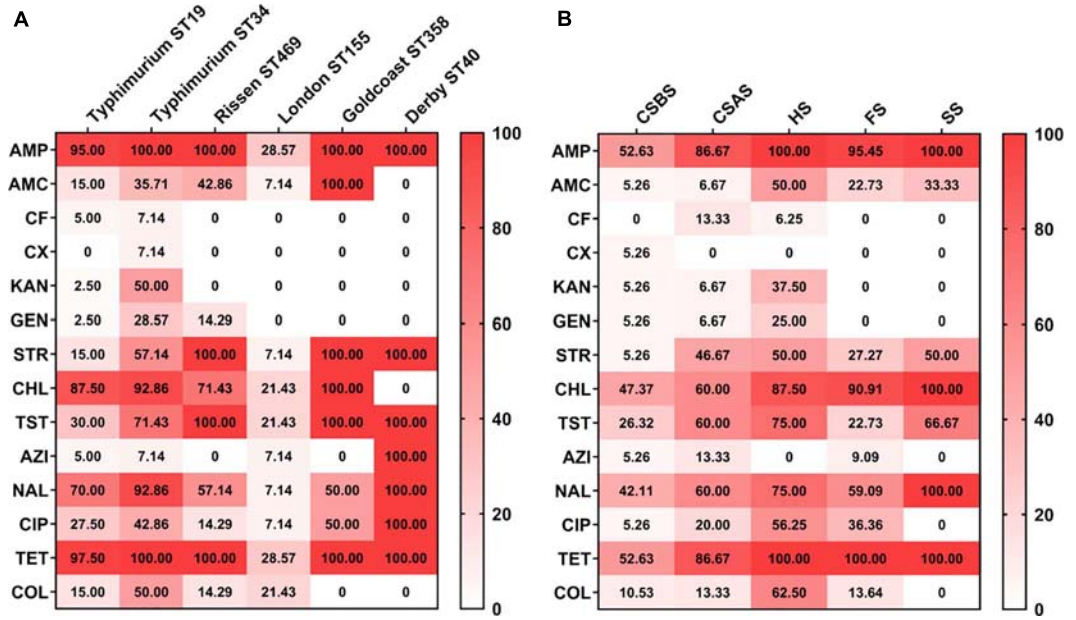

FIGURE 2 | Heatmap of antimicrobial resistance of *Salmonella* isolated from pig slaughtering process according to serovars and sampling sources. The isolates of *Salmonella* Typhimurium ST34 were resistant to all the tested antimicrobial agents **(A)**, while *Salmonella* isolated from HS were the most resistant to the tested antimicrobial agents **(B)**. The numbers in cells correspond to the percentage (%) of antimicrobial resistance isolates. CSBS, carcass swabs before splitting; CSAS, carcass swabs after splitting; HS, hepatobiliary samples; FS, fecal samples; SS, sewer samples.

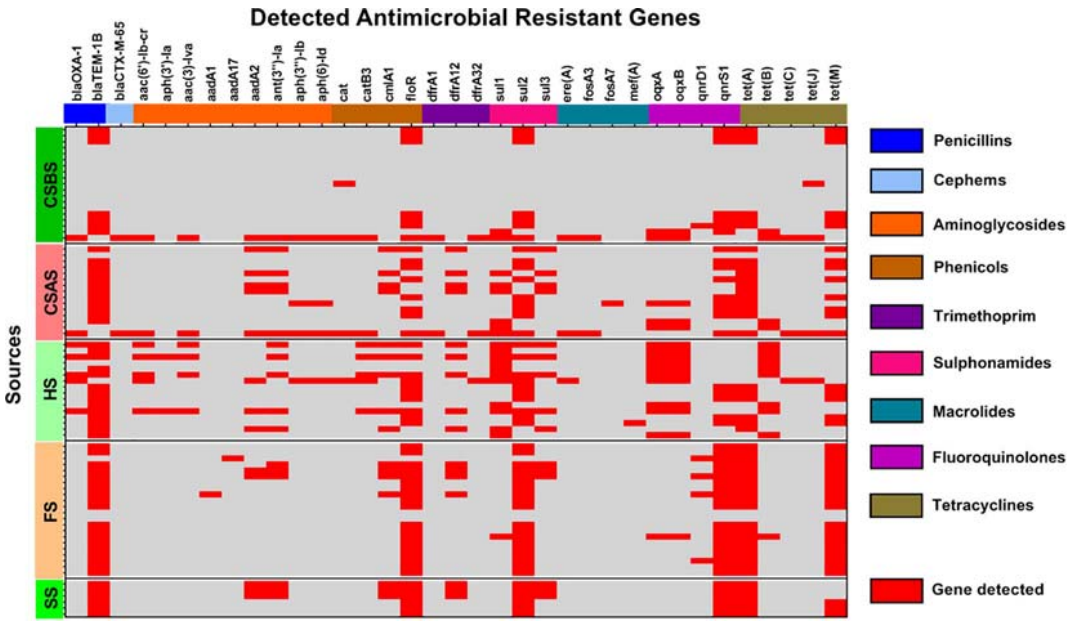

FIGURE 3 | Heatmap of the detection of antimicrobial resistance genes among the studied *Salmonella* isolates (*n* = 78).

Zhou et al., 2017) and in Spain (39.7%) (Arguello et al., 2012), while they were higher than those reported in the slaughtered pigs in Sardinia, Italy (12.9%) (Fois et al., 2017); pig carcasses and intestines from five slaughterhouses in Belgium (14.1%) (De Busser et al., 2011); a pig slaughterhouse in Yangzhou, China (17.43%) (Li et al., 2019); pork and slaughterhouse environment in Ahmedabad, Gujarat, India (13.7%) (Chaudhary et al., 2015); and pig slaughterhouses in two different regions of southwestern Spain (12.93%) (Morales-Partera et al., 2018). According to the slaughtering process, samples recovered from the dehairing area and splitting area were the most contaminated samples. In the dehairing area, the frequently used knife for carcass modification

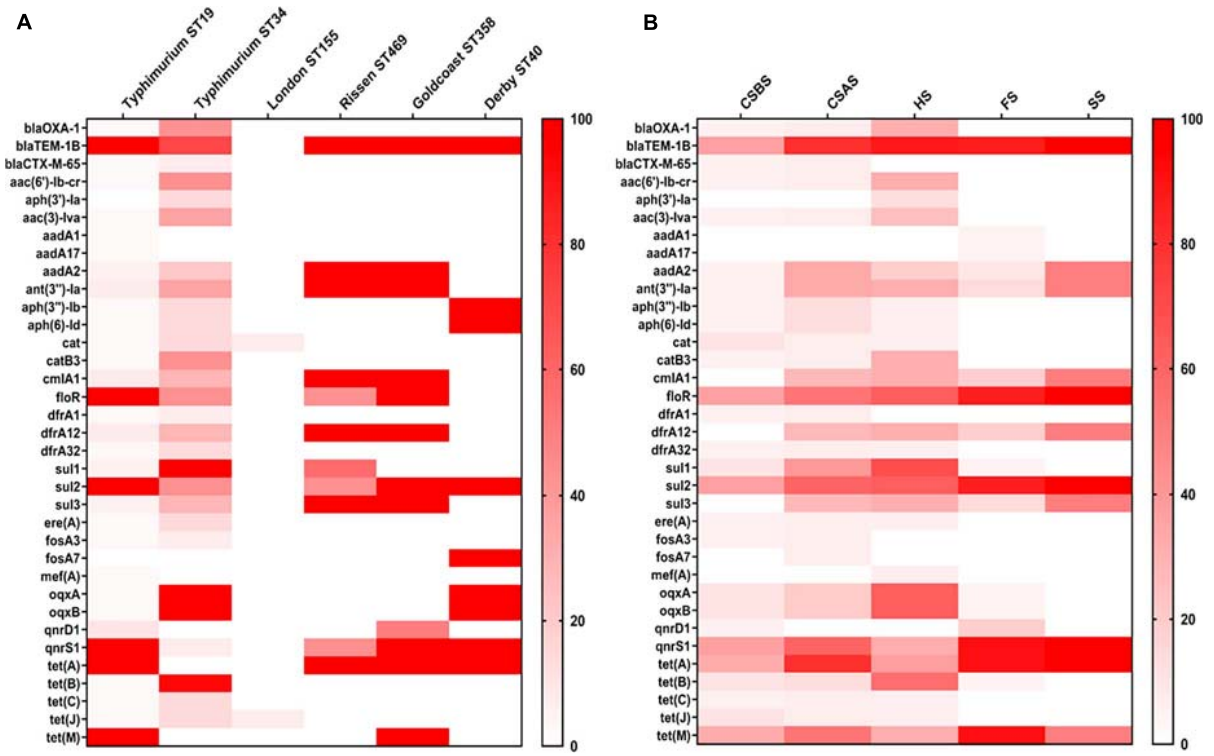

FIGURE 4 | Heatmap of antimicrobial resistance genes according to serovars **(A)** and sampling sources **(B)**. The isolates of *Salmonella* Typhimurium ST34 harbor the most diversified antimicrobial resistance genes. However, *Salmonella* isolates recovered from HS and CSAS contain more resistance genes compared with those isolated from other sources. CSBS, carcass swabs before splitting; CSAS, carcass swabs after splitting; HS, hepatobiliary samples; FS, fecal samples; SS, sewer samples.

TABLE 5 | Phenotypical and genotypical analyses of antimicrobial resistance of *Salmonella* isolates.

Antimicrobial class	Antimicrobial agent	Coherent results		Incoherent results		Percentage of incoherence
		Both resistant	Both susceptible	Phenotype resistant and Genotype susceptible	Genotype resistant and Phenotype susceptible	
Penicillins	Ampicillin	60	11	6	1	8.97% (7/78)
Cephems	Ceftiofur	1	74	2	1	3.85% (3/78)
	Cefoxitin	1	76	0	1	1.28% (1/78)
Aminoglycosides	Kanamycin	7	56	1	14	19.23% (15/78)
	Gentamycin	6	57	0	15	19.23% (15/78)
	Streptomycin	20	52	5	1	7.69% (6/78)
Phenicols	Chloramphenicol	48	13	10	7	21.79% (17/78)
Macrolides	Azithromycin	3	71	2	2	5.13% (4/78)
Fluoroquinolones	Nalidixic acid	48	16	2	12	17.95% (14/78)
	Ciprofloxacin	19	16	2	41	55.13% (43/78)
Tetracyclines	Tetracycline	64	10	3	1	5.13% (4/78)
Polymyxins	Colistin	0	61	17	0	21.79% (17/78)

was considered as the risk factor for the observed carcass cross-contamination. However, the splitting step located at the next step after evisceration has been confirmed as the other step with a higher risk of *Salmonella* contamination (Cai et al., 2016; Zhou et al., 2017). After evisceration, the intestinal content can contaminate a part of animal carcasses; however, during splitting, the splitter could be contaminated and then cross-contaminate other carcasses, resulting in the increase of *Salmonella* prevalence in the splitting area (Cai et al., 2016; Li et al., 2016). Therefore, the implementation of good hygienic practices and management systems to control critical points during the slaughtering process is of high priority to reduce the prevalence of *Salmonella*.

Among the 55 positive samples, 78 different *Salmonella* isolates were identified in this study. These isolates belong to

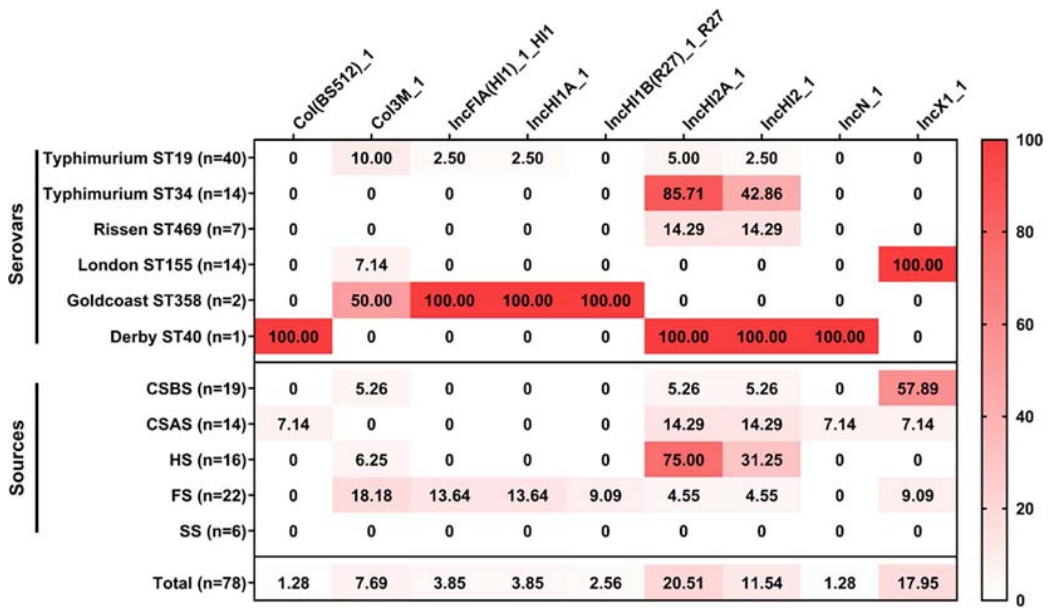

FIGURE 5 | Heatmap of plasmid replicon distribution in the studied *Salmonella* isolates (n = 78). The numbers in cells correspond to the prevalence (%) of plasmid replicons in *Salmonella* isolates according to serovar distribution and sampling sources.

five different serovars and six MLST patterns, namely, with importance degree, *Salmonella* Typhimurium ST19, *Salmonella* Typhimurium ST34, *Salmonella* London ST155, *Salmonella* Rissen ST469, *Salmonella* Goldcoast ST358, and *Salmonella* Derby ST40. In China, *Salmonella* Derby was identified as the most isolated serovar from pig slaughterhouse samples (Li et al., 2016, 2019; Zhou et al., 2017, 2018; Liu et al., 2020). However, *Salmonella* Typhimurium has been reported previously as the dominant serovar in *Salmonella* isolates recovered from pig slaughterhouses in Henan Province (Bai et al., 2015). In fact, it is well known that *Salmonella* Typhimurium was classified among the major serovars causing human salmonellosis worldwide (CDC, 2018; EFSA and ECDC, 2018), especially those with multilocus sequence types ST19 and ST34, which were reported in several cases of human infections (Wong et al., 2013; Carden et al., 2015; Panzenhagen et al., 2018; Luo et al., 2020; Monte et al., 2020). Therefore, the transmission of these isolates to the final meat products along the food chain is of high risk for consumers and may cause severe cases of foodborne diseases.

The infections caused by *Salmonella* are treated with different antimicrobial drugs. However, in the last decades, development of *Salmonella* resistance to many antimicrobials has been observed worldwide, either for the isolates provided from clinical, food, and environmental samples. In this study, the phenotypical and genotypical antimicrobial resistance profiles of the 78 isolated *Salmonella* strains were evaluated. Phenotypical results classified tetracycline and ampicillin as the less effective antimicrobial agents. In fact, the high resistance of *Salmonella* isolates to tetracycline and ampicillin has been reported over the world in samples collected along the animal food chain (Ed-Dra et al., 2017; Jiang et al., 2019, 2021; Wang et al., 2019; Liu et al., 2020, 2021), since they were frequently used in animal farms (Lekagul et al., 2019). In fact, the abuse and the misuse of antimicrobial drugs in animal livestock for therapeutic, prophylaxis, and growth promotions have led to the development of antimicrobial resistance. Moreover, our results showed that 85.90% of isolates/strains were resistant to more than two antimicrobial classes (MDR), which is considered a serious threat to public health that leads to therapeutic failure after a simple infection by MDR isolates.

Genotypical antimicrobial resistance prediction showed the detection of 35 resistance genes encoding resistance to nine antimicrobial classes, with a high prevalence of $bla_{\mathrm{TEM-1B}}$ gene encoding resistance to penicillins, *sul2* gene encoding resistance to sulfonamides, *tet(A)* gene encoding resistance to tetracyclines, *floR* gene encoding resistance to phenicols, and *qnrS1* gene encoding resistance to fluoroquinolone. The presence of these genes in bacterial genomes could be responsible for the acquisition of resistance to the corresponding antimicrobial classes. However, the analysis of coherence between genotypic and phenotypic antimicrobial resistance showed that phenotypic resistance cannot always be linked to the presence of resistance genes. Our results are in agreement with those reported previously in *Salmonella* isolates, showing a difference between phenotypic and genotypic resistance profiles (Liu et al., 2020, 2021). Hence, the phenotypic test remains the gold method for the assessment of bacterial behavior toward antimicrobial agents.

The prediction of virulence genes implicated in virulence and pathogenicity mechanisms reveals the detection of 117 different genes, particularly the detection of *cdtB* gene encoding typhoid toxins in two isolates of *Salmonella* Goldcoast ST358 and one isolate of *Salmonella* Typhimurium ST19 and the detection of genes encoding for the siderophore "yersiniabactin" in one isolate of *Salmonella* London ST155, and this isolate also harbors the

gene encoding for the enterotoxin TieB (*senB*). In fact, it has been reported that the presence of *cdtB* in the *Salmonella* genome was linked to isolates implicated in human bloodstream and invasive infections (Miller et al., 2018; Xu X. et al., 2020). Additionally, yersiniabactin siderophore that was initially described in *Yersinia* spp. is required for iron uptake and growth of the bacteria in an iron-restricted environment (Perry and Fetherston, 2011; Khan et al., 2018). However, the enterotoxin TieB was initially described in enteroinvasive *E. coli* (EIEC) (Nataro et al., 1995) and has been suggested to play a key role in bacteria virulence in humans (Meza-Segura et al., 2020). Indeed, the presence of these virulence genes in the genome of *Salmonella* isolated from the pig slaughtering process may lead to severe disease outcomes in humans.

In this study, nine different plasmid replicons were detected among the 78 *Salmonella* isolates. The most abundant plasmids were IncHI2A_1, IncX1_1, and IncHI2_1. IncHI2A_1 and IncHI2_1 were predominant in *Salmonella* Typhimurium ST34, while IncX1_1 was detected only in *Salmonella* London ST155. These plasmids were identified previously in *Salmonella* isolates recovered from the animal food chain, especially pork production chains (Liu et al., 2020, 2021; Viana et al., 2020). Interestingly, it has been demonstrated that these plasmids were associated with resistance to different antimicrobial classes, including β-lactams, aminoglycosides, sulfonamides, tetracyclines, and polymyxins (Elbediwi et al., 2020b,a; Gu et al., 2020; McMillan et al., 2020). Consequently, these plasmids may mediate the horizontal transmission of antimicrobial resistance genes during this slaughtering process.

CONCLUSION

In this study, we provided the dynamic prevalence of *Salmonella* during the pig slaughtering process. Additionally, we demonstrated the use of whole genome sequencing as a cost-effective approach for routine surveillance of foodborne pathogens, especially *Salmonella*. The prediction of serovar distribution, MLST patterns, antimicrobial resistance genes, plasmid replicons, and virulence factors in *Salmonella* isolates recovered from the pig slaughtering process showed the isolation of MDR isolates harboring different antimicrobial resistant determinants and virulence factors like *cdtB* gene encoding typhoid toxins, *senB* gene encoding for the enterotoxin production, and several genes encoding for the siderophore "yersiniabactin." Therefore, it is time to prevent the use of antimicrobials in animal livestock in order to avoid the dissemination of antimicrobial resistance determinants along the food chain and to implement management systems to control critical points in order to avoid the transmission of foodborne pathogens to humans.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI Bioproject with the accession number no. PRJNA686895.

AUTHOR CONTRIBUTIONS

BW, AE-D, and HP contributed equally to this work. AE-D and HP analyzed the data and finalized the figures. AE-D and MY were wrote the manuscript. BW, HP, CD, and CJ did the experiment and data collection. MY conceived the idea and assisted with data analysis and writing. All authors read, revised, and approved the final manuscript.

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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.2021.704636/full#supplementary-material

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Whole Genome Analysis of Three Multi-Drug Resistant *Listeria innocua* and Genomic Insights Into Their Relatedness With Resistant *Listeria monocytogenes*

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Listeria innocua are Gram-positive rod-shaped bacteria, which are not generally infectious as opposed to *Listeria monocytogenes*. However, the comparatively high genomic similarity between both along with on occasion, their coexistence in similar ecological niches may present the opportunity for resistance or virulence gene transfer. In this study, three multi-drug resistant *L. innocua* originally cultured from food were put forward for long-read genome sequencing. Chromosome and plasmid genomes were assembled and annotated. Analysis demonstrated that the resistant phenotypes correlated well with genotypes. Three plasmids pLI42, pLI203, and pLI47-1 were identified which harbor resistance islands. Sequence alignments suggested that plasmids pLI42 and pLI203 were highly similar to a previously sequenced *L. monocytogenes* plasmid pLR1. Similarly, another three types of resistance gene islands were observed on chromosome, including *tet*(M) gene islands (transposon Tn*916* orthologs), *dfrG* gene islands and *optrA-erm*(A) gene islands. All three *L. innocua* isolates possessed listeria pathogenicity island-4 (LIPI-4) which is linked to cases of mengitis. Further genome environment and phylogenic analysis of regions flanking LIPI-4 of *L. innocua* and *L. monocytogenes* showed that these may have common origins and with the potential to transmit from the former. Our findings raise the possible need to include both *L. monocytogenes* and *L. innocua* in food surveillance programs so as to further understand of the origins of antimicrobial resistance and virulence markers of public health importance in *L. monocytogenes*.

Keywords: *Listeria innocua*, multi-drug resistance, whole genome sequencing, listeria pathogenicity island 4, genome environment analysis

INTRODUCTION

Listeria species are Gram-positive and facultative anaerobic bacteria that exist in soil, water and the animal gut. Members of this genus are found to contaminate certain types of foods and the associated food processing environment thereby representing a risk for public health (Finlay, 2001). Nineteen species of *Listeria* have been reported (Orsi and Wiedmann, 2016). Among them,

L. monocytogenes is considered as the only one that can cause listeriosis both in humans and animals (Vivant et al., 2013). Previously, *Listeria innocua* was generally considered as a non-virulent species with a closer evolutionary relationship than other members of the genus to *L. monocytogenes* (Buchrieser et al., 2003).

To better describe the genomic evolution and potential for horizontal gene transfer (HGT) between *L. monocytogenes* and *L. innocua*, the findings of comparative genomics analyses were reported in previous studies (Buchrieser et al., 2003; Hain et al., 2006). These data highlighted the close genetic relationship existing between *L. monocytogenes* and *L. innocua*. Phylogenetic studies using amplicons or *Listeria* house-keeping genes provided evidence that *L. innocua* and *L. monocytogenes* are indeed related genetically (Glaser et al., 2001b; Orsi and Wiedmann, 2016). The orthologous genes identified between both species are highly conserved. Further, data indicated that *L. innocua* may have evolved through gene elimination and acquisition from the same pathogenic ancestor of *L. monocytogenes* (Chen et al., 2009).

Although few in number, early studies described plasmid-mediated AMR and their transmission in *L. innocua* (Bertsch et al., 2014; Gomez et al., 2014; Escolar et al., 2017). Based on recent sequencing data all *L. monocytogenes* possessed listeria pathogenicity island-1 (LIPI-1) and *inlAB* (Reddy and Lawrence, 2014). Hypervirulent isolates of *L. monocytogenes* harbor LIPI-3 and LIPI-4 (Maury et al., 2016), in which LIPI-3 encodes listeriolysin S, a second hemolysin that enhances the survival of *L. monocytogenes* in polymorphonuclear neutrophils (PMN) while LIPI-4 encodes a cellobiose-family phosphotransferase system (PTS) (Cotter et al., 2008), that enhances invasion of the central nervous system (CNS) along with maternal-neonatal infection (MN). Most *L. innocua* isolates lack LIPI-1 and several important virulence genes including *inlA and inlB*, while other data reported on atypical *L. innocua* that harbored LIPI-1 or LIPI-3 (Volokhov et al., 2007; Clayton et al., 2014; Moura et al., 2019). Unlike other LIPIs, LIPI-4 orthologous has been reported to be found in many *L. innocua* isolates (Moura et al., 2019). Furthermore, few examples of HGT involving resistance and virulence genes between these two species have been reported (Bertsch et al., 2014).

In this study, we describe three MDR *L. innocua* LI42, LI47, and LI203, isolated from food samples in China. In order to extend our understanding of the genetic relationships and antibiotic resistance and virulence, all three were sequenced and compared with the closely related *L. innocua* and *L. monocytogenes* reference genomes, including two resistant *L. monocytogenes* in our previous study (Yan et al., 2019).

MATERIALS AND METHODS

Bacterial Isolates

Three *L. innocua isolates* were isolated from food in China from year 2015 to 2016. The source information was listed in **Supplementary Table 1**. All isolates were confirmed by API listeria (Suarez et al., 2001).

Antibiotics Susceptibility Testing (AST)

All isolates were tested for antimicrobial susceptibility using broth microdilution against a panel of nine antimicrobial compounds commonly used in veterinary and human therapy and these data were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) M45 (3rd edition) (Clsi Institute, 2015), where appropriate. Drugs tested included ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), erythromycin (ERY), gentamicin (GEN), meropenem (MEM), trimethoprim-sulfamethoxazole (SXT), tetracycline (TET), and vancomycin (VAN). All antibiotics were purchased from Sigma-Aldrich, Germany.

DNA Purification and Sequencing

Each isolate was grown in brain heart infusion (BHI) broth (Beijing Land Bridge) at 37°C and genomic DNA (gDNA) was purified using Omega EZNA® Bacterial DNA Kit (Omega Biotek, Norcross, GA, United States). The bacterial genomes were sequenced by Tianjin Biochip Corporation, using a PacBio RS II platform (Pacific Biosciences, Menlo Park, CA, United States). The sequencing depth is 1000X. *De novo* assembly was performed by SMRT Link (V6.0.0.47841).

Annotation of Genomes and AMR Genes

The chromosomes and plasmids of three *L. innocua* were annotated with the prokaryotic genome annotation tool Prokka (v1.12). Antibiotic resistance genes were extracted from these genome sequences using the ABRicate[1] software package, where a combination of three reference databases CARD (Jia et al., 2017), ResFinder (Zankari et al., 2012), and NCBI AMRFinderPlus (Feldgarden et al., 2019) were used. Gene names were unified to the NCBI AMRFinderPlus references. All resistance genes were screened using the BLASTN algorithm with minimum nucleotide identity and alignment length coverage of 80%.

Assessment of Virulence Factors

The presence and integrity of virulence factors was assessed using *L. monocytogenes* EGD-e (NC_003210) as the reference genome for Internalin A (*InlA*), Internalin B (*InlB*), listeria pathogenicity island 1 (LIPI-1) (Glaser et al., 2001b; Toledo-Arana et al., 2009). *L. monocytogenes* F2365 (NC_002973) was used as reference genome for LIPI-3 with the protein sequences (LMOf2365_1113 to LMOf2365_1119) (Nelson et al., 2004). *L. monocytogenes* LM9005581 (CYWW00000000) was used as reference for LIPI-4 with the protein sequences (LM9005581_70009 to LM9005581_70014). Analysis was performed using the BLASTN algorithm with a minimum identity of 80%, coverage of 80%.

Genomic Comparison

Sequence comparison was executed between chromosomes or plasmids on average nucleotide identity (ANI) based on BLASTN alignment, using pyani (v0.2.7). ANIb values of each pair of samples was calculated and classified into two groups *via* species. A one-tailed student *t*-test was performed. A circular genome

[1]https://github.com/tseemann/abricate

comparison graph was performed with BRIG (v0.95). Sequence comparisons were done using BLASTN and visualized using EasyFig (v2.2.3).

Core Genome Alignment and Phylogenetic Tree

Core genomes of all assemblies were calculated using Roary (v3.11.0). The core genomes were aligned with MAFFT (v7.313). Maximum Likelihood phylogenetic tree of the aligned genomes was performed using FastTree (v2.1.10). The phylogenetic tree was illustrated by adjusting the mid-point as root.

Sequence Data Accession Numbers

Accession numbers for complete genome sequences are SAMN18079989 (LI42), SAMN18080006 (LI47), and SAMN18080009 (LI203).

Conjugation Experiments

Conjugation experiments were performed using *L. innocua* LI42, LI47, and LI203 as the donors, *L. monocytogenes* ATCC 19115, *L. monocytogenes* ST9 isolate and *E. coli* J53 (NaN_3 resistant) as recipients. For the selection of the transconjugants between *L. innocua* and *L. monocytogenes*, blood agar plate was supplemented with 4 mg/L tetracycline, and the colonies were separated by hemolysis test. For the selection of the transconjugants between *L. innocua* and *E. coli* J53, MacConkey Agar (MAC) plate was supplemented with 4 mg/L tetracycline and 100 mg/L NaN_3. The Colonies grew on these selective plates were further confirmed by PCR amplification of *hly* and *tet*(S) genes for *listeria spp*., and *tet*(S) for *E. coli* J53.

RESULTS

Pheno- and Genotypic Characterization of Three *L. innocua* Isolates

Listeria innocua LI42, LI47, and LI203 were found to express the same MDR phenotype resistant to chloramphenicol, erythromycin, tetracycline and trimethoprim-sulfamethoxazole as shown in **Figure 1**, albeit somewhat different minimum inhibitory concentration (MIC) values. AMR genotypes corresponded well with the phenotypes described by AST analysis, where the three MDR isolates harbored resistance genes, respectively, for aminoglycoside, macrolide-lincosamide-streptogramin B (MLS_b), phenicol, tetracycline, and sulfamethoxazole resistance. Complete LIPI-4 orthologs were found in all three *L. innocua* isolates.

Genome Wide Characterization of Three *Listeria innocua* Genomes

As shown in **Table 1**, long-read sequencing of *L. innocua* LI42, LI47, LI203 facilitated the construction of the complete genome sequence in each case including their chromosomes and plasmids. *Listeria innocua* LI42 and LI203 genomes each contain one chromosome and one plasmid (pLI42 or pLI203), while *L. innocua* LI47 contained a chromosome and two plasmids (pLI47-1 and pLI47-2).

Comparative Genomic Analysis of Three *Listeria innocua* Plasmids

Annotation of resistance genes showed that plasmid pLI42, pLI47-1, and pLI203 carried multiple antibiotic resistance genes including *ant*(6)-*Ia, aph(3′)-IIIa, catA8, dfrG, erm*(B), *lnu*(A), *lnu*(B), *lsa*(E), *msr*(D), *mef*(A), *spw* and *tet*(S), associating resistances of amikacin, aminoglycoside, chloramphenicol, kanamycin, lincosamide, macrolide, tetracycline, trimethoprim, streptogramin, and streptomycin. Multiple copies of insertion sequences were also noted (**Figure 1A**).

Our previous study reporting on *L. monocytogenes* from foods in China described a multidrug resistant (MDR) gene island *dfrG-tet*(S)-*lnu*(B)-*lsa*(E)-*spw-ant*(6)*_Ia-erm*(B)-*aphA-catA8* from *L. monocytogenes* LR1 (SAMN10434273) (Yan et al., 2019). The recent long-read re-sequencing of this strain confirmed that this gene island is located on a plasmid, denoted as plasmid pLR1. Average nucleotide identities between each pair of plasmids pLI42, pLI203 and *L. monocytogenes* MDR plasmid pLR1 sequences showed >95.0% coverages and >99.9% identities between each two sequences, demonstrating that plasmids pLI42 and pLI203 are close orthologs of plasmid pLR1.

Linear sequence comparison was performed involving plasmids pLI42, pLI47-1, and pLI203 along with three plasmids pLI100 (*L. innocua*) from *L. innocua* Clip11262 (Glaser et al., 2001a), pLR1 (*L. monocytogenes*), and pPIR00540 (*L. monocytogenes*) as the closest common hits of online BLAST result of the three plasmids against NCBI nt/nr database (Portmann et al., 2018), which clearly displayed the ortholog regions (**Figure 1B**). Although plasmid pLI203 lacked the chloramphenicol resistance gene *catA8*, plasmids pLI42, pLI203, and pLR1 shared a similar overall genetic backbone covering the previously described LR1 MDR gene island. *Listeria innocua* LI47 MDR gene island mapped to plasmid pLI47-1 demonstrated a unique resistance gene arrangement *lnuA-catA8-tet*(S)-*ant*(6)*Ia-msr*(D)-*mef*(A). The conserved flanking region of *L. innocua* LI47 MDR gene island on plasmid pLI47-1 was identical to plasmid pPIR00540.

Insertion sequence (IS) elements IS*3*, IS*6*, IS*21*, IS*1595*, IS*1380*, IS*Lre2,* and Tn*3* were also annotated, along with the resistance gene *dfrG*, which is also known as transposes IS*Ssu9* (Holden et al., 2009). Multiple copies of IS*1216E* were noted on these plasmids. Each of plasmids pLI42, pLI203, and pLR1 had six direct repeats of IS*1216E*. Plasmids pLI47-1 and pPIR00540 contain two direct repeats, while three direct repeats and a single inverted repeat are found in plasmid pLI100.

Comparative Genomic Analysis of Resistance Gene Islands Located on Chromosomes

Listeria innocua LI42, LI47, and LI203 chromosomes were discovered harboring three different types of resistance gene island including *tet*(M) gene island (∼10,925 bp, found on the LI42 and LI203 chromosomes, associated with tetracycline

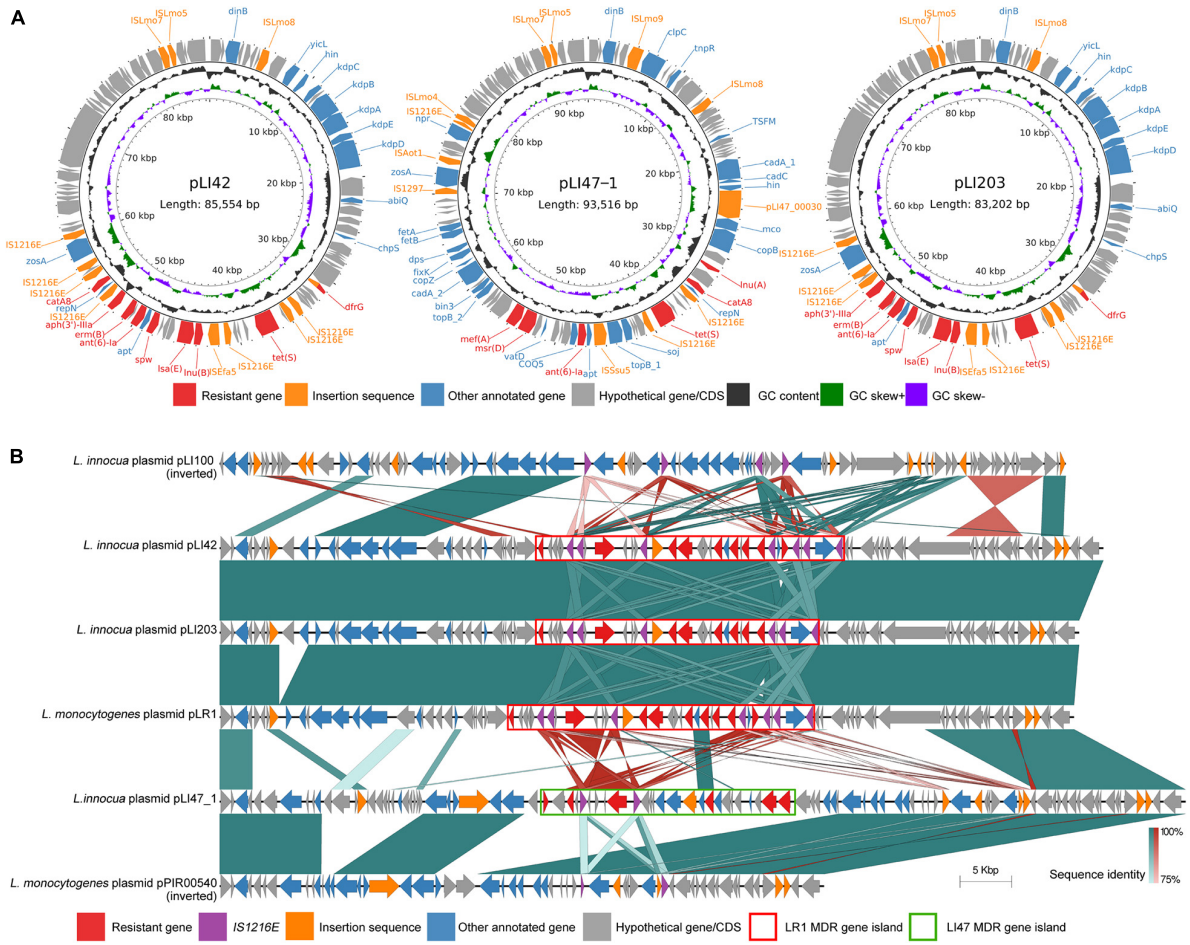

FIGURE 1 | Circular schematic map and linear sequence comparison of three plasmids carrying antibiotic resistance genes. **(A)** The sequence structure of three sequenced *L. innocua* plasmid pLI42, pLI47-1, and PLI203. CDSs were shown as arrows. Antibiotic resistance genes were highlighted as red. Insertion sequences were highlighted by orange. Other annotated genes were denoted by blue. The inner two rings represented GC Skew[(G – C)/(G + C), G and C represents guanine content cytosine content] and GC content. **(B)** Comparison of three *L. innocua* plasmid pLI47-1, pLI203, and PLI42 sequences with a closely related reference *L. innocua* plasmid pLI100 and two *L. monocytogenes* reference plasmid pLR1 and pPIR00540. CDSs were shown as arrows and colored based on gene functions. Copies of insertion sequence IS*1216E* was specially highlighted as purple. Green or red shades between each two sequences represented direct or inverted nucleotide identity between covered regions with at least 75% identity. Reference plasmids pLI100 and pPIR00540 sequences were reversed to be aligned with other sequences. *L. monocytogenes* LR1 MDR gene island and the orthologs were marked in red frames. *L. innocua* LI47 MDR gene island was marked in green frame.

resistance), *dfrG* gene island (∼3,310 bp, mapped on the LI47 chromosome, and associated with trimethoprim resistance) and *optrA-erm*(A) gene island (∼18,861 bp, integrally found on the LI47 chromosome, partially found on LI42 and LI203 chromosome, associated with macrolide, florfenicol and oxazolidinone resistance).

Nucleotide comparison confirmed that the *tet*(M) and *dfrG* gene-containing islands are highly identical (>99%) with their orthologs in *L. monocytogenes* LR8 (SAMN10434278), a feature reported in our earlier study (Yan et al., 2019). Specifically, *tet*(M) is an identical ortholog of transposon Tn*916*, which is found among several different bacteria (Kathariou et al., 1987; Bertsch et al., 2013), while the *dfrG* gene is known to exist as an independent antibiotic resistance gene and also associated with an insertion sequence element IS*1595*. The insert locations for the *tet*(M) gene-containing island in *L. innocua* LI42 and LI203 chromosomes differ from that of *L. monocytogenes* LR8 (as shown in **Figure 2A**). Meanwhile, the *dfrG* gene-containing island also mapped to plasmids pLI42 and pLR1, suggesting horizontal movement between both plasmids and chromosomes. The latter contains a pair of short direct repeats (∼63 bp) flanking both ends, and module inserts reversibly at the corresponding location on the chromosomes of *L. innocua* LR8 and LI42 (**Figure 2B**).

The *L. innocua* LI42 chromosome harbors the complete *optrA-erm*(A) gene island. It is identical to *Staphylococcus simulans* IY19 *optrA* gene cluster (MF805730), which was reported in an earlier study (Sun et al., 2018). In *L. innocua* LI47 and LI203 a partial *optrA-erm*(A) gene island only was noted, and which is devoid of *erm*(A) gene. The insertion locations of this gene island were consistent among *L. innocua* LI42, LI47 and LI203. Flanking regions of all three resistance gene islands were

TABLE 1 | Summary for complete genome sequencing and antimicrobial resistance pheno- and genotypes of three *L. innocua* isolates.

Isolate	Sequence type	Total length (bp)	GC content (%)	Antibiotic resistance genes	MLST	Antibiotic susceptibility (MIC, μg/mL)								
						AMP	CHL	CIP	ERY	GEN	MEM	TET	SXT	VAN
LI42	Chromosome	2,930,429	37.45	*tet*(M), *optrA*, *fexA*	474	S (0.25)	R (>128)	S (1)	R (32)	S (0.25)	S (0.25)	R (32)	R (4/76)	S (0.5)
	Plasmid (pLI42)	85,554	34.64	*dfrG*, *tet*(S), *lnu*(B), *lsa*(E), *ant*(6)-*Ia*, *erm*(B), *aph(3′)-IIIa*, *catA8*										
LI47	Chromosome	2,927,254	37.46	*erm*(A), *dfrG*,*optrA*, *fexA*	602	S (0.25)	R (>128)	S (1)	R (32)	S (1)	S (0.25)	R (16)	R (2/38)	S (0.5)
	Plasmid (pLI47-1)	93,516	35.99	*lnu*(A), *catA8*, *tet*(S), *ant*(6)-*Ia*, *msr*(D), *mef*(A)										
	Plasmid (pLI47-2)	52,798	31.63	–										
LI203	Chromosome	2,940,225	37.47	*tet*(M), *optrA*, *fexA*	474	S (0.5)	R (>128)	S (1)	R (16)	S (0.25)	S (0.1)	R (64)	R (2/38)	S (1)
	Plasmid (pLI203)	83,202	34.77	*dfrG*, *tet*(S), *lnu*(B), *lsa*(E),*ant(6)-Ia*, *erm*(B), *aph(3′)-IIIa*, *catA8*										

The table shows basic information summary of three L. innocua samples and their genome sequences.
The abbreviations were ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), erythromycin (ERY), gentamicin (GEN), meropenem (MEM), trimethoprim-sulfamethoxazole (SXT), tetracycline (TET), and vancomycin (VAN).
Antibiotic susceptibilities are described as S (susceptible)/R (resistant) with the original minimum inhibitory concentration (MIC) value.
MIC breakpoints of AMP, SXT, and MEM were in accordance with CLSI M45 (3rd edition), all other compounds were interpreted using Bacillus spp. as the reference from the same document.

identical in comparison with the susceptible *L. innocua* reference genome Clip11262.

Genome Environment Analysis of LIPI-4

Genomic comparisons were performed on LIPI-4 and its flanking region in *L. innocua* and *L. monocytogenes* in order to discover the degree to which it may be conserved between these species. The LIPI-4 ortholog was located at the corresponding position on the chromosomes of *L. innocua* LI42, LI47,and LI203 and the reference *L. monocytogenes* N2306. The sequence context of LIPI-4 was found to be consistent. The reference *L. monocytogenes* EGD-e was devoid of LIPI-4, while possessing an identical LIPI-4 flanking region (as shown in **Figure 3**).

A 10-kbp upstream and downstream region flanking LIPI-4 were assessed for a comparative analysis of their genomic features. These regions were found to be identical among the *L. innocua* strains, and in *L. monocytogenes* with/without LIPI-4. The LIPI-4 locus of both species shared an identical *lacE-celA-lacF-chbG-manR-pagL* gene arrangement. Average sequence identity of each gene within/between species is shown in **Supplementary Table 2**. *L. monocytogenes* lacking LIPI-4 demonstrated highly similar orthologous regions to the LIPI-4 adjacent regions, especially for the lineage-I strains. The sequence identity between the non-LIPI-4 orthologs and the LIPI-4 containing *L. monocytogenes* lineage-I was approximately 100%.

DISCUSSION

Genomic Features of Antibiotic Resistance

In this study, three *L. innocua* each expressing an MDR phenotype were studied. When sequenced, their corresponding antibiotic resistant-encoding genes were found to map either to the bacterial chromosome or to plasmids contained therein. In the case of these plasmids that were identified, an ortholog of *L. monocytogenes* MDR-expressing plasmid pLR1 were detected in *L. innocua* LI42 and LI203. The same plasmid in a *L. monocytogenes* isolate was also recovered in China, and denoted as plasmid pNH1 (Yan et al., 2020). A novel MDR expressing plasmid pLI47-1 is also described in this study. Sequence comparisons highlighted the mosaic nature of MDR gene islands in both of these plasmids wherein their resistance genes appeared to arise from multiple origins including *Staphylococcus aureus*, *Enterococcus faecium,* and *Lactococcus lactis*, with IS*1216E* likely to play a decisive role in the recombination steps contributing to their formation. Similar observations have been reported earlier by others (Kang et al., 2019; Moroi et al., 2019; Iimura et al., 2020).

The >99% sequence identities between plasmid pLI42 and pLI203 in *L. innocua* and pLR1 in *L. monocytogenes* showed that these plasmids were highly homologous. Meanwhile, the isolates harboring these plasmids were isolated from different years, for *L. monocytogenes* isolate LR1 harboring pLR1 was discovered in 2012, while the *L. innocua* isolates harboring pLI42 and pLI203 were discovered in 2015 and 2016. These evidences may indicate the potential mobilizing nature of these plasmids between *Listeria* species. However, our conjugation experiment showed that pLI42, pLI47-1 and pLI203 in *L. innocua* were non-conjugative to *L. monocytogenes* or *E. coli* (data not shown). A previous study also reported that the homologous plasmid pNH1 was non-conjugative between *L. monocytogenes* strains (Yan et al., 2020). Thus, the specific mobilizing mechanism of the plasmids still needs to be revealed.

On chromosomes, three different types of antibiotic resistance gene-containing islands were discovered in *L. innocua* in

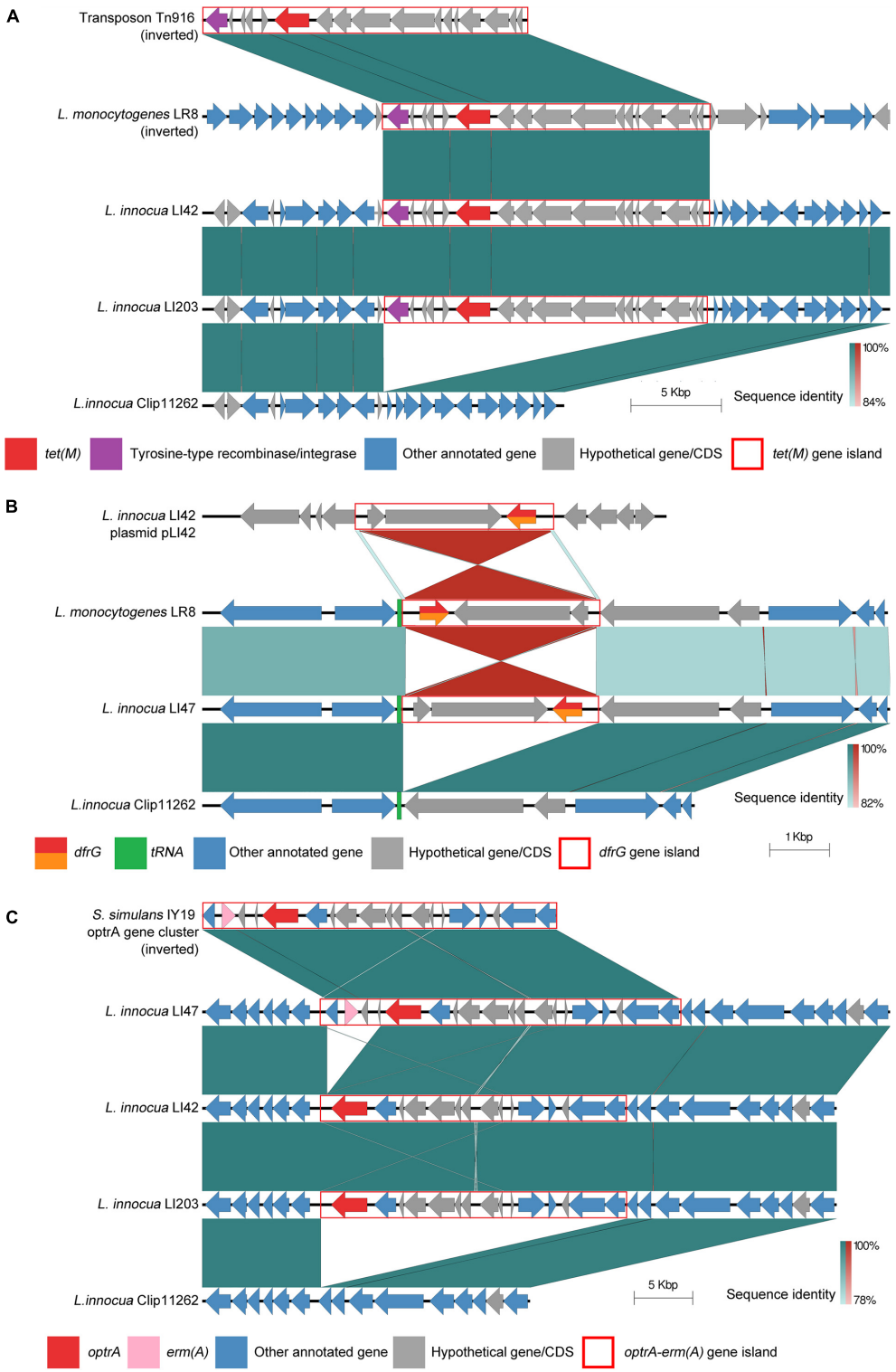

FIGURE 2 | Linear sequence comparison of resistance genes and its flanking region on chromosome. The *tet*(M) gene islands **(A)**, *dfrG* gene islands **(B)**, and *optrA-erm*(A) gene islands **(C)** and their flanking regions of LI42, LI47 or LI203 chromosomes was compared with their most closely related orthologs from nr/nt database and the susceptible *L. innocua* reference genome Clip11262. **(A)** Comparison of the *tet*(M) gene islands on LI42 and LI203 chromosomes with *L. monocytogenes* LR8 and transposon TN916. **(B)** Comparison of *dfrG* gene island in LI47 chromosome with plasmid pLI42 and *L. monocytogenes* LR8 chromosome. **(C)** Comparison of *optrA-erm*(A) gene islands in LI47, LI42, and LI203 chromosomes with *S. simulans* IY19 optrA gene cluster. Resistance genes and genes related to insertion or recombination were highlighted. Resistance gene islands were marked in red frames. Green or red shades between each two sequences represented direct or inverted nucleotide identity between covered regions.

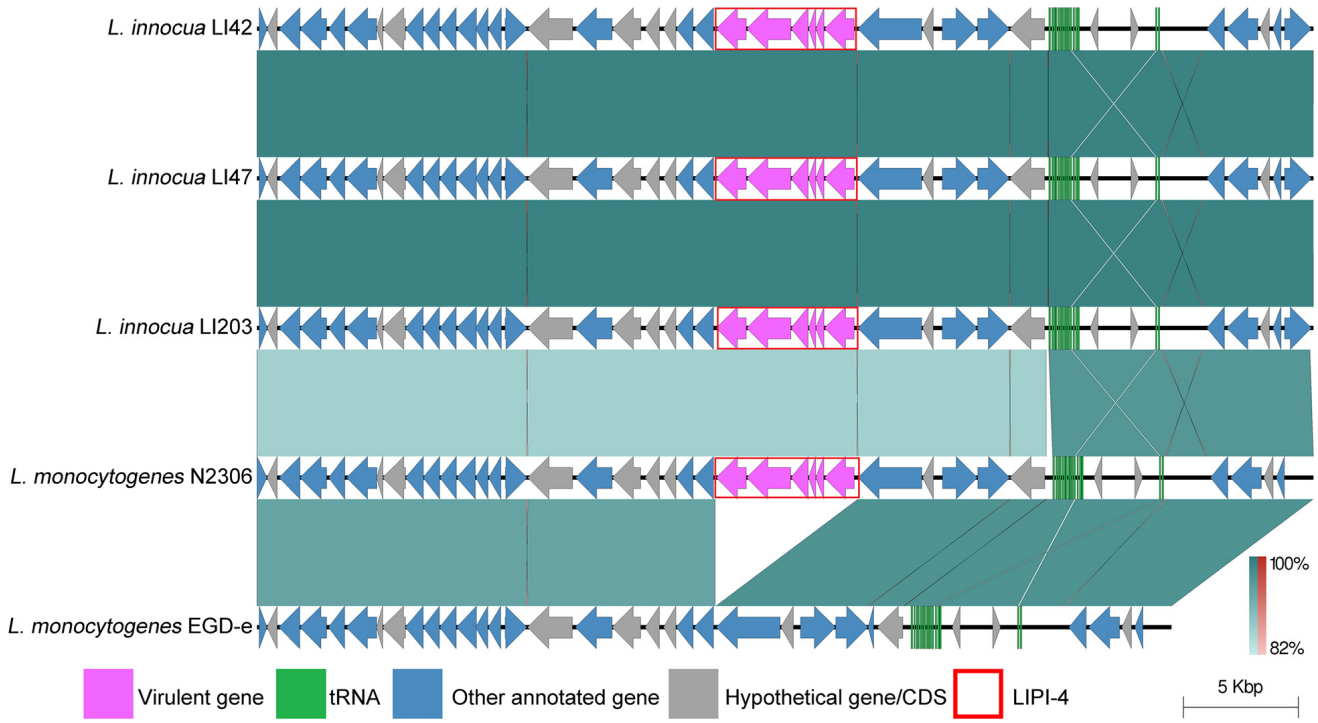

FIGURE 3 | Linear sequence comparison of LIPI-4 and its flanking regions. Sequences of LIPI-4 and their flanking regions on LI42, LI47, and LI203 genomes were compared with their ortholog in *L. monocytogenes* reference genome N2306 and EGD-e. Virulence genes were highlighted in pink arrows and LIPI-4 regions were marked in red frame. Green or red shades between each two sequences represented direct or inverted nucleotide identity between covered regions.

this study. The *tet*(M) gene-containing islands were found to be orthologs of transposon Tn*916*. The differences in the sequence context identified in *L. monocytogenes* LR8 and the two *L. innocua* isolates suggested its ability to transfer and recombine at different positions on bacterial chromosome. The short direct repeat sequences flanking *dfrG* gene-containing islands may facilitate insertion into the genome, a feature noted on both chromosomes as well as plasmids. The flanking regions of *dfrG* islands in *L. innocua* LR8 and LI47 are identical, while the insertional orientation of the module is opposite, reflecting the flexibility of this step. There were no orthologs of *optrA-erm*(A) gene-containing islands found in any *L. monocytogenes* genomes analyzed to date. However, its identification in *S. simulans* (Sun et al., 2018), a non-closely related species of *L. innocua*, as well as their significant identity, suggested a potentially recent horizontal gene transfer, which possibly hints at its horizontal transmitting nature. Additionally, the GC content of *optrA-erm*(A) gene island is 35.1%. Comparing with *L. monocytogenes* (∼38.1%) and *L. innocua* (∼37.5%), it is closer to *S. simulans* (∼35.9%, *S. simulans* strain NCTC11046, NZ_LS483313). Thus *S. simulans* is more likely to be the original host of this gene island.

Discovery of *L. monocytogenes* MDR plasmids and resistance gene-containing islands on chromosome in *L. innocua* confirmed the fact that the latter can act as a gene sink, collecting AMR determinants from a range of sources. Antimicrobial resistant *L. innocua* have the potential to constitute a serious threat to public health through possible transferring of resistance genes to susceptible *L. monocytogenes*.

Genomic and Evolutionary Features and of Virulence

L. innocua containing LIPIs are usually considered as "atypical"' (Volokhov et al., 2007; Clayton et al., 2014; Moura et al., 2019). Nonetheless, LIPI-4 is recently reported widespread among this species (Moura et al., 2019). In this study, LIPI-4 was identified in all three *L. innocua* studied. The existence of the LIPI-4 orthologs in *L. innocua* suggested a possibility of gain or loss of virulence genes during evolution.

A LIPI-4 phylogenetic tree created using 10 *L. innocua* and 11 *L. monocytogenes* clearly clustered by species (**Supplementary Figure 1**). The tree was split precisely into two branches in accordance with their species. The *L. innocua* branch had longer internal evolutionary distances compared to the other, implying a longer evolutionary history. The core genome phylogenetic tree of *L. innocua* and *L. monocytogenes* indicated an explicit clustering by species. The *L. monocytogenes* branch was clustered by lineages, while *L. monocytogenes* LIPI-4 were interspersed in the *L. monocytogenes* lineage -I cluster (**Supplementary Figure 2**). All *L. monocytogenes* containing LIPI-4 were found in lineage -I, and in this case the branch containing *L. monocytogenes* lineages -II and -III collapsed. There

was no explicit common ancestor for the *L. monocytogenes* containing LIPI-4.

To better discover the evolutionary pathway of LIPI-4, K_a/K_s values were calculated for both *L. innocua* and *L. monocytogenes*, except wherein the gene had no mutation among all pairs (**Supplementary Table 3**; Wang et al., 2009, 2010). When the K_a/K_s ratio was less than 1 for all *L. innocua* and monocytogenes LIPI-4 genes, this implied that LIPI-4 was under purifying selection for both species. All genes of LIPI-4 orthologous were in identical order and shared high similarity in gene sequence individually. All *L. monocytogenes* harboring LIPI-4 belonged to lineage -I. Both genomes and LIPI-4 containing regions of *L. monocytogenes* were less diverse comparing with those of *L. innocua*. Additionally, no LIPI-4-absent *L. innocua* strain had been reported. Combining the above observations, it may imply that LIPI-4 of both species may originate from same ancestor. The obviously slower differentiation rate of *L. monocytogenes* LIPI-4 than that of *L. innocua*, suggested *L. innocua* may acquire the LIPI-4 earlier than *L. monocytogenes*.

Since phylogeny of LIPI-4 and genome showed no branch crossing, together with the result of the sequence comparison where average identity of LIPI-4 was 4% lower than the overall genome average identity between the two species, it is less likely that horizontal gene transfer of LIPI-4 arose in from contemporary *L. innocua*. Moreover, as no other species besides *L. innocua* and *L. monocytogenes* have been found to harbor a LIPI-4 ortholog or found through online BLASTN toward NCBI nt/nr database. It could be assumed that LIPI-4 may have transferred from a progenitor of *L. innocua* to a later *L. monocytogenes* lineage -I. However, the origins and transferring path of LIPI-4 remains unknown.

CONCLUSION

L. innocua, shared resistance and virulence genes with its infamous close relative *L. monocytogenes,* is not totally innocuous. As reports have highlighted, *L. innocua* and *L. monocytogenes* are commonly detected together in the same ecological niches (Franco et al., 1995; Wagner et al., 2007; Kim et al., 2017; Zhao et al., 2021). This implies the possibility of virulence/resistance gene transferring between these two species on the other side. Therefore, it would be better for public health that *L. innocua* need to be taken into consideration to refine the risk assessment of *L. monocytogenes* during future food surveillance and monitoring.

DATA AVAILABILITY STATEMENT

Accession numbers for complete genome sequences are SAMN18079989 (LI42), SAMN18080006 (LI47), and SAMN18080009 (LI203).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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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.2021.694361/full#supplementary-material

Supplementary Figure 1 | LIPI-4 phylogenetic tree of 4 *L. innocua* isolates with reference *L. innocua* and *L. monocytogenes*. Phylogenetic tree was built with FastTree and modified by FigTree using LIPI-4 sequences of 4 *L. innocua* and from NCBI nt/nr database. Root was set as midpoint.

Supplementary Figure 2 | Genome phylogenetic tree of 4 *L. innocua* isolates with reference *L. innocua* and *L. monocytogenes*. Phylogenetic tree was built with FastTree (v2.1.10) and modified by FigTree using whole genome sequences of 4 *L. innocua* and completely assembled reference genomes of *L. monocytogenes* and *L. innocua* from NCBI assembly database. Root was set as midpoint. *L. innocua* strains were marked in blue. *L. monocytogenes* strains with LIPI-4 were marked in red, while the strains without LIPI-4 were marked in yellow.

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Characterization of Tigecycline Resistance Among Tigecycline Non-susceptible *Klebsiella pneumoniae* Isolates From Humans, Food-Producing Animals, and *in vitro* Selection Assay

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Emergence of extensively drug-resistant isolates of *Klebsiella pneumoniae* has prompted increased reliance on the last-resort antibiotics such as tigecycline (TGC) for treating infections caused by these pathogens. Consumption of human antibiotics in the food production industry has been found to contribute to the current antibiotic resistance crisis. In the current study, we aimed to investigate the mechanisms of TGC resistance among 18 TGC-non-susceptible (resistant or intermediate) *K. pneumoniae* (TGC-NSKP) isolates obtained from human (n = 5), food animals (n = 7), and *in vitro* selection experiment (n = 6). Isolates were genotyped by multilocus sequence typing (MLST). *ramR*, *acrR*, *rpsJ*, *tetA*, and *mgrB* (for colistin resistance) genes were sequenced. The presence of *tetX*, *tetX1*, and carbapenemase genes was examined by PCR. Susceptibility to different classes of antibiotics was evaluated by disc diffusion and broth macrodilution methods. The expression level of *acrB* was quantified by RT-qPCR assay. The 12 TGC-NSKP isolates [minimum inhibitory concentrations (MICs) = 4–32 mg/l] belonged to 10 distinct sequence types including ST37 (n = 2), ST11, ST15, ST45, ST1326 (animal isolates); ST147 (n = 2, human and animal isolates); and ST16, ST377, ST893, and ST2935 (human isolates). Co-resistance to TGC and colistin was identified among 57 and 40% of animal and human isolates, respectively. All human TGC-NSKP isolates carried carbapenemase genes (bla_{OXA-48}, bla_{NDM-1}, and bla_{NDM-5}). *tetX/X1* genes were not detected in any isolates. About 83% of TGC-NSKP isolates (n = 15) carried *ramR* and/or *acrR* alterations including missense/nonsense mutations (A19V, L44Q, I141T, G180D, A28T, R114L, T119S, Y59stop, and Q122stop), insertions (positions +205 and +343), or deletions (position +205) for *ramR*, and R90G substitution or frameshift mutations for *acrR*. In one isolate *ramR* amplicon was not detected using all primers used in this study. Among seven colistin-resistant isolates, five harbored inactivated/mutated MgrB due to premature termination by nonsense mutations, insertion of IS elements, and frameshift mutations. All isolates

revealed wild-type RpsJ and TetA (if present). Increased expression of *acrB* gene was detected among all resistant isolates, with the *in vitro* selected mutants showing the highest values. A combination of RamR and AcrR alterations was involved in TGC non-susceptibility in the majority of studied isolates.

Keywords: tigecycline resistance, *Klebsiella pneumoniae*, food animals, ramR, AcrR, AcrAB efflux pump

INTRODUCTION

Antibiotic resistance is rising to dangerously high levels in both human and veterinary medicine. The limited number of antibiotics coming to the market and new threats arising from extensively drug-resistant bacteria bring us perilously to the end of antibiotic era in which common treatable infections and minor injuries can once again be fatal. The use of human antibiotics in food animals as growth promoters or prophylactic agents has been identified as a significant contributing factor to increasing antimicrobial resistance (Van et al., 2020). Food animals have the potential to serve as reservoir for antibiotic-resistant bacteria, which can be transmitted to humans via direct contact or the food chain (Founou et al., 2016). *Klebsiella pneumoniae* is among the most problematic pathogens that have acquired much public health concern due to developing resistance to most clinically important antibiotics. It is an opportunistic pathogen that commonly causes a variety of the community- and hospital-acquired infections including urinary tract infection, pneumonia, and bloodstream infection. The emergence and dissemination of carbapenemase-producing strains of *K. pneumoniae* in healthcare facilities constitute a serious threat to public health (Pitout et al., 2015). The management of infections caused by carbapenem-resistant *K. pneumoniae* (CRKP) is complicated and often requires the use of last-resort antibiotics such as tigecycline (TGC) and colistin (Renteria et al., 2014; Sader et al., 2015). TGC is the first member of the novel class of glycylcyclines with expanded-spectrum antibacterial activity against Gram-negative and Gram-positive bacteria. It is approved by the Food and Drug Administration (FDA) for use in complicated skin and skin structure infections, complicated intra-abdominal infections, and community-acquired bacterial pneumonia (Beabout et al., 2015). TGC acts by inhibition of bacterial protein synthesis and has the ability to evade the classical mechanisms mediating resistance to tetracyclines including ribosomal protection and active efflux mediated by Tet proteins (Livermore, 2005).

In *K. pneumoniae*, TGC resistance is being increasingly reported since its approval (Spanu et al., 2012; Chiu et al., 2017a). TGC resistance in *K. pneumoniae* is believed to be mainly mediated by the overexpression of AcrAB efflux pump, which is regulated by local repressor AcrR and transcriptional activator RamA. The latter protein is also negatively regulated by RamR whose mutations have been found to contribute to significant increases in *ramA* and subsequently *acrAB* expression upon resistance occurrence (Hentschke et al., 2010; Chiu et al., 2017b). Decreased susceptibility to TGC has been also found to be related to alterations in the efflux pump encoding gene *tetA* (Du et al., 2018), or *rpsJ* (Beabout et al., 2015), the gene that encodes the ribosomal S10 protein. Moreover, enzymatic inactivation by TetX protein, a flavin-dependent monooxygenase, has been described to confer TGC resistance among some clinical pathogens (Moore et al., 2005; Deng et al., 2014). We aimed in the current study to investigate the TGC resistance determinants from a diverse group of TGC-non-susceptible (intermediate or resistant) *K. pneumoniae* (TGC-NSKP) isolates of clinical and animal origins and *in vitro* developed TGC-NSKP mutants. Also, the sequence types of TGC-NSKP isolates were determined by multilocus sequence typing (MLST) to identify major types associated with TGC non-susceptibility in each group of bacterial isolates from different host origins.

MATERIALS AND METHODS

Bacterial Isolates

Klebsiella pneumoniae isolates from animal and human sources were included in this study. Animal isolates were obtained by taking cloacal swabs (using sterile cotton swab) from randomly selected broilers at a major chicken slaughterhouse. Taken samples were seeded on Eosin Methylene Blue (EMB) agar plates and were incubated at 37°C for 24 h. Screening for TGC-non-susceptible isolates among the grown colonies on EMB agar was performed using Mueller–Hinton broth (MHB) supplemented with 3 mg/l of TGC according to the method described in our previous study (Pishnian et al., 2019). Moreover, five clinical TGC-NSKP isolates obtained from patients hospitalized in two teaching hospitals of the country were included as human isolates. Identification of isolates was performed by conventional biochemical methods (Mahon et al., 2018).

In vitro Selection of Tigecycline-Resistant Bacteria

To identify the genetic alterations mediating TGC resistance, upon TGC exposure, an *in vitro* resistance induction experiment was performed by exposing three TGC-susceptible (TGC-S) isolates to elevated concentrations of TGC. The Mueller–Hinton agar (MHA) plates supplemented with a sub-inhibitory concentration of antibiotic [1/2 × of minimum inhibitory concentrations (MICs)] were inoculated with 3×10^5 CFU/ml TGC-S bacterial suspension and were incubated for 24–72 h. Colonies appearing on each plate were randomly picked and reisolated on media with the same concentration of TGC or concentrations, which were within 1.25–1.5× of previous concentration. In cases where no growth was observed at higher concentrations, colonies appeared at the same concentration were picked and transferred to MHA supplemented with the same and higher concentration of TGC. The obtained *in vitro*

induced TGC-NSKP isolates were subjected to antimicrobial susceptibility testing.

Antimicrobial Susceptibility Testing

The MICs of TGC (Glentham Life Sciences, Corsham, United Kingdom) (batch nos. 389SOI and 081GRB), colistin (colistin sulfate, Glentham Life Sciences, United Kingdom; batch no. 844WZQ), and imipenem (IPM) (Glentham Life Sciences, United Kingdom; batch no. 205WLC) were determined by broth macrodilution method using freshly prepared (less than 12-h-old) MHB from Difco (BD Diagnostic Systems, Sparks, MD, United States). The susceptibility to other classes of antibiotics was determined by disc diffusion method (Kirby–Bauer) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines using the following antibiotics: gentamicin, amikacin, ampicillin, ceftriaxone, cefepime, nalidixic acid, ciprofloxacin, levofloxacin, gatifloxacin, tetracycline, doxycycline, minocycline, chloramphenicol, nitrofurantoin, and fosfomycin (BBL Sensi-Disc^{TM}, Becton-Dickinson, Sparks, MD, United States). Due to the lack of established CLSI breakpoints for TGC at this time, the FDA breakpoints issued for Enterobacteriaceae (susceptible $\leq$ 2 mg/l, intermediate = 4 mg/l, and resistant $\geq$ 8 mg/l) were applied for interpretation of results. Isolates characterized with colistin MIC values greater than 2 mg/l were categorized as resistant according to guidelines described by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). *Escherichia coli* ATCC 25922 was used as a quality-control strain for antimicrobial susceptibility testing.

Bacterial Genotyping by Multilocus Sequence Typing

MLST with seven housekeeping genes (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB*, and *tonB*) was carried out for TGC-NSKP isolates of human and animal origins following the methods described previously (Diancourt et al., 2005). The allelic profiles and sequence types (STs) were assigned by using the *K. pneumoniae* MLST database provided by the Institut Pasteur, Paris, France.[1]

Molecular Determinants of Tigecycline, Co*listin, and* Carbapenem Resistance

The TGC-NSKP isolates were screened for the presence of *tetX* and *tetX1* genes by performing PCR with gene-specific primers (**Table 1**) [a second pair of primers were used for detection of *tetX1* gene (forward 5′-GCGACATTCCTGAACCAGAAACG and reverse 5′-CGGACGATTACTCTTCCAAGG)]. The coding regions of *ramR*, *acrR*, *rpsj*, *tetA*, and *mgrB* [for colistin (Col) resistance] were amplified and sequenced using the primers listed in **Table 1**. For isolates that did not yield a PCR product using primers targeting amplification of *ramR* coding sequence as well as some flanking regions (ramR-ext), amplification with other pairs of primers targeting an internal region of *ramR* coding sequence was repeated (ramR-int). Mutations were characterized by comparing the sequences with those of *K. pneumoniae* ATCC 700603 and three TGC-susceptible isolates (TGC MICs = 0.25 mg/l). The impact of the identified amino acid substitutions on the biological function of the protein

[1]http://bigsdb.pasteur.fr/klebsiella/klebsiella.html

TABLE 1 | Nucleotide sequences of primers used in this study.

Primer name	Sequence (5′–3′)	Size of product (bp)	References
Sequencing or detection			
ramR-ext-F	TGGTCAGACGTGCCAAGATC	654	This study
ramR-ext-R	CAGTGTTTCCGGCGTCATTAG		
ramR-int-F	GCAAGCGTTACTGGAAGCTG	515	This study
ramR-int-R	CAAAGCCAAGGGCGATAATCT		
acrR-F	GTAAAGTCATTAACCTATGGCACG	667	This study
acrR-R	TTAAGCTGACAAGCTCTCCG		Rosenblum et al., 2011
rpsj-F	CAATCGTAATGGGTATGAGGAGT	514	This study
rpsj-R	CCTGAGTAACACGGTTTGCTT		
tetA-F	ACCCAACAGACCCCTGATCGT	1,133	This study
tetA-R	GCAAGTAGAGGGCAGCGCCT		
tetX-F	TTAGCCTTACCAATGGGTGT	243	Bartha et al., 2011
tetX-R	CAAATCTGCTGTTTCACTCG		
tetX1-F	TCAGGACAAGAAGCAATGAA	150	Bartha et al., 2011
tetX1-R	TATTTCGGGGTTGTCAAACT		
mgrB-F	ACCACCTCAAAGAGAAGGCGTT	347	Haeili et al., 2017
mgrB-R	GGCGTGATTTTGACACGAACAC		
RT-qPCR			
acrB-F	CAGCTTAACGCCTCGATCATC	127	This study
acrB-R	CCAGCTCAATTTTGGCGACATC		
rpsl-F	CCGTGGCGGTCGTGTTAAAGA	109	Cannatelli et al., 2013
rpsl-R	GCCGTACTTGGAGCGAGCCTG		

(i.e., neutral or deleterious) were further predicted by using the Protein Variation Effect Analyzer tool (PROVEAN) (Choi and Chan, 2015). The IS Finder database[2] was used to identify and analyze insertion sequences. Moreover, the presence of carbapenemase-encoding genes (bla_{KPC}, bla_{VIM}, bla_{NDM}, and bla_{OXA-48}) were examined by PCR using the primers and amplification conditions described previously (Poirel et al., 2011). The nucleotide sequences of bla_{NDM} were determined using the primers NDM-F-5′-GCCCAATATTATGCACCCGGTC and NDM-R-5′-AGCGCAGCTTGTCGGCCAT (Jafari et al., 2019).

Assessment of acrB Expression

To investigate the association between TGC non-susceptibility and overexpression of AcrAB efflux pump, the expression level of *acrB* gene was measured using RT-qPCR analysis. The total RNA from all TGC-S and TGC-NS bacterial cells was harvested using a GeneAll RiboEx Total RNA extraction kit (GeneAll Biotechnology, Seoul, South Korea). cDNA was synthesized from 1 μg of RNase-free DNase I (Takara Biotechnology, Dalian, China)-treated total RNA using Revert Aid first-strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, United States). Real-time PCR amplification was performed using a Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA, United States) on a Eco Real-Time PCR system (Illumina, San Diego, CA, United States). The relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ formula with *rpsL* housekeeping gene as internal control. A TGC-S isolate with TGC MIC of 0.25 mg/l was used as a reference strain. In the case of *in vitro* selected mutants, expression levels of *acrB* were compared with those of parental TGC-S isolates.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of the studied genes have been deposited at GenBank nucleotide sequence database under the following accession numbers:

MW653710 to MW653712 (mutated/altered *mgrB*), MW653713 and MW653714 (wild-type *mgrB*), MW653715 to MW653725 (mutated/altered *ramR*), MW653726 to MW653730 (wild-type *ramR*) MW653731 to MW653736 (mutated/altered *acrR*), MW653737 (HK10-S, AK88-S, AK298, AK299, and HK98), MW653738 (HK2-S), MW653739 (AK294 and AK297) (wild-type *acrR*), MW653740 (*rpsJ*, all isolates had identical sequences), and MW653741 (*tetA*, all isolates had identical sequences).

RESULTS

Bacterial Isolates, Molecular Typi*ng, and* Antimicrobial Susceptibility Testing

Among the 1,430 samples taken from healthy broilers (collected from 83 different farms), six TGC-resistant (TGC-R) bacteria were detected, all corresponding to *K. pneumoniae*. The TGC-R *K. pneumoniae* (TGC-RKP) isolates displayed TGC MICs ranging from 8 to 32 mg/l. Moreover, one TGC-R isolate (AK513, TGC MIC = 8 mg/l) was included from our previous work (Pishnian et al., 2019), which was obtained from a turkey during the screening for colistin-resistant bacteria (**Table 2**). The seven TGC-R animal isolates belonged to six different sequence types including ST37 (n = 2 isolates) and ST11, ST15. ST45, ST147, and ST1326 (n = 1 each). Testing susceptibility to other antimicrobials revealed that TGC resistance was linked to a multidrug-resistant phenotype, and all animal TGC-RKP isolates showed resistance to quinolones, chloramphenicol, other members of tetracycline family and nitrofurantoin. The full resistance rate to gentamycin, ceftriaxone, and fosfomycin was found to be 42.8, 28, and 28%, respectively. One isolate, AK294, was found to be extended-spectrum β-lactamase (ESBL) producer using the combination disc method. Co-resistance to colistin and TGC was observed among 57% of animal isolates belonging to ST37, ST11, and ST15. All TGC-R animal isolates were susceptible to amikacin and IPM. Among the five studied human isolates, one was TGC-R (MIC = 8 mg/l), and the remaining isolates showed intermediate susceptibility to TGC (MICs = 4 mg/l). The MLST distributed the five TGC-NSKP human isolates into five distinct sequence types, including ST16, ST147 (also found in one animal isolate), ST377, ST893, and ST2935. Two human isolates showed simultaneous resistance to TGC (nonsusceptible), colistin, and IPM, with the remaining three isolates being characterized with co-resistance to IPM and TGC. Fosfomycin and amikacin were among the few antimicrobials that showed 40% activity against the TGC, IPM, and ±Col NSKP human isolates. The antimicrobial susceptibility profiles of the studied isolates are described in **Table 2**.

In vitro Selection of *Klebsiella pneumoniae* Mutants With Reduced Susceptibility to Tigecycline

Three TGC-susceptible *K. pneumoniae* isolates (TGC MICs = 0.25 mg/l) were exposed to increasing concentrations of TGC. As 50% of the TGC-NSKP isolates in this study were characterized with co-resistance to colistin and TGC, we included one TGC-S but Col-R isolate among the *in vitro* selected isolates to see if resistance to colistin facilitates development of TGC resistance. Overall, it took 21–23 selection cycles to obtain six TGC-NSKP isolates with TGC MICs of 32 (n = 1 mutant), 16 (n = 3 mutants), 8 (n = 1 mutant), and 4 mg/l (n = 1 mutant) from three parental TGC-S isolates (**Table 2**). There was no significant difference between the selection cycles required to reach a TGC-NSKP isolate among the ColR and ColS isolates. Interestingly, all progenitor TGC-S isolates that were susceptible to tetracycline, minocycline, doxycycline, nalidixic acid, and chloramphenicol before induction became fully resistant (or showed intermediate susceptibility) to these antibiotics upon TGC non-susceptibility induction.

Detection and Sequence Anal*ysis of* Resi*stance* Conferring Genes

The plasmid-encoded *tetX* and *tetX1* genes were not detected in any of the TGC-NSKP isolates of both origins. All TGC-NSKP isolates of human, animal, and *in vitro* selected origins revealed

[2]https://isfinder.biotoul.fr

TABLE 2 | Genotypic and phenotypic characteristics of tigecycline susceptible and non-susceptible *K. pneumoniae* isolates obtained from human, food animals, and *in vitro* selection assay.

Isolate	Sequence type	MIC (mg/l)			RamR	AcrR	TetA	MgrB	Carbapenemase	Antimicrobial susceptibility profile		
		Tgc	Col	Ipm						S	I	R
Wild type isolates												
AK88-S	ND	0.25	>128	0.25	WT	WT	–	WT	–	D, LVX, NA, TE, GM, MI, CRO, AN, GAT, F/M, FOS, C, CIP, FEP		AM
HK10-S	ND	0.25	0.25	0.25	WT	WT	–	WT	–	D, LVX, NA, TE, GM, MI, CRO, AN, GAT, F/M, FOS, C, CIP,FEP		AM
HK2-S	ND	0.25	0.25	1	WT	WT	–	WT	–	AN, FOS, TE, D, MI		AM, CRO, F/M, NA, FEP, GM, CIP, LVX, GAT, C
Laboratory induced mutants												
AK88-R1	ND	16	>128	0.25	L44Q	WT	–	WT	–	LVX, NA, GM, CRO, AN, GAT, F/M, FOS, CIP, FEP		AM, D, MI, C, TE
AK88-R2	ND	8	>128	0.25	A28T R114L	WT	–	WT	–	LVX, GM, CRO, AN, GAT, F/M, FOS, CIP, FEP	NA	AM, D, MI, C, TE
HK10-R1	ND	32	0.25	0.25	WT	Δ14nt (g58-t71) (frameshift)	–	WT	–	LVX, GM, CRO, AN, GAT, F/M, FOS, CIP, FEP	NA	AM, D, MI, C, TE
HK10-R2	ND	16	0.25	0.25	WT	WT	–	WT	–	LVX, GM, CRO, AN, GAT, F/M, FOS, CIP, FEP		AM, NA, D, MI, C, TE
HK10-R3	ND	16	0.25	0.25	T119S	WT	–	WT	–	LVX, GM, CRO, AN, GAT, F/M, FOS, CIP, FEP	NA	AM, D, MI, C, TE
HK2-R1	ND	4	0.25	1	Insertion of 7nt at +205 (frameshift)	WT	–	WT	–	AN, FOS		AM, CRO, F/M, NA, FEP, GM, CIP, LVX, GAT, D, C, MI, TE
Animal isolates												
AK267	ST15	32	128	0.25	A19V	△G139[b] (frameshift)	+WT	Insertion of IS*5*-like between +51, +52	–	FOS, FEP, AN, CRO	GM	D, MI, CIP, LVX, NA, AM, GAT, C, F/M, TE
AK291	ST37	8	64	0.25	WT	R90G	+WT	ΔT72 (frameshift)	–	FEP, AN, CRO	FOS	D, MI, CIP, LVX, NA, AM, GAT, C, F/M, TE, GM
AK294	ST11	8	>128	0.25	Δ*ramR*[a] locus	WT	–	WT	–	AN	FEP	D, MI, CIP, LVX, NA, AM, GAT, C, F/M, TE, FOS, CRO, GM
AK297	ST1326	16	0.25	0.25	WT	WT	+WT	WT	–	FEP, AN, CRO	FOS, GM	D, MI, CIP, LVX, NA, AM, GAT, C, F/M, TE
AK298	ST147	8	0.25	0.12	Y59stop	WT	–	WT	–	GM, FEP, AN, CRO		FOS, D, MI, CIP, LVX, NA, AM, GAT, C, F/M, TE
AK299	ST45	8	0.25	0.12	Q122stop	WT	+WT	WT	–	FOS, AN	FEP, C	D, MI, CIP, LVX, NA, AM, CRO, GAT, F/M, GM, TE
AK513	ST37	8	>128	0.25	WT	R90G	+WT	Insertion of IS*3*-like between +112, +113	–	FOS, FEP, AN, CRO	GM	D, MI, CIP, LVX, NA, AM, GAT, C, F/M, TE
Human isolates												
HK5	ST377	4	0.12	16	Insertion of 3nt at +343	WT	–	WT	OXA-48	AN	FOS	LVX, D, MI, CIP, AM, NA, GM, CRO, GAT, FEP, TE, C, F/M
HK6	ST16	4	0.12	8	I141T	△G139[b] (frameshift)	+WT	WT	OXA-48	FOS		LVX, D, MI, CIP, AM, AN, NA, GM, CRO, GAT, FEP, F/M, TE, C

(Continued)

TABLE 2 | Continued

Isolate	Sequence type	MIC (mg/l)			RamR	AcrR	TetA	MgrB	Carbapenemase	Antimicrobial susceptibility profile		
		Tgc	Col	Ipm						S	I	R
HK98	ST147	4	0.12	128	G180D	WT	–	WT	NDM-1		FOS	D, MI, LVX, CIP, AM, NA, GM, CRO, GAT, FEP, TE, AN, C, F/M
HK156	ST893	4	64	32	WT	ΔG139[b] (frameshift)	+WT	Premature termination by nonsense mutation at nt88	OXA-48	AN, C	FOS, F/M	MI, D, CIP, LVX, AM, NA, GM, CRO, GAT, FEP, TE
HK157	ST2935	8	>128	64	Δ12nt (a205-c216), I141T	Δ12nt (a430-g441)	–	Premature termination by nonsense mutation at nt88	OXA-48 NDM-5	FOS	C	MI, D, CIP, LVX, AM, NA, GM, CRO, GAT, FEP, TE, AN, F/M

WT, wild type; ND, not determined, S, susceptible; I, intermediate; R, resistant.
IPM, imipenem; TGC, tigecycline, Col, colistin; FOS, fosfomycin; D, doxycycline; TE, tetracycline; MI, minocycline; NA, nalidixic acid; CIP, ciprofloxacin; LVX, levofloxacin; GAT, gatifloxacin, GM, gentamicin; AN, amikacin; AM, ampicillin; CRO, ceftriaxone; FEP, cefepime; C, chloramphenicol; F/M, nitrofurantoin.
[a] *ΔramR locus, not amplifiable with all primers used in this study.*
[b] *Deletion of one of the guanines at positions 134–139.*

wild-type RpsJ and TetA (if present). The *ramR* amplicons were obtained for 17 out of 18 TGC-NSKP isolates with the exception of isolate AK294, which did not yield any PCR product using the two pairs of primers used in this study. Eleven out of eighteen TGC-NSKP isolates (61%) (n = 3 animal, 4 human, and 4 *in vitro* selected mutants) revealed mutated/altered *ramR* gene. The observed RamR alterations included premature termination by nonsense mutations at amino acid positions 59 [TAC(Y) > TAA] and 122 [CAG (Q) > TAG]; a 12-nt deletion at positions 205–216; a 3-nt (AGC, serine) insertion at position +343 (between +342, +343); substitutions A19V, G180D, and I141T found among human and animal TGC-NSKP isolates and frameshift mutation due to insertion of 7 nt at position +205; and substitutions L44Q, A28T+R114L, and T119S observed among *in vitro* selected mutants. Moreover, AcrR alterations were observed among seven (38%) TGC-NSKP isolates (three animal isolates, three human isolates, and one *in vitro* selected mutant). A frameshift mutation resulting from deletion of one of the six guanines at positions 134–139 was the most common AcrR alteration identified among both animal (n = 1) and human isolates (n = 2). AcrR R90G substitution was found among two animal isolates, both of which carried a wild-type RamR protein. Also a frameshift mutation resulting from 14-nt deletions (g58-t71) was detected in one *in vitro* selected mutant (TGC MIC = 32 mg/l) carrying a wild-type RamR. One TGC-NSKP human isolate co-harbored two 12-nt deletion in *ramR* (a205-c216) and *acrR* (a430-g441) genes (**Table 2**). Among seven colistin-resistant isolates, five harbored inactivated/mutated MgrB due to premature termination by nonsense mutations, insertion of IS elements, and frameshift mutation as shown in **Table 2**. The genes encoding for carbapenemases were detected in all IPM-resistant human isolates, with three isolates harboring $bla_{\text{OXA-48}}$, one carrying $bla_{\text{NDM-1}}$, and one co-harboring $bla_{\text{OXA-48}}$ and $bla_{\text{NDM-5}}$. Two TGC-RKP isolates with TGC MIC = 16 mg/l carried wild-type RamR and AcrR proteins (**Table 2**).

Expression Levels *of the AcrAB* Pump

To see if TGC non-susceptibility was linked to overexpression of AcrAB pump, the expression level of *acrB* gene was quantified by RT-qPCR analysis. A constitutively expressed housekeeping gene *rpsL* was used as a control and the TGC-susceptible clinical strain HK10 as a reference for the data analysis of animal and human isolates. For the *in vitro* selected mutants, the expression level was compared with that of their TGC-S parental strains. The *in vitro* selected TGC-NSKP isolates showed the highest expression levels (18- to 146-fold) among the studied isolates. Since the expression level of *acrB* in all three TGC-S parental isolates (K88-S, K10-S, and K2-S) were similar (representing with similar Δ_{CT}), there was no significant difference when *acrB* expression of *in vitro* selected mutants was compared with that of parental TGC-S or with the TGC-S clinical isolate HK10-S. The TGC-RKP isolates of animal and human origins displayed levels of *acrB* expression that were 5- to 62-fold and 2- to 26-fold higher than those of control HK10, respectively (**Figure 1**). These data support the hypothesis that increased expression of *acrB* is associated with increased MICs of TGC.

DISCUSSION

Multidrug-resistant isolates of *K. pneumoniae* have emerged in past years as results of wide application of antibacterial agents in both human and veterinary medicine. Indeed, consumption of human antibiotics in veterinary practices has been blamed for contributing to the magnitude of current antibiotic resistance crisis. Emergence of antibiotic resistance among commensal bacteria propagated in food animals, posing a great challenge for human health since they have the potential to reach human hosts through the food chain or transmit their mobile resistance

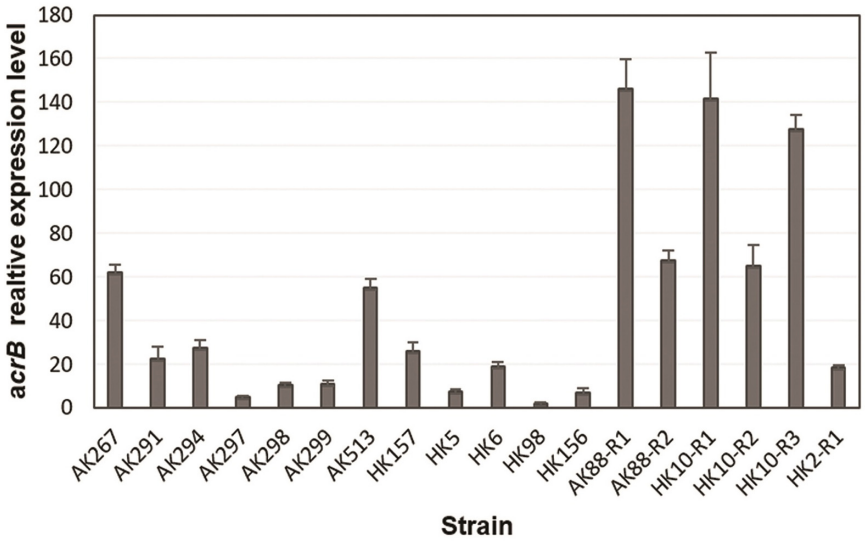

FIGURE 1 | Relative expression of *acrB* gene from tigecycline (TGC)-non-susceptible isolates of *Klebsiella pneumoniae* from different origins determined by RT-qPCR.

elements to human pathogens (Founou et al., 2016). Resistance of *K. pneumoniae* to various classes of antibiotics can be mediated by a variety of mechanisms, among which extrusion of antibiotics using efflux machineries such as AcrAB has been well-studied. Due to its broad substrate range property, AcrAB pump has been found to mediate resistance to a different families of antibiotics such as penicillins, cephalosporins, fluoroquinolones, macrolides, chloramphenicol, and tetracyclines (Xu et al., 2021). In the current study, screening among commensal bacteria of broiler chickens revealed six TGC-RKP isolates that were also resistant to quinolones, chloramphenicol, nitrofurantoin, and other tetracyclines. There are currently no TGC-containing products authorized for the veterinary use in Iran, and it is likely that selective pressure caused by application of other antibiotics such as older tetracyclines, florfenicol, or enrofloxacin may contribute to TGC non-susceptibility in animal commensal bacterial isolates. In a recent study from China, five TGC-RKP isolates that all belonged to ST1 were identified from the fecal samples of healthy chickens. Resistance to TGC as well as other antibiotics in these isolates was found to be mediated by a novel plasmid-mediated RND efflux pump gene cluster, designated *tmexCD1-toprJ1* (Lv et al., 2020). Li et al. also reported isolation of TGC-R *Klebsiella aerogenes* (TGC MIC = 32 mg/l) from the feces of a chicken farm, which contained the $bla_{\mathrm{NDM-9}}$ and *tet*(A) variant genes (Li et al., 2021). Analyzing the genetic relatedness of TGC-NSKP isolates of both origins using MLST revealed high genetic diversity among the isolates with 12 TGC-NSKP isolates belonging to 10 distinct STs, among which ST147 was found as a common type between two human and animal isolates. Most of the animal isolates belonged to STs, which are commonly reported from different human infections including ST11 (Gu et al., 2018), ST37 (Zhao et al., 2019), ST15, and ST147 (Yan et al., 2015; Chen et al., 2020). Co-resistance to TGC and colistin was observed among animal isolates belonging to ST11, ST37, and ST15. Other studies have also reported occurrence of TGC and colistin co-resistance among *K. pneumoniae* isolates belonging to ST37 and ST11 (Taniguchi et al., 2017; Xu et al., 2020). A recent study also reported detection of colistin and TGC-RKP (ST29) in municipal wastewater influents from Japan (Hayashi et al., 2021). All human TGC-NSKP isolates showed co-resistance to carbapenems and carried genes encoding for two different carbapenemases. The isolate HK157 belonging to ST2935 revealed co-resistance to three last-resort antibiotics and co-harbored a truncated *mgrB* gene, mutant *ramR* and *acrR* genes, and $bla_{\mathrm{OXA-48}}$ and $bla_{\mathrm{NDM-5}}$ carbapenemase genes. This is the first study reporting detection of $bla_{\mathrm{NDM-5}}$ among clinical CRKP isolates from Iran.

Analysis of the genes, AcrR (AcrAB repressor) and the RamR regulator, revealed inactivating genetic alterations in both proteins. In 83% of isolates (n = 15 out of 18) mutation/deletions in *ramR* (n = 8 isolates) or *acrR* (n = 4) or both (n = 3) was identified. The *ramR* was not detected in one isolate (AK294) using two pairs of primers used for amplification of the *ramR* gene, suggesting deletion of *ramR* locus in this isolate. In two animal isolates, RamR was truncated due to nonsense mutation, which resulted in production of 58 and 121 amino acid long proteins instead of wild-type protein with 193 amino acids. The RamR Q122 stop substitution has been previously reported among TGC-NSKP isolates by several other studies presenting this alteration as a common resistance mechanism among *K. pneumoniae* isolates (He et al., 2015; Chiu et al., 2017b; Li et al., 2017; Park et al., 2020). In addition to two previously reported RamR A19V and L44Q mutations (reported as L44R) (Chiu et al., 2017b), five novel amino acid substitutions, including I141T, G180D, A28T, R114L, and T119S, were identified, with the latter three changes being detected after resistance induction in *in vitro* selected mutants. The A19V substitution, which was also predicted by the PROVEAN tool to be a neutral change,

has been previously demonstrated to have no effect on the TGC MICs (Chiu et al., 2017b). While RamR A28T, R114L, and G180D substitutions were predicted by the PROVEAN tool to have a deleterious impact on protein structure (PEOVEAN scores –3.6, –4, and –6.8, respectively), the I141T and T119S substitutions (with PEOVEAN scores 0.21 and –2.44 (very close to prediction cutoff = –2.5), respectively) were predicted to be neutral changes. An isolate-carrying RamR I141T substitution co-harbored an AcrR inactivating mutation (frameshift), indicating that AcrR alteration might be the main mediator of TGC non-susceptibility in this bacterium. However, the exact role of these novel mutations in elevation of TGC MICs requires further studies by confirmatory assays. In one *in vitro* selected mutant and a clinical TGC-R isolate nucleotide insertion (7nt) and deletion (12 nt) at position +205 was observed, respectively, indicating that this position is more prone to alterations upon resistance development. Among the 18 TGC-NSKP, mutations/deletions within AcrR were detected among seven isolates from which six carried a wild-type RamR or RamR substitutions, which were predicted or previously demonstrated to be neutral changes. The novel AcrR R90G substitution detected in two TGC-R animal isolates belonging to ST37 was predicted by the PROVEAN tool to have a deleterious effect on the functionality of the protein (PROVEAN score = –5.9). The AcrR frameshift mutation mediated by deletion of one of the guanines at positions 134–139 was detected in three TGC-NSKP isolates of both origins that belonged to different STs representing this region as a mutation-prone position within *acrR* gene. In two TGC-RKP isolates with overexpressed AcrB (AK297 and HK10-R2, TGC MIC = 16 mg/l), no alteration was identified in any of the studied genes, indicating that alterations in promoter region of the studied genes or other loci (*marR* or *soxR*; Bratu et al., 2009; Veleba and Schneiders, 2012) are probably involved in resistance development in these isolates. Gene expression analysis revealed an association between overexpression of AcrAB efflux pump and TGC non-susceptibility. However, there was no correlation between the TGC MICs and the level of *acrB* expression, as some isolates with similar MICs exhibited different expression levels. This suggests the possibility of contribution of other resistance mechanisms to increased TGC MICs.

CONCLUSION

A combination of genetic alterations in AcrR and RamR mediated the overexpression of AcrAB efflux pump and subsequently TGC non-susceptibility among the majority of human, animal, and *in vitro* selected mutants studied in this work. The most worrisome finding in our study was detection of multidrug-resistant isolates with co-resistance to two last-resort human antibiotics (TGC and colistin) belonging to sequence types commonly implicated in human infections (ST11, ST37, and ST15) among commensal bacteria of food animals. This can be considered a great threat to human health due to probability of transmission of these bacteria to humans through the food chain or direct contact. Acquiring carbapenem resistance among these isolates would be the most troublesome event. While the TGC exposure history among the human isolates was not clear, emergence of TGC resistance among animal isolates (which are not exposed to TGC) is an issue of great concern. It is speculated that selective pressure caused by other antimicrobials in particular tetracycline families (as one of the most used antimicrobials in food animals) may have contributed to increased TGC MICs in these isolates probably through the overexpression of AcrAB efflux pump. Therefore, immediate actions need to be taken to restrict/minimize the use of human antibiotics (at least not as growth promoters) in food animals to prevent the emergence and dissemination of antibiotic-resistant bacteria through the food chain.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: GenBank; accession numbers MW653710-MW653741.

AUTHOR CONTRIBUTIONS

MM performed the experiments, analyzed the experiment data, and drafted the manuscript. MH and HM designed the experiments, analyzed the experiment data, and wrote the manuscript. All authors read and approved the final manuscript.

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Antibiotic Resistance Profiles and Molecular Characteristics of Extended-Spectrum Beta-Lactamase (ESBL)-Producing *Escherichia coli* and *Klebsiella pneumoniae* Isolated From Shrimp Aquaculture Farms in Kerala, India

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This study was undertaken to evaluate the prevalence of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* in selected shrimp aquaculture farms (n = 37) in Kerala, South India and to characterize the isolates using molecular tools. Overall, a low prevalence of ESBL-producers was found in the farms, most likely due to the reduced antibiotic usage in the shrimp farming sector. Out of the 261 samples (77 shrimp and 92 each of water and sediment), 14 (5.4%) tested positive for ESBL-*E. coli* or ESBL-*K. pneumoniae*. A total of 32 ESBL-*E. coli* and 15 ESBL- *K. pneumoniae* were recovered from these samples. All ESBL isolates were cefotaxime-resistant with minimal inhibitory concentration (MIC) ≥32 μg/ml. Of all isolates, 9 (28.1%) *E. coli* and 13 (86.7%) *K. pneumoniae* showed simultaneous resistance to tetracycline, ciprofloxacin, and trimethoprim-sulfamethoxazole. PCR analysis identified CTX-M group 1 ($bla_{CTX-M-15}$) as the predominant ESBL genotype in both *E. coli* (23, 71.9%) and *K. pneumoniae* (15, 100%). Other beta-lactamase genes detected were as follows: bla_{TEM} and bla_{SHV} (11 *K. pneumoniae*), $bla_{CTX-Mgroup9}$ (9 *E. coli*), and bla_{CMY-2} (2 *E. coli*). Further screening for AMR genes identified *tetA* and *tetB* (13, 40.6%), *sul1* (11, 34.4%), *sul2* (9, 28.1%), *catA* and *cmlA* (11, 34.4%), *qepA* and *aac(6′)-Ib-cr* (9, 28.1%) and *strAB* and *aadA1* (2, 6.3%) in *E. coli,* and *qnrB* (13, 86.7%), *qnrS* (3, 20%), *oqxB* (13, 86.7%), *tetA* (13, 86.7%), and *sul2* (13, 86.7%) in *K. pneumoniae* isolates. Phylogenetic groups identified among *E. coli* isolates included B1 (4, 12.5%), B2 (6, 18.8%), C (10, 31.3%), D (3, 9.4%), and E (9, 28.1%). PCR-based replicon typing (PBRT) showed the predominance of IncFIA and IncFIB plasmids in *E. coli*; however, in *K. pneumoniae*, the major replicon type detected was

IncHI1. Invariably, all isolates of *K. pneumoniae* harbored virulence-associated genes viz., *iutA*, *entB*, and *mrkD*. Epidemiological typing by pulsed-field gel electrophoresis (PFGE) revealed that *E. coli* isolates recovered from different farms were genetically unrelated, whereas isolates of *K. pneumoniae* showed considerable genetic relatedness. In conclusion, our findings provide evidence that shrimp aquaculture environments can act as reservoirs of multi-drug resistant *E. coli* and *K. pneumoniae.*

Keywords: shrimp aquaculture, extended-spectrum beta-lactamase, *Escherichia coli*, *Klebsiella pneumoniae*, multidrug resistance

INTRODUCTION

Antimicrobial resistance (AMR) is undoubtedly a huge public health crisis across the globe. AMR had long been regarded as an issue of human health alone, but recent years have witnessed a growing recognition of the imprudent use of antibiotics in multiple sectors (agriculture, food animals, aquaculture, and environment) as important drivers of resistance. However, our understanding of the extent and magnitude of the contribution of each sector to the overall burden of AMR is quite limited. Of particular concern are the intensive animal production practices, both in livestock and aquaculture sectors where antibiotics are regularly used for therapeutic, prophylactic or growth promotion purposes. Aquaculture is a rapidly growing food sector with the majority of production taking place in low and middle income countries (LMICs) (FAO, 2016). Global antimicrobial consumption in aquaculture in 2017 was estimated at 10,259 tons, with India (11.3%) being the second-largest consumer after China (Schar et al., 2020). In particular, shrimp aquaculture industry has gained prominence in recent decades owing to the growing demand in the global market. India was the top exporter of farmed shrimps in 2018 with the United States, Vietnam, and EU countries as the leading markets for Indian shrimps (FAO, 2019). This sector faces many challenges, the most important being the high disease burden caused by various viruses, bacteria, fungi, and parasites. Despite the fact that most of the devastating diseases of shrimps are due to viral or parasitic infections, a wide range of antibiotics are used in shrimp hatcheries and farms. There were recent incidences of rejection of Indian consignments of shrimp by EU countries owing to the presence of banned antibiotics such as furazolidone and chloramphenicol (Salunke et al., 2020). Antibiotics, when used appropriately, i.e., on the basis of proper diagnosis, can be helpful. But this does not seem to happen in most of the farms as farmers usually have poor access to diagnostic facilities. Moreover, the decision to use antibiotics in farms is often influenced by the advice given by neighboring farmers, drug suppliers, feed companies, private veterinarians and others.

Many factors are known to favor the emergence of AMR in aquaculture and its spread to other sectors. This includes high stocking densities leading to elevated stress and infections in shrimp, widespread use of various chemicals, nutrient-rich environment in the ponds, occupational human exposure to AMR bacteria, release of untreated water/waste to local environment, etc. (Thornber et al., 2020). Antibiotic-resistant bacteria, including human and zoonotic pathogens have been reported from various aquaculture settings (Cabello et al., 2013; Miranda et al., 2013; Watts et al., 2017). Among these, members of *Enterobacteriaceae* are of particular concern owing to their considerable ability to acquire resistance to various antimicrobials and to disseminate widely. Multidrug resistance in *Enterobacteriaceae* has become an escalating problem in healthcare as well as community settings worldwide. This in large part is due to the highly diverse and rapidly evolving group of beta-lactamases such as extended-spectrum beta-lactamases (ESBLs) and carbapenemases. ESBLs, generally found in *Enterobacteriaceae* and *Pseudomonas aeruginosa,* are a class of enzymes conferring resistance to penicillins, first-, second- and third-generation cephalosporins, and aztreonam, and are usually inhibited by beta-lactamase inhibitors such as clavulanic acid (Paterson and Bonomo, 2005). Enzyme families with ESBL phenotype are mainly described in class A (TEM, SHV, CTX-M, GES, and VEB families) and class D (OXA family) beta-lactamases (Paterson and Bonomo, 2005). Most of the ESBLs prevalent initially were TEM or SHV variants possessing amino acid substitutions which changed their substrate profile to include extended-spectrum cephalosporins. Currently, there are 183 variants of TEM and 178 variants of SHV enzymes, although not all of them are ESBLs (Bush and Bradford, 2020). In contrast, the CTX-M type ESBLs originated by the mobilization of chromosomal *bla* genes of *Kluyvera* spp., an innocuous rhizosphere bacterium (D'Andrea et al., 2013). Since 2000, CTX-M type enzymes gained prominence over other ESBLs and disseminated widely around the world resulting in a "CTX-M pandemic" with *Escherichia coli* being the predominant pathogen producing these enzymes (Cantón et al., 2012). AmpC beta-lactamases, another group of enzymes classified as class C beta-lactamases, possess broader hydrolytic spectrum (including cephamycins), and are not inhibited by class A enzyme inhibitors such as clavulanate.

Apart from their widespread occurrence in clinical settings, ESBL/AmpC-producing bacteria are increasingly being reported from livestock, companion animals, and environmental sources (Ewers et al., 2012; Haberecht et al., 2019; Hordijk et al., 2019). Wide dissemination of AMR genes in *Enterobacteriaceae* is mainly achieved by plasmids belonging to various incompatibility groups (Inc) such as F, A/C, L/M, I1, HI2, and N (Zurfluh et al., 2015). Though human to human transmission is shown to be the major route of transfer of these genes, the complex dynamics involved in the dissemination of AMR genes underpins the importance of continuously monitoring non-human sources for potential events of transmission to humans (Mughini-Gras et al., 2019).

With respect to aquaculture settings, very little data is available, particularly from this geographical region, on the prevalence of ESBL-producing bacteria. Thus, we undertook the present study with an aim to screen shrimp, water, and sediment samples from different shrimp farms in Kerala for ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae,* and to further characterize them using molecular methods.

MATERIALS AND METHODS

Study Sites and Sample Collection

Sampling was done during the period November 2018–January 2020 at randomly selected shrimp aquaculture farms in two major shrimp farming zones in the state of Kerala: Kodungallur (in Thrissur district) and Thuravoor (in Alappuzha district) (**Figure 1**). For each farm, site description data viz., GPS co-ordinates, size of the farm, number of ponds, type of the cultured species, etc., were collected. Also, interviews were carried out with farmers as part of design ethnography to better understand the daily practices followed in the farms and also to identify the challenges for farmers within the system. This included information on seed procurement, feed types and feeding routine, antibiotic usage, natural remedies used in disease management, recording and testing practices, etc.

A total of 37 farms (29 from Kodungallur and eight from Thuravoor) were screened in the study, with the majority of farms (n = 25) having monoculture of *Litopenaeus vannamei* (whiteleg shrimp). The remaining 12 farms reared *Penaeus monodon* (tiger prawn) cultures. All the farms had been in operation for a minimum of 1 year at the time when sampling was conducted. Sizes of the farms ranged from 2 to 17 acres with at least two ponds in each farm. Samples mainly included shrimp, water, and sediment. All ponds stocked with shrimps were chosen for sampling. Water samples were collected in sterile sample containers (500 ml) from three different spots at some distance from the edge of the pond. These spots were selected in such a manner that influent and effluent points of the ponds were included. Sediment samples from the selected spots were scooped out and put in sterile plastic covers. An appropriate number of adult shrimps (3–4 nos) were collected using cast-net and transferred to sterile polythene bags and covered with ice. All the samples were labeled properly with information on farmer's name, pond number, and date of collection. Samples were then brought to laboratory on ice (within 4–5 h).

Sample Processing and Bacterial Isolation

All samples were processed at the Microbiology, Fermentation and Biotechnology Division of ICAR-Central Institute of Fisheries Technology (ICAR-CIFT), Cochin, Kerala. Water samples collected at different spots of the same pond were pooled together and homogenized by inverting the bottles several times. Similarly, sediment samples were also pooled and mixed well. After removing the carapace, shrimps were aseptically cut into head, body and tail and a mixture of these parts were taken for bacterial isolation.

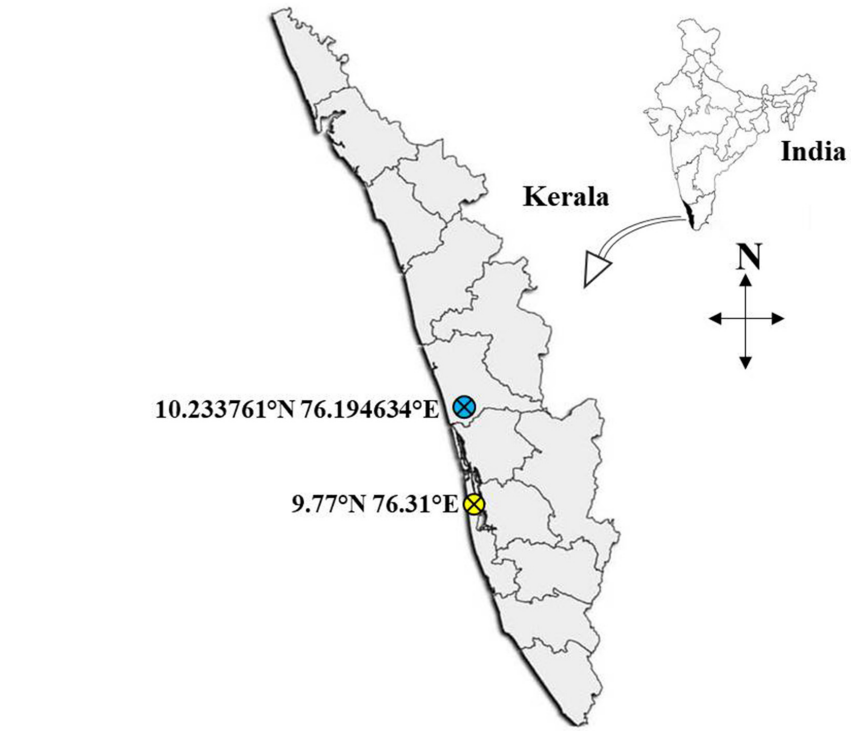
EE broth Mossel (BD Difco, United States), a modified form of brilliant green bile lactose broth was used for the selective enrichment of *Enterobacteriaceae*. Samples (10 ml of water; 10 g each of sediment and shrimp) were incubated in 90 ml of EE broth (pH 7.2) for 18–24 h at 37°C. To isolate ESBL-producing strains, a loopful of the enriched culture was streaked onto MacConkey agar plates (BD Difco, United States) supplemented with 1 μg/ml cefotaxime (Sigma-Aldrich, United States) and incubated for 18–24 h at 37°C. Lactose-fermenting colonies (pink in color) with morphological characteristics of *E. coli* or *K. pneumoniae* were picked and stored on tryptic soy agar (TSA) (BD Difco, United States) slants for identification and further characterization.

Bacterial Identification and Antibiotic Susceptibility Testing (AST)

Bacterial species were identified using BD Phoenix^{TM} M50 automated system (BD Diagnostics, United States). The ID-AST combo panel, NMIC/ID55 designed for identification and susceptibility testing (MIC determination) of Gram-negative bacteria was used in this study. The panel consists of an ID side containing wells with dried substrates for bacterial identification and an AST side containing wells having varying concentrations of antimicrobial agents. With regard to ESBL detection, NMIC/ID55 panel has a "BD Phoenix ESBL screening test" which is based on the growth response of bacteria to selected second or third generation cephalosporins in the presence or absence of the beta-lactamase inhibitor, clavulanic acid. Procedures were performed according to manufacturer's instructions. Briefly, bacterial colonies from pure cultures were transferred to the ID broth (BD Difco diagnostic systems, United States) and the inoculum density was adjusted to 0.5 McFarland using BD PhoenixSpec nephelometer. Twenty five microliters of the adjusted ID broth suspension was transferred to the AST broth with the AST indicator which is a resazurin-based dye. The suspensions (ID broth inoculum and AST broth inoculum) were then poured to the corresponding fill ports in the panel. Panels were sealed and loaded into the instrument for incubation at 35°C for around 16 h. Quality control was also performed using the reference strain *E. coli* ATCC 25922. BD Phoenix system is connected to EpiCenter, the data management software to analyze test results and generate reports. Further, BD Phoenix uses a rule-based expert system namely BDXpert which can interpret AST results and provide recommendations based on CLSI guidelines. Thus, on identifying specific resistance markers such as ESBL, BDXpert can alter, if necessary, the initial raw categorization (S, I, or R) for selected antibiotics and provide a "final SIR." However, the MIC values are never altered.

PCR Screening for Resistance Genes

Isolates of *E. coli* and *K. pneumoniae* alerted as "ESBL-producers" by BD Phoenix were screened for the presence of genes encoding various beta-lactamases. For this, DNA extraction was performed using DNeasy Blood and Tissue kits (Qiagen, Germany) as per manufacturer's instructions. Two Multiplex PCRs (I and II) were employed for detecting CTX-M, TEM, SHV, and OXA-1-like



Site of sample collection	shrimp farms (n)	shrimp samples (n)	Sediment samples (n)	Water samples (n)
Kodungallur, Thrissur	29	62	75	75
Thuravoor, Alappuzha	08	15	17	17
Total	37	77	92	92

FIGURE 1 | Map of the study region showing the locations of sampling sites. Below the map is a table giving information on the number of farms surveyed and the samples collected.

genes using primers and amplification conditions as described by Dallenne et al. (2010). Multiplex I targeted CTX-M group 1, group 2, and group 9 enzymes, whereas multiplex II aimed at the simultaneous detection of bla_{TEM}, bla_{SHV}, and bla_{OXA} genes. Additionally, for positive isolates of CTX-M group 1, further PCR to detect the most common group 1 enzyme, $bla_{CTX-M-15}$ was performed according to Gundran et al. (2019). Isolates were also tested for the presence of genes encoding AmpC enzymes by multiplex PCR that can detect the six major families of plasmid-mediated AmpC beta-lactamases (ACC, CIT, MOX, FOX, DHA, and EBC) (Dallenne et al., 2010). Presence of one of the most common pAmpC genes, bla_{CMY-2} (CIT-type) was also investigated using previously described primers (Kozak et al., 2009).

Further, based on phenotypic resistance patterns, PCRs were performed to identify various genes associated with resistance to different non-beta-lactam drugs. This included detection of genes conferring resistance to tetracycline (*tetA* and *tetB*), chloramphenicol (*cmlA* and *catA*), fluoroquinolones [*qnrA*, *qnrB*, *qnrS*, *qepA*, *oqxA*, *oqxB*, and *aac(6′)-Ib-cr*], aminoglycosides (*aadA1*, *strA*, and *strB*), and sulfonamides (*sul1* and *sul2*). All PCR reactions were carried out in a final volume of 25 μl containing 1X JumpStart RedTaq ReadyMix (Sigma-Aldrich, United States), primers in varying concentrations and 2 μl of DNA extract. PCR products were resolved on a 2% agarose gel containing ethidium bromide at a final concentration of 1 μg/ml. Details of the primers and PCR conditions used in this study for the detection of various resistance genes are given in the **Supplementary Table 1**.

PCR-Based Replicon Typing (PBRT)

Plasmid incompatibility (InC) types were determined using the protocol described by Carattoli et al. (2005) which employs 18 primer pairs in five multiplex and three uniplex PCRs to distinguish the major InC groups (prevalent in *Enterobacteriaceae*) namely HI1, HI2, I1-Ic, X, L/M, N, FIA, FIB,

W, Y, P, FIC, A/C, T, FIIAs, F, K, and B/O. All multiplex PCRs were run with the following conditions: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 s and elongation at 72°C for 1 min and a final extension at 72°C for 5 min. Uniplex PCRs were also performed with the same conditions as above except that the annealing temperature was set at 52°C.

Phylogenetic Grouping of ESBL-*E. coli* Isolates

Distribution of phylo-groups (A, B1, B2, C, D, E, F, and cryptic clade) among the ESBL-producing *E. coli* isolates was analyzed using the PCR method described by Clermont et al. (2013). This included an initial quadruplex PCR performed on the isolates to test the presence/absence of four genes: *arpA* (400 bp), *chuA* (288 bp), *yjaA* (211 bp), and *TspE4.C2* (152 bp). Based on the amplification pattern, an isolate either can be assigned to a specific phylogroup (B1, B2, and F) or needs additional PCRs to confirm the phylogroup (A, C, D, E, and clade I). All PCRs were performed with the following cycling conditions: initial denaturation at 94°C for 4 min, 30 cycles of denaturation at 94°C for 5 s, annealing at 59°C (quadruplex and group C) or 57°C (group E) for 20 s and extension at 72°C for 1 min, and a final extension at 72°C for 5 min.

Detection of Virulence Genes in ESBL-*K. pneumoniae*

A multiplex PCR targeting the serotype-specific genes, *magA* (K1 serotype) and *wzi* (K2 serotype), and the virulence genes *rmpA*, *entB*, *ybtS*, *kfu*, *iutA*, *mrkD*, and *allS* was performed according to Compain et al. (2014). Cycling conditions included an initial denaturation at 95°C for 15 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 90 s and extension at 72°C for 60 s, and a final extension at 72°C for 10 min.

Pulsed-Field Gel Electrophoresis (PFGE)

Pulsed-field gel electrophoresis was performed according to the standard operating procedure by PulseNet International[1] to analyze the clonal relatedness between the isolates. Briefly, plugs containing whole genomic DNA of the *E. coli* or *K. pneumoniae* isolates were digested with *XbaI* (50 U/sample, New England Biolabs) at 37°C for 1.5–2 h. DNA fragments were separated on a 1% Megabase agarose gel (Bio-Rad, United States) in 0.5X TBE buffer at 14°C in a CHEF-Mapper XA device (Bio-Rad, United States). Electrophoresis conditions included a constant voltage of 6 V/cm, run time of 19 h, pulse time ranging from 6.76 to 35.38 s and an included angle of 120°. *Salmonella* serotype Braenderup H9812 was used as the standard molecular size marker. The gels were stained with ethidium bromide and observed under UV illumination. Gel images were exported to BioNumerics software package 7.6.3 (Applied Maths, Belgium) and cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) and Dice coefficient. Isolates which exhibited PFGE profiles with ≥85% similarity were considered genetically related.

[1]https://www.cdc.gov/pulsenet/pdf/ecoli-shigella-salmonella-pfge-protocol-508c.pdf

RESULTS

Sampling and Design Ethnographic Assessment

The present study analyzed shrimp (n = 77), water (n = 92), and sediment (n = 92) collected from a total of 37 farms. All the farmers consented to be interviewed and were willing to share the details of the culture and routine farming activities. Most of the farm owners were middle class with many of them having experience of working abroad and having access to other sources of income. Except for four farms which followed traditional farming practices, all other farms (n = 33) adhered to scientific farming. This included regular monitoring and management of water and soil quality, selection of good quality hatchery seeds for stocking, usage of commercially available high nutritive pelleted feeds, usage of aerators and harvesting only at the end of a crop season. On average, each farmer had 5.8 ± 4.5 (mean ± SD) acres of farm. Stocking density varied in the range between 30 and 60 nos/m^2. Nearly 45% of the farmers in this study had experienced disease outbreaks in their farms. Infections caused by white spot syndrome virus (WSSV), a microsporidian parasite namely *Enterocytozoon Hepatopenaei* (EHP), and *Vibrio* bacteria were the most common. All the participating farmers reported that they hadn't used any antibiotics in their farms. Probiotic usage was found to be a common practice among the farmers, with 62% of the farmers using at least one commercially available probiotic preparation. It is also noteworthy that natural remedies such as garlic paste and jaggery were cited by many farmers as supporting the health of their stock.

Detection of ESBL-Producers and Their Resistance Profile

A total of 32 isolates of *E. coli* and 15 isolates of *K. pneumoniae* recovered from the samples were alerted as "ESBL-producers" by BD Phoenix^{TM} M50 automated system. Out of the 37 farms screened in the study, eight farms tested positive for ESBL-*E. coli* and five farms for *K. pneumoniae*. None of the farms harbored both the pathogens. Overall, five sediment samples and two each of shrimp and water samples yielded ESBL-*E. coli*, whereas for ESBL-*K. pneumoniae*, the following distribution was observed: sediment (0), shrimp (4), and water (1). Invariably all isolates of *E. coli* and *K. pneumoniae* from this study were resistant to ampicillin, piperacillin, cefazolin, and cefotaxime. MIC of cefotaxime was found to be ≥32 μg/ml for all isolates. However, MICs of other cephalosporins (ceftazidime and cefepime) and the monobactam drug, aztreonam varied among the isolates (**Table 1**). It is important to note that 18 (56.3%) *E. coli* isolates showed resistance (MIC ≥ 16 μg/ml) toward cefepime, a fourth generation cephalosporin and 14 (43.8%) isolates belonged to SDD category (susceptible-dose dependent, MIC 4–8 μg/ml). Notably, two isolates of *E. coli* were resistant to amoxicillin-clavulanate and intermediate to cefoxitin. In the

TABLE 1 | Distribution of MICs of different cephalosporins and aztreonam among the ESBL-producing isolates.

Antibiotic	Cefazolin	Cefepime		Cefotaxime	Ceftazidime			Aztreonam		
MIC range (μ g/ml)	>16 (R)	4–8 (SDD)	≥16 (R)	≥32 (R)	4 (S)	8 (I)	≥16 (R)	4 (S)	8 (I)	≥16 (R)
ESBL$^+$ EC (n)	32 (100%)	14 (43.8%)	18 (56.3%)	32 (100%)	13 (40.6%)	17 (53.1%)	2 (6.3%)	2 (6.3%)	11 (34.4%)	19 (59.4%)
ESBL$^+$ KP (n)	15 (100%)	0	15 (100%)	15 (100%)	0	2 (13.3%)	13 (86.7%)	0	2 (13.3%)	13 (86.7%)

ESBL$^+$ EC, ESBL-producing E. coli; ESBL$^+$ KP, ESBL-producing K. pneumoniae; R, resistant; I, intermediate; S, susceptible; SDD, susceptible dose dependent.

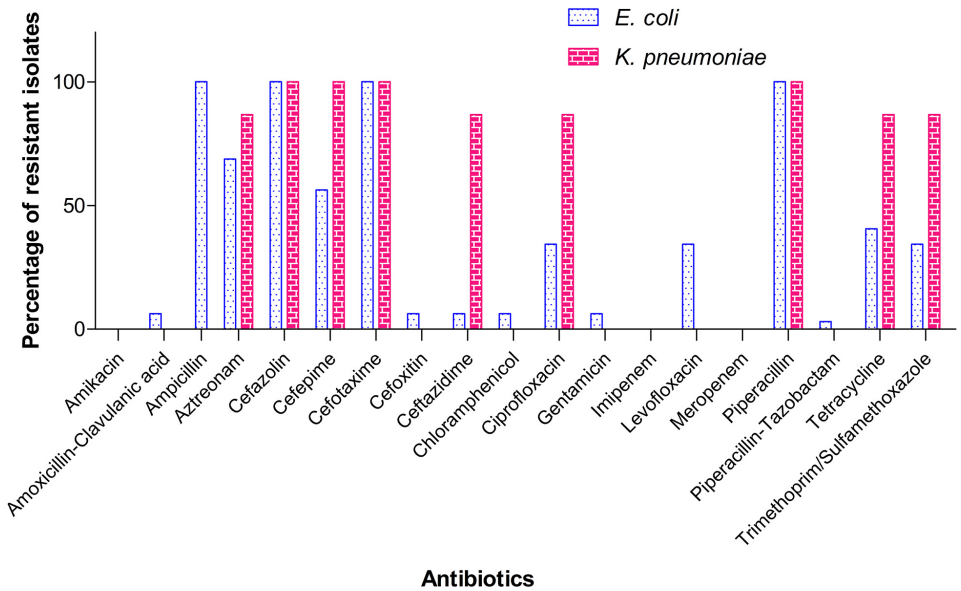


FIGURE 2 | Resistance pattern of the ESBL-producing *E. coli* and *K. pneumoniae* isolates toward different antibiotics. *K. pneumoniae* is intrinsically resistant to ampicillin and therefore not shown here.

case of *K. pneumoniae,* all isolates were resistant to cefepime (MIC ≥ 16 μg/ml). Percentage of resistance among *E. coli* isolates toward other antibiotics was as follows: tetracycline (40.6%), trimethoprim-sulfamethoxazole (34.4%), ciprofloxacin and levofloxacin (34.4%), chloramphenicol and gentamicin (6.3%). Of all isolates of *K. pneumoniae*, 13 (86.7%) showed resistance to tetracycline, ciprofloxacin, and trimethoprim/sulfamethoxazole (**Figure 2**). However, all ciprofloxacin-resistant isolates of *K. pneumoniae* remained susceptible to levofloxacin. Notably, multidrug resistance (resistance to at least one agent in ≥3 classes of antibiotics) was observed in all ESBL-positive isolates. Hundred per cent susceptibility was recorded for amikacin, imipenem, and meropenem drugs. MICs of all the tested antibiotics for *E. coli* and *K. pneumoniae* isolates are given in the **Supplementary Tables 2**, **3**, respectively.

Detection of Resistance-Conferring Genes

Among the ESBL genes screened, CTX-M group 1 was found to be the predominant type, with 23 *E. coli* (71.9%) and 15 *K. pneumoniae* (100%) isolates testing positive for the same. CTX-M group 9 was identified in 9 (28.1%) isolates of *E. coli,* but not in any of the *K. pneumoniae* isolates. Other beta-lactamase genes detected included blaTEM & blaSHV (11 *K. pneumoniae*, 73.3%) and blaCMY-2 (2 *E. coli*, 6.3%). No ESBL-positive isolate from our study carried OXA-1 type gene. Screening for tetracycline resistance genes (*tet*) identified the co-occurrence of *tetA* and *tetB* genes in 13 isolates (40.6%) of *E. coli,* whereas tetracycline resistance in *K. pneumoniae* isolates (13, 86.7%) was attributed to the presence of *tetA* alone. Other major resistance genes identified in *E. coli* included sulfonamide resistance genes, *sul1* (11, 34.4%) and *sul2* (9, 28.1%); chloramphenicol resistance genes, *catA+cmlA* (11, 34.4%); fluoroquinolone resistance genes, *qepA+ aac(6′)-Ib-cr* (9, 28.1%); and aminoglycoside resistance genes, *strA+strB+aadA1* (2, 6.25%). In contrast to *E. coli*, fluoroquinolone resistance in *K. pneumoniae* was mediated by *qnrB+oqxB* (13, 86.7%) and *qnrS* (3, 20%). Sulfonamide resistance in *K. pneumoniae* was attributed to *sul2* (13, 86.7%) alone. The distribution of resistance genes identified among the isolates is shown in **Figure 3**. A master chart detailing the phenotypic and genotypic characteristics of all isolates is provided as **Supplementary Table 4**.

Replicon Typing

The predominant plasmid replicon types in *E. coli* were IncFIB and IncFIA, as observed in 21 (65.6%) and 15 (46.9%) isolates

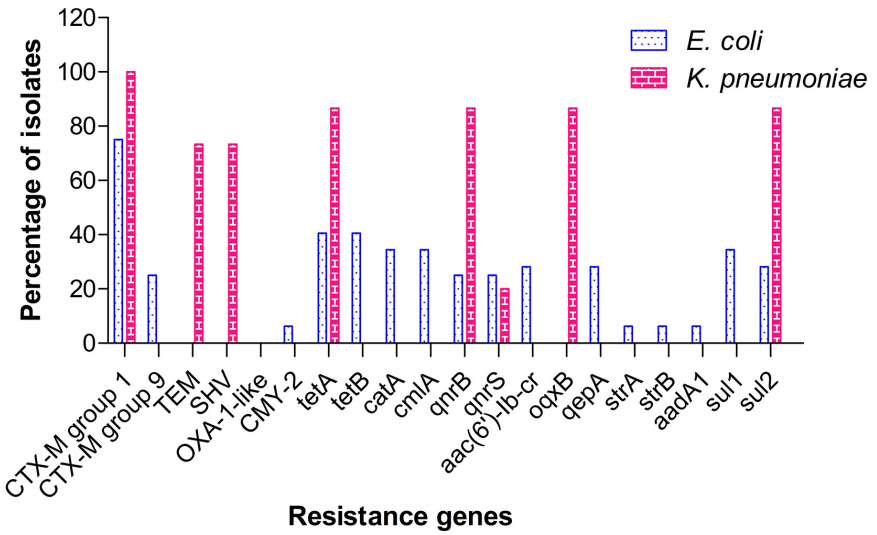


FIGURE 3 | Distribution pattern of resistance genes among isolates of ESBL-producing *E. coli* and *K. pneumoniae*.

respectively. All isolates which carried IncFIA type had also harbored FIB type plasmids. However, six isolates were found to possess only FIB type plasmid. The employed primer sets could not determine plasmid types in 11 *E. coli* isolates. All isolates of ESBL-*K. pneumoniae* uniformly harbored IncHI1 type plasmids.

Phylogenetic Grouping of ESBL-*E. coli* Isolates

Phylogenetic groups identified among the *E. coli* isolates included B1 (4, 12.5%), B2 (6, 18.8%), C (10, 31.3%), D (3, 9.4%), and E (9, 28.1%). With respect to the source of the isolates, the distribution of phylogroups was as follows: shrimp (B1, B2, and C), sediment (B1, B2, C, D, and E), and water (E).

Detection of Virulence Genes in ESBL-*K. pneumoniae*

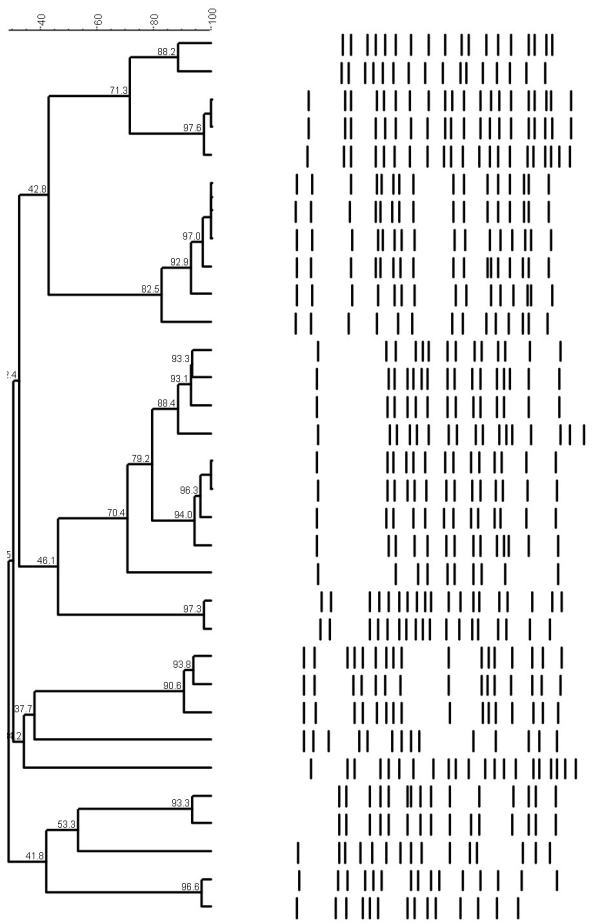
In the multiplex PCR employed to detect capsular serotypes K1 and K2, and seven major virulence factors, no K1- or K2-specific loci could be amplified in any of the *K. pneumoniae* isolates tested. Among the virulence-associated genes tested, *iutA*, *entB*, and *mrkD* were found in all the isolates.

Molecular Typing by PFGE

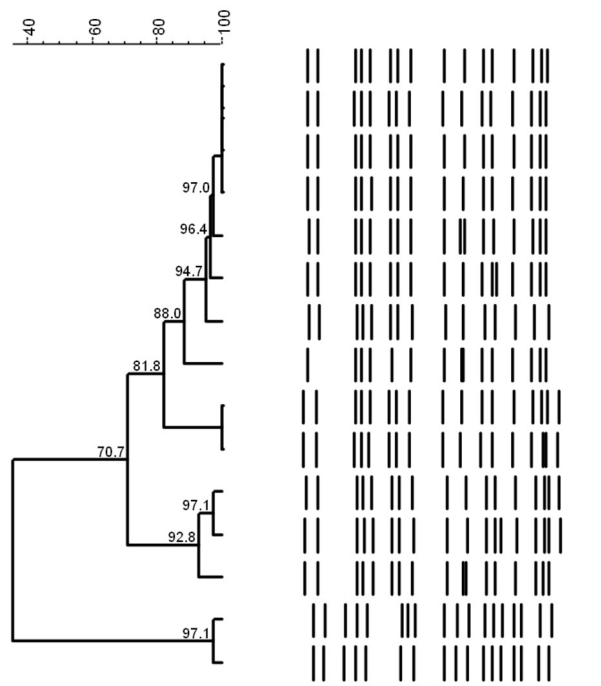
All isolates from this study were typeable by *XbaI*-PFGE. Applying a similarity cut-off value of 85%, isolates of *E. coli* ($n = 32$; 28 pulsotypes) and *K. pneumoniae* ($n = 15$, 11 pulsotypes) were grouped into 14 and four clusters, respectively (**Figure 4**). At this cut-off, five isolates of *E. coli*, namely K18S3_2, K17A4_6, K18S6_1, K11A1_1, and PR1W2_6 -all from different farms- did not cluster with other isolates. Overall, shrimp-, water-, and sediment-derived isolates of *E. coli* were scattered across 6, 7, and 2 clusters, respectively. Exclusively in one event, isolates of *E. coli* (K17A4_1, K17S3_1, K17S3_2, and K17S3_3) from two different farms were found clustered. In another instance, *E. coli* recovered from the water (PR1W1) and sediment (PR1S1) samples of the same farm were found genetically unrelated. In contrast to *E. coli*, isolates of *K. pneumoniae* from different sample types and/or farms were found clustered. The largest cluster had eight isolates recovered from both shrimp and water samples. All the four clusters of *K. pneumoniae* had shrimp-derived isolates; however, isolates recovered from water samples were grouped into two clusters.

DISCUSSION

There is a growing perception that aquaculture settings are contributing significantly to the development and dissemination of AMR. Although antibiotics are not used in aquaculture for growth promotion purposes, their prophylactic use is not uncommon in shrimp farms (Watts et al., 2017). A recent meta-analysis which studied data from nearly 40 countries, majority of which are LMICs, has shown that multi-antibiotic resistance index (MAR) of aquaculture-derived bacteria correlates strongly with that of human clinical isolates (Reverter et al., 2020). ESBL-producing *E. coli* is found widespread in clinical as well as other environments such as sewage, meat, food animals, etc. However, most of the studies on ESBL from India are confined to clinical settings, with little information available on the prevalence and epidemiology of ESBL-producing strains in aquaculture and other aquatic environments (Gandra et al., 2017). The low frequency of ESBL-positive isolates observed in our study could probably be attributed to the proper management of the ponds and general cleanliness maintained in the shrimp farms and surroundings. It is noteworthy that, in most cases, a given farm (which tested positive) had only one sample type -i.e., shrimp or sediment or water- yielding ESBL-positive isolate. Antibiotic usage appeared less-likely in the farms as all the farms we surveyed used



Isolate ID	Source	Beta-lactamase genes	Phylogroup
PR1S1_5	Sediment	CTX-M-group 1	B2
PR1S1_6	Sediment	CTX-M-group 1	B2
K11A1_8	Shrimp	CTX-M-group 1	B2
K11A1_4	Shrimp	CTX-M-group 1	B2
K11A1_11	Shrimp	CTX-M-group 1	B2
K18S3_1	Sediment	CTX-M-group 1	E
K18S3_4	Sediment	CTX-M-group 1	E
K18S3_5	Sediment	CTX-M-group 1	E
K18S3_3	Sediment	CTX-M-group 1	E
K18S3_6	Sediment	CTX-M-group 1	E
K18S3_2	Sediment	CTX-M-group 1	E
K17S3_1	Sediment	CTX-M-group 9	C
K17S3_3	Sediment	CTX-M-group 9	C
K17S3_2	Sediment	CTX-M-group 9	C
K17A4_1	Shrimp	CTX-M-group 9	C
K17A4_2	Shrimp	CTX-M-group 9	C
K17A4_4	Shrimp	CTX-M-group 9	C
K17A4_3	Shrimp	CTX-M-group 9	C
K17A4_5	Shrimp	CTX-M-group 9	C
K17A4_6	Shrimp	CTX-M-group 9	C
K11A1_6	Shrimp	CTX-M-group 1	B1
K11A1_13	Shrimp	CTX-M-group 1	B1
K16S2_3	Sediment	CTX-M-group 1	D
K16S2_4	Sediment	CTX-M-group 1	D
K16S2_1	Sediment	CTX-M-group 1	D
K18S6_1	Sediment	CTX-M-group 1	C
K11A1_1	Shrimp	CTX-M-group 1	B2
K16S2_2	Sediment	CTX-M-group 1	B1
K16S2_5	Sediment	CTX-M-group 1	B1
PR1W2_6	Water	CTX-M-group 1	E
PR1W1_1	Water	CTX-M-group 1, CMY-2	E
PR1W1_2	Water	CTX-M-group 1, CMY-2	E



Isolate ID	Source	Beta-lactamase genes
K8W2_3	Water	CTX-M-group 1, TEM, SHV
K9A1_2	Shrimp	CTX-M-group 1, TEM, SHV
K9A1_5	Shrimp	CTX-M-group 1, TEM, SHV
K9A3_8	Shrimp	CTX-M-group 1, TEM, SHV
K8W2_5	Water	CTX-M-group 1, TEM, SHV
K9A3_7	Shrimp	CTX-M-group 1, TEM, SHV
K8W2_1	Water	CTX-M-group 1, TEM, SHV
K9A1_6	Shrimp	CTX-M-group 1, TEM, SHV
K9A1_8	Shrimp	CTX-M-group 1
K9A3_10	Shrimp	CTX-M-group 1
K8W2_2	Water	CTX-M-group 1, TEM, SHV
K8W2_4	Water	CTX-M-group 1, TEM, SHV
K9A1_7	Shrimp	CTX-M-group 1, TEM, SHV
K19A2_2	Shrimp	CTX-M-group 1
K19A3_2	Shrimp	CTX-M-group 1

FIGURE 4 | Dendrograms showing cluster analyses of *Xba1*-PFGE patterns of the ESBL-producing *E. coli* (top) and *K. pneumoniae* (below) isolates.

commercial feeds certified as "antibiotic free" and most of the farmers were aware of the issues related to the imprudent use of antibiotics in aquaculture. However, the usage of any other supplements pre-mixed with antibiotics cannot be completely ruled out.

Though the majority of farms in our study tested negative for ESBL-producers, resistance traits observed in the isolates is a cause of concern. All isolates of *E. coli* and *K. pneumoniae* tested in our study were found to be resistant to at least four and five antibiotics, respectively. Moreover, nearly 41% of *E. coli* and 87% of *K. pneumoniae* isolates from this study were resistant to $\geq$8 out of the 19 antibiotics tested. In addition to beta-lactam and monobactam drugs to which ESBL-producing bacteria are generally resistant, 28.1% of *E. coli* isolates and 86.7% of *K. pneumoniae* isolates from our study showed simultaneous resistance to tetracycline, ciprofloxacin, and trimethoprim-sulfamethoxazole. Several previous studies reported increased frequency of bacteria resistant to tetracycline, fluoroquinolones and trimethoprim-sulfamethoxazole in aquaculture farms and surrounding regions (as reviewed by Cabello et al., 2013). Also, incidences of tetracycline-resistant *E. coli* and other bacterial spp. in shrimps imported from Asian countries have been reported (Ellis-Iversen et al., 2019; Khan et al., 2019). While acknowledging the fact that the major driver of AMR in aquaculture is the indiscriminate use of antibiotics in this sector, detection of resistant bacteria in aquaculture environment cannot always have direct link to the use of antimicrobials in the farm/setting in question. Aquatic systems are highly complex and dynamic in nature, and can receive effluents containing antibiotics/AMR bacteria from various other sources including hospitals, wastewater treatment plants, runoff from agricultural/livestock activities, etc. It is also possible that the source of the resistant bacteria is the hatchery where antibiotics are routinely used.

In recent years, *K. pneumoniae* has emerged as a major public health threat owing to high prevalence of MDR-strains producing ESBLs and/or carbapenemases. However, data pertaining to the occurrence, population structure and transmission routes of this pathogen in major animal, food and environmental reservoirs is quite limited (Chi et al., 2019; Wyres et al., 2020). In our study, multidrug resistance was observed at higher frequency in *K. pneumoniae* isolates when compared to *E. coli*. Moreover, higher MIC was observed for ceftazidime and cefepime in the majority of *K. pneumoniae* isolates. Similar to our observations, a previous study involving shrimp farms with intensive farming practices in Vietnam also reported multidrug-resistant isolates of *K. pneumoniae* (resistant mainly to sulfonamides, fluoroquinolones, and tetracycline) (Pham et al., 2018). On account of its broader ecological niche and high burden of plasmids, *K. pneumoniae* serves as a major transmitter of resistance from environment to various human or animal pathogens (Wyres and Holt, 2018).

Regarding the ESBL genotypes of our isolates, we observed the predominance of CTX-M-type ESBLs in both *E. coli* (100%) and *K. pneumoniae* (100%) followed by an equal representation of TEM and SHV enzymes in *K. pneumoniae* (73.3%). Thus, the majority (73.3%) of *K. pneumoniae* isolates from our study carried multiple beta-lactamases ($bla_{CTX-M-15}$+ bla_{TEM} + bla_{SHV}). As the primer set we employed in the present study detects both ESBL and non-ESBL variants of TEM and SHV enzymes, we cannot rule out the possibility of some of the TEM and SHV genes identified here being non-ESBL type. Co-occurrence of multiple ESBL determinants in *E. coli* or *K. pneumoniae* has been well documented in clinical settings (Hassan and Abdalhamid, 2014; Jena et al., 2017; Gautam et al., 2019). There is a paucity of data on the prevalence and molecular epidemiology of ESBL-producing bacteria from aquaculture environments; however, there are a couple of studies which reported the presence of ESBL genes in aquaculture settings or imported shrimps. In a study conducted on fishes from aquaculture farms in China, various ESBL genes such as $bla_{CTX-M-14}$, $bla_{CTX-M-79}$, and bla_{SHV-27} were detected (Jiang et al., 2012). A previous study by Khan et al. (2019) reported CTX-M (56%) as the predominant ESBL gene, followed by TEM (16%) in *E. coli* isolates recovered from shrimps imported to United States from Asian countries. Similar incidence was also reported from Denmark where *E. coli* isolates from imported shrimps from Asia were found to harbor $bla_{CTX-M-15}$ and $bla_{CTX-M-55}$ genes (Ellis-Iversen et al., 2019). Many recent studies from Vietnam, where shrimp aquaculture is a major industry, reported ESBL-positive bacteria in retail shrimps and attributed this mainly to the inappropriate usage of antibiotics in shrimp farms (Le et al., 2015; Nguyen et al., 2016; Yen et al., 2020). Also, a study from Mumbai, India reported for the first time the detection of multiple ESBL genes in sea food isolates of *E. coli* (Singh et al., 2017).

Studies undertaken in various settings in India showed different patterns of distribution of ESBL genes. A study conducted in Vellore, South India reported high prevalence (91.8%) of CTX-M among ESBL-*E. coli* recovered from human (patients and healthy volunteers) and environmental samples (stagnant water bodies, sewage, public toilets, and meat markets) (George et al., 2015). In contrary, study by Bajaj et al. (2015) from north India reported the predominance of bla_{TEM} (100%) and low occurrence of bla_{CTX-M} (16%) among ESBL-*E. coli* recovered from aquatic environments. Nevertheless, similar to our findings, isolates from the above study also had $bla_{CTX-M-15}$ as the CTX-M determinant. CTX-M-15, reported for the first time from India in 2011, has now become a widespread ESBL genotype and has been reported from humans, livestock and fishes (Palmeira and Ferreira, 2020). Importantly, two of our *E. coli* isolates harbored bla_{CMY-2}, a CIT-type AmpC beta-lactamase. These isolates were resistant to cefotaxime, ceftazidime and amoxicillin-clavulanate, and intermediate towards cefoxitin. CMY-2 is one of the most common AmpC beta-lactamases encountered in *E. coli* isolated from humans and animals (Ewers et al., 2012). Co-expression of ESBL and AmpC has been reported previously in many environmental *E. coli* isolates (Bajaj et al., 2015; Ye et al., 2017).

In aquaculture, the prophylactic and therapeutic use of tetracycline has been well acknowledged and also shown to be a major factor contributing to the spread of tetracycline

genes in the environment. Tetracycline resistance is mainly mediated by efflux pumps encoded by different *tet* genes, which often disseminate easily owing to their location on plasmids and transposons (Miranda et al., 2013). Further, these genes have been shown to persist in aquaculture settings even in the absence of selection pressure imposed by the continuous usage of tetracycline (Tamminen et al., 2011). All our tetracycline-resistant *E. coli* isolates carried both *tet(A)* and *tet(B)* genes and these isolates were from shrimp and sediment samples. Previous studies have also shown the predominance of *tet(A)*, *tet(B)*, and *tet(M)* genes among tetracycline-resistant isolates recovered from aquaculture farms and their environments (Akinbowale et al., 2007; Changkaew et al., 2014; Liyanage and Manage, 2019). Co-occurrence of *tetA* and *tetB* genes, as found in our study, was also reported in earlier studies carried out in aquaculture settings (Akinbowale et al., 2007; Nawaz et al., 2009). A study by Rhodes et al. (2000) showed that plasmids bearing tetracycline resistance determinants such as *tetA* disseminated between *Aeromonas* spp. and *E. coli* and between the aquaculture and human environments. This provides direct evidence that these settings interact more closely than previously thought. Our study also documents the prevalence of the sulfonamide resistance gene, *sul* and the chloramphenicol resistance genes, *catA* and *cmlA*. *Sul1* has been shown to be a potential indicator for assessing contamination by antibiotic resistance genes (ARGs) in aquaculture environments (Su et al., 2017). Recently Wang et al. (2019) reported predominance of *sul1* and *cmlA* among various antibiotic resistance genes (ARGs) in the rearing environments of intensive shrimp farms in South China.

The other major resistance trait among our isolates was the fluoroquinolone resistance, observed in 34.4% of *E. coli* and 73.3% *K. pneumoniae* isolates. Similar to our findings, a study from North India reported co-resistance to fluoroquinolones among ESBL-producing *E. coli* from aquatic sources (Bajaj et al., 2016). Further, a meta-analysis by Wiener et al. (2016) also reported significant association between ESBL phenotype and fluoroquinolone resistance in *Enterobacteriaceae* and cautions the empirical use of quinolones in treating ESBL infections. In our study, quinolone resistance in *E. coli* isolates was found mediated by both *qepA* and *aac(6′)-Ib-cr* which encode, respectively, a multidrug efflux pump and a mutant aminoglycoside-modifying enzyme capable of modifying quinolones as well. However, in *K. pneumoniae* isolates, *qnr genes (qnrB* and *qnrS)* which code for proteins that protect the target enzyme from quinolones action and the efflux pump-encoding *oqxB* gene accounted for resistance. A recent study from Kerala which investigated the prevalence of quinolone-resistant *E. coli* in water bodies contaminated with hospital effluents and in the surrounding aquaculture farms reported the predominance of *qnrB* followed by *qnrS*, *oqxAB*, *qnrA*, and *aac (6')-Ib-cr* (Girijan et al., 2020). Plasmid-mediated quinolone resistance (PMQR) is a growing concern owing to the fact that fluoroquinolones are one of the safer and widely prescribed drugs and the mainstay for treatment of serious Gram-negative infections. PMQR genes such as *qnrA* and *qnrS* are shared between the aquatic *Shewanella*, *Aeromonas*, *Vibrio* and the human pathogens *E. coli* and *K. pneumoniae,* suggesting that many ARGs originated in aquatic environments before their dissemination to terrestrial hosts (Poirel et al., 2012).

Phylogenetic grouping showed that *E. coli* isolates recovered from shrimp samples belonged to B1, B2, and C. This is a worrisome finding from public health point of view as most of the virulent extra-intestinal strains of *E. coli* are primarily from group B2 and to a lesser extent from group D. In a study from Vietnam, B1 was found to be the most prevalent phylogroup in ESBL-*E. coli* isolated from retail shrimps (Le et al., 2015). It has been suggested that B2 *E. coli* isolates are more sensitive toward antibiotics, particularly to quinolones compared to non-B2 *E. coli* isolates (Skurnik et al., 2009). This was evident in our B2 isolates all of which were sensitive to quinolones, tetracycline, and trimethoprim-sulfamethoxazole. The majority of fluoroquinoline-resistant *E. coli* isolates from this study belonged to phylogroup C. With regard to the association of CTX-M group with phylogroups, we noted that CTX-M-15-producing isolates were spread over different phylogroups viz., B1, B2, C, D, and E, whereas all CTX-M-group 9 isolates belonged to phylogroup C.

Concerning the distribution of plasmids, we have found IncF and IncHI1 as the major replicon types among the isolates of *E. coli* and *K. pneumoniae*, respectively. A previous study had reported B/O as the predominant replicon type, followed by FIA in ESBL-*E. coli* isolated from imported shrimp (Khan et al., 2019). It has been shown that resistance genes, harbored on the narrow host range IncF-type plasmids in *Enterobacteriaceae* spread readily in *E. coli* (Mathers et al., 2015). Moreover, IncF plasmids carrying $bla_{CTX-M-15}$ have been found in *Enterobacteriaceae* isolated from clinical, environmental, and livestock settings (Zurfluh et al., 2015). This perhaps indicates the role of IncF plasmids in disseminating CTX-M-15 across different settings. IncHI1, the plasmid type found in the *K. pneumoniae* isolates from our study, generally have broad host range and have been reported in various environmental Gram-negative species (Villa et al., 2012). However, many recent studies including one from India reported $IncFII_k$ and other IncF plasmids as carriers of $bla_{CTX-M-15}$ in *K. pneumoniae* (Shankar et al., 2018; Kakuta et al., 2020). In regard to virulence genes, our *K. pneumoniae* isolates harbored the siderophore genes *entB* (enterobactin) and *iutA* (aerobactin), and the type 3 fimbriae-encoding gene *mrkD*. Siderophore systems consist of extracellular iron-chelating molecules capable of scavenging Fe^{3+} from host proteins, and surface receptors for internalization. Enterobactin is a core siderophore present ubiquitously in *K. pneumoniae*, whereas, aerobactin is an acquired siderophore occasionally encountered in *Klebsiella* with an incidence rate of <10% (El Fertas-Aissani et al., 2013). Type 3 fimbriae, another frequent virulence determinant found in clinical as well as environmental isolates of *K. pneumoniae* aid in intestinal colonization and are also promoters of biofilm formation on biotic and abiotic surfaces (Struve and Krogfelt, 2004; Khater et al., 2015).

Pulsed-field gel electrophoresis analysis revealed high heterogeneity in ESBL-*E. coli* isolates recovered from different shrimp farms. In most cases, multiple isolates from the same sample showed identical banding patterns; however, there were also instances where the same sample yielded isolates with unrelated PFGE profiles, indicating multiple sources contaminating the farm. These isolates also differed significantly with respect to their antibiogram and phylogroups. In a previous study by Jiang et al. (2019), high clonality was observed for *E. coli* isolates recovered from shrimps sold at different open markets in China owing to the fact that these shrimps were reared in the same large-scale shrimp farm. Concerning the *K. pneumoniae* isolates, considerable genetic relatedness was observed, with 86% (n = 13) of the isolates belonging to a single cluster at a 70% similarity cut-off. Presence of one dominant cluster might be indicative of a well-adapted clone in this environment.

In summary, our results indicate the presence of multidrug-resistant isolates of ESBL-producing *E. coli* and *K. pneumoniae,* with resistance mainly toward cephalosporins, fluoroquinolones, tetracycline and trimethoprim-sulfamethoxazole drugs, in samples from various shrimp aquaculture farms in Kerala. To our best knowledge, this study provides the first data on the molecular features of ESBL-producing isolates prevailing in shrimp aquaculture settings of this region. In our study, bla_{CTX-M} was found to be the predominant ESBL genotype in both *E. coli* and *K. pneumoniae*. This, along with a high prevalence of *tet, sul,* and PMQR genes may be a public health concern and emphasizes the need for monitoring aquaculture settings for the possible emergence of antibiotic-resistant bacteria. Though our investigation was limited to a few numbers of farms and the results may only indicate a local trend, it has important implications for public health considering the long and complex nature of the shrimp supply chain starting from the farm worker to the consumer. Moreover, shrimp being an internationally traded commodity, can potentially aid in the transmission of resistant bacteria to different geographical regions.

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 authors.

AUTHOR CONTRIBUTIONS

GKS, RE, TTB, and AP conceived the study and designed the experiments. VR and AV collected the samples and performed the experiments. GKS and VR analyzed and interpreted the results. VR wrote the manuscript. GKS and AP reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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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.2021.622891/full#supplementary-material

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Discerning the Antimicrobial Resistance, Virulence, and Phylogenetic Relatedness of *Salmonella* Isolates Across the Human, Poultry, and Food Materials Sources in Malaysia

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Salmonella enterica subspecies *enterica* serovar Enteritidis is one of the major foodborne zoonotic pathogens globally. It has significantly impacted human health and global trade. In this investigation, whole-genome sequencing was employed to determine the antimicrobial resistance (AMR) pattern of a collection of *Salmonella* Enteritidis isolated from humans, poultry, and food sources. The study also investigated the virulence genes profile of the isolates as well as the phylogenetic relationships among strains. Illumina NextSeq technology was used to sequence the genome of 82 *Salmonella* Enteritidis strains isolated over 3 years (2016–2018) in Peninsular Malaysia. The pattern of resistance showed that tetracycline had the highest frequency (37/82, 45.12%), and isolates from food samples showed the highest rate of 9/18 (50.00%), followed by human 17/35 (48.57%) and then poultry 11/29 (37.93%). The second drug with the highest resistance rate is ampicillin with 5/29 (17.24%) for poultry, 4/35 (11.43%) for human, and 0/18 (0.00%) for food isolates respectively. Similarly, a total of 19 antimicrobial resistance (AMR) genes corresponding to the nine drugs used in the disc diffusion assay were evaluated from the whole genome sequence data. The aminoglycoside resistance gene *aac(6′)-Iy* was detected in 79 of the 82 isolates (96.34%). While the phylogenetic analysis revealed distinct lineages isolated, the three sources indicating possible cross-contamination. In conclusion, the results showed that the genomic profile of *Salmonella* Enteritidis isolated from humans, poultry, and food samples share genetic traits, hence the need to institute measures at controlling the continuous spread of these resistant pathogens.

Keywords: *Salmonella*, whole-genome sequencing, antimicrobial resistance, phylogenetic studies, virulence gene profile, foodborne infections, *Salmonella enteritidis*

INTRODUCTION

Salmonella is a very important zoonotic pathogen that has been reported to cause over 200 million human clinical infections with an estimated mortality of 3 million annually (Coburn et al., 2007; Graham et al., 2018; Moussa et al., 2021). Humans easily acquire infection from contact with animal or environmental reservoirs. The emergence of antimicrobial-resistant *Salmonella enterica* subspecies *enterica* serovar Enteritidis constitutes a serious global health problem (Pan et al., 2018). This phenomenon is believed to be due to the unregulated use and abuse of antimicrobials especially veterinary drugs including the World Health Organization's critically important antimicrobials for prophylaxis, during the management of diseases or as growth promoters (Azmi et al., 2018; Sharma et al., 2018; Yu et al., 2021). *S.* Enteritidis is one of the most important serovars shared between humans and animals in most parts of the world (Mohan et al., 2019). It is notorious for its ability to colonize livestock; wild animals as well as the reproductive system of chickens where they persist without any apparent clinical manifestation, thus continuously contaminate eggs and the immediate environment (Campioni et al., 2018; Abdulhaleem et al., 2019). The sustained and undetected presence of these bacteria in food-producing animals makes it possible to cause prolonged epidemics globally, especially where the consumption of poorly cooked poultry egg and meat is common (Salihu et al., 2015; Judd et al., 2019).

Salmonella Enteritidis is one of the major cause of invasive salmonellosis globally, particularly among malnourished children and adults with debilitating illnesses (Stanaway et al., 2019). Population-based estimates of the burden of enteric *Salmonella* infection in Southeast Asia range from 2.2 to 10.7 cases/100,000 people annually (Whistler et al., 2018). Similarly, *S.* Enteritidis is reported to account for 38% of all clinical cases in the Southeast Asia region (Eng et al., 2015). Recent investigations in Thailand on the causes of bacteremia discovered that *S.* Enteritidis accounts for 51.6% of all cases of invasive non-typhoidal *Salmonella* infections (Phu Huong Lan et al., 2016; Whistler et al., 2018). In a related investigation to determine the major food pathogens isolated in food materials in China, *Salmonella*, alongside *Vibrio parahaemolyticus*, and *Campylobacter* were among the most prevalent (Paudyal et al., 2018). These observations points to the continued risk humans face from these infectious disease pathogens, particularly where they exhibit antimicrobial resistance (AMR).

Gastro-enteric infection as a result of *S.* Enteritidis has persisted in Malaysia and continues to be a serious public health problem (Thung et al., 2016; Zakaria et al., 2020). The incidence rate has steadily increased in recent years, and this may be attributed to the unhygienic mode of handling and processing of food, especially poultry and poultry products (Thung et al., 2018; Mohan et al., 2019). Although considerable measures to improve food safety has been put in place by the relevant public health authorities, pockets of outbreaks involving large populations continue to be recorded (Balkis et al., 2017; Packierisamy et al., 2018). Other possible reasons why *S.* Enteritidis continues to be a health burden in Malaysia include the proliferation of rodent reservoirs in poultry farms, households, and public food restaurants, evolutionary selection, and the emergence of antimicrobial-resistance among other evolutionary traits (Porwollik et al., 2005; Betancor et al., 2009; Campioni et al., 2018; Azmi et al., 2021). In Malaysia like many parts of the world, it is believed that resistant *S.* Enteritidis had been introduced into the country through the importation of poultry meats and poultry products from other endemic countries (Elemile et al., 2019). In light of these explanations, and the need to understand the phenotypic and genomic diversity of circulating *S.* Enteritidis strains in Malaysia, a Whole-genome sequencing approach was used to assess the phylogenetic relationships, virulence factor determinants, and the antimicrobial-resistance profile of a collection of *S.* Enteritidis isolated from the Central region of Peninsular Malaysia from 2016 to 2018, with a view to understand the genetic relationship of this important pathogen across different sources (human clinical samples, live birds, and chicken meat).

MATERIALS AND METHODS

Salmonella Isolates Selection

The 82 *Salmonella* isolates used for this study were obtained from government diagnostic laboratories operating within Peninsular Malaysia as part of an ongoing National Antimicrobial Surveillance Program. The isolates were collected from multiple sources, including humans, poultry, and food. *Salmonella* was isolated at various time points from 2016 to 2018. The human *Salmonella* isolates (n = 35) were obtained from blood and stool samples collected from clinical cases and provided by the Bacteriology Unit, Institute for Medical Research, Ministry of Health Malaysia. Poultry samples (29) originated from fecal samples from commercial poultry farms provided by the Antimicrobial Surveillance Section, Department of Veterinary Services, Ministry of Agriculture Malaysia. At the same time, the food isolates were obtained from ready to eat food from restaurants and chicken meats from wet markets (18) and were provided by the Food Safety and Quality Division, Ministry of Health Malaysia. All the samples were stored in nutrient agar slants until required for further characterization.

Serotype Prediction and Evaluation of Phenotypic AMR

Traditional serotype determination, according to the Kauffmann White Scheme and the *in silico* serotype prediction based on the raw reads and genome assemblies, were conducted using SeqSero2 (SeqSero2 v1.1.0) pipeline (Zhang et al., 2019). While the Kauffmann White Scheme determines *Salmonella* serovars using standard agglutination (SSI Diagnostica *Salmonella* Sero-quick ID kit, UC Bioscience Sdn, Bhd) method according to their antigenic formula (O and H antigens), the SeqSero 2 software uses a k-mer based algorithm to predict serovars from raw reads which ensures improved serotype prediction from draft genome assemblies (Diep et al., 2019; Zhang et al., 2019).

Similarly, all the *Salmonella* isolates serotyped using the traditional Kauffmann White Scheme and then subjected to phenotypic antimicrobial susceptibility test using the

Kirby Bauer Disc Diffusion method. A panel of nine (9) antimicrobials purchased from Oxoid (Thermo Scientific Microbiology Sdn Bhd)including ampicillin (Amp-R < 13 mm), chloramphenicol (C-R < 12 mm), gentamicin (CN-R < 12 mm), streptomycin (S-R < 11 mm), sulfamethazine/trimethoprim (SXT-R < 10 mm), tetracycline (TE-R < 11 mm), ceftiofur (EFT-R < 10 mm), cefotaxime (Ctx-R < 22 mm), and ciprofloxacin (CIP-R < 15 mm) was used (Wayne, 2019). *Escherichia coli* ATCC25922 was used as internal quality control. The *Salmonella* isolates with the zone of inhibition within the intermediate range were considered to be susceptible to avoid overestimation of resistance.

Genome Library Preparation, Sequence Assembly, and Annotation

About 2 ml of an overnight culture (at 37°C) of the *Salmonella* isolates (n = 82) at on Luria-Bertani (L.B.) agar was pelleted by centrifugation at 5000 × g for 10 min. The genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen), and the purity and concentration were determined using the NanoDrop spectrophotometer (Thermo Fisher Scientific). The Nextera^{TM} DNA Flex Library Prep Kit was employed for the preparation of the genomic libraries. The whole-genome sequencing was performed on the NextSeq 550 System (Illumina, United States). Sequencing reads obtained from the Illumina NextSeq sequencer were scanned for adapter sequence and low-quality sequence using BBDuk (BBTools version 36), where adapter trimming, quality trimming, contaminant filtering, and read length filtering was done, and part of the reads containing poor quality sequence were removed. The good quality sequencing reads were then assembled using SPAdes (SPAdes version 3.9.0) to obtain contigs (Bankevich et al., 2012).

Due to the different sequence profiles, the gene annotation was achieved by predicting the rRNA genes using RNAmmer (Lagesen et al., 2007), while the tRNA genes were predicted using ARAGORN (Laslett and Canback, 2004). The protein-coding genes were first predicted using Prodigal (Hyatt et al., 2010), and the predicted sequences were used to predict their function by using BLAST (Camacho et al., 2009) and HMMER (Eddy, 1998) to search against various sequence or domain databases.

Comparative Analysis

All the assembled contig sequences from the 82 *Salmonella* isolates as well as representative reference genomes of closely related *Salmonella* species (CP019177.1, CP036165.1, CP036166.1, CP022500.1, CP037917.1, CP019183.1, CP022489.1, CP003278.1, AE006468.2, NC_003197.2, and CP014996.1) obtained from the NCBI database were used to infer the relationship, while *Salmonella bongori* (NC_015761.1) and *E. coli* (NC_000913.3) were used as outgroup controls. All the sequences were subjected to comparative studies using the EPInod pipeline developed by BioEasy Sdn Bhd. The software package is capable of evaluating the sequences for average nucleotide identity (ANI), multi-locus sequence typing (MLST), Single Nucleotide Polymorphism (SNP)-based phylogenomics estimation, pan-genome gene conservation analysis, virulence factor (V.F.) detection, and AMR gene detection.

Whole-genome was utilized to deduce the genetic relationship among isolates from different species. The identification of pan-genome SNPs and phylogenetic analysis was made using the kSNP3.0 program, as described by Gardner et al. (2015). The FASTA input file of the target genomes for SNPs discovery was first input, and k, which represents the length of the flanking sequence, including the SNPs, was specified. Finally, SNPs positions in the finished genomes were found by matching with MUMmer. The phylogenetic tree was inferred using all genome SNPs aligned multiple FASTA files with MEGA7 (Kumar et al., 2016). The maximum-likelihood phylogenetic tree of all SNPs loci was generated using the Jukes-Cantor/GTI model with calculated SH-like branch support (1,000 iterations), not bootstrapping. This is because SH-like branch support is fast and efficient, and is commonly used as the default method of choice for phylogenetic software.

Similarly, the genome Virulence Factor analysis to determine the major virulence factors of the characterized bacterial pathogen was achieved using the virulence factor database (VFDB), which is an integrated and comprehensive online resource for curating information about virulence factors of bacterial pathogens (Chen et al., 2016). The NCBI BLAST tool was used to scan predicted CDS (nucleotides) against VFDB for virulence factors, which represent the measure of the pathogenicity of the isolates. The virulence factors analysis was done in two sets, where set A includes genes with experimentally verified V.F. only while set B covers all genes related to known and predicted V.Fs in the V.F database. Finally, the Genome Antibiotic Resistance to identify the AMR profile of the isolates was streamlined via the abricate program against ResFinder and the Comprehensive Antibiotic Resistance Database (CARD) to screen contigs for AMR.

Correlation Between the Phenotypic and Genotypic AMR Profiles

The antimicrobial susceptibility pattern of the isolates was determined with the Kirby Bauer disk diffusion test, as mentioned earlier. The AMR pattern of the isolates was correlated with its known corresponding resistance gene for each of the strains detected by the WGS analysis, and the percentage correlation was calculated. This was done by counting the genotypic results and dividing the total by the number of isolates that exhibited phenotypic resistance to determine the sensitivity. While the specificity was calculated by dividing the number of isolates that showed genotypic susceptibility by the total number of isolates showing phenotypic susceptibility (Trevethan, 2017). The percentages of positive predictive values (PPVs) and negative predictive values (NPVs) were also calculated as described by Trevethan (2017).

Statistical Analysis

The association between AMR determinants and virulence genes is calculated using the Chi-square test, and the P-value level <0.05 is considered significant. Similarly, the correlation between the phenotypic and genetic AMR was also deduced. A one-way analysis of variance (ANOVA) was also conducted to determine if there is significant difference between the means of the three

hosts (Human, Poultry and Food), followed by a *post hoc* for multiple comparisons.

RESULTS

The Kauffmann White Scheme serotyping tests indicated that all the isolates from the three different sources (human, poultry, and food) belonged to the *S*. Enteritidis serogroup. However, the overall results suggested that SeqSero2 (SeqSero2 v1.1.0) improved the serotype prediction compared to the Kauffmann White Scheme serotyping scheme. Although 93% of the serotype prediction according to the Kauffmann White Scheme was concordant with the *in silico* WGS prediction, six isolates were found to conflict as they were predicted to be Brancaster (S6-human), Mbandaka (S18-human), Ohio (S72-poultry), Weltevreden (S77-poultry), and Kentucky (S81-poultry), while S63 (poultry) was found to be either Albany or Duesseldorf because they share the same antigenic formula (8: z4, z24). Additionally, the SeqSero2 also identified one unique isolate with antigenic formula I 4:b:- (S87-poultry), which is not listed in the Kauffmann White Scheme. The antimicrobial susceptibility assay, according to the Clinical and Laboratory Standards Institute (CLSI) standards (Patel et al., 2016), showed that thirty (30) isolates were resistant to at least one of the tested antimicrobial drugs, while twenty-four (24) were resistant to multiple drugs tested (MDR: resistance to three or more antimicrobial classes), and the remaining isolates were all susceptible to the tested antimicrobials. However, only one isolate showed resistance to each of ceftiofur (1 human isolate), cefotaxime (1 human isolate), sulfamethazine/trimethoprim (1 food isolate), gentamicin (1 poultry isolate), and chloramphenicol (1 human isolate), while none of the isolates were resistant to ciprofloxacin. The phenotypic and genotypic antimicrobial susceptibility results are summarized in **Tables 1**, **2**.

The pattern of resistance showed that tetracycline had the highest frequency (37/82, 45.12%), and isolates from human samples showed the highest rate with 17/35 followed by poultry 11/29 and then food 9/18 (**Figure 1**). The second drug with the highest resistance rate is ampicillin with 4/35 (11.43%) for human, 5/29 (17.24%) for poultry, and 0/18 (0.00%) for food isolates respectively (**Figure 2**). However, the chi-square statistic to check for any association between the resistances observed and the source of the samples (human, poultry, or food) was found to be 1.772, and the *p*-value was 0.412. This signifies that the association is not significant at $p < 0.05$. Similarly, no statistically significant difference was observed based on the ANOVA with a *p*-value of 0.704 ($p > 0.05$).

A total of 19 antimicrobial resistance (A.R) genes corresponding to the nine drugs used in the disc diffusion assay were evaluated from the whole genome sequence data. The aminoglycoside resistance gene *aac(6′)-ly* was detected in 79 of the 82 isolates (96.34%). This observation is in contrast to the phenotypic analysis, which showed that only one gentamicin-resistant isolate (1.22%) was found among all the

TABLE 1 | Distribution of phenotypic antimicrobial resistance (AMR) profile among *S*. Enteritidis isolates.

Isolates	Antimicrobial Resistance (%)								
	AMP	**C**	**CN**	**S**	**SXT**	**TE**	**EFT**	**CTX**	**CIP**
Poultry	5/29 (17.24%)	0/29 (0.00%)	1/29 (3.45%)	1/29 (3.45%)	0/29 (0.00%)	11/29 (37.93%)	0/29 (0.00%)	0/29 (0.00%)	0/29 (0.00%)
Food	0/18 (0.00%)	0/18 (0.00%)	0/18 (0.00%)	0/18 (0.00%)	1/18 (5.56%)	9/18 (50.00%)	0/18 (0.00%)	0/18 (0.00%)	0/18 (0.00%)
Human	4/35 (11.43%)	1/35 (2.86%)	0/35 (0.00%)	1/35 (2.86%)	0/35 (0.00%)	17/35 (48.57%)	1/35 (2.86%)	1/35 (2.86%)	0/35 (0.00%)
Overall	9 (10.98%)	1 (1.22%)	1 (1.22%)	2 (2.44%)	1 (1.22%)	37 (45.12%)	1 (1.22%)	1 (1.22%)	0 (0.00%)

The antimicrobial susceptibility/resistance was determined based zone of inhibition according to the CLSI standard. Ampicillin (AMP), chloramphenicol (C), gentamicin (C.N.), streptomycin (S), sulfamethazine/trimethoprim (SXT), tetracycline (T.E.), ceftiofur (EFT), cefotaxime (CTX), and ciprofloxacin (CIP).

TABLE 2 | Distribution of genotypic AMR genes among *S*. Enteritidis isolates.

Source of isolate	Antimicrobial Resistance (%)								
	AMP (*TEM 33*; *TEM 4*)	**C (*floR*)**	**CN (*aac(6′)-ly*; *aadA*)**	**S (*strA*; *strB*)**	**SXT (*sul1*; *sul2*; *dfrA14*; *dfrA15*)**	**TE (*tetA*; *tetC*)**	**EFT (*blaCMY-2*; *blaTEM-1*)**	**CTX (bla-*TEM*, bla-*CTX-M*)**	**CIP (*qnrS1*; *qnrD1*)**
Poultry	6/29 (20.69%)	−/29 (0.00%)	28/29 (96.55%)	1/29 (3.45%)	−/29 (0.00%)	10/29 (34.48%)	−/29 (0.00%)	−/29 (0.00%)	1/29 (3.45%)
Food	−/18 (0.00%)	−/18 (0.00%)	17/18 (94.44%)	−/18 (0.00%)	1/18 (5.56%)	9/18 (50.00%)	−/18 (0.00%)	−/18 (0.00%)	−/18 (0.00%)
Human	3/35 (8.57%)	1/35 (2.86%)	34/35 (97.14%)	−/35 (0.00%)	2/35 (5.71%)	19/35 (54.29%)	−/35 (0.00%)	1/35 (2.86%)	2/35 (5.71%)
Overall	9 (10.98%)	1 (1.22%)	79 (96.34%)	1 (1.22%)	3 (3.66%)	38 (46.34%)	0 (0.00%)	1 (1.22%)	3 (3.66%)

The table presents multiple genes that confer resistance against the antimicrobial drugs used for the antimicrobial susceptibility test (AST) analysis. Ampicillin (Amp), chloramphenicol (C), gentamicin (C.N.), streptomycin (S), sulfamethazine/trimethoprim (SXT), tetracycline (T.E.), ceftiofur (EFT), cefotaxime (CTX), and ciprofloxacin (CIP).

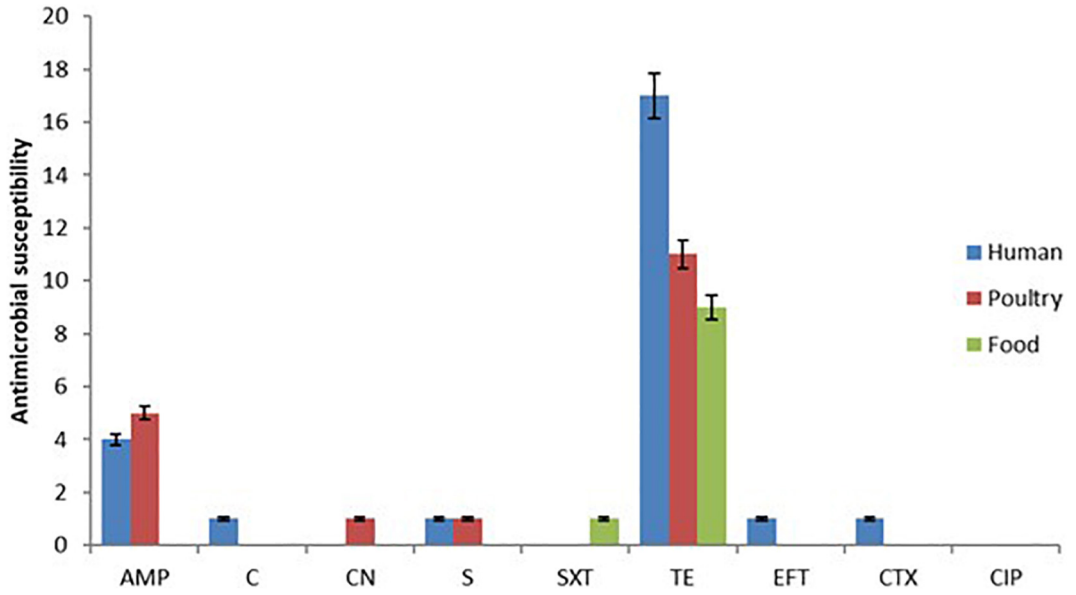

FIGURE 1 | Showing the antimicrobial susceptibility test results based on the Kirby Bauer disk diffusion method indicating high resistance to tetracycline and ampicillin.

isolates. Although, the detection of the tetracycline-resistant genes (*tetA*, *tetC*, and *tetD*) 38/82 (46.34%) was found to correspond with the phenotypic result for tetracycline 37/82 (45.12%), albeit with the genotypic detection being higher by one isolate (**Figure 2**). However, the predominant tetracycline resistance encoding gene was *tetC*, with only one isolate possessing the *tetA* gene.

Ten (10) isolates were resistant to beta-lactams (10.98%), and the two genes encoding beta-lactamases identified in these isolates were *TEM33* (10/82), and *TEM4* (10/82). Not a single isolate was found to show resistance to the quinolone ciprofloxacin based on the disc diffusion method. However, the genes *qnrS*, and *qnrD1* were detected from two human isolates and one poultry isolate. Other resistance genes identified by the whole-genome AMR analysis are *dfr*A14 (3/82), *dfr*A15 (3/82), and *sul2* (2/82) for trimethoprim and sulfonamide resistance as well as one *floR* gene (1/82) that codes for chloramphenicol resistance.

Correlation Between AMR Based on Kirby Bauer Disc Diffusion Test and Whole-Genome Sequence AMR Analysis

The data generated from the whole-genome sequence AMR analysis was correlated with the phenotypic AMR to evaluate the ability of the whole-genome sequence data to predict the phenotypic AMR profiles obtained by the disc diffusion method. The predominant phenotypic AMR resistance observed was resistance against tetracycline (45.12%) and ampicillin (10.98%). However, the analysis did not include ciprofloxacin because none of the isolates studied showed phenotypic resistance to ciprofloxacin. Generally, a strong correlation between the phenotypic resistance by disc diffusion and the AMR gene prediction by WGS was observed, except for gentamicin, which was discordant (**Table 3**).

The overall sensitivity for the WGS based AMR prediction across all the antimicrobial agents evaluated was 50.97%, while the specificity was 55.73%. The post-test probability of the AMR prediction, which measures how well the technique predicts the resistance accurately, was 20.12%, while the NPV was 91.78%.

Analysis of *S.* Enteritidis Virulence Determinants

For this article, only those virulence genes that are associated with experimentally verified virulence factors were considered. The virulence factors included bacterial toxins, cell surface proteins that mediate the attachment, cell surface carbohydrates, and proteins that protect the bacterium and hydrolytic enzymes that may contribute to the pathogenicity of the bacteria. These genes were analyzed using the BLAST program against the VFDB. The parameters used were the Percent identity cut-off (50), Percent query coverage per high-scoring segment pair (hsp) (50), and the number of aligned sequences to keep (Chen et al., 2016). A total of 121 virulence genes were detected by the BLAST search against VFDB. The virulence-associated determinants identified in this study include a group of transport, adhesion and some effector proteins such as; *Salmonella* virulence plasmid determinant protein, Mg^{2+} transport protein, Intimin-like protein, resistance to compliment killing protein, type III secretion system effector and plasmid-encoded fimbria protein, among other (**Supplementary Table 1**).

The virulence genes identified in this study are predominantly genes belonging to the type III secretion system (T3SS), which is encoded by the *Salmonella* pathogenicity sequence 1 (SPI-1) and *Salmonella* pathogenicity sequence 2 (SPI-2). The type III

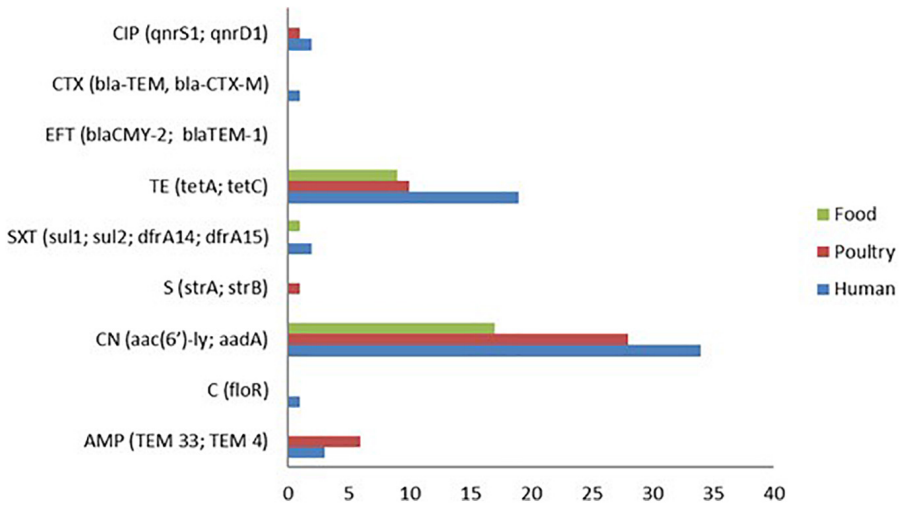

FIGURE 2 | Genotypic antimicrobial resistance (AMR) patterns of *S.* Enteritidis isolates showing aminoglycoside resistance genes (*aac (6')-ly*) and *aadA* as the predominant resistance genes detected followed by tetracycline (*tetA and tetC*) genes.

TABLE 3 | Correlation between AMR phenotype by Kirby Bauer method and genotypic AMR WGS analysis for *S.* Enteritidis isolates (*n* = 82).

Antimicrobials	Resistant isolates		Susceptible isolates		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	Genotypic	Phenotypic	Genotypic	Phenotypic				
AMP	9	9	73	73	10.98	89.02	50.00	50.00
C	1	1	81	81	1.22	98.78	50.00	50.00
CN	79	1	3	81	96.34	98.78	98.75	96.43
S	1	2	81	80	1.22	97.56	33.33	49.69
SXT	3	1	79	81	3.66	98.78	75.00	50.63
TE	38	37	44	43	46.34	53.75	50.67	49.43
EFT	0	1	82	81	0.00	98.78	0.00	49.69
CTX	1	1	81	81	1.22	98.78	50.00	50.00
Total					20.12	91.78	50.97	55.73

Ampicillin (AMP), Chloramphenicol (C), Gentamicin (C.N.), Streptomycin (S), sulfamethazine/trimethoprim (SXT), Tetracycline (T.E.), Ceftiofur (EFT), Cefotaxime (CTX), and Ciprofloxacin (CIP), PPV-Positive Predictive Value, NPV-Negative Predictive Value.

SPI 1 genes include; system effector (*SteA* and *SteB*), type III secretion system accessory cytosolic protein (OrgA and OrgC), type III secretion system regulatory protein (InvA-InvJ), type III secretion system export apparatus switch protein (SpaO-SpaS), and type III secretion system hydrophilic translocator, and pore protein (SipA-SipD) (**Supplementary Table 1**). These genes were detected in all the isolates from the human, poultry, and food samples. Similarly, SPI-2 system effector (SopB, SopD2, and SlrP) chaperone protein-coding (sseB-sseC) *SteC, SseK1, SifB, SseK2,* and type III secretion system gatekeeper (*SsaH-SsaL*) were among the genes detected in all the isolates except S63 isolate from poultry swab.

In addition to the genes within the pathogenicity sequences, virulence plasmid (plasmid-encoded fimbriae chaperone protein PefD), ion acquisition (ferrienterobactin outer membrane transporter), fimbriae (long polar fimbrial chaperone protein, type I fimbriae adaptor protein FimF), as well as the flagella and flagellin genes (flagellar motor protein MotA) were all identified. Within the fimbrial adherence determinants, are genes that codes for the curli fimbriae and curli assembly protein CsgC that mediate the binding to various serum and tissues matrix proteins (Thomas et al., 2017). Other important virulence determinants detected from most of the isolates after the BLAST analysis within the VFDB software are; the antimicrobial peptide resistance protein Mig-14, Mg^{2+} transport protein MgtBC, and the *Salmonella* plasmid virulence which is responsible for the regulation of spv operon.

Phylogenomics of *S.* Enteritidis

Phylogenetic analysis for the 82 genome sequence (WGS data attached as a **Supplementary Document 2** – https://submit.ncbi.nlm.nih.gov/wgs_common/report/SUB8477823) was conducted using the maximum likelihood method. The analysis included the sequence from the 35 human isolates, 18 isolates from food, and 31 isolates obtained from poultry cloacal swabs and an *S.* Brancaster, *S.* Ohio, *S.* Mbandaka, *S.* Kentucky, *S.* Weltevreden, *S.* Typhimurium, *S.* Bongori, and *E. coli* K12 control genome. Two bifurcating clades and one monophyletic clade from the

ancestral node were confirmed from the phylogenetic tree. The rooted *S.* Enteritidis tree indicated multiple bifurcating patterns with varying levels of diversity and placed both *S.* Enteritidis isolates from humans, poultry, and food samples into distinct monophyletic clades, suggesting that the isolates evolved into distinct evolutionary lineages from their common phylogenetic ancestor. The ancestral node branched into two with the *E. coli* (clade A) outgroup diverging away from the *Salmonella* group (clade B). Subsequently, clade B bifurcated into sub-clade B1 occupied by *S.* Bongori and B2 (*S. enterica*), which later splits into multiple clusters with variable genetic properties compared to the reference genomes.

The phylogenetic analysis revealed that sub-clade B2-I contained S6 and S18 from human clinical samples, which clustered with *S.* Brancaster and *S.* Mbandaka, respectively. While S63, S72, S77, and S81 clustered with *S.* Albany, *S.* Ohio, *S.* Weltevreden, and *S.* Kentucky, respectively (sub-clade B2-I). The clustering of these isolates with the reference genome conforms with the SeqSero serotype allocation. Important to note that while the SeqSero analysis identified that S63 shared the same antigenic formula with *S.* Albany and *S.* Duesseldorf, the phylogenetic tree showed it is more genetically related with *S.* Albany rather than *S.* Duesseldorf. *S.* Typhi, which is typhoidal *Salmonella*, was found to share some genetic relationship with *S.* Ohio, *S.* Enteritidis, and *S.* Typhimurium having branched from the same bifurcation. Sub-clade B2-II, on the other which contained the largest convergence of the isolates, implies close genetic relation across the different sources.

DISCUSSION

Salmonella serotyping using the traditional phenotypic method was the first analysis conducted in this study because of its importance in the characterization of *Salmonella*, especially during epidemiological investigations and surveillance programs (Diep et al., 2019). However, because of the ease and superior predictability of WGS based *in silico* methods, SeqSero2 was used to confirm the phenotypic serotyping (Zhang et al., 2019). The results show that the SeqSero2 correctly identified *S.* Enteritidis in concordance with the Kauffmann White Scheme result with 93% similarity. However, the SeqSero2 was able to identify six serovars other than *S.* Enteritidis (Brancaster, Mbandaka, Ohio, Weltevreden, and Kentucky), including Albany and Dusseldorf which had the same antigenic formula (I 4:b:-), while one was not available in the database. This finding agrees with earlier studies that indicated that the same antigenic formula could be shared by different subspecies (Diep et al., 2019). *In silico* WGS-based serotyping has been shown to perform better in identifying *Salmonella* serotype diversity compared to the traditional method and has become popular among molecular epidemiologists and public health scientists (Diep et al., 2019; Elnekave et al., 2020).

The present investigation also evaluated AMR determinants, virulence factor genes, and phylogenetic relationships of the isolates using a whole-genome sequencing approach. This is because comparative genomics studies have indicated that host specificity (humans, animals, birds, and environment) is a very important factor driving the evolution of new lineages of enteric *Salmonella* species (Tasmin et al., 2017; Deblais et al., 2018). Secondly, the choice of WGS is because its application permits a broader inference of pathogen characterization, including the prediction of antibiotic resistance and virulence determinants from the sequence (Gupta et al., 2019). The 82 *S.* Enteritidis, *S.* Brancaster, *S.* Mbandaka, *S.* Albany, *S.* Ohio, *S.* Weltevreden, and *S.* Kentucky isolates identified in this study were cultured from multiple sources, including human clinical samples (blood and feces), poultry (cloacal swab), and food (chicken meat). Upon obtaining the whole genome sequence, EPInod pipeline was used for the analysis of AMR profile, virulence determinants, and the SNP-based phylogenetic analysis.

Antimicrobial usage represents a very critical factor in the emerging public health crisis due to antibiotic resistance. Its extensive application in food animal production to manage clinical infections, as prophylaxis to prevent and control livestock diseases, as well as its use as an additive in feed to enhance growth and productivity have all played roles in the emergence of resistance (Salihu et al., 2013; Wang et al., 2019; Van et al., 2020; Ma et al., 2021). In this study, tetracycline (45.12%) and ampicillin (11.43%) were identified as drugs with the highest phenotypic resistance rate. Similarly, resistance to these drugs was highest among the human isolates (17/35), followed by food isolates (9/18) and then poultry (11/29). These antimicrobials are among those that have been declared critical by the WHO and their use in animals has been restricted. This finding implies that some of these legislation is not being observed and this could lead to the loss of potency of the drugs and progressive emergenc of antimicrobial resistant pathogens. Also, 29.27% of the isolates comprising of *S.* Enteritidis, *S.* Mbandaka, *S.* Ohio, *S.* Kentucky, and *S.* Brancaster exhibited multiple resistances to 2 or more drugs. According to studies sanctioned by the Department of Veterinary Services Malaysia under the Livestock Farm Practices Scheme (SALT) program, for *Salmonella* species isolated from chicken cloacal swabs revealed that 13.5% of the *Salmonella* isolates were resistant to tetracycline, 5.4% to polymyxin and erythromycin. This is similar to the investigation conducted in China where tetracycline resistance was found to predominate among *Salmonella* isolates obtained from slaughtered pigs (Wu et al., 2021). In comparison, 2.7% were resistant to chloramphenicol and trimethoprim (Azmi et al., 2018). Despite the resistance observed in this study, the association between the resistance rate and the source of the sample as well as statistical significance based on ANOVA between their means was found not to be statistically significant ($p > 0.05$). Other drugs that were found to exhibit resistance were chloramphenicol among human isolates (2.86%), gentamicin in poultry (3.45%), sulfamethazine/trimethoprim in food (5.56%), ceftiofur and cefotaxime both in human (2.86%). In a related study conducted in the East Coast of Peninsula Malaysia, *Salmonella* isolates from broiler chicken were found to exhibit resistance to chloramphenicol (76.2%), sulfamethoxazole/trimethoprim (42.9%) (Ibrahim et al., 2021). This further highlights the crucial role of poultry and poultry products in the dissemination of resistant Salmonella pathogens to humans and other susceptible animal species.

On the other hand, the WGS analysis of AMR genes correlated significantly by identifying some genes that encode resistance to the majority of the phenotypic resistance observed. These included tetracycline, ampicillin, streptomycin, sulfamethazine/trimethoprim, cefotaxime, and ciprofloxacin resistance genes such as *tetA*, *tetC*, *TEM33*, *TEM4, floR*, *aac(6′)-ly*, *strA*, *sul1*, *dfrA14*, *dfrA15*, *blaCTX-M*, and *qnrS1*, respectively (**Table 2**). Although no phenotypic resistance was observed for gentamicin, the AMR genotype result showed that the gentamicin gene (*aac(6′)-ly*) was the most frequent (96.34%) with isolates from a human source having the highest percentage (97.14%). This observation goes to show WGS as an excellent tool for the accurate prediction of antimicrobial-resistant phenotype in human, animal, and environment samples (Pornsukarom et al., 2018; Liu et al., 2021). The gene *aac(6′)-ly* is one of the important chromosomal genes responsible for the enzymatic modification of aminoglycoside leading to the development of resistance (Odumosu et al., 2015; El-Badawy et al., 2017). However, it is important to note that mechanism for aminoglycoside resistance include among others decreased uptake and accumulation of the drug in the bacterial pathogen, as well as the expression of aminoglycoside modifying enzymes (AMEs) that causes inactivation of the drug (Mir et al., 2016). Even though, a strong correlation between the phenotypic resistance by disc diffusion and the AMR gene prediction by WGS was observed (sensitivity 20.12% and specificity 91.78%), the result for gentamicin was discordant (**Table 3**). The most common genes conferring resistance to β-lactam antibiotics in this study were *TEM 33*

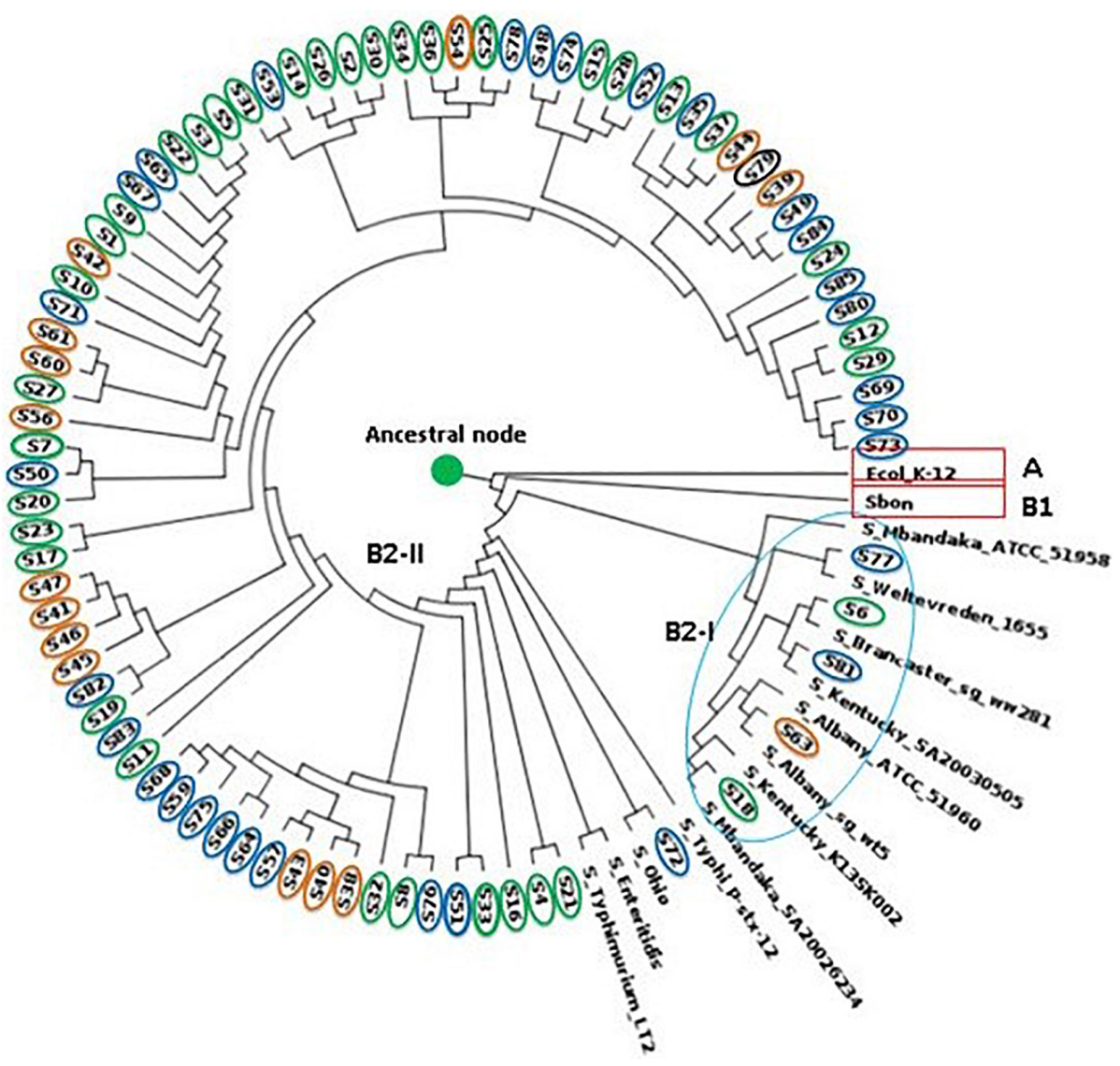

FIGURE 3 | Phylogram showing maximum-likelihood phylogeny of *S.* Enteritidis calculated using aligned all genome SNPs. Human isolates circled in green, food in orange and chicken isolates in blue.

and *TEM 4* without corresponding phenotypic consequence. Qualitative determination of antimicrobial susceptibility based on the zone of inhibition is prone to error due to the instability of antibiotic-containing discs under varying temperature and humidity, interference of the functionality of some antimicrobials by the components of growth agar, and the possibility that an isolate may be falsely classified as susceptible (Neuert et al., 2018; Srivastava et al., 2020). This may explain the relative number of mismatches observed in the present study. Moreover, many of the resistance genes detected by the WGS algorithm are plasmid-encoded. In other words, bacterial plasmids are known to commonly cure during storage and sub-culture of the isolates, such that genes detected during sequencing might have become altered (Neuert et al., 2018). Furthermore, *Salmonella* has been shown to possess silent resistance genes, as well as variants (*aac(6′)*), which only become transcriptionally active in rare cases (Heider et al., 2009; Frye and Jackson, 2013; Adesiji et al., 2014).

The WGS analysis revealed multiple virulence genes among the *Salmonella* isolates across the different sources (**Supplementary Table 2**). These genes included the type III secretion system (T3SS) located within the *Salmonella* Pathogenicity Sequence I and II, intimin-like proteins, plasmid-encoded fimbriae chaperone protein, host recognition/invasion determinants, as well as magnesium uptake, iron acquisition factors. These genes are responsible for *Salmonella* infectivity, transmission, and survival (Pornsukarom et al., 2018). The result of the present study indicates that the *S.* Enteritidis isolated from the poultry cloacal swabs and food sources possessed the same virulence genes as the human clinical isolates. Virulence factors such as adhesins, toxins, and iron ion transport systems are responsible for the adhesion of pathogens to epithelial cells of intestines, which constitute an essential colonization factor during intestinal infections (Sarowska et al., 2019). The detection of a high number of plasmid encoding virulence genes in food, and especially poultry, may serve as a means of transmission of these resistance factors to humans, as observed in this study. The detection of fimbrial genes and a host of other plasmid-encoded genes within the pathogenicity sequences 1–2 among the isolates in this study may signify the potentials of these isolates to cause severe humans infection. Hence, this report will provide valuable reference data that will assist in future investigations of human *Salmonella* infection. In the same vein, the phylogenetic tree obtained from SNPs exhibited diverse clustering of the isolates with an initial one singleton and two clades that subsequently bifurcated. Phylogenetic analysis based on WGS-derived has proven to be a superior cluster resolution method compared to many of the standard subtyping methods (Mohammed and Thapa, 2020; Ford et al., 2021). In order to determine the genetic relatedness among the isolates and infer their evolutionary history based on a genome-wide scale, across the genomes of all *S.* Enteritidis and closely related serovars were identified. Phylogenetic analysis based on the maximum likelihood tree produced strong support for the monophyly of the *Salmonella* isolates with *S.* Enteritidis being the predominant (**Figure 3**). Within the *S.* Enteritidis isolates from the different sources, a split that delineates two sister lineages, Clade B1 and B2 was observed. At the same time, Clade A consisted of 1 branch occupied by the *E. coli* K12 outgroup, Clade B comprised of the *S. bongori* reference strain and the other members of the *S. enterica* serogroup. Sub-clade B2-I which is a branched form the B1 clade, clustered with five of the isolates (S77, S6, S81, S63, and S18) which shared same branches with *S.* Mbandaka, *S.* Weltevreden, *S.* Brancaster, *S.* Kentucky, and *S.* Ohio implying close genetic relationship as determined via the SeqSero serotyping. The major bifurcating clade B2-II, on the other hand, contained one monophyletic clade occupied by *S.* Bandaka and another highly branched sub-clades containing isolates from all the three sources. The fact that samples from poultry, human and food as well as the control *S.* Typhimurium, and *S.* Typhi were located in this well-supported sub-clade may indicate similarity in their gene sequence that can be attributed to convergent evolution due to adaptation to the various sources as earlier reported by Didelot et al. (2007). As deduced from the phylogenetic tree in **Figure 3**, though the split of clade B is suggestive of allele sharing, possibly via horizontal gene transfer and homologous recombination between the clades, which is a common phenomenon in enteric bacteria (Lawrence and Retchless, 2009; Hall et al., 2020). However, the gene transfer may not have occurred to the extent that divergence between the two clades is eliminated. This is in concordance to divergent lineages observed in *E. coli* following high recombination (Touchon et al., 2009).

CONCLUSION

An insight into the genetic profile of enteric *S.* Enteritidis to assess the evolutionary and genetic diversity based on SNPs phylogenomics, as well as understand the virulence and resistance pattern using WGS, was undertaken. The aim was to understand further the genomic diversity existing among *S.* Enteritidis from different host species. The potential transmissibility of genetic elements was evident due to the clustering of multiple isolates from all the three sources. Most of the virulence factors were also detected from the genome of all the isolates across the human, poultry, and food isolates. However, while the genotypic AMR was mostly congruent with the phenotypic profiles of the isolates, slight mismatches were observed with particular reference to gentamicin. This discrepancy was attributed to their silent nature of the *aac(6′)-ly* gene whose transcription occurs intermittently. As a whole, the WGS analysis produced a comprehensive understanding of the resistance profile of S. Enteritidis and the ability of the bacteria to adapt to different host species. However, a more in-depth study, including other *Salmonella* serovars, will be of immense benefit to the understanding of the adaptability and survival mechanism in *Salmonella* serovars.

LIMITATION OF THE STUDY

This investigation was not able to ascertain the geographical details of the various samples/sources because the isolates were

donated as part of stock cultures covering the study period by the agencies involved with the Malaysian National Antimicrobial Resistance Surveillance Program.

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**.

AUTHOR CONTRIBUTIONS

ZZ, LH, SAH, and NA: conceptualization. ZS, NMS, and RA: methodology. ZZ, NMS, and BG: software sequence analysis and interpretation. ZZ and BG: writing—original draft preparation. ZZ, BG, LH, NA, SAH, ZS, NMS, and RA: writing—review and editing. ZZ: funding acquisition. ZZ and LH: supervision.

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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.2021.652642/full#supplementary-material

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Whole Genome Sequencing of Extended-Spectrum- and AmpC- β-Lactamase-Positive Enterobacterales Isolated From Spinach Production in Gauteng Province, South Africa

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The increasing occurrence of multidrug-resistant (MDR) extended-spectrum β-lactamase- (ESBL) and/or AmpC β-lactamase- (AmpC) producing Enterobacterales in irrigation water and associated irrigated fresh produce represents risks related to the environment, food safety, and public health. In South Africa, information about the presence of ESBL/AmpC-producing Enterobacterales from non-clinical sources is limited, particularly in the water–plant-food interface. This study aimed to characterize 19 selected MDR ESBL/AmpC-producing *Escherichia coli* ($n = 3$), *Klebsiella pneumoniae* ($n = 5$), *Serratia fonticola* ($n = 10$), and *Salmonella enterica* ($n = 1$) isolates from spinach and associated irrigation water samples from two commercial spinach production systems within South Africa, using whole genome sequencing (WGS). Antibiotic resistance genes potentially encoding resistance to eight different classes were present, with $bla_{\text{CTX-M-15}}$ being the dominant ESBL encoding gene and bla_{ACT}-types being the dominant AmpC encoding gene detected. A greater number of resistance genes across more antibiotic classes were seen in all the *K. pneumoniae* strains, compared to the other genera tested. From one farm, $bla_{\text{CTX-M-15}}$-positive *K. pneumoniae* strains of the same sequence type 985 (ST 985) were present in spinach at harvest and retail samples after processing, suggesting successful persistence of these MDR strains. In addition, ESBL-producing *K. pneumoniae* ST15, an emerging high-risk clone causing nosocomical outbreaks worldwide, was isolated from irrigation water. Known resistance plasmid replicon types of Enterobacterales including IncFIB, IncFIA, IncFII, IncB/O, and IncHI1B were observed in all strains following analysis with PlasmidFinder. However, $bla_{\text{CTX-M-15}}$ was the only β-lactamase resistance gene associated with plasmids (IncFII and IncFIB) in *K. pneumoniae* ($n = 4$) strains. In one *E. coli* and five *K. pneumoniae* strains, integron In191 was observed. Relevant similarities to human pathogens were predicted with PathogenFinder for all 19 strains, with a confidence of

0.635–0.721 in *S. fonticola*, 0.852–0.931 in *E. coli*, 0.796–0.899 in *K. pneumoniae*, and 0.939 in the *S. enterica* strain. The presence of MDR ESBL/AmpC-producing *E. coli*, *K. pneumoniae*, *S. fonticola*, and *S. enterica* with similarities to human pathogens in the agricultural production systems reflects environmental and food contamination mediated by anthropogenic activities, contributing to the spread of antibiotic resistance genes.

Keywords: WGS, food safety, leafy greens, multidrug resistance, foodborne bacterial pathogens

INTRODUCTION

The discovery of antibiotics in the 1940s led to a new age in medical care. However, the global increase in antimicrobial resistance (AMR) is reducing the effectiveness of clinically important antibiotics (Lobanovska and Pilla, 2017; Dandachi et al., 2019). Examples of shifting resistance profiles in bacteria within the β-lactam class of antibiotics, including penicillins and third generation cephalosporins, which are the most widely used in human and veterinary medicine and widely expressed AMR are being reported (Finton et al., 2020). Persistent exposure to these antibiotics has resulted in bacteria becoming resistant by evolving extended-spectrum β-lactamases (ESBLs), which hydrolyze the β-lactam ring within the antibiotic, thus rendering it inactive (Bush and Jacoby, 2010). Consequently, production of ESBLs is regarded as one of the most clinically significant resistance mechanisms (Bush and Jacoby, 2010), with ESBL-producing Enterobacterales (*Escherichia coli*, *Klebsiella pneumoniae*, and *Serratia* spp., among others) listed as priority pathogens for research and development in the new frontier of antibiotics (WHO, 2017).

Classified into several groups according to their amino acid sequence homology, the CTX-M, TEM, and SHV ESBL variants are the most common β-lactamases identified in Enterobacterales (van Duin and Doi, 2017). In addition, AmpC β-lactamases (AmpCs) are chromosomally encoded by several Enterobacterales species and play a key role in resistance development (van Duin and Doi, 2017). Plasmid encoded AmpC genes have been known since 1989 (Jacoby, 2009) and are now regularly reported in clinical and environmental strains (Khari et al., 2016; Colosi et al., 2020; Tekele et al., 2020). Both chromosomally encoded and plasmid-mediated AmpC β-lactamases confer resistance to a broad spectrum of β-lactams such as penicillins, oxyimino-cephalosporins (including cefotaxime and ceftazidime), cephamycins, and aztreonam at variable levels (Jacoby, 2009; Palzkill, 2018; Furlan and Stehling, 2021; Lopes et al., 2021b).

The increase in antimicrobial resistant strains and effective resistance mechanisms among Enterobacterales has led to numerous global reports of ESBLs, AmpC-, and more recently carbapenemase-producing Enterobacterales not only in clinical settings, but also in the agricultural environment (Ye et al., 2017; Al-Kharousi et al., 2019; Dandachi et al., 2019; Hassen et al., 2020; Richter et al., 2020). Although members of the Enterobacterales family occur naturally in human and animals' gastrointestinal tracts as well as in the environment (water, soil, and plants; Blaak et al., 2014; Ye et al., 2017), the occurrence of multidrug-resistant (MDR) strains in the different habitats is concerning. Inadequately treated or untreated effluents from industries, households, and zootechnical farms are reported as one of the main contamination causes of South African surface- and ground water resources (Verlicchi and Grillini, 2020). It is also well-documented that the three principal antibiotic contamination channels in the environment are animal-, human-, and manufacturing waste (O'neill, 2016). Consequently, contamination of soil, irrigation-, and drinking water as well as crops can occur, adding additional exposure routes to humans (Finton et al., 2020; Lopes et al., 2021a).

Previous surveillance studies have shown prevalence of MDR ESBL/AmpC-producing Enterobacterales in fresh vegetables sold in South Africa (Richter et al., 2019) and in other countries e.g., the Netherlands, Switzerland, and Germany (Reuland et al., 2014; Zurfluh et al., 2015; Reid et al., 2020). Occurrence of ESBL-producing Enterobacterales has also been reported in corresponding irrigation water sources and cultivated crops (Blaak et al., 2014; Njage and Buys, 2014; Ye et al., 2017). Furthermore, Richter et al. (2020) reported the occurrence of ESBL/AmpC-producing Enterobacterales in different spinach supply chains from irrigation water and produce at harvest, throughout processing and at retail in the Gauteng Province of South Africa.

The high discriminatory power of whole genome sequencing (WGS) has led to an increase in use of this method for detecting points of contamination, source tracking, pathogen surveillance, and outbreak investigations (Oniciuc et al., 2018; CDC, 2019). WGS provides information regarding multiple AMR genes, genomic mutations, mobile genetic elements, and association with resistance genes, as well as molecular typing like multi-locus sequence typing (MLST; Oniciuc et al., 2018; CDC, 2019; Kim et al., 2020). Consequently, the WGS results can aid in elucidating the genetic relationship among isolates from different environments and along the food chain (Adator et al., 2020). Surveillance of antimicrobial resistant strains through WGS is increasingly being used due to increasing accessibility and affordability (Adator et al., 2020). In South Africa, WGS has been used for characterization of clinical ESBL-producing *K. pneumoniae* strains among others (Founou et al., 2019), as well as typing of *Listeria monocytogenes* from environmental and clinical settings during the 2017 listeriosis outbreak (Thomas et al., 2020). However, the use of WGS for surveillance of antimicrobial resistant potential pathogenic Enterobacterales in retailed fresh produce and the production environment has not been reported locally.

The World Health Organization (WHO) developed the Global AMR Surveillance System (GLASS) in 2015 supporting research

and surveillance as well as a global data sharing through a standardized analysis approach (WHO, 2020). Initially, the GLASS focus was mainly on surveillance of human priority pathogens, but has since shifted to include AMR in foodborne pathogens (WHO, 2020). Moreover, the One Health framework for understanding AMR in pathogenic gram-negative bacteria is increasingly attracting attention (Collignon and McEwen, 2019). In South Africa, information regarding AMR in fresh produce production systems and specifically focusing on the Enterobacterales is lacking. The aim of this study was thus to use WGS for analysis of AMR genes, associated mobile genetic elements, virulence factors, serotypes, multi-locus sequence types, and pathogenicity of selected, partially characterized, ESBL/AmpC-producing environmental Enterobacterales from commercial spinach production systems (Richter et al., 2020). These isolates included four different species (*E. coli*, *K. pneumoniae*, *S. fonticola*, and *S. enterica*) listed by the WHO as a particular threat for gram-negative bacteria that are resistant to multiple antibiotics (WHO, 2017), while isolates harbouring integrons as described in Richter et al. (2020) were preferentially selected. The results of this study will contribute to address the problem of antimicrobial drug resistance at the water–plant-food interface and how it might impact human health and disease.

MATERIALS AND METHODS

Isolation and DNA Extraction of ESBL/AmpC-Producing Enterobacterales

Irrigation water and fresh produce samples from spinach production systems were collected and ESBL-producing Enterobacterales were isolated as described (Richter et al., 2020). A selection of 19 isolates were further characterized (**Table 1**). The genomic DNA of each isolate was extracted with the DNeasy PowerSoil kit (Qiagen, South Africa) according to the manufacturer's instructions. Following gDNA extraction, the concentrations were determined using the Qubit dsDNA Broad Range Assay and a Qubit 2.0 fluorometer (Life Technologies, Johannesburg) and quantification was determined on a Nanodrop 2000 (ThermoScientific, Johannesburg).

DNA Sequencing and Whole Genome Analysis

Sequencing was performed on an Illumina MiSeq instrument (2 × 300 bp) with 100× coverage by the National Institute for Communicable Diseases Sequencing Core Facility, South Africa, following preparation of multiplexed paired-end libraries with the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, United States). The resultant reads were quality trimmed using CLC version 20[1] and *de novo* assembled. The contiguous sequences were then submitted to the National Centre for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline.[2] The AMR gene presence was corroborated using ABRicate[3] that included the Comprehensive Antibiotic Resistance Database (CARD), ARG-ANNOT, ResFinder, NCBI AMRFinder Plus, and MEGARes databases (Zankari et al., 2012; Gupta et al., 2014; Jia et al., 2017; Feldgarden et al., 2019; Doster et al., 2020). Plasmid replicon types were determined with PlasmidFinder (version 2.1; Carattoli et al., 2014). Using the Centre for Genomic Epidemiology (CGE) platform[4], mobile genetic elements for all four species, sequence types of *E. coli*, *K. pneumoniae*, and *S. enterica* as well as the *E. coli* serotypes based on lipopolysaccharide (O-antigen) and capsular flagella (protein; H-antigen), and virulence genes of *E. coli* were determined with MGEFinder, Multilocus Sequence Typing (MLST; version 2.2), SeroTypeFinder (version 2.0), and VirulenceFinder (version 2.0), respectively (Larsen et al., 2012; Joensen et al., 2014, 2015; Johansson et al., 2021). The following parameters were used in the Serotype Finder Web-based tool: 85% threshold for %ID and 60% minimum length (the number of nucleotides in a sequence of interest that must overlap a serotype gene to count as a hit for that gene; Joensen et al., 2015). The *in silico* serotyping based on the capsule polysaccharide (K-antigen) of *K. pneumoniae* strains was conducted using Kaptive Web (Wick et al., 2018), while the presence of virulence genes for *K. pneumoniae* was

TABLE 1 | Isolates selected for whole genome sequence analysis from the agricultural environment in spinach supply chains, Gauteng Province, South Africa.

Strain	Organism identity	Source	Isolation point from spinach production systems
		Water (W) or spinach (S)	
UPMP2117	*Escherichia coli*	W	Water reservoir
UPMP2120	*Escherichia coli*	S	Unwashed spinach bunches at retailer
UPMP2130	*Escherichia coli*	W	Holding dam water (source water)
UPMP2112	*Klebsiella pneumoniae*	W	Irrigation pivot point water
UPMP2114	*Klebsiella pneumoniae*	S	Spinach at harvest
UPMP2118	*Klebsiella pneumoniae*	W	Irrigation pivot point water
UPMP2121	*Klebsiella pneumoniae*	S	Unwashed spinach bunches at retailer
UPMP2122	*Klebsiella pneumoniae*	S	Spinach at retailer
UPMP2115	*Salmonella spp.*	W	River water
UPMP2116	*Serratia fonticola*	W	River water
UPMP2119	*Serratia fonticola*	W	Irrigation pivot point water
UPMP2123	*Serratia fonticola*	S	Unwashed spinach punnet at retailer
UPMP2124	*Serratia fonticola*	S	Spinach at receival
UPMP2125	*Serratia fonticola*	S	Spinach after pack
UPMP2126	*Serratia fonticola*	S	Spinach at receival
UPMP2127	*Serratia fonticola*	S	Unwashed spinach at retailer
UPMP2128	*Serratia fonticola*	S	Unwashed spinach at retailer
UPMP2129	*Serratia fonticola*	S	Spinach at receival
UPMP2131	*Serratia fonticola*	S	Unwashed spinach at retailer

[1]https://digitalinsights.qiagen.com
[2]https://pubmed.ncbi.nlm.nih.gov/27342282/
[3]https://github.com/tseemann/abricate
[4]https://cge.cbs.dtu.dk/services/

identified by using the Institut Pasteur's *Klebsiella* database.[5] Additionally, paired reads of the WGS raw data files for the *S. enterica* strain were uploaded to the online SeroSeq tool version 1.0 which predicted the *Salmonella* serotype of the requested isolate (Zhang et al., 2015; Thompson et al., 2018). The *Salmonella* Pathogenicity Islands (SPI) were identified with SPIFinder 2.0 (Roer et al., 2016). Next, the existence of virulence factors in each SPI was analyzed by performing BLAST analysis on the predicted SPIs against the virulence factor database (VFDB; Chen et al., 2016; Ashari et al., 2019). The virulence factors of *S. fonticola* were determined using the VFDB with ABRicate (Chen et al., 2016). All sequences were submitted to the INTEGRALL database[6] for annotation and integron number assignment. Using PathogenFinder (version 1.1) on the CGE platform, the strains' pathogenicity towards humans was predicted (Cosentino et al., 2013).

Data Availability

The nucleotide sequences of the 19 Enterobacterales strains described in this paper were deposited in the National Center for Biotechnology Information GenBank database in the BioProject number: PRJNA642017, accession numbers NZ_JACAAL010000000, NZ_JACBIV000000000-NZ_JACBJE000000000, and NZ_JACNY M000000000-NZ_JACNYT000000000 (**Table 2**).

RESULTS

Detection of Antimicrobial Resistance Genes

The selected 19 ESBL/AmpC producing Enterobacterales isolates all harboured at least one β-lactamase encoding gene in addition to the ESBL/AmpC genetic determinants, accompanied by resistance genes from different antibiotic classes including fluoroquinolone, sulfonomide, fosfomycin, aminoglycoside, trimethroprim, phenicol, and/or tetracycline (**Figure 1**). The β-lactamase resistance genes included chromosomally encoded AmpC in the *S. enterica* strain as well as all three *E. coli* strains. Plasmid-mediated AmpC genes ($bla_{\text{CMY-113}}$ and $bla_{\text{CMY-101}}$) were present in two *E. coli* strains from irrigation water and $bla_{\text{ACT-13}}$, $bla_{\text{ACT-38}}$, $bla_{\text{ACT-6}}$, and/or $bla_{\text{ACT-58}}$ were present in 10 *S. fonticola* strains from irrigation water ($n=2$) and spinach ($n=8$) samples (**Figure 1**). Additionally, $bla_{\text{FONA-5}}$ ($n=8$) from irrigation water and spinach and $bla_{\text{FONA-6}}$ ($n=2$) from spinach were present in *S. fonticola* strains. The ESBL genes included $bla_{\text{SFO-1}}$ in all 10 *S. fonticola* strains, $bla_{\text{CTX-M-15}}$ in five *K. pneumoniae* strains from irrigation water and spinach, and one *E. coli* strain from spinach. It also included $bla_{\text{CTX-M-14}}$ in an *E. coli* strain from irrigation water, while $bla_{\text{SHV-187}}$ ($n=3$), $bla_{\text{SHV-106}}$ ($n=1$), and $bla_{\text{SHV-178}}$ ($n=1$) were present in *K. pneumoniae* strains (**Figure 1**).

Interestingly, a greater number of resistance genes across more classes were seen in all the *K. pneumoniae* strains ($n=5$), compared to the other genera tested. All five *K. pneumoniae* strains had chloramphenicol (*catB3*), aminoglycosides [*aac(6')-Ib-cr*, *aph(6)-Id* and *aph(3")-Ib*], fosfomycin (*fosA6*), and sulfonomide (*sul2*) resistance genes present (**Figure 1**). Other resistance genes included fluoroquinolone *oqxA* ($n=4$), *oqxB* ($n=4$), and *qnrB1* ($n=4$) in *K. pneumoniae* from spinach and water, *qnrS1* ($n=1$) in *E. coli* from spinach and *qnrB6* ($n=3$), *qnrB37* ($n=5$), *qnrE1* ($n=10$) in *S. fonticola* from spinach and water, while *mdtK* ($n=4$), and *mdtH* ($n=3$) were present in *S. fonticola* from water only. The *qnrB17* resistance gene was present in *K. pneumoniae* ($n=4$) and *S. fonticola* ($n=2$) strains from spinach and water (**Figure 1**). The *S. enterica* strain isolated from irrigation water also harboured *aac(6')-Iaa* and *aac(6')-Iy* aminoglycoside resistance genes (**Figure 1**) and a *S. fonticola* strain from irrigation water harboured an aminoglycoside [*aph(3")-Ib*] and sulfonomide (*sul2*) resistance gene (**Figure 1**).

Detection of Mobile Genetic Elements and Association to Antimicrobial Resistance Genes

Known resistance plasmid replicon types of Enterobacterales including IncFIB, IncFIA, IncFII, IncB/O, and IncHI1B were observed in all strains following analysis with PlasmidFinder (data not shown). The β-lactamase gene, $bla_{\text{CTX-M-15}}$, was the only resistance gene associated with plasmids (IncFII_pKP91 and/or IncFIB(K)_1_Kpn3) in four *K. pneumoniae* strains upon further analysis (**Table 3**). The IS6 family elements (IS6100) have been reported to play a pivotal role in the dissemination of resistance determinants in gram-negative bacteria (Partridge et al., 2018), and were observed in relation to the *dfrA14b* resistance gene in all five *K. pneumoniae* strains (**Table 3**). The $bla_{\text{CTX-M-14}}$ and *sul2* resistance genes were related to the ISEcp1 element within the IS1380 family in one *E. coli* and three *K. pneumoniae* strains, respectively, while one *S. fonticola* strain carried a *sul2* gene that was related to IS110 (**Table 3**). One *E. coli* strain carried the *qnrS1* resistance gene that was related to ISKra4. Other insertion sequences detected belonged predominantly to the IS3 and IS110 families (data not shown), with one *K. pneumoniae* strain carrying the $bla_{\text{SHV-80}}$ broad spectrum β-lactamase that was related to IS3 (**Table 3**). In all *K. pneumoniae* strains ($n=5$) where the *qnrB1* resistance gene was present, association to Tn5403 was seen (**Table 1**). In one *E. coli* and five *K. pneumoniae* strains, integron In191 was observed, with *dfrA14* in the cassette array (**Table 3**).

In silico Analysis of Serotypes, Multi-Locus Sequence Types, and Virulence Factors

The *in silico* MLST analysis, predicted serotypes, and pathogenicity probability of all 19 strains are shown in **Table 2**. Three different sequence types (ST58, ST117, and ST10) and three different serotypes (O75:H9, O11:H4, and O8:H17) were observed in the three *E. coli* strains. The five *K. pneumoniae* strains belonged to three different sequence types and three different serotypes (KL27, KL24, and KL39) which were observed based on the K-antigen, while the O-serotype included O4 and O1 (**Table 2**). The predicted antigenic profile of the *S. enterica* strain was O11:k:1,2. Furthermore, the *S. enterica* strain contained 11 *Salmonella* SPI, namely SPI-1,

[5]https://bigsdb.pasteur.fr/klebsiella/klebsiella.html
[6]http://integrall.bio.ua.pt

TABLE 2 | *In silico* multi-locus sequence typing (MLST) analysis, predicted serotypes, and pathogenicity probability of Enterobacterales isolated from irrigation water and spinach throughout production from farm to retail.

Accession	Strain	Source	Species	Sequence type	Serotype	Pathogenicity probability
NZ_JACNYS000000000	UPMP2120	S	*Escherichia coli*	ST58	O75:H9	0.888
NZ_JACNYT000000000	UPMP2117	W	*Escherichia coli*	ST117	O11:H4	0.931
NZ_JACNYN000000000	UPMP2130	W	*Escherichia coli*	ST10	O8:H17	0.852
NZ_JACAAL010000000	UPMP2112	W	*Klebsiella pneumoniae*	ST3559	KL27:O4	0.899
NZ_JACBJB000000000	UPMP 2118	W	*Klebsiella pneumoniae*	ST15	KL24:O1v1	0.889
NZ_JACBJE000000000	UPMP2114	S	*Klebsiella pneumoniae*	ST985	KL39:O1v2	0.885
NZ_JACBIZ000000000	UPMP2121	S	*Klebsiella pneumoniae*	ST985	KL39:O1v2	0.796
NZ_JACBIY000000000	UPMP2122	S	*Klebsiella pneumoniae*	ST985	KL39O1v1	0.885
NZ_JACBJD000000000	UPMP2115	W	*Salmonella enterica*	ST4924	Pretoria	0.939
NZ_JACBJC000000000	UPMP2116	W	*Serratia fonticola*	N.D	N.D	0.721
NZ_JACBJA000000000	UPMP2119	W	*Serratia fonticola*	N.D	N.D	0.699
NZ_JACBIX000000000	UPMP2123	S	*Serratia fonticola*	N.D	N.D	0.692
NZ_JACNYR000000000	UPMP2124	S	*Serratia fonticola*	N.D	N.D	0.635
NZ_JACNYQ000000000	UPMP2125	S	*Serratia fonticola*	N.D	N.D	0.645
NZ_JACNYP000000000	UPMP2126	S	*Serratia fonticola*	N.D	N.D	0.659
NZ_JACNYO000000000	UPMP2127	S	*Serratia fonticola*	N.D	N.D	0.659
NZ_JACBIW000000000	UPMP2128	S	*Serratia fonticola*	N.D	N.D	0.674
NZ_JACBIV000000000	UPMP2129	S	*Serratia fonticola*	N.D	N.D	0.659
NZ_JACNYM000000000	UPMP2131	S	*Serratia fonticola*	N.D	N.D	0.705

W, water; S, spinach; and N.D., not determined.

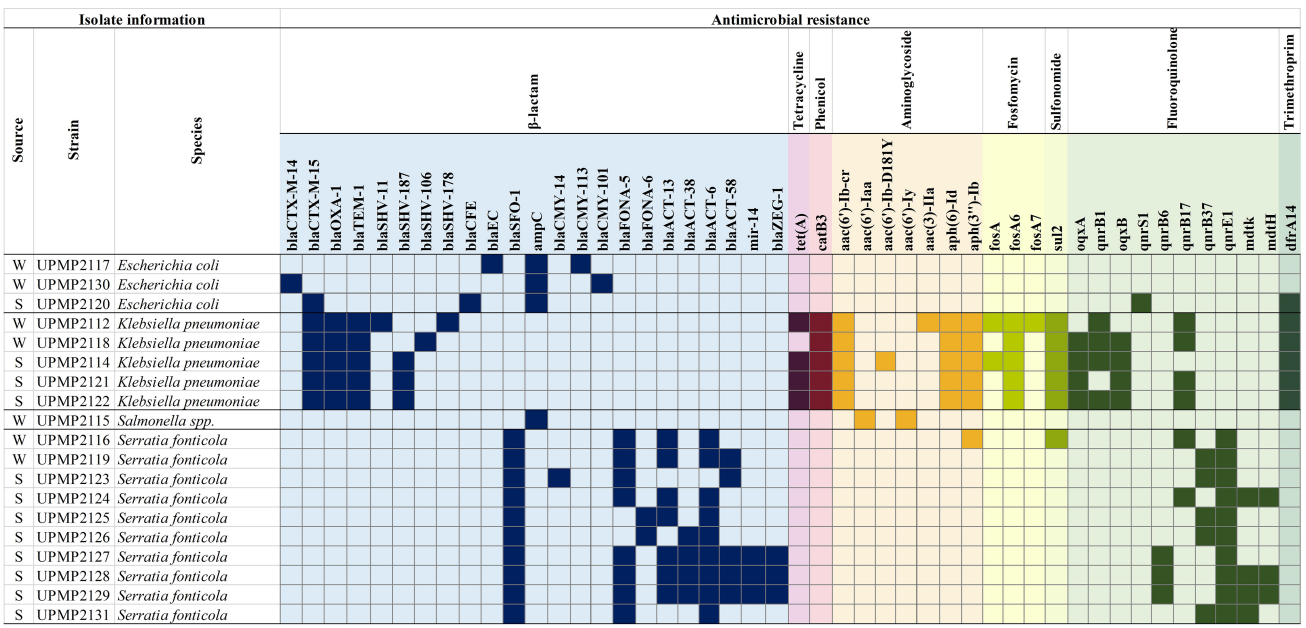

FIGURE 1 | Antimicrobial resistance (AMR) genes present in Enterobacterales isolated from water and spinach from farm to retail. W, water; S, spinach.

SPI-2, SPI-3, SPI-4, SPI-5, SPI-9, SPI-13, SPI-14, one unnamed, as well as the centisome 63 (C63PI) and 54 (CS54) pathogenicity islands, each harbouring between 20 and 60 virulence factors (**Supplementary Table S1**). A total of 42 virulence genes were identified in the *E. coli* and *K. pneumoniae* strains (**Supplementary Tables S2, S3**). Of these, 20 were detected in *E. coli* strains only and 20 in *K. pneumoniae* strains only, while *fyuA* (iron uptake associated with siderophores) and *irp2* (iron uptake) virulence factors were detected in two *E. coli* strains from irrigation water as well as three *K. pneumoniae* strains from spinach samples. All three *E. coli* strains carried the *terC* (tellurite resistance) virulence gene (**Supplementary Table S2**) and in all five *K. pneumoniae* strains, the *mrkA*, *mrkB*, *mrkC*, *mrkD*, *mrkE*, (main structural subunit and assembly machinery for type 3 fimbriae) *mrkH* (regulatory protein), and *mrkI* (DNA binding protein) virulence factors were present (**Supplementary Table S3**). No shiga-toxin producing genes were present in the *E. coli* strains. A total of 89 virulence factors were identified in the *S. fonticola*

TABLE 3 | Extended-spectrum β-lactamase (ESBL)/AmpC β-lactamase (AmpC)-producing Enterobacterales with resistance genes related to mobile genetic elements.

Isolate information			Resistance genes associated with mobile genetic elements					
			Genes		Mobile genetic elements			
Source	Strain	Species	β-lactamase	Other	Plasmids	Insertion sequence families	Transposons	Integron
W	UPMP2130	*Escherichia coli*	CTX-M-14			IS1380		
S	UPMP2120	*Escherichia coli*		qnrS1 dfrA14b		ISKra4		In191
W	UPMP2112	*Klebsiella pneumoniae*	SHV-80 CTX-M-15	 sul2 qnrB1 dfrA14b	IncFIB(K)_1_Kpn3	IS3 IS1380 IS6	 Tn5403	 In191
W	UPMP2118	*Klebsiella pneumoniae*	TEM-1B	 dfrA14b qnrB1		IS1380 IS6	 Tn5403	 In191
S	UPMP2114	*Klebsiella pneumoniae*	CTX-M-15	 sul2 qnrB1 dfrA14b	IncFII_pKP91 IncFIB(K)_1_Kpn3	 IS1380 IS6	 Tn5403	 In191
S	UPMP2121	*Klebsiella pneumoniae*	CTX-M-15 TEM-1B	 qnrB1 dfrA14b	IncFII_pKP91	 IS1380 IS6	 Tn5403	 In191
S	UPMP2122	*Klebsiella pneumoniae*	CTX-M-15	 sul 2 qnrB1 dfrA14b	IncFII_pKP91 IncFIB(K)_1_Kpn3	 IS1380 IS6	 Tn5403	 In191
W	UPMP2116	*Serratia fonticola*		sul2		IS110		

W, water; S, spinach.

strains (**Supplementary Table S4**). This included 25, 18, 16, and 6 of the virulence factors present in 100% ($n=10$), 90, 80, and 70% of the selected *S. fonticola* strains, respectively, while the remaining 24 virulence factors were present in varying numbers in 1–6 of the strains (**Supplementary Table S4**). The *iroN* salmochelin siderophore receptor which plays a role in disease establishment was present in three *S. fonticola* strains (two from unwashed baby spinach samples at the retailer and one from the irrigation pivot point water), one *E. coli* strain from the ground water, as well as in the SPI-13 in the *S. enterica* strain from river irrigation water. Relevant similarities to human pathogens were predicted for all 19 strains with a confidence of 0.635–0.721 in the *S. fonticola* strains ($n=10$), 0.852–0.931 in the *E. coli* strains ($n=3$), 0.796–0.899 in the *K. pneumoniae* strains ($n=5$), and 0.939 in the *S. enterica* strain (**Table 2**).

DISCUSSION

To the authors knowledge, this is the first study to use WGS for in-depth molecular characterization of ESBL/AmpC-producing *E. coli*, *K. pneumoniae*, *S. enterica*, and *S. fonticola* isolates, previously identified and partially characterized, from spinach and irrigation water samples in commercial production chains (Richter et al., 2020). Characterization included AMR, mobile genetic elements (e.g., insertion sequences, plasmids, and integrons), serotypes, and determining the pathogenicity. All these factors are crucial in defining and attributing infection sources of food-related outbreaks caused by resistant microorganisms (Oniciuc et al., 2018). Overall, the results corresponded with main global findings where AMR genes and associated mobile genetic elements have been reported in Enterobacterales from fresh produce and irrigation water, with the potential to pose a health risk to humans upon exposure (Jones-Dias et al., 2016; Finton et al., 2020). Previously, the presence of *intI3* was reported in a high percentage of isolates from the current study following conventional PCR and sequencing (Richter et al., 2020). However, in-depth WGS analysis showed that no *attI* fragment preceded the *IntI3* genes; consequently, the *IntI3* genes detected did not form part of complete integrons, which typically include an integrase *intI* gene encoding a site-specific recombinase, a recombination site *attI* as well as a promoter (P_c; Kaushik et al., 2018). Overall, six isolates in the current study

were positive for Class 1 integrons (In191), similar to In191 positive clinical ESBL-producing Enterobacterales from an academic teaching hospital in Pretoria, SA (Sekyere et al., 2020). Additionally, these MDR environmental isolates harbored various virulence factors central to pathogenicity, including genes associated with urinary tract infections and iron sequestering systems crucial for disease establishment. All isolates had relevant similarity to human pathogens and form part of the WHO 3rd generation cephalosporin resistant critical priority pathogens (WHO, 2017).

Two of the *E. coli* strains from the current study harboured plasmid-mediated AmpC $bla_{\text{CMY-2-like}}$ genes ($bla_{\text{CMY-113}}$ and $bla_{\text{CMY-101}}$), which correspond to the phenotypic profile of resistance to expanded-spectrum cephalosporins previously reported for these isolates using traditional PCR analysis (Richter et al., 2020). The $bla_{\text{CMY-2}}$ pAmpC genes are the most commonly reported in *E. coli* and other Enterobacterales species and have clinical relevance, as it inactivates third generation cephalosporins and mediates resistance to carbapenems (Jacoby, 2009; Bortolaia et al., 2014). Three different multi-locus sequence types, namely ST58, ST10, and ST117, were identified in the *E. coli* isolates. Isolated from the retailed unwashed spinach samples in the current study, ST58 *E. coli* have previously also been associated with human extra-intestinal infections including sepsis, and have emerged worldwide in wild and food-production animals (Reid et al., 2020). As an example, ST58 *E. coli* with serotype O75:H9 corresponded to an *E. coli* strain of bovine origin from Pakistan and also carried the IncFIB plasmid (Ali et al., 2020). Although the strain from the current study had less AMR genes than reported in ST58 *E. coli* with serotype O75:H9 by Ali et al. (2020), the trimethoprim (*dfrA14*), fluoroquinolone (*qnrS1*), and β-lactam ($bla_{\text{CTX-M-15}}$) genes corresponded. Similarly, uropathogenic ST58 *E. coli* with resistance to fluoroquinolone and trimethoprim have previously been isolated from hospital patients in Australia (McKinnon et al., 2018). The $bla_{\text{CTX-M-15}}$ gene identified in the ST58 *E. coli* strain from the current study was associated with the ISKra4 insertion sequence, previously identified in *K. pneumoniae* harbouring $bla_{\text{CTX-M-15}}$, and was responsible for the movement to different parts of the genome through a replicative transposition mechanism (Razavi et al., 2020). In contrast to Hauser et al. (2013) who identified food-associated shiga-toxin producing *E. coli* ST58, no *stx* genes were present in the strains. The *E. coli* ST58 from the current study harboured the *gad* (glutamate decarboxylase) virulence gene, similar to *E. coli* ST58 strains isolated from aragula (rocket; Reid et al., 2020). However, the presence of *lpfA* (long polar fimbriae) and *terC* (tellurium ion resistance protein) virulence factors in the strain from the current study contrasted the virulence gene profiles reported by Reid et al. (2020). *Escherichia coli* ST10 have previously been associated with human clinical infections and has been isolated from different sources including recreational and/or wastewater samples (Falgenhauer et al., 2019). From the current study, the *E. coli* ST10 with serotype O8:H17 was isolated from borehole water used for irrigation. Although this sequence type has previously been associated with shiga-toxin-producing *E. coli* (STEC; Gonzalez-Escalona and Kase, 2018), no *stx* genes were detected in the current study. The virulence factors present were *terC* (tellurium ion resistance protein), *astA* (EAST-1 heat-stable toxin), *fyuA* (ferric yersiniabactin uptake receptor), *irp2* (nonribosomal peptide synthetases), *iss* (increased serum survival), and *sitA* (iron transport protein). Previously, *E. coli* ST10 with similar virulence gene profiles were isolated from human blood cultures and reported as extra-intestinal pathogenic *E. coli* (ExPEC; Maluta et al., 2017). Additionally, ESBL-producing *E. coli* ST10 of the same serotype have been isolated from wastewater and are depicted as a probable environmental reservoir of $bla_{\text{CTX-M}}$ genetic determinants (Tanaka et al., 2019). In the current study, the ST58 *E. coli* strain harboured the $bla_{\text{CTX-M-15}}$ genetic determinant, while $bla_{\text{CTX-M-14}}$ was present in the ST10 *E. coli* strain. Globally, the CTX-M type ESBLs (especially $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$) have become the dominant genotype and the most widely distributed (Cantón et al., 2012; Adamski et al., 2015). *Escherichia coli* $bla_{\text{CTX-M-14}}$ positive strains have previously been isolated from store-bought produce in Germany and South Africa (Richter et al., 2019; Reid et al., 2020), food producing animals in China (Liao et al., 2015) and clinical settings in Brazil and South Africa (Cergole-Novella et al., 2010; Peirano et al., 2011).

The third *E. coli* sequence type (ST117) detected from irrigation source water in the current study has previously been reported as part of a group of multi-serotype extra-intestinal pathogenic *E. coli* (ExPEC) and avian pathogenic *E. coli* (APEC) strains (Kim et al., 2017). The *E. coli* ST117 strain from the current study harboured 20 virulence factors including the ExPEC *hlyF* (Hemolysin F) virulence gene. In previous studies, *stx* genes were identified in *E. coli* strains with the same STs detected in the current study, yet the virulence gene content and serotypes differ from the strains in the current study (Gonzalez-Escalona and Kase, 2018). However, the three non-STEC *E. coli* strains (ST58, ST10, and ST117) from the current study had a 93, 89, and 85% probability of being human pathogens, based on the pathogenic protein families.

In addition to *E. coli*, other Enterobacterales isolates harbouring $bla_{\text{CTX-M-15}}$ have also been detected in different environments. In the current study, all five *K. pneumoniae* strains harboured the $bla_{\text{CTX-M-15}}$ genetic determinant. The prevalence and dissemination of $bla_{\text{CTX-M}}$ throughout various environments globally underlines the different contamination routes through which fresh produce may also become contaminated with these MDR organisms. For instance, Gekenidis et al. (2020) have demonstrated the long-term persistence of *E. coli* harbouring $bla_{\text{CTX-M-15}}$ in soil and lettuce after its introduction *via* irrigation water. Similarly, $bla_{\text{CTX-M-15}}$ positive ST985 *K. pneumoniae* strains were present in spinach at harvest on the farm as well as retail samples after processing in the current study, suggesting successful persistence of these MDR strains. In four *K. pneumoniae* strains (ST3559, $n = 1$ and ST985, $n = 3$), the $bla_{\text{CTX-M-15}}$ genes were associated with IncF replicons (IncFII_{K} and IncFIB) which have previously been linked to diverse *K. pneumoniae* outbreak strains (Dolejska et al., 2012, 2013; Löhr et al., 2015). Moreover, in *K. pneumoniae* ST3559, $bla_{\text{CTX-M-15}}$ was also associated with *ISEcp1* (also called *ISEc9*), a member of the widely reported IS1380 family, and can enable the independent transposition with insertion mutation and

genetic relocations (Partridge, 2011). The *K. pneumoniae* strains in the current study also harboured bla_{SHV} ESBL encoding genes ($bla_{SHV-187}$, $bla_{SHV-106}$, and $bla_{SHV-178}$). Previously, SHV genetic determinants were reported in *K. pneumoniae* from hospitals and receiving wastewater treatment plants in Romania (Surleac et al., 2020) as well as irrigation water and agricultural soil in South Africa (Iwu et al., 2020; Richter et al., 2020). Interestingly, the *K. pneumoniae* ST15 strain isolated from water in the current study harboured $bla_{SHV-106}$ which Liakopoulos et al. (2016) previously reported to be geographically constrained and have only been described in *K. pneumoniae* isolates from Portugal together with bla_{TEM-1}. Similarly, the *K. pneumoniae* ST15 strain from the current study also harboured $bla_{SHV-106}$ together with bla_{TEM-1}. *Klebsiella pneumoniae* ST15 is regarded as an emerging international high-risk clone causing nosocomial outbreaks worldwide with high-levels of antibiotic resistance including production of ESBLs, mainly CTX-M-15 (Han et al., 2021).

The *K. pneumoniae* ST3559 strain isolated from irrigation water in the current study was capsular type 27 and serotype O4, which is similar to an O4 serotype MDR *K. pneumoniae* outbreak strain from a neonatal care unit in sub-Saharan Africa (Cornick et al., 2020). In addition, *K. pneumoniae* ST3559 harboured the $bla_{SHV-178}$ gene which, to the best of our knowledge, have previously only been reported in clinical *Enterobacter hormaechei* strains from the First Affiliated Hospital of Zhejiang University in Hangzhou (Gou et al., 2020). Apart from β-lactamase genes, the *K. pneumoniae* strains also harboured aminoglycoside, fosfomycin, fluoroquinolone, tetracyline, phenicol, trimethoprim, and sulfonomide resistance genes, which is a greater diversity of resistance genes than previously reported in Enterobacterales isolates from German surface waters (Falgenhauer et al., 2019). Similar to the results of clinical *K. pneumoniae* strains reported by Mbelle et al. (2020), In191, harbouring *dfrA14* was identified in the three different *K. pneumoniae* sequence types of the current study, reiterating that it is not a narrow spectrum integron. In addition, *dfrA14b* was associated with *IS6* that has previously been reported as having a vital role in the rearrangement and dissemination of antibiotic resistance (Varani et al., 2021). The presence of *fosA* and *sul2* in all the *K. pneumoniae* strains of the current study also corresponds to the results reported by Mbelle et al. (2020) from clinical *K. pneumoniae* strains in Pretoria. The high-level of trimethoprim resistance globally has however led to trimethoprim-sulfamethoxazole no longer being recommended for outpatient treatment of urinary tract infections and similarly, the use of fosfomycin might not be efficacious anymore (Mbelle et al., 2020). Four MDR *K. pneumoniae* isolates from irrigation water (ST15, $n = 1$) and spinach (ST985, $n = 3$) had O1 serotypes, previously reported as the most commonly isolated serotypes from human hosts and dominant in human disease (Follador et al., 2016). However, it is noteworthy that no genes encoding carbapenamases nor resistance to colistin were identified in the current study. All five characterized *K. pneumoniae* strains also harbored several virulence factors including those that coded for an iron uptake system (*kfu*) and type 3 fimbrial adhesins (*mrk*) that play an important role in adhesion to medical devices such as catheters (Albasha et al., 2020; Finton et al., 2020).

Serratia spp. are opportunistic pathogens that may pose a health threat to immunocompromised and hospitalized patients (Petersen and Tisa, 2013). The *S. marcescens* species is most often associated with nosocomial infections; however, *S. fonticola* has been reported to function as a human pathogen when detected alone or may be a bystander and act as a carrier of resistance genes when discovered with other organisms (Petersen and Tisa, 2013; Aljorayid et al., 2016). Characterizing virulence genes of the MDR environmental strains therefore becomes important within the plant-food producing environment. In the current study, all *S. fonticola* strains harboured bla_{SFO-1} and numerous plasmid incompatibility (Inc) groups were identified in these *S. fonticola* strains (data not shown). However more in-depth plasmid typing and analysis will be required to fully understand the risk/probability of bla_{SFO-1} dissemination in the environment where *S. fonticola* naturally occurs. In certain Enterobacterales species, ESBL genes are inherently carried on chromosomes (Naas et al., 2008). This includes the bla_{SFO-1} ESBL gene from *S. fonticola* that differs from most class A ESBLs, as the β-lactamases' production can be induced by a high level of imipenem (Naas et al., 2008). The bla_{SFO-1} ESBL does not form part of the most clinically relevant ESBLs and are therefore rarely reported. Zhou et al. (2020) reported in contrast an increasing trend of the co-existence of plasmid-borne bla_{SFO-1} and carbapenemase genes in clinical *Enterobacter* spp. in China. All the *S. fonticola* strains also harboured numerous fluoroquinolone resistance genes, raising a health concern for treatment options, as fluoroquinolones are often used for management of conditions including typhoid fever and MDR tuberculosis (Richards et al., 2019). Interestingly, one *S. fonticola* strain harboured an acquired trimethoprim (*sul2*) resistance gene associated with IS110, corresponding to *K. pneumoniae* from a German university hospital (Schwanbeck et al., 2021). The *Serratia* genus naturally lacks resistance genes for trimethoprim and sulfonamides (Sandner-Miranda et al., 2018). Previous reports of potential pathogenic *S. fonticola* primarily focused on the antibiotic resistance profiles (Tasić et al., 2013; Aljorayid et al., 2016; Hai et al., 2020). The strains from the current study additionally harboured various virulence factors. This included flagellar biosynthesis- and chemotaxis-related genes as well as genes encoding iron uptake systems corresponding to those previously reported in important MDR nosocomial pathogenic *S. marcescens* (Iguchi et al., 2014).

Only one *S. enterica* strain isolated from river irrigation water was characterized in the current study. Irrigation water is well documented as a source for fresh produce contamination of foodborne pathogens including *Salmonella* spp. (Liu et al., 2018). The strain harboured an AmpC resistance gene, similar to *S. enterica* characterized from surface water in the United States (Li et al., 2014). In addition, the *S. enterica* from the current study carried aminoglycoside resistance genes [*aac(6')-Iaa* and *aac(6')-Iy*], similar to results reported by Nair et al. (2016) for non-typhoidal *Salmonella* spp. isolated from a United Kingdom population. Of the 23 known *Salmonella* SPIs previously described (Mansour et al., 2020), the isolate from

the current study carried 11 SPIs. This included SPIs that are commonly reported in *S. enterica* and encode genes responsible for enabling invasion of epithelial cells (SPI1), facilitating the replication of intracellular bacteria (SPI2), adhesion to epithelial cells (SPI3, 4, 5, and 9; Waterman and Holden, 2003; Velásquez et al., 2016; Mansour et al., 2020), as well as SPI13 and 14 which corresponds to being part of the core genome of invasive non-typhoidal *Salmonella* spp. (Suez et al., 2013). Additionally, pathogenicity islands C63PI and CS54 were present in the *S. enterica* strain in this study, which has previously been found in the S. Typhimurium and S. Typhi genomes (Sabbagh et al., 2010; Jibril et al., 2021). Since no phenotypic indication of virulence was investigated, the prediction of virulence genes using *in silico* tools should be regarded with care; however, using PathogenFinder, the *S. enterica* strain from the current study showed 94% probability of being a human pathogen.

CONCLUSION

This is the first WGS analysis study of MDR ESBL/AmpC-producing *E. coli*, *K. pneumoniae*, *S. fonticola*, and *S. enterica* isolates from spinach production systems within South Africa. The selected isolates represent potential pathogenic genera listed by the WHO as a priority for surveillance of AMR screening. Numerous clinically relevant resistance genes were detected in the screened samples. This study showed the potential of using WGS in metadata studies for detailed molecular characterization of potential pathogenic Enterobacterales. Furthermore, the study highlighted the importance of the agricultural production environment as a source of antibiotic resistance genes within Enterobacterales at the water-plant-food interface. A more in-depth and controlled analysis, with a greater number of sequenced isolates from the farm-to-retail supply chain is required to better understand the prevalence and resistance gene transmission through the supply chain. The results from this study further highlight the need for expanded surveillance in agricultural systems.

DATA AVAILABILITY STATEMENT

The nucleotide sequences of the 19 Enterobacteriaceae strains described in this paper were deposited in the National Center for Biotechnology Information GenBank database in the BioProject number: PRJNA642017, accession numbers NZ_JACAAL010000000, NZ_JACBIV000000000-NZ_JACBJE000000000, and NZ_JACNYM000000000-NZ_JACNYT000000000 (**Table 2**).

AUTHOR CONTRIBUTIONS

EP, SD, LR, and LK contributed to the conception and design of the study. LR performed the experiments. LR, SD, MA, and AI analyzed the data. LR, EP, and SD contributed to interpretation and presentation. SD, EP, and LK were involved in funding acquisition. All authors contributed to the article and approved the submitted version.

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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.2021.734649/full#supplementary-material

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Aminoglycoside Resistance and Possible Mechanisms in *Campylobacter* Spp. Isolated From Chicken and Swine in Jiangsu, China

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Campylobacter is a major food-borne pathogen in humans, and previous studies reported a high prevalence of gentamicin-resistant *Campylobacter* isolates from food-producing animals in China. This study aimed to investigate the aminoglycoside resistance of *Campylobacter* isolated from chicken and swine in Jiangsu province, China and understand the possible mechanisms responsible for aminoglycoside resistance. One hundred and eighty-five *Campylobacter* isolates of chicken and swine origins in 2017 and 2018 were analyzed for gentamicin and kanamycin resistance. Some aminoglycoside resistance genes were selected for PCR detection in all strains. The genomic DNAs of two strains with high resistance to gentamicin were used as donors to subject *C. jejuni* NCTC11168 to natural transformation. The transformants were investigated by whole-genome sequencing and analyzed comparatively with *C. jejuni* NCTC11168. In total, 30.5% (29/95) of *C. jejuni* isolates and 42.2% (38/90) of *C. coli* isolates were resistant to gentamicin and kanamycin. The prevalence of the *aph(2")-If* gene and *aac(6')-Ie/aph(2")-Ia* gene was 65.4% (121/185) and 36.2% (67/185) in *Campylobacter* isolates, respectively. The *aadE-sat4-aphA-3* cluster was identified in 8.7% (8/92) and 20.4% (19/93) of all *Campylobacter* isolates in each year. With each donor DNA, aminoglycoside-resistant transformants were obtained. The transformants showed ≥128-fold increases in the MICs of gentamicin, kanamycin, and tobramycin. A 5200-bp segment was found to be inserted between the highly conserved genes *Cj0299* and *panB* of *Campylobacter*. A total of 9.7% (18/185) strains showing high resistance to aminoglycosides had this segment by PCR detection. The genetic diversity of the insertion-fragment positive strains was determined by MLST, and seven sequence types were identified for these strains.

Keywords: *Campylobacter*, aminoglycoside resistance, natural transformation, MLST, Food Safety

INTRODUCTION

Campylobacter jejuni and *Campylobacter coli* are the main pathogens that cause sporadic gastroenteritis worldwide (Costa and Iraola, 2019; Man, 2011). In 2010, *Campylobacter* was estimated by laboratory confirmation to cause the highest number of food-borne bacterial infections globally (Noordhout et al., 2017). With a laboratory-modified isolation kit based on a membrane filter method (ZC-CAMPY-002, Qingdao Sinova Biotechnology Co., Ltd., Qingdao, China) has been extensively used to isolate *Campylobacter* from diarrheic patients in Chines CDC's surveillance project, the *Campylobacter* isolation in sporadic diarrheal cases significantly increased (Li et al., 2018). But compared with other countries, the outbreak caused by *Campylobacter* in China is rare (Li et al., 2020). Most *Campylobacter* enteritis cases are usually mild and self-limiting and do not require antimicrobial therapy; however, for severe or prolonged cases, antibiotic treatment is needed. Fluoroquinolones and macrolides are commonly used to treat campylobacteriosis, but aminoglycosides are used in systemic infections, such as bacteremia (Payot et al., 2006; Blaser and Engberg, 2008). Aminoglycosides are important veterinary antimicrobials in all major food-producing animals to treat infections and are classified by the WHO as important antimicrobials for human medicine (Giguère et al., 2013; World Health Organization, 2019). The extensive use of antibiotics in food-animal production has led to an increase in antimicrobial-resistant strains of *Campylobacter* (Alfredson and Korolik, 2007).

Among the known mechanisms of acquired aminoglycoside resistance, the enzymatic modification is the most common mechanism for the inactivation of aminoglycosides in many bacterial species, including *Campylobacter* spp. (Vakulenko and Mobashery, 2003; Ramirez and Tolmasky, 2010). The aminoglycoside-modifying enzymes are divided into three main classes based on the reactions they catalyze as: aminoglycoside acetyltransferases (AAC), aminoglycoside nucleotidyltransferases, and aminoglycoside phosphotransferases (APH) (Ramirez and Tolmasky, 2010). Numerous aminoglycoside resistance genes have been reported on mobile genetic elements and transposons (Lee et al., 2002; Nirdnoy et al., 2005). Gentamicin resistance genes including *aacA4*, the bifunctional gene *aac(6')-Ie/aph(2")-Ia* (also named *aacA-aphD*), *aph(2")-If*, and *aph(2")-Ig* have been detected in *Campylobacter* (Chen et al., 2013; Zhao et al., 2015). A unique genetic structure containing the aminoglycoside resistance gene cluster *aadE-sat4-aphA-3* and *aacA-aphD* has been identified on the chromosome of *C. coli* (Qin et al., 2012). Recently, a high prevalence and predominance of the *aph(2")-If* gene has been reported in *Campylobacter* (Yao et al., 2017). The *aph(2")-If* shows resistance to gentamicin and other aminoglycosides, such as kanamycin, sisomicin, and tobramycin (Toth er al., 2013). A novel streptomycin resistance gene has also been described, and its widespread presence among *C. coli* may partly account for the streptomycin resistance (Olkkola et al., 2016).

Gentamicin resistance in *Campylobacter* isolated from various livestock commodities along the food-producing continuum ("farm to fork") and in humans is rare and stable in most countries. In Netherlands in 2017, no gentamicin resistance was found in *Campylobacter* isolates from cattle, pigs, and poultry. The resistance to streptomycin varies between 0 and 2.6% in *C. jejuni* isolates from broilers (European Food Safety Authority (EFSA), 2017). The level of resistance to streptomycin and gentamicin is low (0–1%) for *C. jejuni* from broilers and cattle in Denmark (DANMAP, 2019). Based on a report of the National Antimicrobial Resistance Monitoring System, gentamicin resistance in *Campylobacter* in the United States was rare before 2007 but has increased rapidly and been detected in 12.2% and 18.1% of human isolates and retail isolates, respectively (National Antimicrobial Resistance Monitoring System: Enteric Bacteria, 2011; National Antimicrobial Resistance Monitoring System: Retail Meat Annual Report, 2011). In China, several aminoglycoside agents have been used in conventional broiler and swine productions, and the prevalence of gentamicin-resistant *Campylobacter* from broiler and swine is much higher than that in the United States. A recent study has shown that over 60% of *Campylobacter* isolated from swine and chicken are resistant to gentamicin (Yao et al., 2017). Up to 95% of *C. coli* isolated from chicken and 23% from swine are gentamicin resistant (Chen et al., 2010; Qin et al., 2011; Ma et al., 2014).

In the present study, the prevalence of gentamicin and kanamycin resistance and the associated aminoglycoside resistance genes were analyzed in *Campylobacter* isolated from chicken and swine in Jiangsu province, China. We found a gene segment which could be transferred from *C. jejuni* or *C. coli* to a *C. jejuni* strain by natural transformation, resulting in a drastic increase in aminoglycoside resistance. These findings suggested that the responsible use of aminoglycosides is highly important in safeguarding public health in China.

MATERIALS AND METHODS

Campylobacter Strains and Aminoglycoside-Susceptibility Testing

A total of 185 *Campylobacter* isolates (95*C. jejuni* and 90*C. coli* isolates) was investigated in this study. All *Campylobacter* isolates were recovered in 2017 and 2018 from cloacal swabs of chickens and feces of swine from Jiangsu province, eastern China, during our laboratory annual antimicrobial resistance surveillance program. Strains in 2017 were isolated from chicken cloacal swabs and chicken meat: 67 *Campylobacter* strains from chicken cloacal swabs (samples number = 150) and 25 *Campylobacter* strains from chicken meat (samples number = 80). The strains in 2018 were isolated from chicken cloacal swabs and pig animal feces: 73 *Campylobacter* strains from chicken cloacal swabs (samples number = 150) and 22 *Campylobacter* strains from pig fresh feces (samples number = 100). Chicken cloacal swabs were collected from 15 chicken farms in each year. From each farm, 10 cloacal swabs were taken from randomly selected animals. Chicken meat samples were collected from 16 supermarkets located in seven districts in Jiangsu Province. From each supermarket, five chicken meat samples were selected randomly. The pig fresh feces were collected from randomly selected animals in 10 pig farms located in Jiangsu province. All isolates were frozen at −80°C in a brain heart infusion broth with 20% glycerol. *Campylobacter* were

grown on Mueller Hinton (MH) agar (Difco, MD, United States) supplemented with 5% sheep blood under microaerophilic conditions (85% nitrogen, 10% carbon dioxide, and 5% oxygen) at 42°C. The MICs of gentamicin and kanamycin for *Campylobacter* were determined by the standard agar dilution method according to the guidelines of the (Clinical and Laboratory Standards Institute, 2016). The isolates which showed resistance to gentamicin and kanamycin were further examined for their susceptibility to amikacin, tobramycin, and streptomycin. The reference strain *C. jejuni* ATCC 33560 was used as a quality-control strain. The above experiments were repeated twice to confirm the reproducibility of the MIC data. The CLSI MIC interpretive criteria for resistance of antimicrobial agents were used. Antimicrobial agents were obtained from the Biomed Biotechnology Company.

Identification of Aminoglycoside Resistance Genes in *Campylobacter* Isolates

The known aminoglycoside resistance genes *aph(2")-If*, *aacA4*, and *aac(6')-Ie/aph(2")-Ia*, as well as the *aadE-sat4-aphA-3* gene cluster, were selected for PCR detection in all strains. Genomic DNA was isolated from the strains by using a TIANamp Bacteria DNA purification kit (TIANGEN, Beijing, China). Amplifications were carried out in a 25 μl PCR mixture composing 12.5 μl of Ex-Tag (Takara, Dalian, China), 1 μl of each primer, 1 μl of chromosomal DNA template, and 9.5 μl of sterile distilled water. The amplifications were carried out on a thermal cycler using the following parameters: 94°C for 5 min, followed by 30 cycles of 94°C for 40 s, annealing temperature specific to the primer pair for 30 s and 72°C for 1.5 min, and final extension at 72°C for 10 min. The primers of these genes and the annealing temperatures for the different target genes are listed in **Table 1**. The amplified products were separated by gel electrophoresis on 1.0% agarose gels, stained with GelRed, and visualized under UV light.

Natural Transformation and Whole-Genome Sequencing

A natural transformation assay was performed according to the method described by Wang and Taylor with minor modifications (Wang and Taylor, 1990). The genomic DNAs of aminoglycoside-resistant *Campylobacter* isolates served as the donors, whereas the aminoglycoside-sensitive strain of *C. jejuni* NCTC11168 served as the recipient. In a typical procedure, the fresh recipient cells were spread on MH agar at about 1×10^8 cells per plate and cultured for 6–8 h (at 42°C under microaerobic conditions). Then, 1 μg of genomic DNA of the donor strain was added to the inoculated agar followed by 5 h of continuous incubation at 42°C under microaerobic conditions. The cells were harvested and plated on the selective MH agar plate containing kanamycin (60 μg/ml), and the plate was further cultured for 48–72 h at 42°C under microaerobic conditions. The recipient without donor DNA cultured on the same MH agar served as a negative control. Single colonies of transformants were selected and sub-cultured on gentamicin-containing plates for purity. The MICs of the aminoglycoside (gentamicin, kanamycin, amikacin, tobramycin, and streptomycin) resistance for the transformants were determined by the standard agar dilution method according to the guidelines of the CLSI (Clinical and Laboratory Standards Institute, 2016). Subsequently, two transformants were selected for whole-genome sequencing on an Illumina HiSeq 2,500 platform (Novogene, Beijing, China). The generated 150 bp pair-end reads were trimmed and quality controlled, and the clean reads were assembled using SPAdes software. The draft genomes of the transformants were compared with NCTC11168 using Mauve.

PCR Detection of the Insert Fragment

According to the insertion site of the fragment, the primers were synthesized following a previous method (Yao et al., 2017) (**Table 1**). The unknown insertion fragment was detected in *Campylobacter* isolates by PCR. The EmeraldAmp PCR Master Mix (RR300A) (TaKaRa, Dalian, China) was composed of 12.5 μl of Hot start DNA polymerase, 1 μl of each primer, 1 μl of chromosomal DNA template prepared as previously described, and 9.5 μl of sterile distilled water. PCR was carried out according to the instructions of RR300A. The PCR products were separated as described above.

MLST and Aminoglycoside-Susceptibility Analysis of Insertion-Fragment Positive Isolates

MLST analysis of insertion-fragment positive *Campylobacter* strains was performed following a previously described method (Dingle et al., 2005). DNA was extracted from the selected strains by using a commercial DNA Kit (Tiangen Biotech Inc., Beijing, China). Primer sequences were obtained from http://pubmlst.org/*Campylobacter*. The nucleotide sequences of the amplicons were determined by Sangon Biotech (Shanghai, China). Allele numbers and sequence types (STs) were assigned using the *Campylobacter* PubMLST database. Subsequently, the resistance to multiple aminoglycoside antimicrobials of the insertion-fragment positive strains was investigated.

RESULTS

Aminoglycoside Resistance in *Campylobacter* Isolates

A total of 36.2% (67/185) *Campylobacter* isolates was resistant to both gentamicin and kanamycin. Among the 92 *Campylobacter* isolates tested in 2017 (*C. jejuni*, 51; *C. coli*, 41), 28 (30.4%, including 13*C. jejuni* and 15*C. coli*) were resistant to the above two antibiotics. However, in 2018, among 93 *Campylobacter* isolates tested (*C. jejuni*, 44; *C. coli*, 49), 39 (41.9%, including 16*C. jejuni* and 23*C. coli*) showed resistance to these two drugs. The proportions of gentamicin and kanamycin-resistant *Campylobacter* strains ranged from 28.1% to 60.0% among the seven regions. The rate of resistance of *Campylobacter* to gentamicin and kanamycin in 2018 was higher than that in 2017. A higher

percentage of *C. coli* than *C. jejuni* isolates was resistant to gentamicin and kanamycin (**Table 2**). The distributions of gentamicin MICs of gentamicin-resistant *Campylobacter* are shown in **Figure 1**, which revealed that the strains with an MIC value of 128 μg/ml accounted for a large proportion.

Presence of Aminoglycoside Resistance Genes

Various aminoglycoside resistance genes were examined by PCR in all 185 *Campylobacter* isolates. The prevalence of the *aph(2")-If* gene was 57.6% (53/92) and 73.1% (68/93) in *Campylobacter* isolates in 2017 and 2018, respectively. Moreover, *aph(2")-If* gene was more prevalent in *C. coli* than in *C. jejuni* in 2017. Similar to the *aph(2")-If* gene described above, *aac(6')-Ie/aph(2")-Ia* gene was identified in 31.5% (63.4% in *C. coli* and 5.9% in *C. jejuni*, 2017) and 40.9% (49.0% in *C. coli* and 31.8% in *C. jejuni*, 2018), showing its common presence and higher prevalence in *C. coli*. Among all gentamicin-resistant strains, 44.8% (30/67) contained two of the resistant genes, and 14.9% (10/67) did not have any of these four tested genes. The prevalence of the *aadE-sat4-aphA-3* cluster in *Campylobacter* isolates was identified to be ≤10% in 2017, but it increased to about 20% in 2018 (**Table 2**).

Transfer of Aminoglycoside Resistance Through Natural Transformation

The genomic DNAs of the two strains with high resistance to gentamicin (*C. jejuni* 165 and *C. coli* 254) were used as the donor DNA to transform the recipient strain (*C. jejuni* NCTC11168) by natural transformation under laboratory conditions. Compared with NCTC11168, the transformants NT-165 and NT-254 were obtained, showing 512- and 128-fold

TABLE 1 | PCR primers used in this study.

Primers	Sequence (5'-3')	Annealing temperature (°C)	Product size (bp)	References
aph(2")-If-F	AAGGAACTTTTTTAACACCAG	50	420	Zhao et al., 2015
aph(2")-If-R	CCWATTTCTTCTTCACTATCTTC			
aacA4-F	ATCTCATATCGTCGAGTGGAC	50	440	Zhao et al., 2015
aacA4-R	CGTGTTTGAACCATGTAC			
aac(6')-Ie/aph(2")-Ia-F	ACAGAGCCTTGGGAAGATGAAG	54	1,106	Zhao et al., 2015
aac(6')-Ie/aph(2")-Ia-R	TGTTCCTATTTCTTTCTTCACTATC			
aadE-sat4-aphA-3-F	CGAGGATTTGTGGAAGAGGCTT	55	1,000	Qin et al., 2012
aadE-sat4-aphA-3-R	TTCCTTCCAGCCATAGCATCATG			
cj0299-F	GTGCCGCTTGTATTATACTC	55	unknown	Yao et al., 2017
panB-R	GGCATATCAGCAAGTACGAAAGAC			

TABLE 2 | Prevalence of gentamicin and kanamycin resistance and the associated resistance genes.

Year	Location of isolates	Host	No. of gentamicin and kanamycin-resistant isolates/total no. of isolates (%)		No. of *aadE-sat4-aphA-3*-positive isolates/total no. of isolates (%)		No. of *aph(2")-If*-positive isolates/total no. of isolates (%)		No. of *aac(6')-Ie/aph(2")-Ia*-positive isolates/total no. of isolates (%)	
			C. jejuni	*C. coli*	*C.jejuni*	*C. coli*	*C. jejuni*	*C. coli*	*C. jejuni*	*C. coli*
2017	YZ	chicken	3/10(30.0)	4/18(22.2)	0/10(0)	1/18(5.6)	5/10(50.0)	14/18(77.8)	2/10(20.0)	14/18(77.8)
	HA	chicken	3/12(25.0)	4/12(33.3)	1/12(8.3)	4/12(33.3)	5/12(41.7)	8/12(66.7)	0/12(0)	4/12(33.3)
	SQ	chicken	3/10(30.0)	2/4(50.0)	0/10(0)	2/4(50.0)	6/10(60.0)	4/4(100.0)	1/10(10.0)	4/4(100.0)
	YC	chicken	1/6(16.7)	3/3(100.0)	0/6(0)	0/3(0)	1/6(16.7)	3/3(100.0)	0/6(0)	2/3(66.7)
	XZ	chicken	2/6(33.3)	1/3(33.3)	0/6(0)	0/3(0)	3/6(50.0)	2/3(66.7)	0/6(0)	1/3(33.3)
	CZ	chicken	1/5(20.0)	1/1(100.0)	0/5(0)	0/1(0)	1/5(20.0)	1/1(100.0)	0/5(0)	1/1(100.0)
	NT	chicken	0/2(0)	0/0(0)	0/2(0)	0/0(0)	0/2(0)	0/0(0)	0/2(0)	0/0(0)
Total			13/51(25.5)	15/41(36.6)	1/51(2.0)	7/41(17.1)	21/51(41.2)	32/41(78.1)	3/51(5.9)	26/41(63.4)
			28/92 (30.4)		8/92 (8.7)		52/92 (57.6)		29/92 (31.5)	
2018	YZ	chicken	4/19(21.1)	5/10(50.0)	0/19(0)	0/10(0)	17/19(89.5)	10/10(100.0)	12/19(63.2)	6/10(60.0)
	HA	chicken	1/1(100.0)	0/0(0)	0/1(0)	0/0(0)	1/1(100.0)	0/0(0)	0/1(0)	0/0(0)
	SQ	chicken	2/5(40.0)	2/7(28.6)	1/5(20.0)	1/7(14.3)	5/5(100.0)	2/7(28.6)	5/5(100.0)	2/7(28.6)
	YC	chicken	1/11(9.1)	2/3(66.7)	0/11(0)	1/3(33.3)	7/11(63.6)	1/3(33.3)	3/11(27.3)	2/3(66.7)
		swine	1/1(100.0)	11/22(50.0)	0/1(0)	11/22(50.0)	1/1(100.0)	11/22(50.0)	1/1(100.0)	10/22(45.5)
	CZ	chicken	5/5(100.0)	2/4(50.0)	1/5(20.0)	2/4(50.0)	5/5(100.0)	3/4(75.0)	2/5(40.0)	2/4(50.0)
	NT	chicken	2/2(100.0)	1/3(33.3)	2/2(100.0)	0/3(0)	2/2(100.0)	3/3(100.0)	2/2(100.0)	2/3(66.7)
Total			16/44(36.4)	23/49(46.9)	4/44(9.1)	15/49(30.6)	38/44(86.4)	30/49(61.2)	14/44(31.8)	24/49(49.0)
			39/93 (41.9)		19/93 (20.4)		68/93 (73.1)		38/93 (40.9)	
Total			29/95(30.5)	38/90(42.2)	5/95(5.3)	22/90(24.4)	59/95(62.1)	62/90(68.9)	17/95(17.9)	50/90(55.6)
			67/185(36.2)		27/185(14.6)		121/185(65.4)		67/185(36.2)	

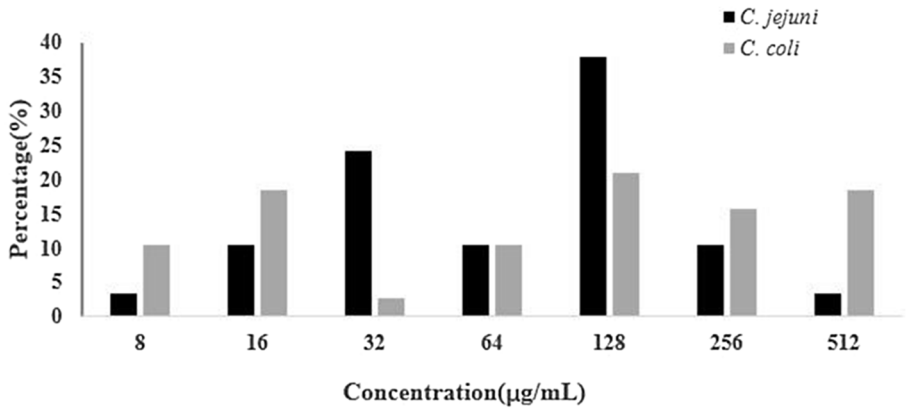

FIGURE 1 | Distributions of gentamicin MICs of gentamicin-resistant *Campylobacter*.

increases in the MICs of gentamicin, kanamycin, and tobramycin, respectively. The transformant NT-254 showed slightly decreased susceptibility to amikacin. However, the transformant NT-165 did not show any resistance to amikacin or streptomycin. The MICs of gentamicin, kanamycin, tobramycin, amikacin, and streptomycin in the recipient, donors, and transformants are revealed in **Table 3**.

The two transformants were then investigated by whole-genome sequencing. Subsequently, the draft genomes of NT-165 and NT-254 were compared with NCTC11168, showing that the backbones of the transformants were NCTC11168. Moreover, a 5,200-bp segment was inserted between the *Campylobacter* highly conserved genes *Cj0299* and *panB*. The same insertion region was already identified in 2017 (Yao et al., 2017). The inserted segment contained six open reading frames (ORFs). It included genes encoding AAC/APH (a bifunctional enzyme), dTDP-fucosamine acetyltransferase, cytidylate kinase, IS1595 family transposase, and two hypothetical protein genes (**Figure 2**). Combined with the MICs results of the transformants above, it suggested that the six-gene cluster can transferred among *Campylobacter* strains naturally, and it conferred a high-level resistance to aminoglycoside antibiotics.

Molecular Typing and Aminoglycoside Resistance of the Insertion-Fragment Positive *Campylobacter* Isolates

Using the primers *cj0299*-F and *panB*-R, a 5.2-kb fragment was amplified in 9.7% (18/185) *Campylobacter* isolates. Some examined isolates, including aminoglycoside- resistant isolates and susceptible ones, did not yield this 5.2-kb fragment but showed a 750-bp fragment (**Figure 3**). These positive strains originated from five different locations in Jiangsu province (**Table 4**).

Subsequently, the resistance to multiple aminoglycoside antimicrobials of the insertion-fragment positive strains was investigated. Most strains showed high MICs to gentamicin and kanamycin. About 72.2% (13/18) showed high MICs to all five tested aminoglycosides (**Table 4**).

To understand if the insertion-fragment positive isolates were genetically related, 18 isolates above were selected for MLST analysis. Seven STs were identified for these strains: a new ST (165 in location HA), ST860 and ST829 (254 and 53–1, respectively, in location HA), ST10062 (246 in location YC and 55–1, 58–3, 58–4, 58–6, 58–8, 61–4, 62–2, and 62–4 in location YZ), ST1666 (59–2 in location YZ), ST9627 (1–1, 1–2, 2–1, and 2–2 in location CZ), and ST832 (52–1 in location NT). Except for a new ST and ST10062 strains whose clonal complexes were not assigned, others belonged to clonal complex 828. ST10062 appeared in different years and different locations, although the number of ST10062 strains in 2017 was relatively small (**Table 4**).

DISCUSSION

Aminoglycosides use in veterinary medicine is associated with increased resistance to aminoglycosides and other antimicrobial classes in bacteria from animals. *C. jejuni* and *C. coli* isolates of clinical and animal origin display resistance to aminoglycoside streptomycin (van Duijkeren et al., 2019). In China, antimicrobial use records reveal that aminoglycoside agents, such as amikacin and neomycin, are commonly used to prevent bacterial diseases in food-producing animals (Wang et al., 2016). Although the level of resistance to gentamicin in *Campylobacter* is low in other countries, an increasing trend has been observed in recent years, and the gentamicin resistance rate of *Campylobacter* in China has become higher than those in other countries (Zhao et al., 2015; Yao et al., 2017; DANMAP, 2019). In the present study, the prevalence of gentamicin and kanamycin resistance in *Campylobacter* is ≥30.0%, consistent with previous reports in China (Qin et al., 2012; Ma et al., 2014; Yao et al., 2017). Previous studies have shown that gentamicin resistance is much more prevalent in *C. coli* than in *C. jejuni* strains (Wang et al., 2016; Yao et al., 2017). In the present study, a higher percentage of resistance to gentamicin and kanamycin was found in *C. coli* than in *C. jejuni* isolates. The resistance to gentamicin and kanamycin

TABLE 3 | MICs of aminoglycosides in the transformants and donor strains.

Antimicrobial agent	MIC (μg/ml)				
	NCTC11168	165	NT-165	254	NT-254
Gentamicin	1	512	512	512	512
Kanamycin	8	1,024	1,024	1,024	1,024
Tobramycin	2	256	256	256	256
Amikacin	4	256	4	32	16
Streptomycin	4	256	4	4	4

NT-165 and NT-254 are transformants of NCTC11168 with donor DNA from C. jejuni 165 and C. coli 254, respectively.

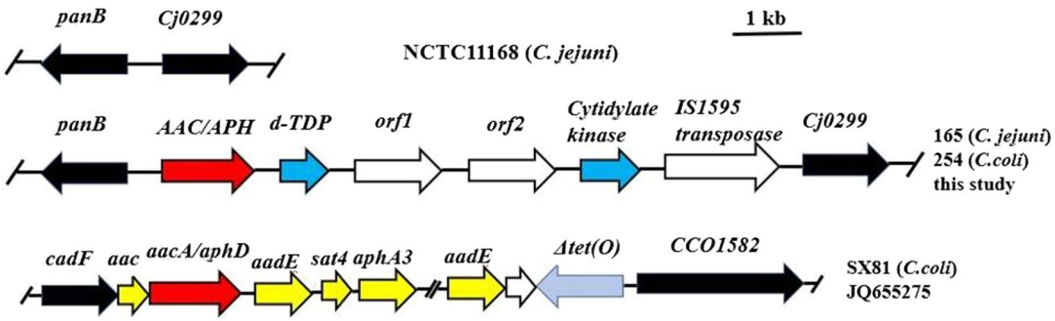

FIGURE 2 | Genomic organization of the aminoglycoside resistance insertion segment in *C. jejuni* 165 and *C. coli* 254 in comparison with the multidrug resistance genomic island of *C. coli* SX81 and *C. jejuni* 11,168. Arrows indicate the positions and directions of transcription of the genes. The locations of primers *panB*-F and *cj0299*-R used to detect the insertion segment are indicated.

of *C. jejuni* and *C. coli* increased by more than 10.0% in 2018 compared with that in 2017 in Jiangsu province. The increasing trend suggested that we should pay attention to rational drug use and strengthen the monitoring of aminoglycoside drug resistance in China.

Resistance genes can be located on the plasmids, chromosome, transposons, or other mobile elements, increasing the aminoglycoside resistance and the co-resistance to other compounds (Ramirez et al., 2013). There is an apparent trend toward multidrug resistance, particularly among *C. coli* which harbored different antimicrobial resistance genes within the genome of a single isolate (European Food Safety Authority (EFSA), 2019). Four variants of aminoglycoside-resistant genes were analyzed by PCR. The bifunctional enzyme aac(6')-Ie/aph(2")-Ia confers resistance to almost all aminoglycosides except streptomycin (Zhao et al., 2015). In clinical *Enterococcus* isolates, it is the most important enzyme associated with high-level gentamicin resistance (Toth et al., 2013). A previous study has reported the recent emergence of *aph(2")-If* and has become the predominant gentamicin resistance determinant in *Campylobacter* in Shandong province in China (Yao et al., 2017). In the present study, the prevalence rates of the *aac(6')-Ie/aph(2")-Ia* gene in *Campylobacter* isolates were 31.5% and 40.9% in 2017 and 2018, respectively. A*ph(2")-If* gene was also more prevalent in *Campylobacter* herein than in another previous study in China. The reason for the increased prevalence of these two genes may be the different years and regions of the isolated strains. Further studies are needed to show if an annual increasing trend occurs in the presence of these two gentamicin resistance genes.

The gene cluster *aadE-sat4-aphA-3*, which confers resistance to streptomycin, streptothricin, and kanamycin, has been detected on a plasmid and in the chromosome in *Campylobacter* (Chen et al., 2013; Zhao et al., 2015). It was identified in a *C. coli* genomic island which harbors genes conferring resistance to multiple aminoglycoside antibiotics in China (Qin et al., 2012). The *aadE-sat4-aphA-3* gene cluster was observed in about ≤15.0% isolates in the present study. Among all cluster-positive strains, 13 isolates did not show any resistance to gentamicin or kanamycin. This result was not consistent with the previously reported results showing that all clusters carrying *C. coli* were resistant to gentamicin (Qin et al., 2012). But in another paper, the florfenicol-resistant gene *cfr(C)*-carrying *C. coli* isolates were susceptible to chloramphenicol and florfenicol (Liu et al., 2019). The gentamicin resistance gene *aacA4* has been reported in *C. jejuni* isolated from the water lines of a broiler-chicken house environment, and it is associated with class 1 integron (Lee et al., 2002). However, no *aacA4* gene was detected in the *Campylobacter* isolates examined in this study, consistent with a previous one (Yao et al., 2017). About 15.0% (10/67) gentamicin-resistant isolates contained none of the above four detected aminoglycoside-resistant genes which may harbor other unknown resistance mechanisms.

Campylobacter are well known for their ability to acquire exogenous DNA by natural transformation (Wang and Taylor,

1990). Some genes associated with high-level gentamicin resistance in *Campylobacter* have probably resulted from horizontal transfer from other microorganisms (Zhao et al., 2015). Herein, transformants with ≥128-fold increase in the MICs of gentamicin were obtained by natural transformation under laboratory conditions. This difference in aminoglycoside MIC values suggested that some genes of the genome were transferred from *C. jejuni* or *C. coli* to *C. jejuni* and intensively increased the aminoglycoside resistance in the recipient strain *C. jejuni*.

Genomic islands harboring aminoglycoside resistance genes and multidrug resistance have been previously detected between *cadF* and *CCO1582* on the chromosome in *C. coli*, and multiple aminoglycoside resistance genes have been found to be located between *Cj0299* and *panB* (Qin et al., 2012; Wang et al., 2014; Yao et al., 2017). Herein, we compared the draft genomes of two transformants with NCTC11168 and found a 5,200-bp segment inserted between the highly conserved *Campylobacter* genes *Cj0299* and *panB*. The presence of this segment was associated with elevated MIC values for aminoglycosides. In recent years, different multidrug resistance genomic islands (MDRGIs) conferring resistance to aminoglycosides, macrolides, and tetracyclines were characterized (Qin et al., 2012; Wang et al., 2014; Liu et al., 2019). Unlike MDRGIs in previous reports, the inserted segment in this study just contained six ORFs (**Figure 2**). *AAC/APH* which encoded a bifunctional enzyme was in it. Gene *aacA-aphD* was the only gene identified to encode a bifunctional aminoglycoside-modifying enzyme responsible for gentamicin and kanamycin resistance in *Campylobacter*. This insertion fragment could spread by horizontal gene transfer. In 18 insertion-fragment positive strains, about 72.0% (13/18) showed high MICs to all five tested aminoglycosides (**Table 4**). This result suggested that the insertion fragment was associated with aminoglycoside resistance, the prevalence of which could confer a fitness advantage under selection by continued aminoglycoside usage.

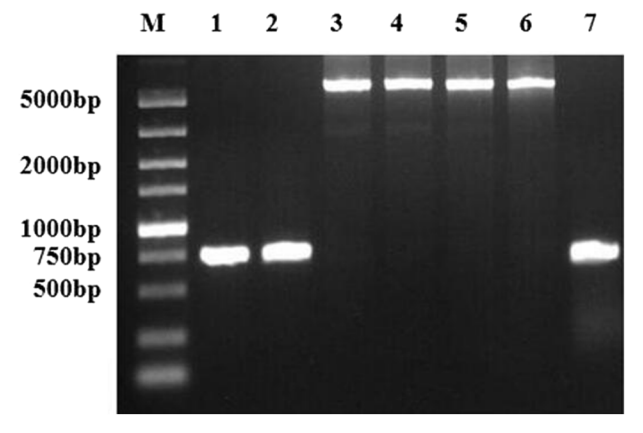

FIGURE 3 | PCR detection of the region between *panB* and *Cj0299* in various strains. Lanes 1 and 2, PCR products from aminoglycoside-susceptible strains 28 and 31, respectively; Lanes 3 and 4, PCR products from the *C. jejuni* transformants of NT-165 and NT-254, respectively; Lanes 5 and 6, PCR products from aminoglycoside-resistant strains 165 and 254, respectively; and Lane 7, PCR products from *C. jejuni* NCTC11168.

In a previous study, an aminoglycoside resistance island was reported to spread by both horizontal gene transfer and clonal expansion by PFGE and MLST analysis in Shandong province, China (Qin et al., 2012). To understand if the insertion-fragment positive *Campylobacter* isolates in the present study were genetically related or had any clonal expansion, 18 fragment positive isolates were selected for MLST analysis. Seven STs were identified for these strains. The 18 strains had no particular clonal expansion. Except for a new ST and ST10062 whose clonal complexes had not been assigned, all

TABLE 4 | MICs of aminoglycoside antibiotics and STs for insertion-fragment positive isolates.

Strain	Species	Year	Location	MIC (μg/ml)					ST	ST-clonal
				Gen	K	AK	TOB	S		
165	*C.jejuni*	2017	HA	512	1,024	256	256	256	new	–
254	*C. coli*	2017	HA	512	1,024	32	256	4	860	828
246	*C. coli*	2017	YC	256	512	64	64	2	10,062	–
1–1	*C.jejuni*	2018	CZ	128	1,024	128	64	128	9,627	828
1–2	*C.jejuni*	2018	CZ	128	1,024	64	64	128	9,627	828
2–1	*C.jejuni*	2018	CZ	128	1,024	128	64	128	9,627	828
2–2	*C.jejuni*	2018	CZ	128	1,024	128	64	64	9,627	828
52–1	*C.coli*	2018	NT	128	1,024	8	32	64	832	828
53–1	*C.jejuni*	2018	HA	64	1,024	16	64	32	829	828
55–1	*C.jejuni*	2018	YZ	128	512	64	64	128	10,062	–
58–3	*C. coli*	2018	YZ	256	1,024	128	64	32	10,062	–
58–4	*C. coli*	2018	YZ	256	1,024	128	64	64	10,062	–
58–6	*C. coli*	2018	YZ	256	1,024	64	64	64	10,062	–
58–8	*C.jejuni*	2018	YZ	128	512	64	64	64	10,062	–
59–2	*C. coli*	2018	YZ	512	1,024	64	64	64	1,666	828
61–4	*C.jejuni*	2018	YZ	128	1,024	64	64	128	10,062	–
62–2	*C.jejuni*	2018	YZ	256	1,024	128	64	64	10,062	–
62–4	*C. coli*	2018	YZ	256	1,024	128	64	64	10,062	–

other strains belonged to clonal complex 828, consistent with a previous study (Qin et al., 2012). Interestingly, ST10062 appeared in different years and locations. More strains are needed to further determine whether this type of ST is a special clonal expansion for the dissemination of aminoglycoside resistance in China.

CONCLUSION

This study provided the prevalence of gentamicin and kanamycin resistance and the associated aminoglycoside resistance genes in *Campylobacter* isolated from chicken and swine in Jiangsu province, China. A gene segment which could drastically increase aminoglycoside resistance by natural transformation was found. Owing to the use of aminoglycosides in poultry and swine production, *Campylobacter* in poultry and swine may be able to deal with the toxicity and selective pressure from these antibiotics. These findings offered insights into the prevalence and spread of the aminoglycoside resistance of *Campylobacter* in China, thereby highlighting the need for concerning and taking measures to reduce the dissemination of aminoglycoside resistance in *Campylobacter*.

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: MZ593442-MZ593447.

ETHICS STATEMENT

This study was carried out in accordance with the principles of the Animal Welfare and Ethical Censor Committee of Jiangsu Institute of Poultry Science. No chickens and pigs were killed for the present study. When collecting cloacal swabs, well-trained farm workers hold the chickens. Fresh feces from pigs were collected without any manipulation of the pigs.

AUTHOR CONTRIBUTIONS

QZ, JZ, and JP performed the antibiotic susceptibility tests. XZ and QZ did the aminoglycoside genes detection. MT and XZ performed natural transformation and MLST. YZ and JL did the data analysis. XZ prepared the manuscript. YG supervised and assisted in the manuscript preparation. All authors contributed to the article and approved the submitted version.

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Methicillin- and Vancomycin-Resistant *Staphylococcus aureus* From Humans and Ready-To-Eat Meat: Characterization of Antimicrobial Resistance and Biofilm Formation Ability

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Methicillin-resistant and vancomycin-resistant *Staphylococcus aureus* (MRSA and VRSA) are zoonotic life-threatening pathogens, and their presence in food raises a public health concern. Yet, scarce data are available regarding MRSA and VRSA in both ready-to-eat (RTE) meat and food handlers. This study was undertaken to determine the frequency, antimicrobial resistance, and biofilm-forming ability of MRSA and VRSA isolated from RTE meat (shawarma and burger) and humans (food handlers, and hospitalized patients) in Zagazig city, Sharkia Governorate, Egypt. We analyzed 176 samples (112 human samples: 72 from hospitalized patients and 40 from food handlers, 64 RTE meat samples: 38 from shawarma and 26 from burger). Using phenotypic, PCR-based identification of *nuc* gene and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), 60 coagulase-positive *S. aureus* (COPS) isolates were identified in the samples as follow: RTE meat (15/64, 23.4%), hospitalized patients (33/72, 45.8%) and food handlers (12/40, 30%). All the COPS isolates were *mecA* positive (and thus were classified as MRSA) and multidrug resistant with multiple antibiotic resistance indices ranging from 0.25 to 0.92. Overall, resistance to cefepime (96.7%), penicillin (88.3%), were common, followed by ampicillin-sulbactam (65%), ciprofloxacin (55%), nitrofurontoin (51.7%), and gentamicin (43.3%). VRSA was detected in 30.3% of COPS hospitalized patient's isolates, 26.7% of COPS RTE meat isolates and 25% of COPS food handler's isolates. *VanA*, *vanB,* or both genes were detected in 64.7, 5.9, and 29.4% of all VAN-resistant isolates, respectively. The majority of the COPS isolates (50/60, 83.3%) have biofilm formation ability and harbored *icaA* (76%), *icaD* (74%), *icaC* (50%), and *icaB* (46%) biofilm-forming genes. The *bap* gene was not detected in any of the isolates. The ability of MRSA and VRSA isolates to

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produce biofilms in addition to being resistant to antimicrobials highlight the danger posed by these potentially virulent microorganisms persisting in RTE meat, food handlers, and patients. Taken together, good hygiene practices and antimicrobial surveillance plans should be strictly implemented along the food chain to reduce the risk of colonization and dissemination of MRSA and VRSA biofilm-producing strains.

Keywords: VRSA, MRSA, multidrug resistance, biofilm, ready-to-eat meat, *S. aureus*, food handlers, patients

INTRODUCTION

Staphylococcus aureus is an opportunistic human and animal pathogen causing food intoxication and a variety of infections ranging from skin and soft tissue infections to serious diseases including endocarditis, septicemia, osteomyelitis, pneumonia and toxic shock syndrome (Chen and Huang, 2014; Otto, 2014). The drug resistance of *S. aureus* has gradually increased in the recent decades due to the misuse of antibiotics (e.g., using antibiotics without prescription, uncontrolled doses, and useless application of drugs), which resulted in bacterial evolution (Gharieb et al., 2020; Guo et al., 2020). Consequently, multidrug-resistant *S. aureus* especially methicillin-resistant (MRSA) strains are a major human health concern causing severe morbidity and mortality, in particular in hospitals (hospital-associated MRSA; HA-MRSA), and in healthy persons (community-associated MRSA; CA-MRSA) (Weber, 2005). MRSA, one of twelve priority pathogens that threaten human health as classified by the World Health Organization (WHO), has been increasingly detected in food products (Craft et al., 2019; Li et al., 2019). Animal-derived foods serve as vehicles for the transmission of livestock-associated MRSA (LA-MRSA) (Mossong et al., 2015; Anjum et al., 2019). Further, human-mediated contamination of carcasses, meat product or ready-to-eat (RTE) foods at abattoirs, meat processing plants or during handling may be a source of MRSA and could represent potential risk for consumers (Hadjirin et al., 2015; Li et al., 2019).

Apart from the studies focused on LA-MRSA, the prevalence of staphylococci in RTE foods is receiving widespread attention due to the increasing number of food poisoning cases (Chajecka-Wierzchowska et al., 2015; Yang et al., 2017). MRSA strains exhibit resistance to various antimicrobials such as penicillins, cephalosporins, and carbapenem through the acquisition of the mobile staphylococcal cassette chromosome mec (SCCmec) that carries *mecA* gene, which encodes for an altered penicillin binding proteins (PBP2a or PBP2′), thereby making their treatment difficult (Hiramatsu et al., 2014). Vancomycin has long been considered the last-resort treatment for MRSA infections (Holmes et al., 2015). However, its excessive use resulted in the emergence of vancomycin-intermediate *S. aureus* (VISA), vancomycin-resistant *S. aureus* (VRSA), and heterogeneous vancomycin-intermediate *S. aureus* (hVISA) strains (Amberpet et al., 2019). In addition to antimicrobial resistance, MRSA strains are able to form biofilm as a fitness and survival mechanism that is mediated by the strong adhesion, increase in drug resistance and reduction in the effectiveness of sanitizers (Craft et al., 2019). In the presence of biofilm, resistance of *S. aureus* to antimicrobials was reported to increase to 1,000 times that of planktonic cells (Guo et al., 2020). Therefore, MRSA biofilm formation is an important persistence and dissemination mechanism of food contamination (Vergara et al., 2017; Avila-Novoa et al., 2021). Biofilm development is thought to be a two-step process in which bacteria adhere to a surface via a capsular antigen, capsular polysaccharide/adhesin (PS/A), and then multiply to form a multilayered biofilm, which is triggered by the synthesis of polysaccharide intercellular adhesin (PIA) from β-1, 6- linked N-acetyld-glucosamines (Periasamy et al., 2012). The intercellular adhesion (*ica*) locus is comprised of *icaA*, *icaD*, *icaB*, and *icaC* genes that mediate the production of PIA and PS/A (McKenney et al., 1998). The *icaA* gene encodes N-acetylglucosaminyltransferase enzyme. Furthermore, *icaD* has been shown to play a key role in the maximum expression of this enzyme, which leads to capsular polysaccharide phenotypic expression (Arciola et al., 2001). The *icaC* functions as a polysaccharide receptor (McKenney et al., 1998). Furthermore, the biofilm-associated protein (*Bap*) is required for *S. aureus* initial attachment and biofilm development (Cucarella et al., 2004). Vaccine candidates consisting of several antigens related to multiple stages of biofilm formation and biofilm-related macromolecules have been proposed as a strategy for prevention and treatment of staphylococcal infection (Mirzaei et al., 2016, 2019, 2021; Bahonar et al., 2019; Gholami et al., 2019).

Indeed, data on the prevalence and biofilm formation by MRSA and VRSA in RTE food of animal origin remain scarce. In Egypt, most of the studies on the prevalence of MRSA isolates in RTE meat has focused on their antimicrobial resistance profile (Saad et al., 2019; Mahros et al., 2021), yet these studies did not analyze biofilm formation potential by the isolates, did not analyze the clustering pattern of such isolates in this important source and no studies exist that included isolates from humans. Here, we provide a characterization of a set of MRSA and VRSA isolates from humans (patients and food handlers) and RTE meat (shawarma and burger) focusing on their frequency, antimicrobial resistance phenotype, biofilm formation ability and how antimicrobial resistance and biofilm formation, as fitness traits, could be linked.

MATERIALS AND METHODS

Study Design and Sample Collection

Figure 1 shows the overall study design with respect to sampling, bacterial isolation, and characterization. During the period from February 2018 to January 2019, 176 samples were collected for isolation and characterization of *S. aureus*. These

included 64 RTE meat samples from both shawarma (n = 38) and burger (n = 26) sandwiches that were collected from six different restaurants in Zagazig city, Sharkia Governorate, Egypt. Moreover, 112 human samples were collected; including 72 samples from patients hospitalized at Zagazig University hospital (62 pus samples from abscesses and wounds and 10 sputum samples) and 40 hand swab samples from food handlers, who, at the time of examination, were clinically free from any bacterial skin infections. All samples were collected aseptically and immediately transferred in an icebox to the laboratory for isolation of *Staphylococcus* species. All participants were informed about the nature of the study and signed informed consent approving the use of their specimens for research purposes. The protocol of this study has been reviewed and approved by ZU-IACUC Committee (approval number ZU-IACUC/2/F/11/2019).

Isolation and Identification of *S. aureus*

The RTE meat and human samples were subjected to the standard microbiological techniques for isolation and identification of *S. aureus* (Cheesbrough, 2006; Aureus, 2016). Following National Food Safety Standards of China document GB 4789.10-2016 (Aureus, 2016), 25 g of each RTE meat sample was homogenized in 225 mL of tryptic soy broth (TSB, Thermo Fisher Scientific Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) with 10% sodium chloride. The homogenate (1 mL) was added to 9 mL of TSB in a sterile tube then incubated at 37°C for 24 h. One hundred μL of food homogenate, hand swabs, swabs from pus as well as sputum samples were plated on Baird-Parker agar (BPA) medium supplemented with an egg yolk–tellurite emulsion (Thermo Fisher Scientific Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) with and without acriflavine supplementation (7 μg/mL; Acros Organics, Morris Plains, NJ, United States) and incubated at 37°C for 48 h. The presumptive black-colored colonies surrounded by a clear halo zone were subcultured onto mannitol salt agar medium (Thermo Fisher Scientific Oxoid Ltd., Basingstoke, Hampshire, United Kingdom), blood agar, and subjected to Gram staining, catalase, coagulase, and oxidation/fermentation tests (Roberson et al., 1992). Also, the recovered isolates were identified using the MALDI TOF MS (BioMeriéux, Marcy l'Etoile, France) and MALDI-TOF MS running MYLA 3.1.0-15 software (BioMérieux, Inc., Marcy l'Etoile, France) according to Luo et al. (2015) and were further confirmed by *nuc* gene amplification as described previously (Brakstad et al., 1992) (**Supplementary Table 1**). The identified isolates were preserved at −20°C in brain heart infusion broth (BHI, Oxoid, Ltd., Basingstoke, Hampshire, United Kingdom) with 30% glycerol for further analysis.

Antimicrobial Susceptibility Testing

Kirby-Bauer disk diffusion test recommended by the Clinical and laboratory standards institute (CLSI) (CLSI, 2018) was employed for phenotypically testing the susceptibility of *S. aureus* isolates to 12 antimicrobial agents commonly used in clinical and veterinary medicine including: penicillin (P, 10 μg, Oxoid, Basingstoke, United Kingdom), methicillin (Me, 5 μg), nitrofurantoin (F, 300 μg), ampicillin/sulbactam (SAM, 20 μg), cefuroxime (CXM, 30 μg), ciprofloxacin (CIP, 5 μg), cefepime (FEP, 50 μg), trimethoprim/sulfamethoxazole (SXT, 23.75/1.25 μg) gentamycin (CN, 10 μg), clindamycin (DA, 2 μg), erythromycin (E, 15 μg), and vancomycin (VA, 30 μg). The results were interpreted according to CLSI guidelines in the reference mentioned before. *S. aureus* ATCC 25923 reference strain was included in the test as a control. The multiple antibiotic resistance (MAR) index was estimated by dividing the number of antimicrobial agents to which the isolate displayed resistance by the total number of tested antimicrobials (Krumperman, 1983). MAR index value > 0.2 identifies the isolate that originates from high-risk source of contamination where antibiotics are extensively used. The isolates were also examined using broth microdilution test for determination of vancomycin (Sigma-Aldrich, United States) minimum inhibitory concentrations (MICs) according to the CLSI guidelines (CLSI, 2018). Isolates, which have MIC ≤ 2 μg/mL was considered as vancomycin-susceptible *S. aureus* (VSSA), VISA: 4–8 μg/mL, and VRSA: MIC >8 μg/mL.

PCR Detection of Methicillin- and Vancomycin- Resistance Genes

Genomic DNA was extracted from 24 h cultures of phenotypic MRSA and VRSA isolates in BHI broth using the QIAamp DNA Mini kit (Qiagen, GmbH, Germany) according to the manufacturer's instructions. The DNA quantity and purity were assessed using NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). Methicillin resistance gene (*mecA*) and vancomycin resistance genes (*vanA* and *vanB*) were detected by PCR using oligonucleotide primers (**Supplementary Table 1**) as previously described (Patel et al., 1997; Kariyama et al., 2000; McClure et al., 2006). PCR amplification was performed in a T3 Thermal cycler (Biometra GmbH, Göttingen, Germany) using 6 μL of the extracted DNA (equalized at 100 ng/μL), 12.5 μL of 2X EmeraldAmp GT PCR master mix (Takara, Japan), and 1 μL (20 pmol) of both forward and reverse primers (Metabion, Germany), and nuclease-free water was added up to 25 μL. *S. aureus* ATCC 33591 (MRSA) and ATCC 29213 (methicillin-susceptible) strains were used as positive and negative controls, respectively. *Enterococcus faecium* ATCC 51559 and *E. faecalis* ATCC 51299 were used as a *vanA*- and *vanB*-positive control strains, respectively. The PCR-amplified products were analyzed by electrophoresis on a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide and visualized using AlphaDigiDoc®RT gel documentation system (Alpha Innotech Corp., San Leandro, CA, United States). A 100 bp ladder (Cat. No. SM0243, Fermentas, United States) was used as a molecular size marker.

Detection of Biofilm Formation Ability and Biofilm Genes

Biofilm formation ability of coagulase-positive *S. aureus* (COPS) isolates (n = 60) was assessed and analyzed using 96 wells flat-bottom polystyrene microtiter plates (Techno Plastic Products, Switzerland) as previously described (Stepanovic et al., 2007). The fresh culture of each isolate in TSB (200 μL, 10^6 CFU/mL) was

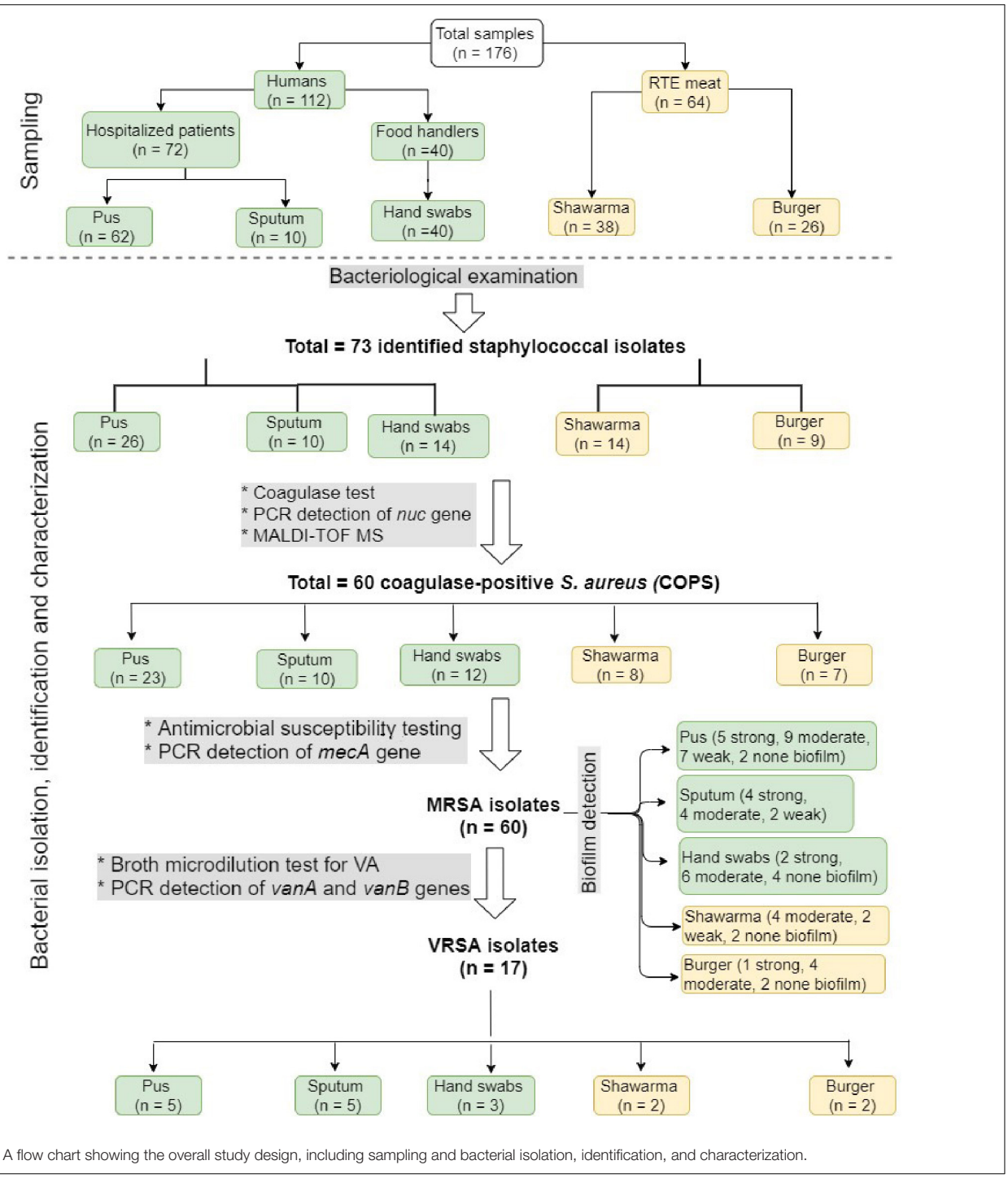

FIGURE 1 | A flow chart showing the overall study design, including sampling and bacterial isolation, identification, and characterization.

inoculated into wells of a sterile microtiter plate and incubated at 37°C for 24 h. TSB without bacteria and *S. aureus* ATCC 25923 were used as negative and positive controls, respectively. The content of wells was aspirated, and each well was washed three times with 200 μL of phosphate buffer saline (PBS, pH 7.3) to remove non-adherent cells. The plates were drained and air-dried for 15 min. The biofilms were stained with 150 μL of 0.1% crystal violet (Fluka AG, Buchs, Switzerland) for 30 min, and thereafter washed twice with PBS and the plate was air-dried. The stain bound to the cells was resolubilized with 150 μL of 95% ethanol for 45 min and the optical density (OD) was determined at a wavelength of 570 nm by ELISA reader (Awareness Technologies stat fax 2100, CA, United States). The isolates were tested in triplicate and the test was performed three times. Therefore, the average OD values ± standard deviations (SD) were calculated for the tested isolates and negative controls. For interpretation

of biofilm production, cut-off value of the OD (ODc) was calculated: ODc = average OD of negative control + (3 × SD of negative control) and accordingly the isolates were classified into the following categories: non-producer OD ≤ ODc, weak (ODc < OD ≤ 2 × ODc), moderate (2 × ODc < OD ≤ 4 × ODc), and strong biofilm producers (4 × ODc < OD). Biofilm-producing isolates were further investigated for biofilm-related genes listed in supplementary **Table 1** (*icaA*, *icaB, icaD*, *icaC*, and *bap*) as previously described (Vasudevan et al., 2003; Cucarella et al., 2004; Kiem et al., 2004).

Data Analyses and Bioinformatics

The significance of association between biofilm production ability and appearance of antimicrobial resistance was determined using Fisher's exact test applied on contingency tables. Odds ratio was estimated to give indication for the influence of biofilm production on the appearance of antimicrobial resistance. The confidence interval of odds was computed using Baptista-Pike method. Heatmap and dendrogram were generated to visualize the overall distribution and clustering of isolates based on their antimicrobial resistance profile and biofilm production ability entered as binary data (1 = present, 0 = absent). This analysis was done using "pheatmap, gplots, RColorBrewer" packages in R software (Csárdi and Nepusz, 2006). Correlation between antimicrobial resistance patterns and biofilm formation ability was determined using R packages corrplot, heatmaply, hmisc, and ggpubr (Friendly, 2002; Galili et al., 2018; Harrell, 2020) and the degree of correlation was reported following a previous study (Mukaka, 2012). To determine how isolates from human and RTE meat would be similar or dissimilar and thus estimate the potential of among-host transmission events, the antimicrobial resistance and biofilm formation profiles for each isolate were used as inputs to calculate single and averaged values of binary distance (using *dist* function within R environment) among isolates. The distances among isolates were then visualized as network diagrams, which were generated using cytoscape software version 3.8.1 (Otasek et al., 2019).

RESULTS

Occurrence of *Staphylococci* in RTE Meat and Human Samples

As shown in **Figure 1**, we isolated 73 staphylococci isolates from the analyzed 176 samples. In the 50 isolates recovered from humans, pus samples revealed the highest number of isolates (n = 26) followed by hand swabs from food handlers (n = 14), and sputum (n = 10). There were 23 isolates identified from RTE meat samples, including 14 and 9 isolates from shawarma and burger, respectively. The recovered isolates, which grow onto Baird Parker and mannitol salt agar media, were Gram positive cocci forming grape-like clusters, non-spore formers, fermentative, and catalase test positive. Out of the 73 isolates, 60 (82.2%) were β-hemolytic on blood agar, grow on modified Baird Parker media, and coagulase positive, so they were identified as *S. aureus* and further confirmed by PCR amplification of *nuc* gene and MALDI TOF MS with a confidence value of 99.9%. The highest number of isolates were identified in pus (n = 23) followed in a descending order by hand swab (n = 12), sputum (n = 10), shawarma (n = 8), and burger (n = 7).

Antimicrobial Resistance Profile of *S. aureus* Isolates

The antibiogram profiles of *S. aureus* isolates revealed that all 60 isolates from RTE meat products and human samples were MDR **(Table 1**, **Supplementary Table 2**, and **Figure 2**), being resistant to 3–11 antimicrobials with MAR indices ranged from 0.25 to 0.92. MDR isolates showed 49 distinct antibiogram patterns. Isolates exhibiting concurrent resistance to P, ME, SAM, FEP, F, CIP (pattern A32 in **Table 1**) and those that showed concurrent resistance to P, ME, FEP, CIP (pattern A44 in **Table 1**) were the major antibiotypes being represented by 4 isolates. The isolates from RTE meat samples showed resistance to 4–11 antimicrobials with MAR indices ranged from 0.33–0.92. The hospitalized patient's isolates (pus and sputum isolates) were resistant to 3–10 antimicrobial agents with MAR indices ranged from 0.25–0.83. Whereas the isolates from hand swabs were resistant to 4–10 antibiotics (MAR indices = 0.33–0.83). Overall, all the isolates exhibited full resistance to methicillin and harbored *mecA* gene, of which only 17 isolate (28.33%) showed resistance to vancomycin with MIC values of 16–512 μg/mL (**Table 1** and **Figure 1**). There were 13 VRSA isolates being identified in patients' samples (5 isolates from pus, 5 from sputum and 3 from hand swabs) and a smaller number of VRSA isolates (n = 4) were identified in RTE meat (shawrama [n = 2] and burger [n = 2]). As depicted in **Table 1** and **Supplementary Table 2**, *vanA*, *vanB,* and both genes were detected in 64.7, 5.9, and 29.4% of VA-resistant isolates, respectively. The majority of MDR isolates were resistant to cefepime (96.7%) and penicillin (88.3%), and more than half (65%) were ampicillin-sulbactam resistant. The resistance to ciprofloxacin was found among 55% of isolates followed by nitrofurontoin (51.7%) and gentamicin (43.3%). The lowest resistance rates were observed for clindamycin (21.67%), sulfamethoxazole - trimethoprim (23.3%), and erythromycin (28.3%).

Biofilm Formation Ability of MRSA and VRSA Isolates

Of the 60 MRSA isolates, 50 (83.3%) were biofilm producers and 10 (16.6%) formed no biofilm. The biofilm-producing isolates were categorized as strong (n = 12, 24%), moderate (n = 27, 54%), and weak (n = 11, 22%) biofilm producers (**Table 1** and **Supplementary Table 2**). Moreover, all the 17 VRSA isolates were biofilm producers (6 strong, 8 moderate, and 3 weak). It was found that the sources of strong biofilm-producing isolates were pus (n = 5) and sputum (n = 4) from patients, hand swabs (n = 2) from food handlers, and burger samples (n = 1). The moderate biofilm producers comprised 9 from pus, 6 from hand swabs and 4 each from shawarma, burger, and sputum. Nine isolates from hospitalized patients (7 from pus and 2 from sputum) and two isolates from shawarma were weak biofilm producers (**Figure 1**). As depicted in **Table 1** and **Supplementary Tables 2**, **3** the *icaADBC* biofilm-producing genes were detected in 92% of the

TABLE 1 | Antibiograms, biofilm formation ability, vancomycin MIC, and resistance genes of the 60 MRSA isolates.

Antibiotype No.	No. of isolates/ pattern	Source	No. of resistant antimicrobials	Resistance pattern	MAR index	Vancomycin MIC	Vancomycin resistance genes	Biofilm-forming ability		Biofilm genes
								OD 570 ± SD*	Degree	
A1	1	Burger	11	P, ME, SAM, CXM, FEP, F, CIP, CN, DA, E, VA	0.92	64	*VanB*	0.89 ± 0.03	Strong	*icaA*, *icaB*, *icaC*, *icaD*
A2	1	Pus	10	P, ME, SAM, CXM, FEP, F, SXT, CN, E, VA	0.83	16	*VanA*, *VanB*	0.96 ± 0.04	Strong	*icaA*, *icaB*, *icaC*, *icaD*
A3	1	Sputum	10	P, ME, SAM, CXM, FEP, F, CIP, CN, E, VA	0.83	512	*VanA*, *VanB*	0.58 ± 0.02	Moderate	*icaA, icaB, icaC, icaD*
A4	1	Sputum	10	P, ME, SAM, CXM, FEP, F, CIP, CN, DA, VA	0.83	64	*VanA*	0.51 ± 0.05	Moderate	*icaA, icaD*
A5	1	Hand swab	10	P, ME, SAM, FEP, F, CIP, CN, DA, E, VA	0.83	128	*VanA*, *VanB*	0.45 ± 0.03	Moderate	*icaA, icaB, icaC, icaD*
A6	1	Shawarma	10	P, ME, SAM, CXM, FEP, CIP, CN, DA, E, VA	0.83	16	*VanA*	0.30 ± 0.01	Weak	*icaA, icaD*
A7	1	Sputum	9	P, ME, SAM, CXM, FEP, F, CN, DA, VA	0.75	32	*VanA*	0.81 ± 0.14	Strong	*icaA*, *icaB*, *icaC*, *icaD*
A8	1	Burger	9	P, ME, SAM, CXM, FEP, CIP, SXT, DA, VA	0.75	16	*VanA*	0.39 ± 0.04	Moderate	*icaA, icaB, icaC, icaD*
A9	1	Hand swab	8	P, ME, SAM, FEP, F, CIP, CN, VA	0.67	16	*VanA*	0.37 ± 0.02	Moderate	*icaB, icaC*
A10	1	Sputum	8	P, ME, SAM, CXM, FEP, F, CN, VA	0.67	256	*VanA*, *VanB*	0.92 ± 0.02	Strong	*icaA*, *icaB*, *icaC*, *icaD*,
A11	1	Burger	8	P, ME, SAM, CXM, FEP, CN, DA, E	0.67	0.5		0.42 ± 0.04	Moderate	*icaB, icaC*
A12	1	Pus	8	P, ME, SAM, CXM, FEP, F, SXT, CN	0.67	8		0.69 ± 0.06	Strong	*icaA*, *icaB*, *icaC, icaD*
A13	1	Sputum	8	P, ME, SAM, CXM, FEP, F, CIP, E	0.67	1		0.78 ± 0.1	Strong	*icaA*, *icaB*, *icaC, icaD*
A14	1	Sputum	8	P, ME, SAM, CXM, FEP, F, CIP, CN	0.67	8		0.79 ± 0.03	Strong	*icaA*, *icaB*, *icaC, icaD*
A15	1	Pus	8	P, ME, CXM, FEP, CIP, SXT, CN, E	0.67	0.5		0.48 ± 0.05	Moderate	*IcaA*, *icaB, icaC, icaD*
A16	1	Shawarma	8	P, ME, SAM, CXM, FEP, F, CN, E	0.67	0.25		0.41 ± 0.1	Moderate	*icaA, icaD*
A17	1	Hand swab	8	P, ME, CXM, FEP, F, CN, DA, E	0.67	0.25		0.35 ± 0.06	Moderate	*icaA, icaD*
A18	1	Pus	7	P, ME, SAM, CXM, FEP, F, SXT	0.58	4		0.32 ± 0.05	Weak	*icaD*
A19	1	Pus	7	P, ME, SAM, CXM, CIP, SXT, VA	0.58	64	*VanA*	0.47 ± 0.12	Moderate	*icaA, icaB, icaC, icaD*
A20	1	Pus	7	P, ME, SAM, CXM, FEP, CIP, VA	0.58	32	*VanA*	0.87 ± 0.03	Strong	*icaA*, *icaB*, *icaC*, *icaD*
A21	1	Pus	7	P, ME, FEP, F, CIP, CN, VA	0.58	32	*VanA*	0.44 ± 0.03	Moderate	*icaA, icaD, icaC*
A22	1	Pus	7	ME, FEP, F, CIP, SXT, DA, VA	0.58	16	*VanA*	0.43 ± 0.08	Moderate	*icaA, icaD*
A23	1	Burger	7	P, ME, SAM, FEP, CIP, CN, E	0.58	4		0.21 ± 0.06	Non-producer	
A24	1	Shawarma	7	P, ME, SAM, CXM, FEP, CIP, CN	0.58	0.5		0.38 ± 0.1	Moderate	*icaB, icaC, icaD*
A25	1	Burger	7	P, ME, SAM, CXM, FEP, F, CIP	0.58	0.25		0.40 ± 0.03	Moderate	*icaA, icaD*
A26	1	Pus	7	P, ME, SAM, CXM, FEP, F, SXT	0.58	4		0.43 ± 0.04	Moderate	*icaA, icaB, icaC*
A27	1	Sputum	7	P, ME, SAM, CXM, FEP, F, CN	0.58	8		0.45 ± 0.12	Moderate	*icaA, icaB, icaC, icaD*

(Continued)

TABLE 1 | (Continued)

Antibiotype No.	No. of isolates/ pattern	Source	No. of resistant antimicrobials	Resistance pattern	MAR index	Vancomycin MIC	Vancomycin resistance genes	Biofilm-forming ability		Biofilm genes
								OD 570 ± SD*	Degree	
A28	1	Sputum	7	P, ME, SAM, CXM, FEP, DA, E	0.58	1		0.24 ± 0.01	Weak	*icaA, icaC*
A29	1	Hand swab	7	P, ME, CXM, FEP, F, SXT, E	0.58	0.5		0.18 ± 0.04	Non-producer	
A30	1	Sputum	7	P, ME, SAM, CXM, FEP, F, VA	0.58	16	*VanA*	0.25 ± 0.03	Weak	*icaA, icaD*
A31	2	Pus	6	P, ME, SAM, CXM, FEP, CIP	0.5	0.5		0.28 ± 0.02	Weak	*icaA, icaC*
		Shawarma				0.25		0.38 ± 0.1	Moderate	*icaA, icaB, icaC, icaD*
A32	4	Pus	6	P, ME, SAM, FEP, F, CIP	0.5	1		0.77 ± 0.04	Strong	*icaA*, *icaD*
		Pus				0.5		0.25 ± 0.06	Weak	*icaA*
		sputum				1		0.46 ± 0.12	Moderate	*icaA, icaB, icaC, icaD*
		Hand swab				0.5		0.37 ± 0.02	Moderate	*icaB, icaC*
A33	2	Pus	6	P, ME, SAM, CXM, FEP, CN	0.5	1		0.42 ± 0.08	Moderate	*icaA, icaB, icaC, icaD*
		Hand swab				0.5		0.39 ± 0.01		ND
A34	1	Shawarma	6	ME, FEP, F, SXT, CN, VA	0.5	16	*VanA*	0.32 ± 0.05	Weak	*icaD*
A35	1	Shawarma	6	ME, FEP, CIP, SXT, CN, E	0.5	0.25		0.39 ± 0.2	Moderate	*icaD*
A36	1	Shawarma	6	P, ME, SAM, FEP, CIP, E	0.5	0.5		0.22 ± 0.06	Non-producer	
A 37	1	Burger	6	P, ME, SAM, FEP, F, SXT	0.5	1		0.40 ± 0.04	Moderate	*icaA, icaD*
A38	1	Hand swab	6	P, ME, FEP, F, E, VA	0.5	256	*VanA*, *VanB*	0.61 ± 0.07	Strong	*icaA*, *icaD*
A39	1	Pus	6	P, ME, SAM, FEP, CIP, CN	0.5	1		0.76 ± 0.02	Strong	*icaA*, *icaD*
A40	2	Hand swab	5	ME, FEP, F, CIP, SXT	0.42	0.5		0.39 ± 0.1	Moderate	*icaA, icaD*
						0.25		0.17 ± 0.06	Non-producer	
A41	1	Burger	5	P, ME, SAM, CXM, FEP	0.42	0.5		0.16 ± 0.01	Non-producer	
A42	1	Pus	5	P, ME, FEP, CN, E	0.42	1		0.28 ± 0.01	Weak	*icaA*
A43	1	Pus	5	ME, FEP, F, CIP, CN	0.42	0.25		0.45 ± 0.05	Moderate	ND
A44	4	Pus	4	P, ME, FEP, CIP	0.33	4		0.29 ± 0.2	Weak	ND
		Pus				4		0.41 ± 0.05	Moderate	*icaA, icaD*
		Pus				1		0.15 ± 0.04	Non-producer	
		Hand swab				0.25		0.20 ± 0.06	Non-producer	
A45	1	Pus	4	P, ME, SAM, FEP	0.33	1		0.19 ± 0.02	Non-producer	
A46	2	Hand swab	4	P, ME, FEP, DA	0.33	0.5		0.56 ± 0.04	Strong	*icaA*, *icaD*
		Pus						0.26 ± 0.06	Weak	*icaB*
A47	1	Shawarma	4	P, ME, SAM, CXM	0.33	0.25		0.21 ± 0.01	Non-producer	
A48	1	Hand swab	4	ME, CXM, FEP, DA	0.33	1		0.15 ± 0.04	Non-producer	
A49	2	Pus	3	P, ME, FEP	0.25	0.5		0.30 ± 0.1	Weak	ND
						1		0.43 ± 0.03	Moderate	*icaA, icaD*

*P, penicillin; Me, methicillin; F, nitrofurantoin; SAM, ampicillin/Sulbactam; CXM, cefuroxime; CIP, ciprofloxacin; FEP, cefepime; SXT, trimethoprim/Sulfamethoxazole; CN, gentamycin; DA, clindamycin; E, erythromycin; VA, vancomycin. ND, not detected, *Average optical density value ± standard deviations (SD). Biofilm genes were assigned for biofilm-producing isolates.*

isolates (46/50), while the *bap* gene was not found in all isolates. *icaA* was the most prevalent (76%), followed by *icaD* (74%), *icaC* (50%), and *icaB* (46%). Several biofilm gene combinations have been identified, with all *ica* genes being the most prevalent (n = 17), followed by *icaA* and *icaD* (n = 16) (**Table 1**).

Diversity and Clustering Pattern of the Study Isolates

Based on the profiles of both antimicrobial resistance and biofilm formation, neither isolates from the same hosts nor isolates from the same sample source formed a single cluster (**Figure 2**). The analyses revealed that the 60 MRSA isolates formed 7 clusters, each cluster entails two isolates with identical profiles. Five of these clusters (71.4%) contained isolates that were all from humans and the remaining 2 clusters (28.5%) contained isolates from both humans and RTE meat. Considering all isolates, the majority of studied antimicrobial agents (n = 11/12, 91.6%) were positioned in a single big cluster (cluster A in **Figure 2**), yet only the pattern of VA resistance was separated into a different cluster (cluster B in **Figure 2**). The profile of moderate biofilm formation ability was also different from other biofilm profiles.

Correlation of Antimicrobial Resistance and Biofilm Formation Ability

Irrespective of the sample type, the correlation among resistance to individual antimicrobial ranged from 0.57 to −0.010 (**Supplementary Table 4**). The highest positive correlation was found between resistance to SAM and P (r = 0.57, *P*-value = 1.69E-06) followed by resistance to VA and CN (r = 0.43, *P*-value = 0.0005). The occurrence of phenotypic resistance to VA was more positively correlated (r = 0.6, *P*-value = 6.07E-09) to the presence of *van A* than to that of *van B* genes (r = 0.3, *P*-value = 0.003766). The ability to form biofilm showed variable association with the appearance of antimicrobial resistance (**Table 2** and **Supplementary Table 4**). It was found that all isolates harboring either *van A* or *van B* genes were biofilm producers and >83.3% of the isolates that showed resistance to all antimicrobials were biofilm producers. As shown in **Supplementary Table 4**, forming strong biofilm showed positive correlation with appearance of resistance to higher number of antimicrobials (11/12, 91.6%) than did moderate and weak biofilm formations (6/12, 50%, each). Strong biofilm was most positively correlated with the presence of *van B* gene (r = 0.38, *P*-value = 0.002). Moderate biofilm formation was correlated positively and significantly (r = 0.4, *P*-value = 0.003) with phenotypic resistance to gentamycin. Fisher's exact test analyses revealed that the phenotypic resistance to cefuroxime, nitrofurantoin, gentamicin, and vancomycin was significantly associated with biofilm formation with variable odds ratios (**Table 2**). We also observed that some biofilm-producing isolates were sensitive to certain antimicrobials. For instance, 66.6% of the isolates that were phenotypically sensitive to VA and did not possess *van A* and *van B* genes (n = 33) were biofilm producers. Similarly, 24 (77.4%) isolates from those that were sensitive to SXT, were biofilm producers.

Sample Type–Dependent Association Between Antimicrobial Resistance and Biofilm Formation Ability

As shown in **Figures 3A,B**, the correlation between antimicrobial resistance trait and biofilm formation ability was sample type-dependent (i.e., human vs. RTE meat isolates). Resistance to vancomycin and gentamycin correlated moderately positive (r = 0.4) in human isolates and strongly positive (r = 1) in RTE meat isolates. The correlation between ampicillin/sulbactam and both nitrofurantoin and cefuroxime were significantly positive in human isolates (r = 0.3 and 0.5, respectively), while this was none significant at all in RTE meat isolates. The source of the isolate has also affected the association between antimicrobial resistance and biofilm formation ability. While in RTE meat isolates moderate biofilm formation ability was significant negatively correlated with *van A* (r = −0.6) and *van B* (r = −0.5), there was no significant correlation among these pairs in human isolates. Similarly, in RTE meat isolates strong biofilm formation was significant negatively correlated with VA resistance, whereas in humans such correlation was non-significant. In human isolates, the resistance to penicillin, ampicillin/sulbactam, nitrofurantoin, gentamycin, erythromycin, and vancomycin showed variable significant negative correlation with isolates that are non-biofilm producers, whereas this correlation was non-significant in RTE meat isolates.

Relationship Between Isolates From Various Sample Types

Based on averaged binary distances among isolates and network analyses, it was found that the isolates recovered from pus, sputum, and hand swabs had an average binary distance of 0.36, 0.37, and 0.41, respectively, to those from burger. Furthermore, isolates from pus, sputum, and hand swabs had an average binary distance of 0.39, 0.41, and 0.42, respectively, to the isolates from shawarma (**Figure 4**).

DISCUSSION

Nowadays, there is an increased trend of RTE food afforded by infinite number of restaurants and street vendors all over the world, notably in Egypt. While this provides the advantage of fast food, it comes with the challenge and risk of bacterial infection especially these RTE products are not further exposed to heating. Raw meat contaminated with *S. aureus* is considered to be a major cause of food poisoning worldwide (de Boer et al., 2009; Wang et al., 2014; Raji et al., 2016). The risk of infection is being increased when the contaminated meat is not cooked sufficiently or RTE food is contaminated with this bacterium by cross-contamination (Wang et al., 2017). Recently, RTE food products have been reported as reservoirs for methicillin-resistant coagulase-negative staphylococci (MRCONS) (Yang et al., 2017), but the occurrence, resistance profile, and biofilm formation ability of methicillin-resistant coagulase-positive staphylococci (MRCOPS) and VRSA have received much less attention and require further in-depth investigation. Indeed, the available

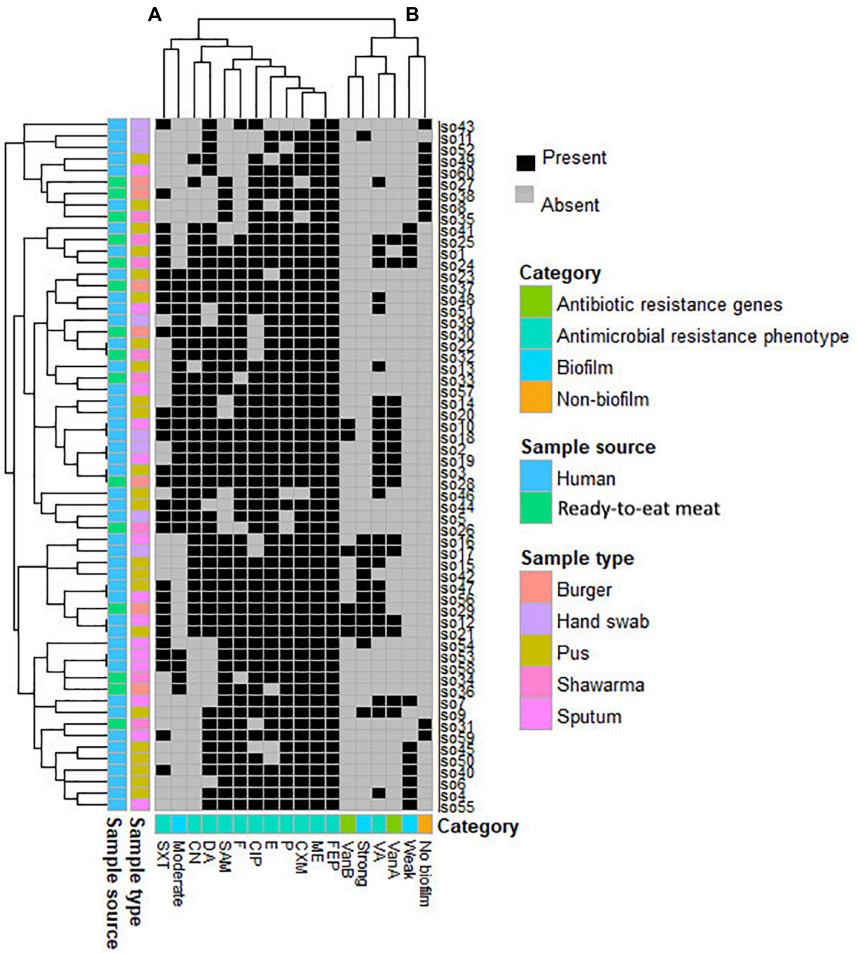

FIGURE 2 | Heatmap supported by dendrogram showing the overall occurrence of antimicrobial resistance and biofilm formation profile in the study MRSA isolates (n = 60). Sample type and sources are shown as color-coded category on the left of the heatmap. **(A,B)** refer to the two clusters of the studied patterns.

studies in Egypt have provided detailed characterization of MRSA strains in raw beef meat (Osman et al., 2015), and the available studies on RTE meat have described the occurrence of antimicrobial resistance without analyzing the biofilm formation ability, although being an important survival feature and none of these studies have analyzed the clustering and association of isolates coming from humans and RTE meat (Saad et al., 2019; Mahros et al., 2021). Here we tried to fill in these gaps by characterizing a set of MRCOPS and VRSA isolates from human and RTE meat with respect to their occurrence, antibiotic resistance profile, and biofilm formation ability.

In this study, 23.4% of RTE meat samples tested positive for *S. aureus*. These findings are higher than that documented in other studies on RTE meat products associated with foodborne illness where isolation rates of *S. aureus* were 9.5% in southern Taiwan (Wei et al., 2006), 11.8% in China (Yang et al., 2016), and 15.4% in Taiwan (Fang et al., 2003), but lower than the rates reported in chicken (40.9%) and pork meat (34.4%) from Shaanxi Province, China (Wang et al., 2014), and Qalubiya Governorate, Egypt (50.8%) (Saad et al., 2019). Our findings also revealed non-significant difference ($P > 0.05$) in the occurrence of *S. aureus* in shawarma (36.8%) and burger sandwiches (34.6%), which were lower than that reported in burger (43.3%) and nearly similar to that observed in shawarma (36.6%) in another Egyptian study (Saad et al., 2019). The differences in the microbiological quality of raw ingredient, the utensils and equipment used for food preparation, cooking, the presence of seasoning ingredients, isolation methods may contribute to the observed differences in the bacterial detection rate (Daelman et al., 2013; Wang et al., 2014). Our study reported a higher frequency of MRSA isolates from the examined COPS isolates recovered from shawarma and burger sandwiches (100%) than that obtained previously in beef burger and hot dog sandwiches (22.2%) (Mahros et al., 2021) and in other RTE meat products (25%) (Saad et al., 2019). This suggests the increased occurrence of MRSA in this kind of RTE meat. The current study showed that high number of MRSA strains were also VRSA (17/60, 28.3%). This is already higher than that obtained previously in Egypt by Mahros et al. (2021). The prevalence of MRSA (23.4%, 15 isolates/64 sample) and VRSA (6.25%, 4/64) in RTE meat samples in our study was higher than

TABLE 2 | The association between biofilm formation and antimicrobial resistance in the investigated 60 MRSA isolates.

Degree/ antimicrobial agent		P	ME	SAM	CXM	FEP	F	CIP	SXT	CN	DA	E	VA	*MecA*	*VanA*	*VanB*
Biofilm producers	R	47*	50	41	49	50	45	42	26	35	40	46	26	50	16	6
	S	3	0	9	1	0	5	8	24	15	10	4	24	0	34	44
None-producers	R	8	10	6	7	10	3	8	3	2	6	7	1	10	1	10
	S	2	0	4	3	0	7	2	7	3	2	6	7	1	10	1
P-value (Fisher's exact test)*		0.1904	>0.9999	0.2011	0.0127	>0.9999	0.0002	0.6677	0.3022	0.0047	0.2221	0.0830	0.0172	>0.9999	0.1585	0.1904
Odds ratio**		3.9	NA	3.0	21.0	NA	21.0	1.3	2.5	9.3	2.7	4.9	9.8	NA	4.7	3.9
Confidence Intervals		0.63–21.0	NA	0.81–11.8	2.5–275.5	NA	4.4–85.6	0.24–6.34	0.56–9.6	1.7–45.8	0.7246–10.04	1.0–22	1.2–110	NA	0.6301–54.02	0.6013–21.06

**The data shown refer to the numbers of isolates representing each category. R = resistance, S = sensitive. P, penicillin; Me, methicillin; F, nitrofurantoin; SAM, ampicillin/Sulbactam; CXM, cefuroxime; CIP, ciprofloxacin; FEP, cefepime; SXT, trimethoprim/Sulfamethoxazole; CN, gentamycin; DA, clindamycin; E, erythromycin; VA, vancomycin. *P-value refer to the significance of association between being a biofilm- forming isolate (irrespective of degree of biofilm formation) or not and the appearance of resistance or susceptibility to the respective antibiotic **odds ratio refer to the odds of being biofilm producer when having resistance to the respective antibiotic.*

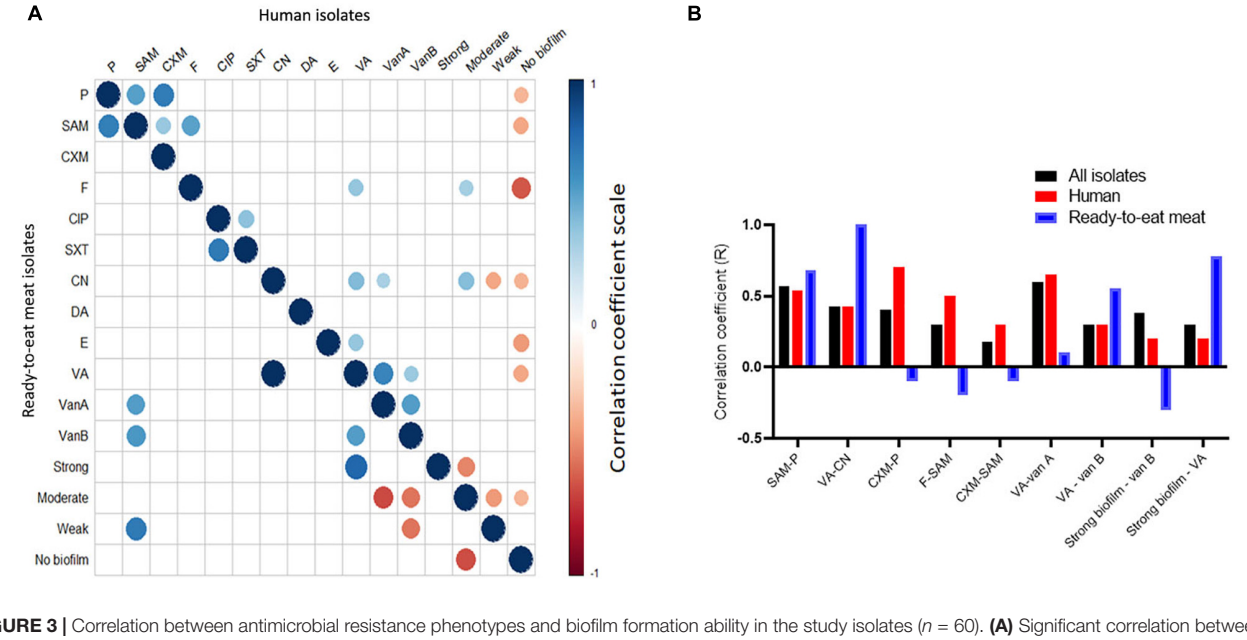

FIGURE 3 | Correlation between antimicrobial resistance phenotypes and biofilm formation ability in the study isolates (n = 60). **(A)** Significant correlation between antimicrobial resistance and biofilm in human (upper triangle) and RTE meat (lower triangle) isolates. Blank cells refer to non-significant correlations. **(B)** Sample source-dependent variation in the correlation between antimicrobial resistance and biofilm formation.

that observed (6.4% MRSA and 0% VRSA) in RTE meat (cooked pork, chicken, and duck) from China (Yang et al., 2016). In addition, a lower prevalence of MRSA (2.3% in chicken meat and 0.6% in pork meat) and VRSA (0%) were detected by Wang et al. (2014). However, Al-Amery et al. (2019) found that 4% (8/200) of camel meat samples from Egypt were positive for VRSA.

Food can become contaminated with *S. aureus* or MRSA from the food-producing animals or from infected food handlers during various processing stages (de Boer et al., 2009; Crago et al., 2012; Al-Amery et al., 2019). Since *S. aureus* is vulnerable to destruction by heating and sanitizing agents, its presence in RTE foods might be related to human contamination rather that contamination from animal origin and is indicative of poor sanitation during processing (U.S. Food and Drug Administration, 1998; Fontes et al., 2013). Generally, infected food handlers are reported to be responsible for causing 20% of bacterial foodborne illnesses. Here, the carriage rate of *S. aureus* in food handlers was 30% (12 isolates/40 samples), all isolates were MRSA and 25% of which was VRSA, indicating that food handlers, which are clinically healthy could pose a serious risk to public health and food safety. Other researches from different countries reported various rates of MRSA carriage

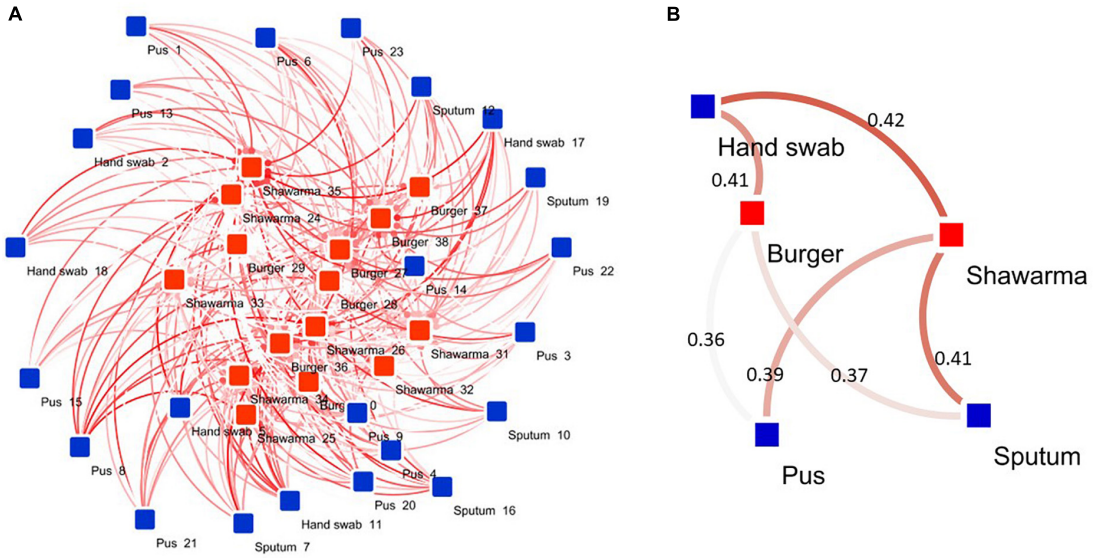

FIGURE 4 | Network diagram showing the association between human and RTE meat isolates. **(A)** Binary distances between isolates (n = 60) belonging to various human and RTE meat sources. **(B)** Averaged binary distance between isolates from humans and RTE meat. In **(A,B)**, the binary distances were visualized as color intensity of the line between two or more isolates. The darker the color the longer the distance between isolates (longer dissimilarity). The numbers shown on the lines in **(B)** refer to average binary distance between isolates belonging to the respective pairs.

on hands and noses of food handlers ranging from 1.6% in Bosnia and Herzegovina (Uzunoviæ et al., 2013), 28.6% in Brazil (Ferreira et al., 2014), and to 79% in Turkey (Sezer et al., 2015). Moreover, Ferreira et al. (2014) reported that 72.9 and 82.5% of the isolates from the handlers' hand and nose were VRSA, respectively. However, VRSA isolates were not identified in the study done in Bosnia and Herzegovina (Uzunoviæ et al., 2013). This variation could be based on different factors including health conditions of food handlers, cleaning habits, level of education, the state of development of the country, and the regulations on inquest (Sezer et al., 2015). This suggests the importance of the continuous survey and proper hygiene of the food handlers, which could decrease contamination by this pathogen and minimize the risk of disease.

Because MRSA and VRSA have been identified in RTE food worldwide, it is supposed that it could be transmitted to humans, causing illness. This study showed that 45.8% of patients' samples were positive for *S. aureus*, all isolates were of MRSA type and 30.3% of patients' isolates were VRSA. In support of our findings, a prospective surveillance study in Egypt has reported an upsurge in the prevalence of HA-MRSA and VRSA as the rate of MRSA has doubled from 48.6 to 86.8% in a 8-year period (from 2005 to 2013) and the proportion of VRSA has increased from 4.2 to 25.8% during the same period (Abdel-Maksoud et al., 2016). Conversely, comparatively lower proportions have been reported in other countries: *S. aureus* isolation rate was 14.3%, MRSA was 17.5%, and VRSA was 5.1% in Ethiopia (Dilnessa and Bitew, 2016) and 63.1, 72, and 15.9%, respectively, in Eritrea (Garoy et al., 2019). The observed variation is possibly attributed to the study population and duration, types of the specimen, and the used laboratory and control procedures.

MRSA isolates pose more risk if they resist treatment by antimicrobials. Alarmingly, all MRSA isolates characterized in the current study from all sources were MDR showing resistance to clinically important antimicrobials including cefepime, penicillin, and ampicillin-sulbactam, ciprofloxacin, nitrofurontoin, gentamicin, vancomycin, erythromycin, sulfamethoxazole - trimethoprim, and clindamycin. Other studies done in Egypt also reported higher resistance rates of HA-MRSA to clindamycin (56%), gentamicin (80.7%), and erythromycin (64.4%) (Abdel-Maksoud et al., 2016). Nearly similar results were observed for penicillin and vancomycin among MRSA isolates from Ethiopian patients but higher resistance was noted for erythromycin and trimethoprim-sulfamethoxazole (Dilnessa and Bitew, 2016). Other authors have reported considerably lower frequency of vancomycin and gentamicin resistance in Eritrea (Garoy et al., 2019).

The higher resistance rates of MRSA isolates cultured from RTE meat for cefuroxime, ciprofloxacin, gentamicin, and erythromycin than those isolated from humans is consonance with results from previous studies in retail foods (Wang et al., 2014) and RTE foods (Yang et al., 2016) in China and food animals in Tanzania (Mdegela et al., 2021). Our findings are even more serious in terms of public health hazard because shawarma and burger sandwiches are consumed without further cooking, which if happened would eliminate or reduce the load of pathogen. Overall, the high frequency of MDR isolates in the study area has been related to prevail, excessive use and imprudent use of antibiotics, including in livestock husbandry, self-medication, and substandard infection control and prevention practices (Garoy et al., 2019). Accordingly,

governments and individuals should pay attention to prevent further spread of MRSA and VRSA.

In addition to exhibiting antimicrobial resistance, MRSA isolates are able to produce biofilm as a defense mechanism enabling further survival in an infection niche (Archer et al., 2011). It is plausible to assume that a successful isolate would be able to form biofilm and resist antimicrobials. Our study supports this idea, where MRSA isolates with strong biofilm producers were resistant to 91.6% of the studied antimicrobials. There was also significant association between phenotypic resistance to cefuroxime, nitrofurantoin, gentamicin, vancomycin and biofilm formation. In agreement, Weigel and coauthors reported that vancomycin resistance is associated with and enhanced by the microenvironment of a biofilm (Weigel et al., 2007). These observations are similar to those reported in a study in Italy, in which food isolates of MRSA (milk and pork) have the ability to produce biofilms, as has been found previously for clinical MRSA (Vergara et al., 2017).

In *S. aureus*, the *ica* genes play a key role in biofilm formation. Strains with the *icaADBC* cluster especially *icaA* and *icaD* have previously been identified as potential biofilm producers (Arciola et al., 2001). These genes were detected in 92% of the biofilm-producing isolates with *icaA* and *icaD* being the most prevalent (76 and 74%, respectively). Our findings are consistent with the results of prior studies that found the *ica* genes in all isolates (Chen et al., 2020) especially both *icaA* and *icaD* genes (Szweda et al., 2012; Dai et al., 2019). However, Bissong and Ateba (2020) detected the *ica* genes in 75.3% of isolates with a lower proportion of *icaA* and *icaD* combination. These findings imply that *S. aureus* strains may differ in their ability to produce biofilms depending on their source (human or animal) and geographical origin. The results of this study, like those of previous research, indicated that the *bap* gene was not detected in biofilm-producing isolates (Szweda et al., 2012; Dai et al., 2019; Chen et al., 2020). Although not all genes involved in biofilm formation were examined, this might imply that the *ica*-dependent pathway is predominantly responsible for adhesion and biofilm formation in these isolates. Other biofilm-associated genes may be responsible for biofilm formation in isolates that tested negative for any of the genes investigated.

Based on the biofilm-forming capability in chronic *S. aureus*-related infection, some chemoprophylaxis procedures such as vaccine candidates were recently documented (Mirzaei et al., 2016, 2019, 2021; Bahonar et al., 2019; Gholami et al., 2019). Gholami et al. (2019) declared that a vaccine containing a mixture of PIA and glycerol teichoic acid (Gly-TA), biofilm related macromolecules, could be effective in preventing *S. aureus* biofilm formation and protect against infection. Furthermore, PIA-rSesC conjugate vaccine has been developed to raise antibodies against PIA in order to eliminate biofilm-forming *S. aureus* and *S. epidermidis* (Bahonar et al., 2019; Mirzaei et al., 2019).

In the absence of advanced sequencing technologies, a comprehensive and deep characterization of bacteria, and thus similarity among isolates from various hosts, is difficult to attain. However, studying targeted antimicrobial resistance and biofilm formation ability in a set of isolates could unravel important isolate's characteristics, especially looking at homogenicity among isolates within and among hosts as shown in our previous investigations in *Campylobacter jejuni* (Abd El-Hamid et al., 2019), *Aeromonas hydrophila* (Tartor et al., 2021), and *Escherichia coli* (Ramadan et al., 2020). This could initiate hypothesizing some zoonotic aspects and transmission scenarios across hosts. Here we showed that considering both antimicrobial resistance and biofilm formation potential, all isolates within most of the *S. aureus* clusters (i.e., the 5 clusters) were from human and none of the RTE meat isolates have identical profiles and thus formed no clusters. In addition, only two clusters were formed that contain mixed RTE meat and human isolates. In support of this, our correlation analyses revealed certain degree of disparity between human and RTE meat isolates, where gene correlation differed between both isolates in the two sources (**Figure 3**). While this should be interpreted using more isolates, this initially reflects the differential use of antimicrobials in each source (i.e., humans vs. RTE meat) (Pokharel et al., 2020). It also suggests the uniqueness of within-hosts isolates and that isolates within a single host (e.g., humans in the current study) could have developed similar fitness strategies such as antimicrobial resistance or biofilm formation ability. There are some clues from previous comprehensive genomic analyses that the majority of the animal-associated *S. aureus* clustered into animal-specific lineages a part from human lineages (Sung et al., 2008). Moreover, human lineages of *S. aureus* were rarely found in animals (Sakwinska et al., 2011). This was also supported by our observation of the host-dependent correlation between presence of antimicrobial resistance and biofilm formation ability (**Figure 3**). These observations motivated us to perform further analyses to determine the extent of similarities and dissimilarities among isolates from human and RTE meat using estimates of average binary distance among all isolates as done previously (Abd El-Hamid et al., 2019). Our observation of high binary distances between human isolates and either burger or shawarma isolates supports the uniqueness of within-host bacterial population.

A shortcoming of this study is the small number of examined samples, which was reflected on the small number of obtained isolates ready for characterization and analyses. However, it was convenient at least as an initial study on these two important sources, to balance the sample number, the multi-sample type nature included in the study and the needed analyses. We acknowledge that the traditional characterization methods applied here are not by any way considered as alternative for advance genomics tools (e.g., whole genome sequencing), and it is becoming clear that introducing modern genomics is vital and inevitable if it comes to comprehensive characterization of bacterial population, which we are planning for in future investigation.

CONCLUSION

The high prevalence of MRSA and VRSA isolates in shawarma and burger sandwiches in the present study suggests that RTE meat could be a potential source of MRSA and VRSA

isolates with significant clinical relevance. The ability of MRSA and VRSA isolates to produce biofilms and the presence of high rates of antimicrobial resistance amongst the isolates highlight the danger posed by these potentially virulent microorganisms persisting in RTE meat, food handlers, and patients. Taken together, good hygiene practices and antimicrobial surveillance plans should be strictly implemented along the food chain to reduce the risk of colonization and dissemination of MRSA and VRSA biofilm-producing strains.

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/s.

AUTHOR CONTRIBUTIONS

RE-M, EA, SE-S, GE, AA, AM, and SA performed investigation, practical work, and supervision. MS performed the statistical and bioinformatics analyses, prepared the figures, and participated with YT in formulating the tables. MS and YT investigated the data and designed the research manuscript. MS, with inputs from YT, took the lead in directing the research manuscript. YT wrote the initial draft of the manuscript. All authors revised the manuscript and agreed to the final version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.735494/full#supplementary-material

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